Effect of Wuziyanzong Pill on sperm quality and calcium ion content in oligoasthenospermia rats

Tongsheng Wang, Jinling Huang, Deling Wu, Qing Li, Xiangguo Liu, Han Chen, Lei Qiao, Dongdong Li, Jun Li

Tongsheng Wang, Jinling Huang, Xiangguo Liu, Han Chen, Lei Qiao, Dongdong Li, College of Pharmacy of Anhui Medical University, Hefei, Anhui 230038, China.
Deling Wu, Department of Chinese Medicine Chemistry of Anhui college of Traditional Chinese Medicine, Hefei, Anhui 230001, China.
Qing Li, Central Laboratory of Anhui Provincial Hospital, Hefei, Anhui 230001, China.
Tongsheng Wang, Jun Li, College of Pharmacy of Anhui Medical University, Hefei, Anhui 230038, China.
Supported by the National Natural Science Foundation of China (Grant No. 81173387) and the Natural Science Foundation of Anhui college of TCM (Grant No. 2010ZR002A)
 Correspondence to: Prof. Jun Li, College of Pharmacy of Anhui Medical University, Hefei, Anhui 230038, China. lijun@ahmu.edu.cn
Telephone: +86-551-5169216
Accepted: June 18, 2012

Abstract
OBJECTIVE: To study the effect of Wuziyanzong treatment on the sperm quality and content of calcium ions (Ca^{2+}) in oligoasthenospermia rats.

METHODS: A model of oligoasthenospermia was induced in 50 Sprague Dawley rats by treatment with tripterygium glycosides at 30 mg/kg per day for 8 weeks. They were divided randomly into a model group, a positive group (Huangjingzanyu capsule, 3.01 g/kg), and low, medium and high dose Wuziyanzong treatment groups (2.30, 4.60, 9.20 g/kg crude drug respectively) with 10 in each group. Another 10 rats were used as a control group. The rats in the control and model groups were administered distilled water, while the rats in the remaining groups were administered Wuziyanzong for 30 d. The epididymides were removed, spermatozoa recovered and the sperm density and viability were measured. The spermatozoa were purified and the contents of Ca^{2+} in the cytoplasm and mitochondria were detected by flow cytometry and atomic absorption spectrometry, respectively.

RESULTS: After 8 weeks of treatment with tripterygium glycosides, the sperm density, sperm activity and the Ca^{2+} content of spermatozoa in the model rats were all significantly decreased compared with the control group (all P<0.05). After 30 d treatment, the sperm density and activity improved and the Ca^{2+} content of sperm were increased significantly in the medium and high dose Wuziyanzong treatment groups in comparison with the model group (all P<0.05).

CONCLUSION: The Wuziyanzong treatment increased sperm density, improved sperm viability and enhanced the content of Ca^{2+} in the sperm cytoplasm and mitochondria in this rat model of oligoasthenospermia.

Keywords: Wuziyanzong; Sperm count; Sperm motility; Asthenozoospermia; Oligozoospermia; Calcium ions

INTRODUCTION
The Wuziyanzong pill, a classic formula in Traditional Chinese Medicine (TCM), is used for treating men with oligoasthenospermia. The formula consists of Ly-
cium barbatum L, Cuscuta chinensis Lam, Schisandra chinensis, Rubus chingii Hu and Plantago asiatica L. Lycium barbatum L. can nourish the kidney and replenish its essence; Cuscuta chinensis Lam can warm the kidney and enrich its essence, and these two medicinal herbs are the principal components of Wuziyanzong. Rubus chingii Hu can warm the kidney without causing dryness and fix sperm without coagulation. Schisandra chinensis has the effects of enriching Qi and easing weakness; these two medicinal herbs act as adjunct medicines. Plantago asiatica L. can remove heat from the liver and lungs and promote diuresis, and acts as a supplementary medicine. Thus, the formula is not cold and dry; it has the overall effects of tonifying the kidney, replenishing its essence and smoothing the kidney’s “Qi”. Treatment with Wuziyanzong could improve semen quality and increase the level of testosterone in patients with oligoasthenospermia, and was shown to increase the pregnancy rate among infertile couples. Treatment with this formula could downregulate 12 genes in azoospermic mice as shown by a gene chip assay. In the present study, we investigated its effect on epididymal sperm quality and Ca²⁺ content and discuss its mechanism.

**MATERIALS AND METHODS**

**Experimental animals**

Male Sprague Dawley rats (specific pathogen-free, weighing 200-220 g) were purchased from the Anhui Animal Breeding Laboratory of the Animal Center, Anhui, P. R. China [Number of animal license SCXK (Anhui) 2011-002].

**Herbs and reagents**

The Wuziyanzong pills, extracted by decoction and concentration, were provided by the Department of TCM Chemistry, Anhui college of TCM (batch No. 20111205). Lycium barbatum L., Cuscuta chinensis Lam, Schisandra chinensis, Rubus chingii Hu and Plantago asiatica L. were bought from the Anhui Ji Ren Pharmaceutical Co. (batch Nos 120201, 110901 and 120201, respectively) and the dosage complied with the “Pharmacopoeia of the People’s Republic of China” (2010 ed). The extracting process was to dilute 10-fold in water, decoct once an hour twice, then filter the mixture and concentrate to 1 g/mL. Tablets of tripterygium glycosides were from Shanghai Fudan Hua Xin Pharmacy Co. (batch No. 110902) and Huangjinzanyu Capsules were from Shanghai Xin Ya Pharmaceutical Co. Ltd. (batch No. 110901).

**Chemicals**

Dimethyl sulfoxide was purchased from Beijing Lvshengyuan Technology Co., Ltd (Sigma, batch No. D5879), Fluo3-AM was from Dojindo Laboratories (batch No. DL740), phosphate buffered saline (PBS, pH 7.2-7.6) was from Wuhu Boster Biological Technology Co., Ltd. (batch No. AR0030) and Tris-HCl was from Shanghai Beyotime Biological Technology Co., Ltd. (batch No. ST774).

**Equipment**

We used an Olympus BX60 light microscope (Olympus Corp., Tokyo, Japan), a BD FACSC ante™ II flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), a 3K15 tabletop centrifuge (Sigma-Aldrich, Germany) and a M6 atomic absorption spectrometer (Thermo Electron Corporation, Waltham, MA, USA).

**Experimental groups and methods**

The 60 rats were divided randomly into six groups: a control group, a model group, a positive group (Huangjingzanyu capsule, 3.01 g/kg), and three Wuziyanzong treatment groups with low, medium and high dose rates (2.30, 4.60, 9.20 g/kg crude drug respectively). Except for the control group, the remaining five groups were administered tripterygium glycosides (suspended in sodium carboxymethylcellulose) orally at 30 mg/kg per day for 8 weeks. After the model be made, the rats were administered drugs orally according to the dosage above (1 mL/100 g body weight) for 30 d, while the rats in the model and control groups were administered distilled water alone. After the last administration, the rats were killed and the epididymides were extracted to prepare sperm suspensions.

**Sperm concentration and viability**

A single-blind method was used in whole experiment. A test tube filled with 6 mL normal saline was preheated to 37°C. The left entire epididymis was put into the tube, then cut into small pieces and shaken at 37°C for 15 min to allow the spermatozoa to disperse. A small amount of the suspension was taken, suitably diluted and put into a hemocytometer to calculate the number of sperm. The number of spermatozoa (N) in five squares was counted under a microscope and the sperm density was calculated. The sperm density per milliliter of suspension was calculated as N´5´20 (dilution factor)´10⁶. Using flat pressing plates method, five fields were chosen randomly and sperm viability was examined in the suspension. For this, “a” grade spermatozoa expressed rapid motility in a straight line and were in perfect condition; “b” grade spermatozoa expressed forward motion in a straight line and were in good form. Motility was expressed as (a+b)% based on all cells counted.

**Calcium concentration in sperm cytoplasm**

A 200 mesh (75 μm) filter was used to filter the sperm suspension and the sperm concentration was adjusted...
to $2\times10^6$/mL. Aliquots of 200 mL were centrifuged at 390 × g for 5 min and the supernatant was removed. The pellet was resuspended in 300 mL PBS (0.01 mol/L), then 20 mL Fluo3-AM (0.1 mmol/L) was added and the suspension was incubated at 37°C for 30 min. The sample was then centrifuged at 390 × g for 5 min and the supernatant removed. The pellet was resuspended in 500 mL PBS (0.01 mol/L) and the calcium concentration in the sperm cytoplasm was determined using flow cytometry. The excitation and emission wavelengths were 480-520 nm and 525-530 nm, respectively. The calcium concentration was estimated from the fluorescence intensity of Fluo3-AM.

**Calcium concentration of sperm mitochondria**
The epididymis was extracted and weighed, then put into nine volumes of saline at 4°C to make a 10% w/v tissue homogenate. The homogenate was centrifuged at 700 × g for 10 min and removed the cell debris. The supernatants were collected and centrifuged at 23 031 × g for 15 min to extract the mitochondria. Added 2 mL solution (0.1 mol/L Tris-HCl, pH 7.4; 1 mmol/L KCl, 1:1 preparation) to the mitochondria as a separate transmitter. Atomic absorption spectrometry was used to determine the calcium concentration in sperm mitochondria.

**Statistical analysis**
All data were expressed as the mean±standard deviation and analyzed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Multiple group means were compared using single-factor analysis of variance and group results were compared using the Student–Newman-Keuls test; $P<0.05$ was considered significant.

**RESULTS**

**Sperm density and sperm viability**
After the model rats had been administered tripterygium glycosides for 8 weeks, the sperm density and activity were significantly decreased compared with the control group (all $P<0.05$). After 30 days treatment, sperm density and activity in the model rats were obviously improved, especially in the Wuziyanzong medium and high dose rate groups compared with the untreated model group (both $P<0.05$; Table 1).

**Calcium content in spermatozoa**
After treatment with tripterygium glycosides for 8 weeks, the Ca$^{2+}$ contents of sperm cytoplasm and mitochondria in model rats were significantly decreased and all groups showed significant differences in comparison with the control group (all $P<0.05$). After 30 d treatment, the Ca$^{2+}$ contents of sperm cytoplasm and mitochondria in the treated groups were obviously increased, especially in the Wuziyanzong medium and high dose rate groups compared with the model group ($P<0.05$) (Table 2 and Figure 1).

**Table 1 Comparison of sperm viability and density among different groups ($\bar{x} \pm s$, n=10)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (g/kg)</th>
<th>Sperm viability (%)</th>
<th>Sperm density ($10^6$/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>-</td>
<td>40±7$^a$</td>
<td>38±9$^a$</td>
</tr>
<tr>
<td>Model group</td>
<td>-</td>
<td>15±6$^b$</td>
<td>16±5$^b$</td>
</tr>
<tr>
<td>Positive group</td>
<td>3.01</td>
<td>23±6$^a$</td>
<td>32±7$^a$</td>
</tr>
<tr>
<td>Wuziyanzong low dose</td>
<td>2.30</td>
<td>19±4$^a$</td>
<td>20±7$^a$</td>
</tr>
<tr>
<td>Wuziyanzong medium</td>
<td>4.60</td>
<td>25±10$^a$</td>
<td>26±8$^a$</td>
</tr>
<tr>
<td>Wuziyanzong high dose</td>
<td>9.20</td>
<td>27±5$^a$</td>
<td>33±9$^a$</td>
</tr>
</tbody>
</table>

Notes: $P<0.05$ compared with the model group; $^aP<0.05$ compared with the control group.

**Table 2 Comparisons of calcium contents of sperm cytoplasm and mitochondria among different groups ($\bar{x} \pm s$, n=10)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (g/kg)</th>
<th>Sperm cytoplasm (fluorescence intensity)</th>
<th>Sperm mitochondria (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>-</td>
<td>227±21$^a$</td>
<td>239±23$^a$</td>
</tr>
<tr>
<td>Model group</td>
<td>-</td>
<td>88±16$^b$</td>
<td>148±34$^a$</td>
</tr>
<tr>
<td>Positive group</td>
<td>3.01</td>
<td>142±44$^a$</td>
<td>176±22$^a$</td>
</tr>
<tr>
<td>Wuziyanzong low dose</td>
<td>2.30</td>
<td>123±43$^a$</td>
<td>150±8$^a$</td>
</tr>
<tr>
<td>Wuziyanzong medium</td>
<td>4.60</td>
<td>160±46$^a$</td>
<td>197±23$^a$</td>
</tr>
<tr>
<td>Wuziyanzong high dose</td>
<td>9.20</td>
<td>198±10$^b$</td>
<td>235±14$^a$</td>
</tr>
</tbody>
</table>

Notes: $P<0.05$ compared with the model group; $^aP<0.05$ compared with the control group.
DISCUSSION

Oligospermia or asthenospermia is one of the most common causes of male infertility. Calcium ions acting as intracellular second messengers play an important role in sperm physiology. Thus, the Ca\(^{2+}\) content is closely related to sperm morphology and reproductive potential and is an important indicator of sperm quality.\(^6\) Research has showed that sperm motility is dependent on an influx of free Ca\(^{2+}\). Thus, Ca\(^{2+}\) channel blockers can lead to a reversible loss in sperm motility. Oligoasthenospermia can arise when the content of Ca\(^{2+}\) in the sperm cytoplasm is decreased.\(^7\) In the present study we found that the sperm quality and the calcium contents in sperm were obviously decreased in response to oral treatment with tripterygium glycosides, consistent with the literature.\(^8,9\)

The main function of mitochondria is to produce energy and provide adenosine triphosphate for sperm movement. The mitochondrial activity and sperm motility are closely related, but the calcium concentration of mitochondria is not often reported. Here we found that the Ca\(^{2+}\) content of mitochondria decreased with the oral treatment with tripterygium glycosides, associated with a decline in sperm motility.\(^10\) Mitochondria act as stores and regulators of intracellular Ca\(^{2+}\). The Ca\(^{2+}\) content in mitochondria is far higher than that of cytoplasm under normal physiological conditions. In pathological conditions, energy metabolism is destroyed, the inner mitochondrial membrane is damaged and the mitochondrial permeability transition pore opens. This leads to mitochondrial Ca\(^{2+}\) outflow and a decline in Ca\(^{2+}\) content, resulting in the destruction of cellular calcium homeostasis. Thus, the oligoasthenospermia induced by tripterygium glycosides might be associated with the damage to sperm mitochondrial structure and collapse of the mitochondrial membrane potential.\(^11,12\)

Wuziyanzong is a classic TCM prescription used to treat reproductive system disorders and has significant effects.\(^13\) Here we found that oral treatment with Wuziyanzong pills could significantly improve sperm quality in a rat model of oligoasthenospermia, by increasing the sperm content of cytosolic Ca\(^{2+}\) and maintaining its homeostasis.

REFERENCES


