Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Effects of the crude extract of *Polygala tenuifolia* Willd on human sperm in vitro*

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Received Sept. 26, 2010; Revision accepted Nov. 29, 2010; Crosschecked May 13, 2011

Abstract: The aim of the present study is to analyze sperm membrane changes and the spermicidal effect in treatment with the crude extract from *Polygala tenuifolia* Willd (PTW) in vitro. The root of PTW was extracted in distilled water. Normal human spermatozoa were used to assess the spermicidal activity (Sander-Cramer assay) of the extract from the PTW root. The hypo-osmotic swelling (HOS) test and the eosin Y (EY) staining were used to detect the integrity of sperm membrane and vitality. The sperm chromatin dispersion (SCD) test was performed to determine sperm DNA integrity. N-9 was used as a reference standard and semen added to physiological saline was used as the control. Semen samples were donated by 42 healthy fertile men. The crude extract from the root of PTW could immobilize and kill 100% spermatozoa within 20 s in vitro at the concentrations of 20.0 and 10.0 mg/ml; at the concentration of 5.0 mg/ml, spermatozoa were immobilized in (39.5±3.2) s. In the groups of the crude extract from the root of PTW and N-9 solution, the rate of the normal HOS (tails swollen) and the white head (unstained) was 0%, and the rate of the abnormal HOS (tails unswollen) and red head (stained) was 100%. Sperm DNA fragmentation showed no change in exposure to the crude extract from the root of PTW and N-9 solution. The sperm revival test did not show any spermatozoa that recovered their motilities. The rapid spermicidal activity of the crude extract from the root of PTW in vitro may occur by the disruption of the sperm membrane integrity.

Key words: *Polygala tenuifolia* Willd, Crude extract, Spermicidal effect in vitro, Sperm membrane, DNA integrity
doi:10.1631/jzus.B1000347 **Document code:** A **CLC number:** R979.2

1 Introduction

Chinese medicinal herbs are an important part of traditional Chinese medical science (Chan *et al*., 2010) and have a long history of indigenous use in China as well as other countries (Gao *et al*., 2011). However, to date, many pharmacological effects of Chinese medicinal herbs have not yet been discovered or exploited. In the recent three decades, a large number of Chinese medicinal herbs have been randomly selected and screened for spermicidal activity in vivo and several species seem to be promising (Huang *et al*., 2009; Li *et al*., 2009). The spermatogenesis is inhibited by *Tripterygium wilfordii* and gossypol in vivo (Waller *et al*., 1980; Qian and Wang, 1984), which acts on different sites and stages of spermatogenesis in testis or sperm activity in epididymis. A low dose of gossypol mainly influences the sperm activity in the epididymis although it also acts on testicular spermatids (Liu, 2005; Huang *et al*., 2009; Li *et al*., 2009; Zhuang and Xie, 2009). However, the immunosuppressive toxicity of *Tripterygium wilfordii* and the hypokalemia of gossypol have limited their clinical use.

The vitality, motility, and morphology of the spermatozoa are important for sperm function. The

* Project (No. 2008-01) supported by the Foundation of Shandong Provincial Committee Family Planning Development, China
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assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperm are immotile, to distinguish between immotile dead sperm and immotile live sperm. The eosin Y (EY) staining and the hypo-osmotic swelling (HOS) test can provide an analysis of the motility evaluation, since the percentage of dead cells should not exceed the percentage of immotile spermatozoa (WHO, 1999). To estimate the sperm vitality of the semen sample treated with spermicidal agents, the HOS test and EY staining are usually used (Primorac *et al*., 1985; Lohiya *et al*., 2000; Venma *et al*., 2002; Chakrabarti *et al*., 2003).

Gandini *et al*. (2000) reported that the presence of DNA fragmentation in ejaculated spermatozoa might correlate with defects in spermatogenesis. Elevated percentages of apoptotic spermatozoa have also been found after infections of the reproductive tract, cancer, and other pathologies (Baccetti *et al*., 1996; Sharma *et al*., 1999; Gandini *et al*., 2000). However, the sensitivity of the cells to DNA damage or the mechanism involved in cell death of ejaculated sperm from normozoospermic donors (Maione *et al*., 1997; Blanc-Layrac *et al*., 2000) is important to elucidate especially in those pathologies where normal spermatozoa are exposed to non-physiological damaging agents (Ramos and Wetzels, 2001). Laforest *et al*. (2004) reported that tetradecyl-dimethyl-benzyl-ammonium fluoride is a powerful new spermicide. In cases of failure of local contraception, there is a theoretical risk that a spermatozoon exposed to a sublethal concentration of spermicide might suffer DNA strand breaks (Laforest *et al*., 2004).

In this study, we screened more than 300 Chinese medicinal herbs such as *Platycodon grandiflorum*, *Herba taraxaci*, *Flos lonicerae japonicae*, and *Radix paeoniae rubra*, and found that the crude extracts from the roots of *Polygala tenuifolia* Willd (PTW) and *Platycodon grandiflorum* had strong sperm immobilizing activity in vitro. The sperm membrane integrity was detected by using the HOS test and the EY staining, and the sperm DNA fragmentation was determined by the sperm chromatin dispersion (SCD) test. In the present study, we detailed the spermicidal effect of the crude extract of PTW in vitro.

2 Materials and methods

2.1 Preparation of the crude PTW extract

The dried Chinese roots of PTW were purchased from the Traditional Chinese Herbal Pharmacy (Jianlian, Jinan, China) and crushed into a coarse powder (<1 mm) in the grinding machine. The powdered roots of PTW materials were subjected to distilled water extraction in the laboratory. Briefly, 5 g of the crushed PTW was mixed with 120 ml distilled water and placed in the distilled water at constant temperature of 100 °C for 60 min. The decoction was allowed to cool to room temperature (20 °C), after which it was filtered through quality filter paper, concentrated by vacuum evaporator (Buchi Rotavapor R-114, Switzerland), and then freeze-dried by using the freeze-drying centrifuge (Christ Alpha 1-2 Model 100200, Germany). Finally, 1.25 g powders were yielded for use in this experiment.

2.2 Semen collection and analytical procedure

Between February 2006 and May 2010, 42 healthy fertile men [(35.9±2.3) years old] were recruited as donors. This study was approved by the Ethical Committees of the Shandong Provincial Institute of Science and Technology for Family Planning. Semen samples were collected by masturbation in sterile glass cups after 3 to 5 d of abstinence and analyzed on-site within 1 h for both macroscopic and microscopic characteristics. Routine semen analysis was carried out according to the Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (WHO, 1999). All samples were allowed to liquefy for at least 30 min. Samples showing a sperm count less than 50×10^6 ml^{-1} and motility less than 60% were rejected. Using an unstained sample of fresh semen, the number of motile sperm was counted until a total of 200 spermatozoa were assessed. In order to improve measurement accuracy, procedures have been carried out twice. The same donor's semen was divided into a number of equal portions for the following experiments.

2.3 Spermicidal test in vitro

The crude extract powders from the roots of PTW were diluted with physiological saline (9 mg/ml NaCl) serially up to 20.0, 10.0, and 5.0 mg/ml,

respectively. The nonoxynol-9 (N-9) powders were diluted with physiological saline serially to 2.0, 1.0, and 0.5 mg/ml, respectively. N-9 was produced from China Pharmaceutical University Pharmaceutical Co., Ltd. (Nanjing, China) and used as a reference standard. Similarly, sperm samples in physiological saline (1:1, v:v) served as the controls.

A spermicidal test was performed with each dilution, following the modified method of Sander and Cramer (1941) and the standard recommended by the WHO (1999) (also see Chijioke *et al.*, 1986). Briefly, 0.05 ml of human semen was placed on a microscopic slide. Then 0.05 ml of PTW extract or N-9 solution or physiological saline was added and mixed with the semen, and then examined under a phase-contrast inversion microscope (Olympus, IX51, Japan) at 400× magnification. The result was scored as momentary spermicidal activity in vitro if 100% spermatozoa became completely immotile within 20 s. When the crude extract from the root of PTW and N-9 solution were mixed with the semen, the time to immobilize sperm was recorded. The original pH values of the crude extract from the root of PTW and N-9 solution were determined by using pH meter (Mettler Toledo FE20, Switzerland) and adjusted with 0.1 mol/L NaOH.

2.4 Sperm HOS test and EY staining

The HOS test was performed as recommended by WHO (1999). Preparation of the HOS solution was as follows: 0.735 g of sodium citrate dihydrate (S4641, Sigma, USA) and 1.351 g of D-fructose (F3510, Sigma, USA) were dissolved in 100 ml of purified water. A total of 0.1 ml of semen sample was mixed with 1.0 ml of a hypo-osmotic solution (150 mOsm). After incubation for 5 min at 37 °C, ≥200 spermatozoa were analyzed by phase-contrast microscopy (Olympus, IX51, Japan) at 400× magnification, evaluating the modifications of the sperm tail to score swollen sperm (normal HOS) and unswollen sperm (abnormal HOS), which were reported as a percentage of all sperm observed.

Sperm viability was determined by using a modified EY staining test referred to the WHO (1999) criterion. A total of 0.5 g of EY (E6003, Sigma, USA) and 0.9 g of sodium chloride (GB1266-77, Shanghai, China) were dissolved in 100 ml distilled water. Live spermatozoa had white heads (EY unstained) and dead spermatozoa had heads that were stained red or dark pink (EY stained).

2.5 SCD test

The SCD test performed according to Fernández *et al.* (2003) and Zhang *et al.* (2010). The semen sample mixed with 0.1 ml of the crude extract from the root of PTW or N-9 or physiological saline was centrifuged at 1000 r/min for 10 min. The seminal plasma was discarded. The sperm sediment was re-suspended and diluted to 10^7 ml^{-1} with phosphate buffer solution (PBS; pH 6.8). The suspensions were mixed with 1% low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37 °C. Aliquots of 50 μl of the mixture were pipetted onto a glass slide precoated with 0.65% standard agarose dried at 80 °C, covered with a coverslip (22 mm× 22 mm), and left to solidify at 4 °C for 5 min.

Coverslips were carefully removed, and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 mol/L HCl) for 7 min at 22 °C in the dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. The denaturation was then stopped, and proteins were removed by a transfer of the slides to a tray with neutralizing and lysing solution (0.4 mol/L Tris, 0.8 mol/L dithiothreitol (DTT), 1% Triton-100, and 50 mmol/L ethylenediaminetetraacetic acid (EDTA); pH 7.3) for 20 min at room temperature. Slides were thoroughly washed in Tris-borate-EDTA buffer (0.09 mol/L Tris-borate and 2 mmol/L EDTA; pH 7.5; Sigma, USA) for 3 min, dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 min each), and then air dried. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI; 2 mg/ml; Vysis, USA) for fluorescence microscopy or with Wright's-Giemsa's solution (Sigma, USA) for brightfield microscopy. Slides were stained for 10 min, washed with distilled water, and then air dried. After staining, the results were observed immediately. Strong staining is preferred to aid in visualization of the periphery of the dispersed DNA loop halos.

A total of 500 spermatozoa were evaluated manually on each slide at right-field optics at 1000× magnification and oil immersion (Leica, DM4000B, Germany) for halo size and dispersion patterns.

The nuclei with large-to-medium size halos were considered sperm with non-fragmented DNA, whereas

nuclei with small size halos or without a halo and degraded were considered sperm with fragmented DNA.

2.6 Sperm revival test

After completion of the spermicidal test in vitro, 0.5 ml of semen sample mixed with the crude extract from the root of PTW or N-9 was added to 1.0 ml physiological saline, and then spermatozoa were washed twice in physiological saline and incubated once again in the Ham's F-10 (N6635, Sigma, USA) medium for 30 min at 37 °C to observe the reversal of sperm motility. The sperm vitality test was used as in the HOS and the EY steps above.

2.7 Statistical analysis

Data were analyzed with SPSS system (Version 12.0) and presented as mean±standard deviation (SD). Statistical significance was evaluated with Student's paired *t*-test and the difference was considered statistically significant at $P<0.05$.

3 Results

3.1 pH values of the crude extract from the root of PTW and N-9 solution and their spermicidal activities

The original pH values of the crude extract from the root of PTW and N-9 solution were 6.6 and 0.3, respectively. When the crude extract from the root of PTW and N-9 solution were mixed with semen (1:1, v:v), the pH values were changed to 6.9±0.2 and 4.2±0.3, respectively. There was a significant difference in the mean value of pH between the crude extract from the root of PTW and N-9 solution ($P<0.001$). To avoid the influence of acidity on sperm motility, the pH values of the crude extract from the root of PTW and N-9 solution were adjusted to 7.4.

A rapid spermicidal activity was exhibited in the crude extract from the root of PTW (pH 7.4) at the concentrations of 20.0 and 10.0 mg/ml, immobilizing all spermatozoa at (5.2±1.8) and (16.7±2.2) s, respectively, and at the concentration of 5.0 mg/ml, all spermatozoa were immobilized within (39.5±3.2) s. In the N-9 group, all spermatozoa were immobilized in (7.8±1.1), (14.2±2.9), and (37.9±6.8) s at the concentrations of 2.0, 1.0, and 0.5 mg/ml, respectively.

3.2 Sperm membrane changes in the HOS test and EY staining, and the rate of sperm DNA fragmentation after treatment with PTW or N-9

In the PTW and N-9 groups, the percentage of normal HOS sperm (curled tails, tail membrane-intact) and EY unstained sperm (head membrane-intact) was 0. In contrast, the percentage of abnormal HOS sperm (no curled in tails, tail membrane-damaged) and EY stained sperm (head membrane-damaged) was 100% (Table 1). The mean percentage of sperm DNA fragmentation was not significantly different among all groups ($P>0.05$).

3.3 Results of sperm revival test

None of the spermatozoa, once immobilized, recovered their motilities following removal of the crude extract from the root of PTW and N-9 solution, and 30 min of incubation in the Ham's F-10 medium. The results of sperm revival test (HOS and EY) of the PTW and N-9 groups were similar to those of the previous spermicidal test of both groups.

4 Discussion

Root of PTW is distributed throughout the wilderness of Northeast Asia, including China, Japan, and Korea. It is an important herb prescribed in the formulas of Chinese medicinal herbs to exhibit sedative and antitussive effects. In traditional Chinese medicine, PTW calms the spirit and has been used for insomnia, palpitations with anxiety, restlessness, and

Table 1 Results of sperm HOS, EY and SCD tests in control, PTW and N-9 groups

Group	*n*	Sperm membrane changes (%)				DNA fragmentation (%)
		Normal HOS	Abnormal HOS	EY stained	EY unstained	
Control	42	77.6±5.8	22.4±2.1	24.8±4.6	75.2±9.9	11.8±3.2
PTW	42	0*	100*	100*	0*	12.0±3.1
N-9	42	0*	100*	100*	0*	12.3±2.2

Values are expressed as mean±SD. * $P<0.0001$, compared with the control group

disorientation. The root of PTW is a well-known traditional Chinese medicine used as an expectorant, a tonic, a tranquillizer, and an anti-dementia drug. Although widely used in Asian countries, spermicidal action of the crude extract from the root of PTW had not been previously reported.

In this study, we have carried out an investigation to evaluate the mechanism of spermicidal activity of the crude extract from the root of PTW. The role of the crude extract from the root of PTW for spermicidal activity was assessed by measuring pH, assaying the sperm membrane integrity by using the HOS and EY test, and analyzing sperm DNA fragmentation by using the SCD test when spermatozoa were exposed to the crude extract from the root of PTW.

The semen pH and osmolarity are important for sperm quality (WHO, 1999). Gradually acidifying the pH can lead to a progressive loss of sperm motility. However, sperm velocity is slightly increased by mild alkalinization (Kamischke and Nieschlag, 1999; WHO, 1999). In the present study, the spermicidal tests of the crude extract from the root of PTW and N-9 were carried out under the original pH and the adjusted pH on the motility of sperm. In order to eliminate the impacts of osmotic pressure and pH on the sperm, physiological saline solution was applied to dissolve the crude extract powder from the root of PTW and N-9.

Our study shows that the crude extract from the root of PTW at the concentrations of 20.0 and 10.0 mg/ml had a momentary (within 20 s) spermicidal activity, and at the concentration of 5.0 mg/ml, the time of complete sperm immobilization was 39.5 s. The results indicate that the crude extract from the root of PTW possesses strong spermicidal activity in vitro and may be as a barrier to sperm penetration through the cervix for birth control.

The percentage of live spermatozoa is assessed by identifying those with an intact cell membrane, from dye exclusion or by hypotonic swelling. The dye exclusion method is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-impermeant stains. The HOS test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions (WHO, 1999). Firstly described for the use with human sperm (Jeyendran *et al.*, 1984), the HOS test enables the identification of sperm with functionally-intact membranes and is one of a range of tests commonly used to determine sperm viability. The osmotic stress caused by the chosen hypo-osmotic medium must be sufficient to induce an influx of water into the cell to result in an increase in volume and hence cause a curling of the tail, but to prevent lysis of the sperm membrane (Matson *et al.*, 2009). The simplified one-step technique, exposing sperm to a mixture of eosin and nigrosin, was introduced on various mammalian sperm (Dott and Foster, 1972) and was standardized internationally by Björndahl *et al*. (2002).

After treating with the crude extract from the root of PTW and N-9, sperm membrane had changed, which was clearly observed under the microscope by using the HOS and EY tests. All spermatozoa (100%) treated with the crude extract from the root of PTW and N-9 were positively stained by EY and tails-unswollen by HOS. The sperm revival test showed that none of the spermatozoa recovered their motilities following the removals of the extract from the root of PTW and N-9. These results indicate that sperm head and tail membranes were injured by the extract from the root of PTW, which caused sperm death. Most of the spermicidal agents of plant extract induce disruption of the plasma membrane of sperm and act on the sperm surface (Lohiya *et al.*, 2000; Souad *et al.*, 2007). Plant derivatives also cause drastic inhibition in sperm membrane-specific enzymes, such as acrosin and hyaluronidase, the most important enzymes in the process of fertilization (Chakrabarti *et al.*, 2003; Souad *et al.*, 2007).

The SCD test is a simple, reliable procedure that does not require elaborate or expensive methodologies (Björndahl *et al.*, 2003; Fernández *et al.*, 2003; Muriel *et al.*, 2007; Qiu *et al.*, 2008; 2009). In our experiment, we used the SCD test to analyze whether sperm DNA was damaged by exposing it to the extract from the root of PTW and N-9. The mean percentage of sperm DNA fragmentation was not significantly different among all groups ($P>0.05$). This indicates that the sperm chromatin is not damaged by exposing it to the crude extract from the root of PTW and N-9 solution in a short period of time.

The advantages of Chinese medicinal herbs include ease of collection, its low cost, and practicality of application. The traditional Chinese medicine plays an indispensable role in the prevention and

treatment of the diseases. However, the partial pharmacologic actions of the Chinese medicinal herbs are still unclear or undiscovered. Therefore, further investigation is necessary and important to understand the unknown pharmacologic effects of Chinese medicinal herbs.

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