Regular Article

Protective Effect of Taohong Siwu Decoction on Abnormal Uterine Bleeding Induced by Incomplete Medical Abortion in Rats During Early Pregnancy

Yanyan Zhang, a,b,c Jichen Wang, a,c Chijing Zuo, a,c Weidong Chen, a,c Qian Zhu, d,e Dongdong Guo, a,c Huanru Wu, a,c Huizhuo Wang, a,c Daiyin Peng*, a,c and Lan Han*, a,c

Affiliation
aSchool of Pharmacy, Anhui University of Chinese Medicine, Hefei 230012, China: bDepartment of Pharmacy, Anhui Medical College, Hefei 230601, China: cAnhui Provincial Key Laboratory for Chinese Medicine Research and Development, Hefei 230012, China: dAnhui Key Laboratory of Bioactivity of Natural Products, Hefei, 230032, China and eSchool of Pharmacy, Institute for Liver Diseases of Anhui Medical University, Anhui Medical University, Hefei, 230032, China

*Correspondence and requests for materials should be addressed to Daiyin Peng (email: pengdaiyin@163.com) or to Lan Han (email: hanlan2007@126.com).
Summary

Abnormal uterine bleeding (AUB) induced by incomplete abortion is a common gynecological disease. Taohong Siwu decoction (TSD) is a traditional Chinese medicine (TCM) formula, which has been developed to treat AUB for hundreds of years. In this study, rats had incomplete abortion induced in early pregnancy using mifepristone and misoprostol. The duration and quantity of uterine bleeding were recorded and measured. The pathologic histologic grade was evaluated by hematoxylin-eosin staining (HE). Estradiol (E2) and progesterone (P) levels were measured by enzyme linked immunosorbent assays (ELISA). The expression levels of estrogen receptor alpha (ERα) and progesterone receptor (PR) were detected by immunohistochemistry and western blotting analysis. We demonstrated that TSD significantly reduced the duration and quantity of uterine bleeding. Meanwhile, TSD promoted endometrial repair and significantly up-regulated the E2 levels and the ERα expression. These results suggest that TSD have a protective effect on the uteri; the mechanism may be concerned with up-regulation of the levels of E2 and the ERα expression.

Keywords

abnormal uterine bleeding; incomplete medical abortion; estrogen; Taohong Siwu Decoction
Introduction

Medical abortion induced by mifepristone and misoprostol is a very common procedure in reproductive health. Medical abortion is considered to be less painful and more efficient and convenient method comparing with surgical abortion, which has been accepted by many women who want to termination of pregnancy. However, one of great side effects of medical abortion is incomplete abortion, which may cause abnormal uterine bleeding (AUB) at any time\textsuperscript{1-2}).

AUB, affecting up to one-third of women of child bearing age, will interfere with the physical, emotional, and social quality of life of women\textsuperscript{3-4}). Patients with clinically diagnosed AUB were treated by means of medicines or operations. Surgical treatments are suggested in the presence of medical therapies failure, which cause severe anemia, and other accompanying uterine pathology because of the influence on fertility. For medical options, the management of AUB can be divided into hormonal and non-hormonal therapies. Non-hormonal, including non-steroidal anti-inflammatory drugs (NSAIDs) and anti-fibrinolytics, hasn't achieved any effective treatment outcomes\textsuperscript{5-6}). The selection of hormonal therapy mainly depends on the patients' preferences. The combined oral contraceptive pills are widely used to regulate bleeding and reduce blood loss, although side effects may limit longer term use.

Traditional Chinese medicine (TCM) therapy has advantages and provides an alternative option in treating the AUB patients. Taohong Siwu decoction (TSD) as a TCM formula has been developed to treat AUB for hundreds of years, which was first recorded in a well-known medical book \textit{Yizong Jinjian (Golden Mirror of Medicine, 1749)} (Qing Dynasty of China) by Wu Qian and widely used for postpartum hemorrhage with a history of several centuries. It is
still used in treating AUB patients in clinics\textsuperscript{7}. However, the underlying therapeutic mechanisms of TSD on AUB induced by medical abortion are still unclear. Some researchers reported that the components of caffeine and caffeic acid in Dang Gui and Chuan Xiong can modify estrogen receptor\textsuperscript{8}. Many components of TSD could elevate estradiol (E\textsubscript{2}) and estrogen receptor alpha (ER\textalpha) levels\textsuperscript{9-10}. In this research, we explore if TSD is relevant to the hormone. The study will provide the evidence for the mechanism of TSD to treat AUB.

Materials and Methods

**Composition and Preparation of TSD** Six medicinal plants which purchased from The First Affiliated Hospital of Anhui University of Chinese Medicine were examined at least twice by Professor Dequn Wang in the School of Pharmacy, Anhui University of Chinese Medicine. According to the *Formulas of Chinese Medicine*, the ratio of six herbs were shown in Table 1\textsuperscript{11}.

<table>
<thead>
<tr>
<th>Components</th>
<th>Ratio</th>
</tr>
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<tbody>
<tr>
<td>Shu Di Huang (<em>Rehmannia glutinosa</em> Libosch.)</td>
<td>4</td>
</tr>
<tr>
<td>Dang Gui (<em>Angelica sinensis</em> (Oliv.) Diels)</td>
<td>3</td>
</tr>
<tr>
<td>Bai Shao (<em>Paeonia lactiflora</em> Pall.)</td>
<td>3</td>
</tr>
<tr>
<td>Chuan Xiong (<em>Ligusticum chuanxiong</em> Hort.)</td>
<td>2</td>
</tr>
<tr>
<td>Tao Ren (<em>Prunus persica</em> (L.) Batsch)</td>
<td>3</td>
</tr>
<tr>
<td>Hong Hua (<em>Carthamus tinctorius</em> L.)</td>
<td>2</td>
</tr>
</tbody>
</table>

TSD is prepared as follows: six herbs were mixed in proportion, and extracted twice in 10 and 8 times (v/w) of 75% ethanol for 2 h, respectively. As a result, 1000mL of extraction
solution was extracted from 2397g of crude Chinese herbs, so the final concentration of TSD was taken as 2.40g/mL. The main components (ferulic acid, hydroxysafflor yellow A, ligustilide, amygdalin) were used as quality control of TSD12-13).

**Animals** Sprague-Dawley (SD) rats, weighing 240 ± 20 g, were obtained from the Laboratory Animal Centre of Anhui Medical University [License No. SCXK (Jin) 2016-0006] and allowed free access to food and water. The protocol was performed in accordance with the Guidelines for Animal Experiments and approved by the Ethical Committee of Anhui Medical University.

**Incomplete medical aborting model and drug administration** An incomplete medical abortion model was established as previously described14-16). The clinical dosage of TSD was 0.80 g/Kg. According to the “Meeh-Rubner equation”, the middle dose of rats was 9 g/kg17). Pregnant rats were randomly divided into six groups, including the pregnant, medical aborting, TSD-treated with 4.5, 9, 18 g/kg (TSD 4.5, TSD 9, TSD 18) group and Leonurus Granule (Qianjin Pharmaceutical Co., Ltd, Zhuzhou, China) - treated with 4.5 g/kg (LG 4.5) group. There were eight rats in each group. In addition, another 8 female rats without mating were used as the non-pregnant group. Each rat in the medical aborting, TSD 4.5, TSD 9, TSD 18 and LG 4.5 group was induced into an incomplete medical aborting model, except for non-pregnant group and pregnant group. The second day of the model success, each rat was treated with drugs (1mL/100g) for seven days as follows: the rats in the non-pregnant, the pregnant, the medical aborting group were treated with distilled water; the rats in groups TSD 4.5, TSD 9, TSD 18 were treated with TSD (4.5, 9, 18 g/kg) and the rats in group LG 4.5 were treated with LG (4.5 g/kg).

**Determination of uterine bleeding quantity** From the day of modeling, a cotton ball of 85-90 mg was tucked into the vagina of each rat. Every 6 h later, it was replaced by a new cotton ball until no blood stain was observed. The cotton balls were collected and stored at

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-20°C for the measurement of the volume of uterine bleeding. Meanwhile, the time of uterine bleeding was recorded. After seven days' administration, the 0.02 mL blood sample was taken out from the tail vein of a female rat with the hemoglobin pipet, then added to the 4 mL NaOH solution (50 g/L, V₁) and mixed. The cotton balls of rats were placed in a small beaker, soaked and washed by an appropriate amount of NaOH solution according to the quantity of the bleeding. The washed solution was then preserved in an additional beaker. The process was repeated 1-2 times and the total volume (V₂) of consumptive NaOH solution was recorded. After mixing of the washed solution of each rat with every 6 h, a 5 mL sample was filtrated for the determination of the absorbance value (A) at 546nm. The equation of volume of uterine bleeding as follows:

\[
\text{Volume (mL)} = \frac{\text{tail vein blood (0.02 mL)} \times \text{A of filtered extract} \times V_2}{\text{the A of tail vein blood} \times V_1}
\]

**Measurement of the uterine index and hematoxylin-eosin staining** On the 7th day of administration, all rats were anesthetized with chloral hydrate and their uteri were removed and weighted. Uterine index of each rat was subsequently obtained by calculating the ratio of the uterine weight to the body weight. The dextro-uteri were fixed in 4% paraformaldehyde for 24-48 h at 4°C and then embedded in paraffin. Tissue sections were serially cut off with 4 μm, and mounted on glass slides, then restained with hematoxylin-eosin (HE). After sealing with neutral gum, the pathologic histologic grade was evaluated under a microscope. The criteria of each uterine pathologic histologic grade was as follows: grade I (score 0), no remains of decidua or conceptus; grade II (score 1), a small decidua tissue or residue of chorionic villi; grade III (score 3), a large number of residue of decidua and villi; grade IV (score 3), part of residual of embryo sac; grade V (score 4), the embryo sac was not discarded\(^{16}\).

**Measurement of estradiol and progesterone** Blood samples which were collected through postcava of rats after the last administration were placed at 4°C until completely

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solidified. After 3000 rpm centrifugal speed for 10 min, the serum were stored at -20°C. Enzyme linked immunosorvent assay (ELISA) was used to analyze the E2 (Shanghai Yuanye, Shanghai, China) and progesterone (P) (Shanghai Yuanye, Shanghai, China) levels according to the instructions.

Immunohistochemistry  Paraffin-embedded tissues sections were cut into slides, then blocked in 5% bovine serum albumin at 37°C for 30 min. Incubating with primary rabbit monoclonal anti-ERα antibodies (1:200, ab32063, Abcam, Shanghai, China) and a mice monoclonal anti-P receptor (PR) antibody (1:50, ab2765, Abcam, Shanghai, China) at 4°C overnight, the samples were incubated with corresponding biotinylated secondary antibodies for 30 min. Next, the samples were stained with diaminobenzidine for microscopic examination. Each slide was counterstained with hematoxylin. Images were obtained with an inverted microscope.

Western blot analysis  Soluble proteins were extracted from 100 mg uterine tissue lysed in RIPA buffer (50 mM Tris-HCl buffer PH 7.4; 5 mM EDTA; 150 mM NaCl; 1% NP-40; 0.25% sodium deoxycholate) with 1 mM PMSF (Beyotime, Shanghai, China) for protease inhibition and for western blotting and immunoprecipitation (Beyotime, Shanghai, China). After centrifuge to remove cell debris, the samples were boiled in 5× sodium dodecylsulfate (SDS) sample loading buffer for 10 min in order to make the protein fully denatured, then the samples were separated on a 10% SDS-polyacrylamide gel (Sigma-Aldrich, Zwijndrecht, The Netherland), and transferred to polyvinylidene difluoride membranes (millipore, Boston, USA). The membranes were blocked for 120 min at room temperature in phosphate buffer saline containing 0.05% Tween 20 (PBST) and 5% non-fat dried milk and probed with a rabbit monoclonal anti-ERα (1:1000, ab32063, Abcam, Shanghai, China), a mouse monoclonal anti-PR (1:1000, ab2765, Abcam, Shanghai, China), a mouse monoclonal anti-β-actin (1:1000, Beijing Zhongshan, Beijing, China), respectively. After incubating at 4°C overnight, the
membranes were incubated with the secondary antibody at room temperature for 120 min. The positive bands were detected by enhanced chemiluminescence analysis (Thermo, Massachusetts, USA). Densitometric analysis of the bands were calculated with ImageJ software\textsuperscript{19}.

**Statistical analysis** The results were presented as mean ± standard deviation (S.D.). Adopting student's t-test and analysis of variance (ANOVA) followed by the Student-Newman-Keuls test to compare the significance between two groups and among more than two groups, respectively. Statistical significance was accepted at a P<0.05.

**Results and Discussion**

**TSD reduced the time and volume of uterine bleeding** Incomplete abortion induced by mifepristone is a common side effect of medical abortion\textsuperscript{20}, which brings the results of the residual of necrotic decidual, change of uterine index and ultimately lead to AUB\textsuperscript{21}. The main features of AUB are a large amount of bleeding and a long bleeding time. In our research, the medical abortion reduced the uterine index (Fig. 1). Rats in the medical aborting group had a significant prolongation and aggravation of uterine bleeding (p<0.05). TSD and LG treatment dramatically reduced the duration of hemorrhage remarkably (Fig. 2) and the volume of uterine bleeding (Fig. 3) comparing with the medical aborting group (p<0.05). These results indicated that TSD and LG could significantly improved the symptoms of AUB. LG is the first line of treatment for AUB in clinics. LG mainly invigorates blood circulation to improve blood stasis syndrome in traditional Chinese medicine\textsuperscript{16}. Due to the similar pharmacological action of TSD, we choose LG as the positive control. The treatment of TSD can not only invigorate blood circulation but also nourish blood in clinics. Previous reports indicated that Semen Persicae and Flos Carthami invigorated blood circulation\textsuperscript{22}, plus Si Wu
Tang nourished blood\textsuperscript{23). So, the effectiveness of TSD has multiple pathways, target points and elements.

Fig. 1. Uterine index of each group. The unilateral uterus weight was measured as a wet weight and the uterine contents were not removed. The unit of weight was in g. Uterine index of each rat was subsequently obtained by calculating the ratio of the uterine weight to the body weight. The results were presented as the mean ± S.D., n=8. ▲\textit{P}<0.05 vs non-pregnant group, ^\textit{P}<0.05 vs pregnant group.

Fig. 2. Duration of uterine bleeding. The duration of uterine bleeding was recorded from the beginning of uterine bleeding after the administration of mifepristone to the cessation of bleeding completely. The unit of duration was measured in hours. The results were presented as the mean ± S.D., n=8. *\textit{P}<0.05 vs medical aborting group.
TSD promoted the endometrial repair

The implanting embryo triggers the decidual process during the pregnancy. A large number of trophoblast cells appear in the endometrium, which provide the nutrition to the embryo\(^{24-25}\). In the pregnant group, we found that uteri with 8-16 embryos were rosy with nodular enlargement, and the surrounding blood supply was abundant (Fig. 4a). From microscopic inspection, we found a large number of trophoblastic cells in pregnant group comparing with the non-pregnant group (Fig. 4c).

In medical aborting group, a dark red residue and blood stasis was observed, because hormone withdrawal triggered the influx of inflammatory cells into the endometrium and led to the degradation and necrosis of decidua and chorion\(^{26-27}\). However, there was minor surrounding hyperemia and ecchymosis in the TSD 9, TSD 18 and LG 4.5 group (Fig. 4a). In drug treatment groups, the number of necrotic decidual cells and neutrophilic granulocytes were less than medical aborting group (Fig. 4c).

Table 2 showed that the patho-scores of uterine tissues in each group. The lower the patho-score, the less the volume of blood and debris remaining in the uterus. The patho-score was low in the medical aborting group. Comparing with the medical aborting group, the
patho-score in TSD 9, TSD 18 and LG 4.5 treatment group had reduced significantly (P<0.05). TSD had a protective effect on the uterus of incomplete aborting model rats and promoted endometrial repair.

![Fig. 4. Macroscopic (a) and pathological (b,c) observation of uterus tissue. Samples were stained with HE as described in Methods. Black arrows indicate trophoblast cells, red arrows indicate infiltration of inflammatory cells, yellow arrows indicate necrotic decidual cells. (Color figure can be accessed in the online version.)](image)

Table 2. Effects of TSD on patho-grade of uterine tissues in rats (x ± S.D., n=8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (g/kg)</th>
<th>Patho-grade of uterine tissue</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Grade I</td>
<td>Grade II</td>
</tr>
<tr>
<td>non-pregnancy</td>
<td>—</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>pregnancy</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>medical abortion</td>
<td>—</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TSD 4.5</td>
<td>4.5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>TSD 9</td>
<td>9</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>TSD 18</td>
<td>18</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>LG 4.5</td>
<td>4.5</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

▲ P<0.05 vs non-pregnant group, ▲ P<0.05 vs pregnant group, * P<0.05 vs medical aborting group.
TSD up-regulated the E$_2$ levels and the ER$\alpha$ expression  Comparing with the pregnant group, the E$_2$ and P levels were reduced in the medical aborting group (P<0.05). Comparing with the medical aborting group, E$_2$ levels increased in TSD 18 and LG 4.5 group (P<0.05), however, P levels did not change significantly in the TSD and LG group (Fig. 5-6).

In the immunohistochemistry assay, the staining of ER$\alpha$ was predominantly localized in the nuclei of glandular epithelial cells, luminal epithelial cells and stromal cells in the non-pregnant group. When pregnancy, immunohistochemical staining of ER$\alpha$ was predominantly localized in the decidual tissue. However, the expression of ER$\alpha$ in luminal epithelial cells decreased in medical aborting group. Interestingly, the expression of ER$\alpha$ in luminal epithelial cells increased in TSD 9, TSD 18 and LG 4.5 group. For PR, the staining was predominantly localized in the stromal cells in the upper functional layer in the non-pregnant group. When pregnancy, the staining of PR was predominantly localized in the decidual cells. PR levels did not change significantly among the medical aborting, TSD and LG group (Fig. 7).

Western blotting demonstrated that the proteinic expression of ER$\alpha$ was decreased in medical aborting group (P<0.05), whereas the proteinic expression of ER$\alpha$ was increased in the TSD 9, TSD 18 and LG 4.5 group (P<0.05). There were no significant differences in the PR levels among the medical aborting, TSD and LG treatment group (Fig. 8). These results indicated that TSD had a protective effect on the uteri. The mechanism may be concerned with up-regulation of the E$_2$ levels and the ER$\alpha$ expression.

P levels increase in the process of pregnancy. However, medical abortion causes P levels down regulation on account of competitive antagonism by mifepristone$^{28}$. The levels of E$_2$
and ERα are closely related to the mechanisms of AUB. It was reported that estrogen withdrawal triggers the influx of inflammatory cells into the endometrium\cite{26} and tissue degradation\cite{27}, which lead to AUB. Up-regulation of E2 and ERα can reduce inflammatory response\cite{29,30} and promote endometrial repair\cite{31}. According to previous reports, the ingredients of TSD, for example, oleic and linolenic acid\cite{9,32}, catalpol\cite{33}, amygdalin\cite{34}, caffeic, chlorogenic and ferulic acid\cite{35-36}, can elevate the E2 levels. Likewise, the ingredients of TSD, such as Z-ligustilide\cite{10}, ferulic acid\cite{37}, tetramethylpyrazine hydrochloride\cite{38}, paeoniflorin\cite{38}, can elevate the ERα levels. So, we speculated that up-regulation of E2 and ERα levels is one of the main reasons of TSD on treating AUB.

In gynecology of traditional Chinese medicine, AUB leads to blood deficiency and blood stasis syndrome. TSD is a classical Chinese formula for treating gynecological diseases caused by blood stasis syndrome, such as AUB\cite{39}, dysmenorrhea\cite{40}. The effectiveness of TSD has multiple pathways, target points and elements. The study will provide the evidence for the mechanism of TSD to treat AUB. However, further researches about possible mechanisms are still required.

![Fig. 5. The E2 levels in each group. The results were presented as the mean ± S.D., n=8. ▲P<0.05 vs non-pregnant group, *P<0.05 vs pregnant group, *P<0.05 vs medical aborting group.](image)
Fig. 6. The P levels in each group. The results were presented as the mean ± S.D., n=8. ▲ P<0.05 vs non-pregnant group, # P<0.05 vs pregnant group.

Fig. 7. The immunohistochemical staining of ERα and PR in endometrium among different groups. A: non-pregnancy; B: pregnancy; C: medical abortion; D: TSD 4.5; E: TSD 9; F: TSD 18; G: LG 4.5. Scale bars, 50 μm.
Fig. 8. Western blotting of the expression of ERα and PR. The results were presented as the mean ± S.D., n=3. ▲P<0.05 vs non-pregnant group, *P<0.05 vs pregnant group, †P<0.05 vs medical aborting group.

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**Conflict of Interest:** The authors declare no conflict of interest.
References


