Mechanism of action of Tripterygium Wilfordii polyglycoside on experimental endometriosis

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Summary

Purpose of investigation: This study was designed to examine the therapeutic effectiveness and mechanism of action of Tripterygium Wilfordii polyglycoside (TWP) in the treatment of endometriosis.

Methods: An experimental endometriosis model was developed using New Zealand White rabbits where endometrial tissue was autotransplanted into the peritoneum. Six weeks after transplantation, a total of 22 rabbits were randomly placed into two groups: Group 1 (n=17) was treated with TWP (10 mg/kg/day) and Group 2 (n=5) served as the water-fed control for three successive months. The volume of endometrial implants was measured before and after administration of TWP and water. Immune and endocrine systems were investigated in the normal phase, six weeks after induction of endometriosis, and three months after TWP treatment and water administration.

Results: After treatment with TWP, the average volume of endometrial implants significantly decreased ($p < 0.0001$), and the antiendometrial antibody (EmAb) level decreased ($p < 0.05$) to near normal levels, but it did not decrease in the untreated controls. Serum FSH and LH levels also decreased after TWP treatment. Furthermore, electron microscopic examination of the pituitary ultrastructure revealed morphological changes in gonadotropic cells (G-cell) after treatment with TWP, and changes gradually disappeared four weeks after withdrawal of TWP.

Conclusion: This study indicates that TWP has both hormonal and immune system action that is effective as a medical treatment for experimental endometriosis by modulating both reproductive endocrine functions and immunosuppression that results in remission of the disease.

Key words: Tripterygium Wilfordii polyglycoside; Endometriosis; Medical treatment.

Introduction

Endometriosis is one of the most common and refractory diseases afflicting women of reproductive age. Its incidence is about 15-20%, and it is thought to be responsible for a large percentage (30-40%) of cases of infertility [1]. The pathogenesis of endometriosis is still not clear even though several theories have been proposed.

Recently, an increasing number of studies have supported the viewpoint that endometriosis has close links with abnormal immune function [2-4]. Startseva first reported that there were changes in the immune response of women with endometriosis and adenomyosis, which included increased B-cell and reduced T-cell activity as well as changes in immunoglobulins [5]. Additionally, there has been evidence that immune surveillance is altered in women with endometriosis, which may facilitate the implantation of endometrial cells that are shed by retrograde menstruation [2, 3, 6, 7].

With respect to therapy for endometriosis, various drugs have been used including danazol, progestins such as medroxyprogesterone acetate (MPA), and GnRH analogues, and even surgery has been employed to treat this disease. Although all of the agents cited are relatively effective with respect to endometriosis, the treatment course is usually limited and the results of treatment are far from satisfactory because of the high incidence of adverse reactions [8-11]. Surgical therapy such as total abdominal hysterectomy with bilateral salpingo-oophorectomy seems to be the best choice in terms of a radical cure for the disease. In reality, that choice is an unreasonable and unacceptable treatment for women of reproductive age, and especially for those who want to preserve their fertility.

In view of these factors, it is important and necessary to develop new and effective agents for the treatment of endometriosis that are able to modulate both reproductive endocrine functions and the immune system but that do not result in sterility.

Tripterygium Wilfordii polyglycoside (TWP) is an extract of Tripterygium Wilfordii, or what is commonly called Lei Gong Teng, which is a refined mixture with definite quality control criteria (provided by Taizhou Pharmaceutical Factory). TWP is a traditional Chinese medicine with a number of roles including antitumor, immunosuppressive, and antifertility activity [12]. In the last 30 years, it has been widely used in the treatment of a number of diseases with more or less promising results, including rheumatoid arthritis, chronic nephritis, chronic hepatitis, thrombocytopenic purpura, ankylosing spondylitis, and various skin diseases [12]. During the last ten years, the authors have used TWP for the treatment of endometriosis and have compared its effectiveness with

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danazol. Clinical study has shown that TWP obtains a response rate in endometriosis but with fewer adverse effects [13]. However, the mechanism of action of TWP and the mode of its effect on endometriosis is, as yet, unknown.

The purpose of the present study was to confirm the effectiveness of TWP on experimental endometriosis in rabbits and to elucidate its mechanism of action in order to determine if TWP is a useful and practical method of treatment for endometriosis as would be suitable for widespread clinical use in the future.

Materials and Methods

In vivo study

A total of 22 New Zealand White female rabbits weighing approximately 3 kg were used in the experiment. Experimental endometriosis was induced in the rabbits by autotransplantation as previously described [14]. Briefly, laparotomy was performed under sterile conditions using intravenous pentothal anesthesia followed by 10 mg/kg Ketamine administered intramuscularly as basal anesthesia. A 4-cm length of the right uterine horn was resected, and the uterine incision was closed with end-to-end anastomosis using 6-0 nylon suture. The resected uterine horn was incised longitudinally, and the endometrium was immediately dissected free from the myometrium. Then the endometrium was sectioned into five pieces, each measuring 5x5x2 mm^3. The five fragments of endometrial tissue were then sutured into the peritoneal cavity at the following sites: medial to the left and right ovary, at the uterine junction of the left and right fallopian tubes, and in the Douglas cul-de-sac. The remainder of the excised endometrium was stored at -80°C for endometrial antigenic preparation.

Six weeks later, a second laparotomy was performed to determine the viability of the endometrial implants. Results indicated that the endometrial implants in all cases had survived and also appeared as cystic masses filled with serous liquid. The volume (length, width and height) of the endometrial implants was measured and the average volume of each of the five implants was calculated. Moreover, histological examination of the endometrial implants showed evidence of endometriosis in the presence of both endometrial glands and stroma.

After the laparotomy, all rabbits with experimental endometriosis were randomly divided into two groups: an experimental group and a control group. The experimental group (n=17) was administered TWP (provided by Taizhou Pharmaceutical Factory) by gastric intubation at a dose of 10 mg/kg per day, six days a week for three consecutive months. The control group (n=5) was administered equal amounts of water by gastric intubation six days a week for three consecutive months.

Three months after treatment the animals were sacrificed using an intravenous overdose of pentobarbital. The volume of each endometrial tissue implant was measured again, and then the implants were excised and fixed in 10% neutral buffered formalin for histological evaluation after hematoxylin and eosin staining. The pituitary gland was also removed from each rabbit and quickly fixed in 2.5% glutaraldehyde for cytological ultrastructure examination by electron microscopy. Baseline blood samples were taken from each rabbit prior to the initial laparotomy, then at six weeks after transplantation but before administration of TWP or water, and then after three months of treatment before being sacrificed.

All blood samples were immediately centrifuged at 3000 rpm/min for 5 min; then, the supernatant was collected and stored at -20°C for subsequent assay of antiendometrial antibody titer and endocrine hormone determinations including FSH and LH.

In vitro study

Antiendometrial antibody assays

Endometrial tissue for antigenic preparation was obtained from the rabbits undergoing autotransplantation, and the tissue free of myometrium was stored at -80°C for the preparation of endometrial antigen. Endometrial glands were isolated according to the method described by Kennedy *et al.* [15]. Briefly, the endometrial tissue was mechanically disrupted with a tissue grinder at 4000 rpm and then treated with 0.25% collagenase (Type I, 132 U/mg; Sigma, St. Louis, MO, USA) by incubation in a shaking water bath at 37°C for two hours. The collagenase-treated endometrium was first strained through a 250-μm sieve to remove any of the undigested tissue and debris; then, the filtrate was passed through a 40-μm sieve. The endometrial glands retained by the 40-μm sieve were washed three times with phosphate-buffered saline (PBS) to remove the collagenase. Then, the glands were resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) to inhibit any tissue serine proteinases before being homogenized in a glass homogenizer. The homogenate was purified by antisera-Sepharose 4B to remove impurities of different charges.

Enzyme-linked immunosorbent assays for antiendometrial antibody were performed according to a method previously reported [15]. The plastic microtiter plates were coated (100 μl/well) with endometrial antigen (EmAg) which was diluted with PBS (pH 9.6) to a protein concentration of 1:100. The plates were incubated overnight at 4°C and then washed three times with Solution A (PBS containing 0.2% Tween-20 and 0.2% bovine serum albumin). Nonspecific protein binding was blocked by incubation for one hour at 45°C with 200-μl/well PBS (pH 7.4) containing 15% newborn bovine serum before washing three times with solution A. Each serum sample from the three periods (100 μl/well) diluted 1:2 in solution B (PBS containing 0.2% Tween-20 and 10% heat-inactivated newborn bovine serum) was added to coated wells in triplicate and then incubated at 45°C for one hour and washed three times with solution A. Then, 100 μl of horseradish peroxidase-conjugated staphylococci protein A (HRP-SPA), diluted 1:2000 in solution B, was added to each well before incubation at 45°C for one hour and washing three times with solution A. The plates were developed with 200-μl/well OPD-H_2O_2 (O-phenylenediamine, hydrogen peroxide in buffer solution), incubated at 37°C for 15 min. The reaction was stopped after 10 min with 100-μl/well 0.01% sodium azide, and absorption at 492 nm was measured with a spectrophotometer. All samples were run in triplicate.

Hormone measurements

Serum concentrations of FSH and LH were determined by a radio immunoassay (RIA) method as described [16]. Serum hormone concentrations were determined prior to autotransplantation, six weeks after autotransplantation but before treatment, and three months after treatment.

Pituitary ultrastructure morphology

Electron microscopic examination of pituitary ultrastructure was performed on the glands three months after treatment.

Statistical methods

Statistical analyses were performed using Statworks statistical software on a Macintosh computer. Tests included the Student's t-test of means and the Wilcoxon rank sum test; p-values below 0.05 were considered significant.

Results

Measurement of the endometrial implants

The average volume of the five endometrial implants of each rabbit during, before and after administration of TWP and water is summarized in Figure 1. After induction of endometriosis, the volume of the implants in the experimental group and the control group were 2212.82 ± 214.26 mm^3 and 2280.76 ± 350.33 mm^3 (mean ± Standard Error mm^3), respectively. After treatment with TWP, the volume decreased significantly to 1571.82 ± 189.36 mm^3 ($p < 0.001$, Figure 1A), while in the control group, the volume was 2628.82 ± 435.39 mm^3, which was still significantly larger than before being fed with water ($p < 0.05$, Figure 1B).

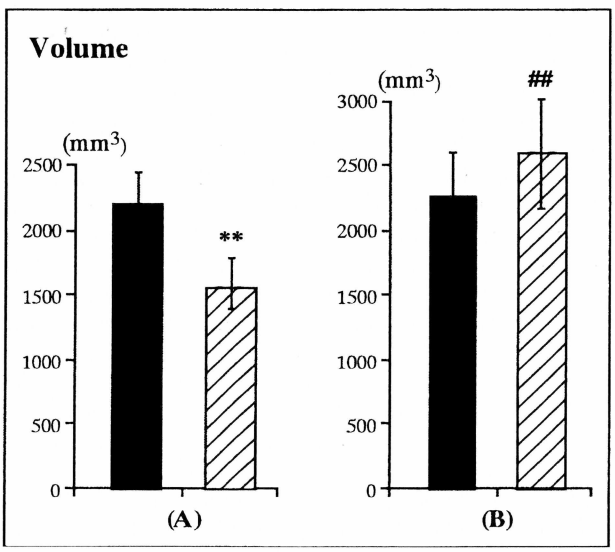

Figure 1. — Comparison of the average volume of the endometrial implants before ■ and after ▨ administration of TWP and water for (A) the experimental group (n=17) and (B) the control group (n=5). Data represents the mean ± SE. **p < 0.001, ##p < 0.05.

Hormone concentrations

The mean basal concentrations of FSH in the experimental group and in the control group were 15.18 ± 3.50 ng/ml and 16.70 ± 3.85 ng/ml (mean ± SE ng/ml), respectively, and three weeks after induction of endometriosis they were almost unchanged (16.97 ± 4.96 ng/ml and 18.20 ± 4.54 ng/ml, respectively) (Figures 2A and B). Three months after treatment with TWP, the concentrations of FSH (10.16 ± 3.19 ng/ml) were significantly lower than the basal concentrations and the concentrations before treatment ($p < 0.05$, Figure 2 A). However, the concentrations of FSH in the control group after three months displayed no difference in comparison to basal concentrations and concentrations before treatment (Figure 2 B). This finding indicates that TWP was effective in suppressing the production of FSH.

LH data for each group and time period are shown in Figures 3A and B. After the induction of endometriosis, the LH level in both the experimental group and control group was essentially unchanged from basal LH levels (4.53 ± 0.99 ng/ml and 4.00 ± 0.94 ng/ml, 4.18 ± 1.01 ng/ml and 5.98 ± 0.88 ng/ml, respectively) (mean ± SE ng/ml) (Figures 3A and B). After treatment with TWP, the concentration of LH in the experimental group was significantly lower than both the basal LH level and the level three months after feeding with water (2.02 ± 1.23 ng/ml, $p < 0.05$, Figure 3A). In contrast, the LH level in the control group after endometriosis was present for three months without treatment and also remained unchanged when compared to basal LH levels and those before treatment (5.10 ± 0.85 ng/ml, Figure 3B). The data indicates that TWP was effective in suppressing the production of LH.

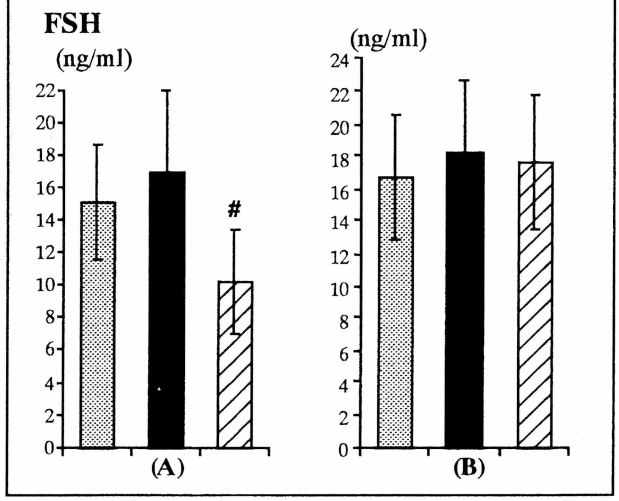

Figure 2. — Comparison of FSH levels during the three phases of the experiment: normal pre-transplant phase ■, after induction of endometriosis but prior to TWP treatment or water administration ■, and after 3 months of endometriosis ▨. (A) the experiemental group (n=17), (B) the control group (n=5). Data represents the mean ± SE. #p < 0.05.

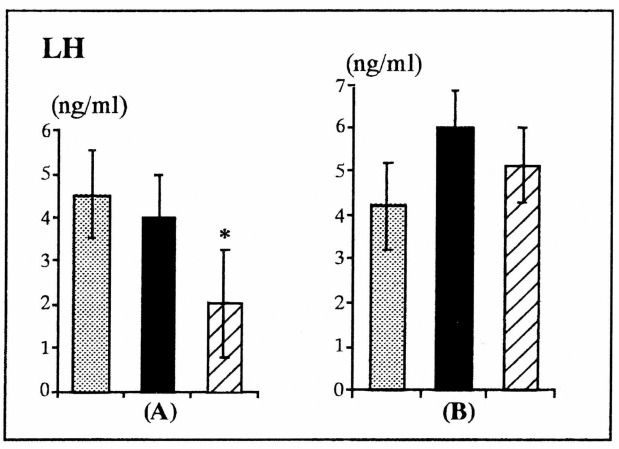

Figure 3. — Comparison of LH levels during the three phases of the experiment: normal pre-transplant phase ■, after induction of endometriosis but prior to TWP treatment or water administration ■, and after three months of endometriosis ▨. (A) the experiemental group (n=17), (B) the control group (n=5). Data represents the mean ± SE. *p < 0.05.

Antiendometrial antibody

Serum antiendometrial antibody titers are shown in Figures 4A and B, represented as the mean absorbance ± SE. After the induction of endometriosis, the endometrial antibody levels of both the experimental group and the control group were significantly higher than before the operation (0.27 ± 0.04 vs 0.15 ± 0.01, $p < 0.001$ and 0.25 ± 0.04 vs 0.14 ± 0.01, $p < 0.05$, respectively) (Figures 4 A and B). After treatment with TWP, however, the antibody titer decreased significantly to pretreatment levels (0.14 ± 0.01 vs 0.27 ± 0.04, $p < 0.001$) (Figure 4A), while in the untreated control group there was no significant difference before and after being fed with water (0.24 ± 0.02 vs 0.25 ± 0.04) (Figure 4B).

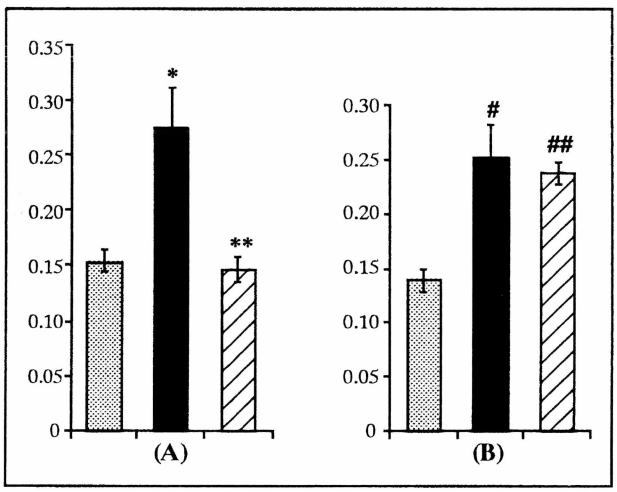

Figure 4. — Comparison of the endometrial antibody levels during the three phases: normal pre-transplant phase ▒, after induction of endometriosis but prior to TWP treatment or water administration ■, and after three months of endometriosis ▨. (A) the experiemental group (n=17), (B) the control group (n=5). Data represents the mean absorbance ± SE. *$p < 0.01$, *vs** $p < 0.05$, #$p < 0.0001$, #vs## $p > 0.05$.

Electron microscopic ultrastructure of the pituitary

Electron microscopic examination of pituitary G-cell ultrastructure three months after treatment with TWP showed that there was increased vacuolization in the cytoplasm, nuclear shrinkage, mitochondrial swelling, and the number of rough endoplasmic reticula had increased (Figure 5).

Discussion

Endometriosis is known not only to be a hormone-dependent disease but also a disease associated with some changes in the immune system [17, 18]. Although it is still not known whether these immunological changes are the cause or the result of endometriosis, it is better for a medical therapy to have two therapeutic effects, namely, an effect on the endocrine functions and on the immune system as well.

In a prior clinical study [13], the therapeutic effect and the adverse effects of TWP and danazol were compared in the treatment of endometriosis. That study showed that the therapeutic-response rate for TWP was almost the same as that for danazol (87.5% vs 84.6%), while the adverse effects of danazol, such as liver dysfunction, were significantly more prevalent than with TWP.

In the present study, the volume of the endometrial implant decreased significantly after treatment with TWP, while that in the control group was still significantly larger than before treatment (Figure 1). Moreover, the high serum antiendometrial antibody titers decreased to normal levels after treatment with TWP, whereas in the control group the antibody levels were unchanged (Figure 4). Since immunoglobulins such as the antiendometrial antibody are known to be produced by B-cells, this finding clearly demonstrated that TWP can inhibit humoral immunity.

The morphologic ultrastructure of the pituitary gland provides some insight about the site of action of TWP because, theoretically, changes in pituitary ultrastructure influence its function. The G-cell of the pituitary is a

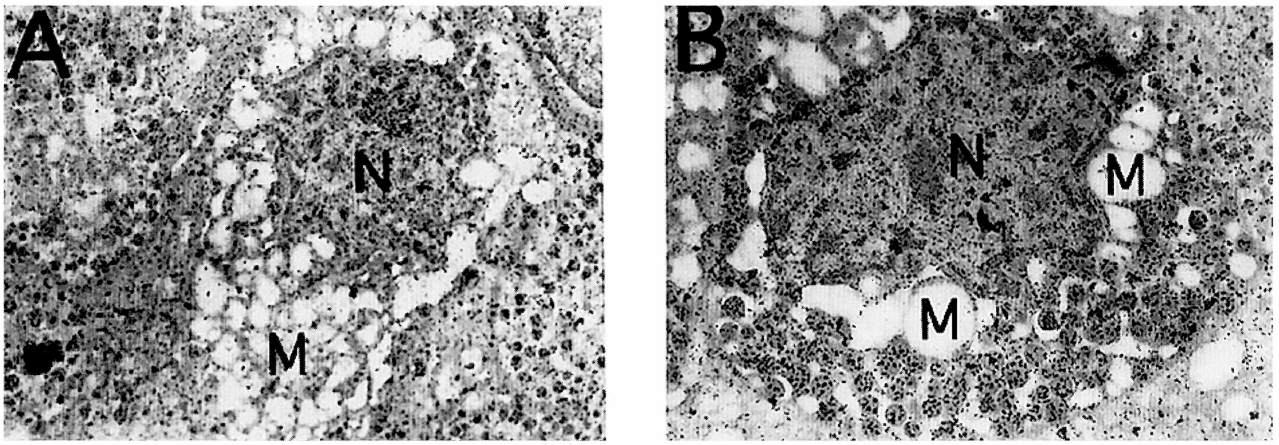

Figure 5. — Appearance of the pituitary ultrastructure 3 months after treatment with TWP (A and B): the cytoplasm of G-cells shows an increase in vacuolization, nuclear shrinkage and mitochondrial swelling. M = mitochondria; N = nucleus.

gonadotrophin-producing cell that secretes FSH and LH. Using electron microscopy of the pituitary glands from the TWP-treated group, changes were observed in the ultrastructure of the G-cells, including nuclear shrinkage, mitochondrial expansion, and vacuolization in the cytoplasm. The serum concentrations of FSH and LH decreased significantly three months after administration of TWP, while FSH and LH were almost unchanged three months later in the water-fed control group when compared with pretreatment levels (Figure 2 and Figure 3). These results indicate that TWP has the ability to inhibit the secretion of FSH and LH by altering G-cell ultrastructure and function in the pituitary. As a result of TWP treatment, ectopic endometrial tissue atrophied and the disease regressed; moreover, treatment was accompanied by minimal adverse effects.

These experimental results seem to clarify the mechanism of action of TWP on the experimental endometriosis by both hormonal and immunosuppressive action. To the authors' knowledge, this is the first experimental study using TWP for the treatment of endometriosis and the first study designed to clarify the mechanism of action of TWP in females.

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