Effects of the Extract of a Chinese Herb

Tripterygium wilfordii Hook f on Rat Pituitary Gland

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Abstract: In China, the ethylacetate extract of the herb Tripterygium wilfordii Hook f (TWEE), containing the major active ingredient triptolide, is often used with favorable effect on rheumatoid arthritis patients, in alternation with the use of prednisone. The mechanism of this therapeutic effect, however, has not been completely delineated. In this study, we studied how TWEE and prednisone affect the pituitary and adrenal glands in rats. Thirty normal male Sprague-Dawley rats (ten per group) were randomly assigned to receive: (1) TWEE (25 mg/kg, twice a day), (2) prednisone (2 mg/kg, twice a day), or (3) vehicle (control) (0.5% sodium carboxymethyl cellulose 1 ml, twice a day), orally for 30 days. Pituitary and trunk blood were collected on day 31. Adrenocorticotropic hormone (ACTH) expression in the pituitary gland was assessed morphologically by immunohistochemical techniques. Plasma ACTH concentrations and serum corticosterone concentrations were quantitatively measured by radioimmunoassay. We found that TWEE significantly increased plasma ACTH concentration and serum corticosterone concentration and dramatically increased the number of ACTH-positive cells in the pituitary. Our findings indicate that TWEE can promote the synthesis and secretion of ACTH cells — in the pars distalis of the rat pituitary gland and the production of corticosterone in the zone fasciculata of the adrenal cortex. Our results indicate that TWEE has a cortical hormone-like function and can promote adrenal cortex function by activating the hypothalamus-pituitary-adrenal axis.

Keywords: Ethylacetate Extract of Tripterygium wilfordii Hook f; Prednisone; Pituitary; ACTH; Corticosterone; Immunohistochemistry; Radioimmunoassay.

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Introduction

The ethylacetate extract of the Chinese herb *Tripterygium wilfordii* Hook f (TW), a vine-like plant common in a wide area of south China, has been reported to be effective in the treatment of several autoimmune diseases, including rheumatoid arthritis, and to have potent anti-leukemia properties and antitumor activity (Tao *et al.*, 2002; Ho and Lai, 2004; Shu *et al.*, 1981; Qin *et al.*, 1982; Li and Wu, 1991; Yang *et al.*, 2003; Kupchan and Schubert, 1974). Experimental and clinical studies conducted over the last 20 years have shown TW affects the neuroendocrine system, especially the hypothalamus-pituitary-adrenal axis (HPAA) (Chen *et al.*, 1999; Chen and Huang, 2001; Hu and Lin, 1997; Zhang *et al.*, 1994; Li and Wei, 1989; Li *et al.*, 1983; Zhou *et al.*, 1983). However, the research has mainly focused on the adrenal glands and not the pituitary gland.

The ethylacetate extract of *Tripterygium wilfordii* Hook f (TWEE) contains triptolide, the major active ingredient of TW. Our aim was to observe how TWEE affects the morphology and function of the pituitary gland and the function of the adrenal gland in Sprague-Dawley rats.

Materials and Methods

Animals and Treatments

Thirty male Sprague-Dawley rats (seven to eight weeks old) were purchased from the Department of Experimental Animals of Shanghai Medical University (Shanghai, China). Rats were housed in wire-bottomed, stainless steel cages (five rats per cage) at 23 ± 2°C and a relative humidity of 40 ± 8%, with a 12-hour light/dark cycle (lights on at 7:00am). All animals were fed a standard rat diet and water *ad libitum*. After 3 days of acclimatization, the rats were randomly assigned to three groups. The TWEE group received TWEE (dry powder containing 0.1% triptolide, suspended in 0.5% sodium carboxymethyl cellulose), 25 mg/kg; the prednisone group received prednisone suspended in 0.5% sodium carboxymethyl cellulose, 2 mg/kg; and the control group received 1 ml of 0.5% sodium carboxymethyl cellulose. All treatments were administered orally twice a day for 30 days. Ten rats were used per group. To adjust the quantity of the medicine, all rats were weighed every 3 days. After 30 days, the rats were weighed, and then killed under general anesthesia with an intraperitoneal injection of sodium pentobarbital solution (45 mg/kg) on the 31st day between 9:30 and 11:30am. To control for body weight differences, the calculation of relative pituitary weight = (pituitary weight/body weight) × 100% was used. Pituitary and trunk blood were collected immediately and handled according to the requirements for each relevant examination as indicated below. Adrenocorticotropic hormone (ACTH) in plasma and corticosterone in serum were measured quantitatively by radioimmunoassay (RIA). The pituitary glands were observed following hematoxylin and eosin (H&E) staining and immunohistochemistry staining with ACTH antibody.
ACTH and Corticosterone Radioimmunoassay Analysis

Trunk blood was collected in cool EDTA plastic tubes (coated with 14% sodium EDTA) immersed in an ice bath at 2–8°C during collection. The plasma was separated from the cells by centrifugation (3000g, 4°C, 20 minutes). The samples were stored at −30°C. Before assay, samples were thawed in an ice bath and kept continually at or below 4°C. Plasma ACTH was estimated by using a double-antibody 125I RIA kit (Diagnostic Products Corporation, Los Angeles, CA). Serum corticosterone levels were determined by RIA (Coat-A-Count, Diagnostic Products Corporation). The Coat-A-Count rat corticosterone procedure is a solid-phase RIA in which rat 125I-labeled corticosterone competes for a fixed time with corticosterone in the sample for antibody sites. The antibody is coated on the wall of a polypropylene tube; simply decanting the supernatant terminates the competition and isolates the antibody-bound fraction of the radiolabeled corticosterone. Counting the tube in a gamma counter then yields a number that converts by way of a calibration curve to a measure of the corticosterone present in the sample.

Immunohistochemical Analysis

The pituitaries were removed, weighed, and fixed in fresh Bouin’s fluid for 6 hours and then embedded in paraffin. Serial sections (5 μm) were cut from the paraffin blocks and mounted on APES-coated slides. ACTH expression was evaluated by using the SABC Kit according to the kit instructions (Strept Avidin-Biotin Complex, a sensitive indirect immunohistochemistry technique, Boster, Wuhan, China). Briefly, sections were deparaffinized in xylene and hydrated through graded ethanol to deionized water. Endogenous peroxidase activity was blocked by 5 minutes incubation in 3% hydrogen peroxide-methanol buffer. Non-specific binding was blocked by incubation for 20 minutes with a dilution of 1:50 normal goat serum. Primary antibody (rabbit anti-human 18-39ACTH antibody, Sigma) was diluted to 1:200 with 0.01M PBS and incubated over the sections in a humidified chamber for 2 hours at 37°C. The slides were washed three times for 3 minutes with 0.01M PBS and then incubated with a biotinylated goat anti-rabbit antibody and SABC reagent (Boster). The reaction product was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (Sigma). Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Negative controls were analyzed using normal goat serum, omitting the primary antibody.

Image Analysis Technology and Statistical Analysis

Image analysis of the pituitary sections was performed by ACTH immunohistochemical staining using the TJTY-400 multimedia color cell image analysis system (Wuhan, China). All results were expressed as group arithmetic means with their standard errors. The data were put into a computer database, and statistical analysis was performed using analysis of variance followed by Student’s t-test (p < 0.05 criteria).
Results

Effects of TWEE and Prednisone on Rat Body Weight, Pituitary Weight, and the Relative Pituitary Weight

The effects of TWEE and prednisone on body weight, pituitary weight, and the relative pituitary weight are shown in Table 1. Values for the relative pituitary weight were significantly greater for the TWEE group than for the prednisone group (p < 0.05) or controls (p < 0.05). In addition, the pituitary weight was significantly greater in the TWEE group than in the prednisone group (p < 0.05), with no difference between the TWEE and control groups. Final body weights did not differ among the three rat groups. These results indicate that rat pituitary weight was significantly greater in the TWEE group after eliminating the effect of body weight.

Table 1. Comparison of Rat Body Weight, Pituitary Weight, and Organ Coefficient Among the Three Groups (Mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Pituitary Weight (mg)</th>
<th>Relative Pituitary Weight* (%) × 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-experiment</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>213.56 ± 23.29</td>
<td>298.67 ± 35.02</td>
<td>9.02 ± 1.70</td>
</tr>
<tr>
<td>Prednisone</td>
<td>213.90 ± 21.79</td>
<td>290.20 ± 29.13</td>
<td>8.87 ± 1.27</td>
</tr>
<tr>
<td>TWEE</td>
<td>213.60 ± 16.29</td>
<td>276.50 ± 47.86</td>
<td>9.89 ± 2.68†</td>
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</table>

Male Sprague-Dawley rats (seven to eight weeks old, ten rats/group) were given vehicle (control), TWEE (containing 0.1% triptolide, 25 mg/kg, twice a day), or prednisone (2 mg/kg, twice a day) orally. All rats were killed on day 31. Statistical analysis was performed using analysis of variance followed by Student’s t-test (p < 0.05 criteria). *Relative pituitary weight = (pituitary weight/body weight) × 100%. †Significantly different from the control group, p < 0.05. ‡Significantly different from the prednisone group, p < 0.05.

Table 2. Comparison of ACTH and Corticosterone Concentrations Among the Three Groups (Mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma ACTH (pg/ml)</th>
<th>Serum Corticosterone (ng/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>50.92 ± 10.94</td>
<td>112.79 ± 19.27</td>
</tr>
<tr>
<td>Prednisone</td>
<td>32.53 ± 8.49†</td>
<td>77.06 ± 14.43†</td>
</tr>
<tr>
<td>TWEE</td>
<td>74.75 ± 12.00‡</td>
<td>142.56 ± 31.34‡</td>
</tr>
</tbody>
</table>

Male Sprague-Dawley rats (seven to eight weeks old, ten rats per group) were given vehicle (control), TWEE (containing 0.1% triptolide, 25 mg/kg, twice a day), or prednisone (2 mg/kg, twice a day) orally. All rats were killed on day 31. Statistical analysis was performed using analysis of variance followed by Student’s t-test (p < 0.05 criteria). †Significantly different from the control group, p < 0.05. ‡Significantly different from the prednisone group, p < 0.05.
Quantitative Measurement of Plasma ACTH and Serum Corticosterone with the Double-Antibody $^{125}$I Radioimmunoassay

ACTH and corticosterone concentrations in the blood increased in the TWEE group and decreased in the prednisone group compared with the controls (Table 2). Analysis of variance indicated that significant differences of plasma ACTH and serum corticosterone were respectively found among three groups (all p < 0.05). Our results indicated that TWEE stimulates ACTH secretion in the rat pituitary and corticosterone secretion in the zone fasciculate of the adrenal cortex, whereas prednisone suppresses ACTH and corticosterone production.

Morphological Changes in ACTH Cells in the Pars Distalis of the Rat Pituitary Gland

Under the light microscope, the pars distalis, pars intermedia, and posterior lobe of the pituitary gland were clearly shown with H&E staining. There were no notable differences among the three groups (data not shown), except for a slight dilation in the capillary vessels and venous sinusoids in the pars distalis (PD) and posterior lobe (LP) of the pituitary gland in the TWEE group (Fig. 1), which was not seen in the control and prednisone groups.

Figure 1. Light microscopic view of the pars distalis (PD), pars intermedia (PI), and posterior lobe (LP) of the rat pituitary gland in the TWEE group. There was a slight dilatation of the capillary vessels and venous sinusoids in the PD and LP of the pituitary gland in the TWEE group but not in the control or prednisone group (H&E staining, 200x).
With the SABC immunohistochemical method, the primary antibody produced evident staining of ACTH in the pars distalis of the rat pituitary gland (Fig. 2). With the use of hematoxylin as counterstaining, all nuclei in the rat pituitary slide were stained blue. The distribution of ACTH positive-staining cells was very interesting. In the control group, many scattered ACTH positive-staining cells were found in the outer two-third regions of the pars distalis of rat pituitary, with no ACTH positive-staining cells in the inner one-third region. Under high-magnification light microscopy, ACTH cells displayed various shapes, such as an irregular astral shape (protrusive of cytoplasm), round, oval shape, and half-moon shape. ACTH positive staining occurred mainly at the cell membrane, with only a little staining in the cytoplasm (Fig. 2A). In the prednisone group, scattered ACTH positive-staining cells were also distributed only in the outer two-third regions of the pars distalis of the pituitary, but the number of the ACTH positive-staining cells was less than in the control group. Moreover, the staining of ACTH cells was very weak, with most staining located at the cell membrane, and a few cells stained in the cytoplasm (Fig. 2B). In the TWEE group, the number of ACTH positive-staining cells in the pars distalis was much greater than in the control or prednisone group. Under high magnification, the ACTH positive staining was dark. Not only did the cell membrane exhibit ACTH positive staining, but there were many dark ACTH staining granules in the cytoplasm as well. In the TWEE group, positive staining occurred in all three regions of the pars distalis of the pituitary. Thus, ACTH positive-staining cells were found in the inner one-third region in the TWEE group. However, in the control and prednisone groups, no ACTH positive-staining cells were found in the inner one-third region (Fig. 2C).

TJTY-400 Multimedia Color Cell Image Analysis System and Statistical Analysis

The intensity of the ACTH positive-staining cells following immunostaining was separately measured as the accumulated absorbency and the staining areas of 160 cells using the TJTY-400 multimedia color cell image analysis system. Darker staining of the ACTH-positive cells indicates higher accumulated absorbency values. The accumulated absorbency was the highest in the TWEE group, intermediate in the control group, and lowest in the prednisone group (all p < 0.05) (Table 3), indicating that the pituitary ACTH cells contain plentiful ACTH in the TWEE group and less in the prednisone group. In addition, the average staining areas of ACTH cells in the control group and the TWEE group were higher than that of the prednisone group (p < 0.05). These results further indicated that TWEE increases ACTH secretion and prednisone decreases ACTH secretion in the pars distalis of the rat pituitary.

Discussion

TW and prednisone are widely used in China to treat many autoimmune diseases, including rheumatoid arthritis (Li and Wei, 1989; Tao and Lipsky, 2000). Although the
Figure 2. Primary antibody (rabbit anti-ACTH) produced evident staining in ACTH cells in the pars distalis (PD) of the rat pituitary gland with the SABC immunohistochemical method. (A) In the control group, many scattered ACTH positive-staining cells were found only in the outer two-third regions of the PD, not in the inner one-third region. ACTH cells displayed various shapes. ACTH-positive staining was located mainly at the cell membrane, with a little in the cytoplasm. (B) In the prednisone group, scattered ACTH positive-staining cells were also located in the outer two-third regions of the PD. Staining of ACTH cells was weak with most staining occurring in the cell membrane. (C) In the TWEE group, the number of ACTH positive-staining cells in the PD was clearly increased. Positive-staining ACTH cells were found in all three regions of the PD. Such positive staining was very dark. In addition to the cell membrane having ACTH positive staining, there were many dark ACTH-staining granules in the cytoplasm. Black arrows (→) indicate the boundary between the PD and PI. Empty arrows (←) indicate representative ACTH positive-staining cells (left, 100x; right, 400x).
long-term use of prednisone alone can effectively control such diseases, there exist side effects, including serious feedback suppression of the hypothalamic-pituitary-adrenal axis. Alternating treatment between prednisone and TW can prevent or reduce side effects in patients who have used prednisone for a long period with beneficial effect. Owing to its toxicity, however, TW has caused cases of poisoning by accidental ingestion or overdosage (Huang, 1982). Liu and Hu (1996) emphasized that alternating administration of TW with prednisone is the best regimen for reducing the side effects of each and ensuring continued long-term treatment. Both TW and prednisone have anti-inflammatory and immunosuppressive effects, but they have obvious differences in their anti-inflammation intensity, pharmacological mechanisms and side effects.

Animal experiments have indicated that TW can promote corticosterone secretion and fascicular zone cell proliferation in the adrenal cortex (Chen et al., 1999; Hu and Liu, 1997; Zhang et al., 1994; Li and Wei, 1989; Li et al., 1983). However, the effect of TW on the morphology and function of the rat pituitary has not been reported before.

The average weight of the pituitary gland in normal male rats has been reported previously as 8.4 mg by Hebel and Stromberg (1986) and as 8.55 ± 1.1 mg by Cai et al. (1994). In the current study, the average was 9.02 ± 1.70 mg. Although we found no statistically significant difference (p = 0.054) in pituitary weight between the TWEE and control groups, there was a significant increase in the relative pituitary weight in the TWEE group compared with the control group. Use of the relative pituitary weight (100% × pituitary weight/body weight) eliminates the effect of body weight differences among different rats and is more meaningful than merely measuring the pituitary weight alone. We can therefore conclude that TWEE significantly increased relative pituitary gland weight.

Various cell types of the pars distalis can be distinguished using immunohistochemical staining with different antibodies (Ciocca et al., 1985). In previous studies, ACTH-positive cells were scattered near the blood sinusoid in the anterior pituitary (Pang et al., 1995), and the proportion of ACTH-positive cells in the pituitary gland was approximately 15%–20% (Kissane and Anderson, 1990). ACTH-positive cells appeared in many shapes. The

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**Table 3. Comparison of Average Accumulated Absorbency and Staining Area in the Pars Distalis of Rat Pituitary (Mean ± SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Accumulated Absorbency</th>
<th>Staining Area</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>17.21 ± 5.85</td>
<td>80.07 ± 32.99</td>
</tr>
<tr>
<td>Prednisone</td>
<td>5.97 ± 3.60</td>
<td>44.21 ± 12.95</td>
</tr>
<tr>
<td>TWEE</td>
<td>29.23 ± 14.50</td>
<td>84.04 ± 26.78</td>
</tr>
</tbody>
</table>

Male Sprague-Dawley rats (seven to eight weeks old, ten rats per group) were given vehicle (control), TWEE (containing 0.1% triptolide, 25 mg/kg, twice a day), or prednisone (2 mg/kg, twice a day) orally. All rats were killed on day 31. Statistical analysis was performed using analysis of variance followed by Student’s t-test (p < 0.05 criteria). *Significantly different from the control group, p < 0.05. †Significantly different from the prednisone group, p < 0.05.
staining granules of ACTH were located mainly in the inner side of the cell membrane (Su et al., 1995). In our study, the ACTH-positive cells were seen only in the outer two-third regions of the anterior pituitary in the control and prednisone groups. In the TWEE group, however, many dark ACTH positive granules in the cytoplasm of the cells of the inner one-third region as well as the outer two-third regions were observed. We therefore assume that TWEE promotes ACTH synthesis and secretion of ACTH in the pars distalis of rat pituitary at least in morphology. Also, it appears as if the distalis of rat pituitary has a potential storage of ACTH, which can be stimulated to synthesize and secrete ACTH if needed.

ACTH, a polypeptide hormone containing 39 amino acids, is synthesized and stored in specific basophils of the anterior pituitary. The first 24 amino acids are common to all species, whereas amino acids 25 to 33 are species-specific (Rosa et al., 1980). Secretion of ACTH from the anterior pituitary is under the control of corticotropin-releasing factor (CRF) from the hypothalamus as well as a negative feedback mechanism dependent on the circulating concentration of cortisol (in humans) or corticosterone (in rats) (Jornot et al., 1985). Plasma concentrations of ACTH exhibit a significant diurnal variation. The plasma concentration of ACTH in normal male Sprague-Dawley rats has been reported as 59.70 ± 6.94 pg/ml by Cai et al. (1994) and as 50.92 ± 10.94 pg/ml by Hu and Liu (1997). Our result was 50.88 ± 7.34 pg/ml. From the data shown in Table 2, we conclude that TWEE can stimulate cells to secrete ACTH in the pars distalis of the rat pituitary and that prednisone has a negative feedback suppression on the pituitary.

Corticosterone is produced in the zone fasciculata of the adrenal cortex. The normal serum corticosterone concentration in Sprague-Dawley rats has been previously given as 107 ± 44.1 ng/ml (plasma) by Cai et al. (1994) and as 190.8 ± 6.8 ng/ml (serum) by Gwosdow-Cohen et al. (1982). Our result was 112.79 ± 19.27 ng/ml (serum). In the current study, the serum corticosterone concentration was significantly greater in the TWEE group than in the prednisone or control groups. From the data shown in Table 2, we conclude that TWEE can stimulate the rat adrenal cortex to secrete corticosterone.

In summary, our study provides experimental data showing that TWEE promotes ACTH secretion in the pituitary gland and stimulates corticosterone secretion in the zone fasciculata of the adrenal cortex in Sprague-Dawley rats indicating that TWEE has a cortical hormone-like function and can promote adrenal cortex function by activating the HPAA. This information will be very useful for a comprehensive appraisal of the effect of TWEE on the HPAA and for the guidance of the reasonable and efficient clinical use of TW.

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References


