CHINESE HERBAL MEDICINE YIN-CHEN-EXTRACT AS AN ADJUNCT TO ANTELMINTIC ALBENDAZOLE USED AGAINST ANGIOSTRONGYLUS CANTONENSIS-INDUCED EOSINOPHILIC MENINGITIS OR MENINGOENCEPHALITIS

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Abstract. The effects of albendazole and Yin-Chen-Extract co-therapy on Angiostrongylus cantonensis-induced eosinophilic meningitis in BALB/c mice were evaluated. Assay indicators for the therapeutic effect include worm recovery, histopathological score of the fourth ventricle, tissue-type plasminogen activator, urokinase-type plasminogen activator, matrix metalloproteinase-9, cerebrospinal fluid total protein, leukocyte counts, and proinflammatory cytokines. As a result, albendazole and Yin-Chen-Extract co-therapy significantly decreased ($P < 0.05$) these factors. Although Yin-Chen-Extract may possess pharmacologic activities beneficial to the inhibition of parasite-induced inflammation, many of these hypothetical benefits await scientific testing and confirmation before application. This study suggests that the combination of Yin-Chen-Extract and albendazole has synergistic effects in managing eosinophilic meningitis or eosinophilic meningoencephalitis.

INTRODUCTION

Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are serine proteinases that play an important role in the pathogenesis of eosinophilic meningitis caused by Angiostrongylus cantonensis. The proinflammatory cytokine tumor necrosis factor (TNF) is a potent activator of neutrophils, mediating adherence, chemotaxis, and degranulation; it is responsible for the severe cachexia that occurs in chronic infections. Matrix metalloproteinase-9 (MMP-9) gene expression by macrophages and polymorphonuclear leukocytes, and hence the release of pro-MMP, can be induced by TNF-$\alpha$ and interleukin (IL)-1$\beta$. Albendazole is an effective compound for the management of angiostrongyliasis through its larvicidal activity and facilitation of an improved inflammatory response via the reduction of MMP-9 activity, but dead worms in the brain may evoke a severe immune response resulting in brain damage. Recently, albendazole has frequently been used in combination with steroids to prevent the inflammatory reaction due to dead larva. However, the use of steroid therapy is still controversial. To replace steroids, this study considers Chinese herbal medicines, which have been used for thousands of years and are now being manufactured in many countries as quality-controlled drugs with standardized quantities of ingredients. Yin-Chen-Extract (Artemisia capillaries Thunb. extract) has been widely used as an anti-inflammatory agent, especially for diseases of the liver. This extract is different from anti-malarial artemisinin. Artemisinin is a drug isolated from extracts of leaves of A. annua and has been used in traditional Chinese medicine for the management of fevers for more than 2000 years.

The present study estimates the efficacy of albendazole and Yin-Chen-Extract co-therapy in a mouse model of parasitic meningitis caused by A. cantonensis infection, and assays the therapeutic indicators of worm recovery, histopathological score of fourth ventricle, tPA, uPA, MMP-9, total protein, and leukocyte counts in parasite-induced eosinophilic meningitis.

MATERIALS AND METHODS

Experimental animals. Five-week-old male BALB/c mice (weighing 20–25 g), were purchased from the National Laboratory Animal Center, Taipei, Taiwan. They were maintained at a 12-hour light/dark cycle photoperiod, provided with Purina Laboratory Chow and water ad libitum. All of the procedures involving animals and their care in this study were approved by the Institutional Animal Care and Use Committee of Chung-Shan Medical University in accordance with institutional guidelines for animal experiments.

Larval preparation. The infective larvae ($L_3$) of A. cantonensis originally obtained from wild giant African snails (Achatina fulica) that were propagated for several months in our laboratory by cycling through rats and snails (Biomphalaria glabrata). The larvae within tissues were recovered using a modification of the method of Parsons and Grieve. Briefly, the shells were crushed, the tissues were homogenized and digested in a pepsin-HCl solution ($\text{pH}$ 1-2, 500 I.U. pepsin/g tissue), and incubated with agitation in a 37°C waterbath for 2 hours. Host cellular debris was removed from the digest by centrifugation at 1,400 g for 10 minutes. The larvae in the sediment were collected by serial washing in double-distilled water and counted under a microscope.

Drug. Albendazole was purchased from Glaxo Smith Kline. Yin-Chen-Extract (Artemisia capillaries Thunb. extract) was purchased from Guanghan Bencao Plant Chemical Co., Ltd (Sichuan, China).

Treatment of animals. Experiment 1. To evaluate the effect of the timing of albendazole and Yin-Chen-Extract co-therapy on worm recovery, infected mice were given albendazole (10 mg/kg/d) and Yin-Chen-Extract (100 mg/kg/d) for 7 consecutive days on days 2, 4, 6, 8, 10, 12, and 14 post-inoculation (PI), respectively. Untreated infected mice served as controls. Each group consisted of 6 infected mice. The mice were killed on day 22 PI, and the brains were removed for worm recovery examination.

Experiment 2. This experiment was conducted to determine the effect of albendazole alone, Yin-Chen-Extract alone, or albendazole and Yin-Chen-Extract co-therapy on worm recovery, histopathological score of the fourth ventricle, tPA, uPA, MMP-9, cerebrospinal fluid total protein,
leukocyte counts, and proinflammatory cytokines on day 10 PI. A total of 100 mice were randomly divided into 5 groups; food and water was withheld for 12 hours before infection. The uninfected control mice were inoculated with distilled water by oral inoculation on day 10 PI; the infected-untreated control mice were infected with 50 larvae and treated as previously indicated; the albendazole treated mice were treated with albendazole (10 mg/kg/d) alone for 7 consecutive days starting on day 10 PI; the Yin-Chen-Extract treated mice were treated with Yin-Chen-Extract (100 mg/kg/d) alone for 7 consecutive days starting on day 10 PI; the albendazole and Yin-Chen-Extract co-therapy mice received a treatment combining albendazole (10 mg/kg/d) and Yin-Chen-Extract (100 mg/kg/d) for 7 consecutive days starting on day 10 PI. All groups were killed on day 22 PI; the brains and CSF-like fluid were collected for histopathological study and biochemical analysis.

**Collection of CSF-like fluid.** The mice were killed by cervical dislocation and their brains removed into a 35-mm dish. The cranial cavity and cerebral ventricles (lateral, third, and fourth ventricles) were rinsed with 1 mL 0.15 M phosphate buffered saline (PBS) and CSF was thus harvested with PBS, the washing solution being so-called “CSF-like fluid”.

**Worm recovery.** Experimental and control mice were killed by cervical dislocation on day 22 PI. Each brain was torn into small pieces and homogenized separately in 15 mL of 0.25% sodium citrate in PBS followed by centrifugation. Larval counts were done under 25 × magnification using a dissecting microscope. The mean number of larvae was compared with the positive control (infected-untreated) group to assess the drug efficacy.

**Histopathological examinations.** The mouse brains were fixed separately in 10% neutral buffered formalin for 24 hours. The fixed specimens were dehydrated in a graded ethanol series (50%, 75%, and 100%) and xylene, and then embedded in paraffin at 55°C for 24 hours. Serial sections were cut at 5-μm thickness for each brain from each mouse. Paraffin was removed by heating the sections for 5 minutes at 65°C. These sections were dewaxed by washing 3 times for 5 minutes each in xylene; and then rehydrated through 100%, 95%, and 75% ethanol for 5 minutes each, and finally rinsed with distilled water. After staining with hematoxylin (Muto, Tokyo, Japan) and eosin (Muto, Tokyo, Japan), pathologic changes were examined under a light microscope.

**Gelatin and casein substrate zymography.** Activity of MMP-9 was determined by gelatin zymography, tPA and uPA activity by caseinzymography. Briefly, the CSF-like fluid was centrifugated at 12,000 g at 4°C for 10 minutes, and the protein contents of the supernatants were determined with protein assay kits (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Protein concentration was determined by absorbencies at 595 nm using a HITACHI U1100 spectrophotometer (Tokyo, Japan).

**Leukocyte count.** The cranial cavity and cerebral ventricles (lateral, third, and fourth ventricles) were rinsed with 1 mL PBS. The CSF-like fluid was centrifuged at 400 g for 10 minutes. The resultant sediments from each mouse were then resuspended with 30 μL PBS to enumerate the total number of leukocytes. The cell count was assessed with Wright-Giemsa staining (Sigma, Taufkirchen, Germany) in 3 μL/ smear.

**MMP-9, tPA, or uPA had migrated and cleaved their respective substrates.** Quantitative analysis of the gelatinolytic and caseinolytic enzyme was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT™ gel Version 5.1 (Silk Scientific, Orem, UT, USA).

**Measurement of total protein.** The CSF-like fluid was centrifuged at 12,000 g at 4°C for 10 minutes, and the protein contents of the supernatants were determined with protein assay kits (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Protein concentration was determined by absorbencies at 595 nm using a HITACHI U1100 spectrophotometer (Tokyo, Japan).

**Effect of albendazole-Yin-Chen-Extract co-therapy on Angiostrongylus cantonensis larvae in BALB/c mice treated for 7 days at different starting medication.**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Days post-inoculation</th>
<th>Number of worms recovered</th>
<th>Worm reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>33.2 ± 3.5</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>1.2 ± 1.0</td>
<td>96.4</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>2.4 ± 1.5</td>
<td>92.8</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>4.6 ± 1.4</td>
<td>86.2</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>6.5 ± 1.3</td>
<td>80.5</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>12.6 ± 1.9</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Each group consists of 6 mice infected with 50 infective larvae and sacrificed on day 22 post-inoculation. The albendazole-Yin-Chen-Extract co-therapy mice received a treatment combining albendazole (10 mg/kg/day) and Yin-Chen-Extract (100 mg/kg/day) for 7 consecutive days. The infected-untreated control mice were infected with 50 infective larvae and inoculated with distilled water by oral inoculation.
Reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The brain homogenates were centrifuged at 12,000 g for 10 minutes to remove supernatants. Total RNA was isolated using Trizol reagent (Invitrogen, Carslbad, CA, USA) from the brains, according to the manufacturer’s instructions. Five μg of total RNA was used for first strand cDNA synthesis in 20 μL of reaction volume using 50 units of Superscript™ II reverse transcriptase (Invitrogen, Carslbad, CA, USA). PCR was performed under standard conditions using Taq DNA polymerase (Invitrogen, Carslbad, CA, USA) and primers. The forward (5’-3’) and reverse (5’-3’) primers, respectively, were 5’- CCC CTT CAT TGA CCT CAA CTA CAT GG-3’ and 5’-GAC ATC ACA GAA AGC ATG GTG AAG CAG GC-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-GCA TCC AGC TAC GAA TCT CCG ACC-3’ and 5’-CAC TTG TTG CTC CAT ATC CGT TCC C-3’ for IL-1β, 5’-ATG AGC ACA GAA AGC AGC ATG ACG CAT -3’ and 5’-TAC AGG CTT GTC ACT CGA ATT -3’ for TNF-α. PCR cycling conditions for GAPDH, IL-1β, and TNF-α were denaturation at 94°C for 45 seconds, annealing at 55°C for 1 minute, primer extension at 72°C for 2 minutes, and then holding at 4°C; this was repeated for 30
cycles for TNF-α and IL-1β; 25 cycles for GAPDH. Ten microliters of the amplified product was then subjected to electrophoresis in 3% agarose gels containing 20 μg/ml ethidium bromide in Tris borate-EDTA buffer. Gels were visualized on a UV transilluminator (Taipei, Taiwan), and digital images were taken using DGIS-5 Digital Gel Image System (Taipei, Taiwan). Quantitative analysis was performed with a computer-assisted imaging densitometer system, UN-SCAN-IT™ gel Version 5.1 (Silk Scientific, Orem, UT, USA).

**Statistical analysis.** Results from the different groups of mice were compared using the nonparametric Kruskal-Wallis test, followed by post-testing using Dunn’s multiple comparison of means. All results are presented as mean ± SD (S.D.), and P values of < 0.05 were considered statistically significant.

### RESULTS

**Influence of treatment on larvae recovery.** The timing of albendazole and Yin-Chen-Extract co-therapy is critical for successful treatment of *A. cantonensis*-infected mice. The highest reductions (100%) were obtained when mice were treated on days 2 or 4 PI. This was followed by treatment on days 6 or 8 PI (96.4–92.8%). Reductions (86.2–80.5%) were observed when mice were medicated on days 10 or 12 PI. Only a slight reduction (62.1%) was observed when mice were medicated on day 14 PI (Table 1). There were fewer recovered larvae in the treated population, compared with the infected-untreated population. Recovered larvae were virtually absent in the brain tissue of mice treated with albendazole alone or albendazole and Yin-Chen-Extract co-therapy. In contrast, no significant difference (P < 0.05) in worm recovery was observed between nontreated group and the group treated by Yin-Chen-Extract alone. The degree of brain inflammation coincided with the larva numbers of stay in CNS. The highest level of worm reduction was observed after co-therapy with albendazole and Yin-Chen-Extract (Figure 1).

**Histologic observations.** Optical microscopic examination of tissues stained using haematoxylin and eosin revealed that inflammation of the fourth ventricle was induced in BALB/c mice by *A. cantonensis* infection. Invasion of the fourth ventricle by larvae was evident 22 days after the inoculation of the larvae. Larvae were significantly decreased in number by co-therapy with albendazole and Yin-Chen-Extract, moderately reduced after albendazole treatment, but no reduction after Yin-Chen-Extract treatment alone was observed (Figure 2).

**Influence of treatment on the activities of tPA and uPA.** The tPA migrating electrophoretically as a 70 kDa species was significantly increased in CSF-like fluid of BALB/c mice infused with *A. cantonensis*, compared with control. The lytic area of the 70 kDa tPA band was significantly reduced by albendazole (ABZ) or albendazole-Yin-Chen-Extract (ABZ+YCE) co-therapy. There was no significant reduction by Yin-Chen-Extract alone. Similarly, the molecular weight of 55 kDa uPA was significantly reduced by albendazole alone or albendazole-Yin-Chen-Extract co-therapy, whereas no significant inhibition was observed by Yin-Chen-Extract alone. (B) Quantitative analysis of the PA enzymes were performed with a computer-assisted imaging densitometer system (*P < 0.05*).

**Influence of treatment on total protein.** The total protein was significantly increased in the CSF-like fluid of BALB/c mice infected with *A. cantonensis*, compared with control. The lytic area of the 70 kDa band was mildly reduced (P < 0.05) by Yin-Chen-Extract alone. MMP-9 was significantly reduced (P < 0.05) by albendazole alone or albendazole-Yin-Chen-Extract co-therapy. Co-therapy with albendazole-Yin-Chen-Extract was associated with a stronger reduction in MMP-9 activity than anthelminthic albendazole alone (Figure 4).

**Influence of treatment on total protein.** The total protein was significantly increased in the CSF-like fluid of BALB/c mice infected with *A. cantonensis*, compared with control. The concentrations were significantly decreased (P < 0.05) by albendazole or by albendazole-Yin-Chen-Extract co-therapy compared with infected-untreated mice, although there was not a significant decrease by Yin-Chen-Extract treatment alone (Figure 5).
Influence of treatment on eosinophil counts. The eosinophils were significantly increased in CSF-like fluid of BALB/c mice infected with *Angiostrongylus cantonensis*, as compared with control. Eosinophils were significantly reduced (*P* < 0.05) by albendazole alone or albendazole-Yin-Chen-Extract (ABZ+YCE) co-therapy, and there was no significant difference (*P* < 0.05) by Yin-Chen-Extract (YCE) alone. Co-therapy with albendazole-Yin-Chen-Extract was more effective than albendazole alone in reducing eosinophilic inflammation (Figure 6).

Influence of treatment on the mRNA expression of TNF-α and IL-1β. Mice infected with *A. cantonensis* exhibited a significant increase in IL-1β and TNF-α mRNA levels as compared with uninfected mice. Albendazole or albendazole-Yin-Chen-Extract co-therapy significantly lowered (*P* < 0.05) the mRNA levels of TNF-α and IL-1β compared with *A. cantonensis*-infected mice. Additionally, the mRNA levels of TNF-α were also significantly lowered (*P* < 0.05) by Yin-Chen-Extract alone (Figure 7).

**DISCUSSION**

One of the most important goals in medicine is to continue searching for newer, more effective treatments. Even in parasitic treatment, better and more effective treatment is replacing the older forms of treatment. Traditional medicine has played its role in the past. Advocates of Chinese herbal medicine generally regard this approach as both safe and effective, and there are many patients who report dramatic benefits to their health from treatment. The present study has actually supported the herbalists' observation of a reduction of inflammation. The number of *A. cantonensis* larvae was significantly decreased when mice were treated with albendazole and Yin-Chen-Extract co-therapy. The highest reductions (100%) of timing were obtained when mice were treated on days 2 or 4 PI. However, the clinical symptoms were pre-Figure 5. Influence of treatment on total protein. The total protein of CSF-like fluid was significantly increased in mice infected with *Angiostrongylus cantonensis*, as compared with uninfected control. The concentrations of total protein were significantly less in mice treated by albendazole (ABZ) alone or albendazole-Yin-Chen-Extract (ABZ+YCE) co-therapy mice when compared with concentrations found in untreated infected mice. There was no significant change by Yin-Chen-Extract (YCE) alone (*P* < 0.05).

Figure 6. Influence of treatment on eosinophil counts. The eosinophils were significantly increased in CSF-like fluid of mice infected with *Angiostrongylus cantonensis*, as compared with uninfected control. Eosinophils were significantly decreased by albendazole or albendazole-Yin-Chen-Extract (ABZ+YCE) co-therapy, whereas there was no significant difference between infected-untreated group and Yin-Chen-Extract (YCE) alone.
sent 10 days after infection with A. cantonensis. Thus, in the present study, we have found that therapeutic indicators such as worm recovery, tPA, uPA, MMP-9, total protein, and leukocyte counts occur on day 10 PI in mice treated with albendazole and Yin-Chen-Extract co-therapy. Additionally, albendazole and Yin-Chen-Extract co-therapy almost completely inhibited PAs activity and inflammation. Only partial inhibition of PAs and inflammation were produced by Yin-Chen-Extract alone. It can be concluded from these findings that PAs inhibition may promote the process of healing from eosinophilic meningitis and therefore, PAs may make useful diagnostic tools to evaluate healing from meningitis.

In the present study, total protein in CSF-like fluid was significantly decreased when mice were treated with albendazole-Yin-Chen-Extract co-therapy. These results suggest that CSF protein contents might play an important indicator in eosinophilic meningitis. Thus, treatment groups may improve the damage of blood-CSF barrier by reducing activity of PAs and increasing the flow rate of CSF.

The pro-inflammatory cytokines TNF-α, and IL-1β can be induced during A. cantonensis infection, and it is clear that basal levels of TNF-α are essential for normal growth and development. Co-therapy with albendazole and Yin-Chen-Extract significantly decreased the mRNA levels of TNF-α and IL-1β, yet the mRNA levels were not completely inhibited. The possibility of co-therapy with albendazole and Yin-Chen-Extract targeting cytokines (TNF-α and IL-1β) may offer an effective management of eosinophilic meningitis or meningooencephalitis.

According to in vitro correlates of therapeutic efficacy plus Yin-Chen-Extract is more effective than anthelminthic albendazole alone. Although Yin-Chen-Extract may possess pharmacologic activities that could be beneficial to the inhibition of parasite-induced inflammation in general many of these hypothetical benefits await scientific testing and confirmation before application. The findings of this study suggest that the combination of Yin-Chen-Extract and albendazole may have a synergistic effect in embattling eosinophilic meningitis. A clinical trial is warranted to assess the utility of Yin-Chin extract as an adjunctive tool in treating A. cantonensis-induced eosinophilic meningitis.

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