CHINESE HERBAL MEDICINE YIN-CHEN-EXTRACT AS AN ADJUNCT TO ANTHELMINTIC 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 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-α and interleukin (IL)-1β.

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 on Angiostrongylus 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.

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-α and interleukin (IL)-1β.

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.
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 x 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 then 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,10 and tPA and uPA activity by casein zymography.1 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.

**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).

**Gelatin and casein substrate zymography.** Activity of 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).

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**Gelatin and casein substrate zymography.** Activity of MMP-9 was determined by gelatin zymography,10 and tPA and uPA activity by casein zymography.1 Briefly, the CSF-like fluid was centrifugated at 12,000 g for 10 minutes to remove debris. The protein contents of the supernatants were analyzed on SDS-PAGE gels copolymerized with 0.1% gelatin (Sigma, St. Louis, MO, USA) for gelatinase activities, 0.1% casein (Sigma, St. Louis, MO, USA) for plasmin activities, and plasminogen (13 μg/mL, Sigma, St. Louis, MO, USA) for PAs activities. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature at 120 V for 1 hour. The gel was washed twice at room temperature for 30 minutes each in 2.5% Triton X-100, and then washed twice with double-distilled water for 10 minutes each. The gel was incubated in reaction buffer at 37°C for 18 hours. The gel was then stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA) for 1 hour and destained in 15% methanol/7.5% acetic acid. The final gel had a uniform background except in regions to which 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).

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Reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The brain homogenates were centrifugated at 12,000 g for 10 minutes to remove supernatants. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, 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, Carlsbad, CA, USA). PCR was performed under standard conditions using *Tag* DNA polymerase (Invitrogen, Carlsbad, 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 AAG AAG GTG 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 CTG TCC C-3’ for IL-1β, 5’-ATG AGC ACA GAA ATG 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.
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 com-
puter-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 com-
parison 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 al-
bendazole 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 inocula-
tion 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
infected with A. cantonensis, compared with control. The lytic
area of the 70 kDa band was significantly reduced (P < 0.05)
by albendazole or albendazole-Yin-Chen-Extract co-therapy;
there was no significantly reduced by Yin-Chen alone. Simi-
larly, the lytic area of the region of 55kDa uPA was signifi-
cantly reduced (P < 0.05) by albendazole and Yin-Chen-
Extract co-therapy or albendazole treatment alone, whereas
no significant inhibition was observed by Yin-Chen-Extract
alone (Figure 3).

Influence of treatment on the activity of MMP-9. This en-
zyme migrates electrophoretically as a 94 kDa species. This
metalloproteinase activity was significantly increased (P < 0.05)
in CSF-like fluid of BALB/c mice infected with A. cantonensis,
compared with control. The lytic area of the MMP-9 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 anthelmin-
thic 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, as compared with the
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. The 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 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-
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Angiostrongylus cantonensis

significant decreased when mice were treated with alben-
dazole-Yin-Chen-Extract co-therapy. These results sug-
ggest that CSF protein contents might play an important indi-
cator in eosinophilic meningitis. Thus, treatment groups may
improve the damage of blood-CSF barrier by reducing activ-
ity 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 inhib-
ted. The possibility of co-therapy with albendazole and Yin-
Chen-Extract targeting cytokines (TNF-α and IL-1β) may of-fer an effective management of eosinophilic meningitis or me-
ningoencephalitis.

According to in vitro correlates of therapeutic efficacy plus
Yin-Chen-Extract is more effective than anthelminthic al-
bendazole alone. Although Yin-Chen-Extract may possess
pharmacologic activities that could be beneficial to the inhi-
bition of parasite-induced inflammation in general many of
these hypothetical benefits await scientific testing and confir-
mation 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 men-
ingitis. A clinical trial is warranted to assess the utility of
Yin-Chin extract as an adjunctive tool in treating A. canton-
ensis-induced eosinophilic meningitis.

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FIGURE 7. Influence of treatment on the mRNA levels of TNF-α
and IL-1β. (A) Angiostrongylus cantonensis-infected mice lead to a
significant increase in TNF-α and IL-1β compared with uninfected
control. Treatment by albendazole (ABZ) or albendazole-Yin-Chen-
Extract (ABZ+YCE) co-therapy significantly lowered the mRNA
levels of TNF-α and IL-1β compared with A. cantonensis-infected
mice. In addition, treatment by Yin-Chen-Extract alone significantly
lowered the TNF-α mRNA level, whereas no significantly change on
IL-1β. GAPDH was used as a loading control. (B) Densitometric
scanning quantification was expressed as the ratio of the signal in-
tensity of TNF-α and IL1β mRNA to that of GAPDH at each group,
respectively (*P < 0.05).

sented 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 leu-
kocyte counts occur on day 10 PI in mice treated with alben-
dazole and Yin-Chen-Extract co-therapy. Additionally,
albendazole and Yin-Chen-Extract co-therapy almost com-
pletely 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

THE PRO-INFLAMMATORY CYTOKINES TNF-α, AND IL-1β CAN BE
INDUCED DURING A. CANTONENSI


