



Evaluation of potential anti-toxoplasmosis efficiency of combined traditional herbs in a mouse model^{*}

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Abstract: *Toxoplasma gondii* is a worldwide spread protozoan and is able to infect almost all warm-blood animals. No effective drugs are available clinically on toxoplasmosis. Chinese traditional herbal medicines have provided remedies for many health problems. There exists a possibility that Chinese herbs may provide protection against *T. gondii*. This work aims to assess the protective efficacy of combined Chinese herbs against *T. gondii*. We screened five herbal medicines that have different pharmacological effects and combined them into a prescription according to the traditional Chinese medicine compatibility principle. The drug potential and protective efficacy were evaluated through a mouse model by determining the survival time, the parasite load in blood and tissues, the change of cell proportions in blood and histological detection. The results showed that the survival time of mice in the 500 mg Chinese herbs group and sulfadiazine group was significantly longer than that of the PBS control group. Also the parasite load in blood and tissues of 500 mg Chinese herbs and sulfadiazine groups was significantly lower than that of PBS group at 7 days post infection (dpi), which was in accordance with the result of histological detection. Monocyte and neutrophil of infected mice were remarkably increased while lymphocyte was dramatically decreased compared to that of blank group at 7 dpi. The results demonstrated that the 500 mg dosage of our Chinese herbs could slow down the replication of *T. gondii* and prolong the survival time of mice and could be considered as possible candidate drug against toxoplasmosis.

Key words: *Toxoplasma gondii*; Chinese herbs; Real-time PCR; Survival; Protection
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1 Introduction

Toxoplasmosis caused by the intracellular protozoan *Toxoplasma gondii* is a ubiquitous worldwide parasitic zoonotic disease (Innes, 2010a). It is usually asymptomatic in immune-competent individuals but may occasionally lead to severe ocular and neurological disorders (Furtado *et al.*, 2013). When it comes to immune-compromised and congenitally

infected individuals, toxoplasmosis can result in lethal systemic disease and eventually death (Shen *et al.*, 2016). Currently, the control of *T. gondii* mainly depends on chemotherapy, such as the combination of sulfadiazine and pyrimethamine, but these drugs have serious side effects (Petersen and Schmidt, 2003). Even though many antigens including rhoptry, surface, and dense granule excreted-secreted antigens were evaluated as potential vaccines (Kur *et al.*, 2009), there is no licensed vaccine against *T. gondii* infection clinically making the situation even worse (Jongert *et al.*, 2009; Innes, 2010b). Therefore, new efficient drugs and safe protective therapies are needed.

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Artemisinin isolated from a Chinese traditional medicine *Artemisia annua* is now included in standard treatment worldwide for *Plasmodium falciparum* malaria. Subsequent to that, interest in Chinese traditional medicine has risen to be considered for providing therapeutic reagents for many infectious diseases. The *Radix clematidis* extract is reported to be a potent inhibitor of matrix metalloproteinase-9 (MMP-9) and notably blocks the nuclear factor- κ B (NF- κ B) pathway in breast carcinoma cells (Noh et al., 2011). Na-Bangchang and Karbwang (2014) found that a Chinese traditional medicine *Atractylodes* possesses various pharmacological effects with anti-cancer, anti-inflammatory and antimicrobial activity, and is now a potential chemotherapeutic for cholangiocarcinoma found in Southeast Asia. *Artemisia anomala* has been widely and in long-term used for the treatment of inflammatory diseases in China. Recent findings suggested that it manifests its anti-inflammatory effects through inhibiting the expression of inducible nitric oxide synthase (iNOS) (Tan et al., 2014). Glycyrrhizin, extracted from *Liquorice*, was reported to have antiviral activity through inhibiting replication of the pathogen (Cinatl et al., 2003).

Throughout history, Chinese traditional herbal medicines have provided many remedies for many health problems. The use of most herbal medicines dates back almost 2000 years in *Shennong's Materia Medica*. However, limited information is available on their pharmacological activity since the use of herbal medicine has been based mainly on empirical treatment. As a consequence, there exists a possibility that many herbal medicines may have unknown effects on different health problems. In this study, we screened five herbal medicines that have different pharmacological effects and combined them into a prescription according to the traditional Chinese medicine compatibility principle. We evaluated the protective efficacy provided by the different dosages of this prescription against the challenge of RH strain of *T. gondii* in a mouse model.

2 Materials and methods

2.1 Animals

Female ICR mice (6–8 weeks old) were kept in an animal experimentation laboratory under standard

conditions according to the guidelines of the Regulation for the Administration of Affairs concerning Experimental Animals of the People's Republic of China. These experiments on mice were approved by Zhejiang University Experimental Animal Ethics Committee (No. ZJU201308-1-10-072).

2.2 Parasites

Tachyzoites of *T. gondii* RH strain were maintained in ICR mice by intraperitoneal (i.p.) serial passage at regular 72 h intervals. The parasites were washed three times by phosphate-buffered saline (PBS) and centrifuged at 1000g for 5 min as previously described (Mack and McLeod, 1984).

2.3 Drugs

Five kinds of Chinese herbal medicines were purchased from Huqingyu Tang Pharmaceutical Co., Ltd., Hangzhou, China. According to the traditional Chinese medicine compatibility principle, they were combined as follows: *R. clematidis* (10 g), *Fructus Ulmi Macrocarpae* (8 g), *Atractylodes* (15 g), *A. anomala* (10 g), and *Glycyrrhizae* (6 g). On the basis of the traditional Chinese medicine decoction method, herbal drugs were boiled for 3 h in water (1 L) followed by concentrating through a rotary evaporator into 2 g/ml of the whole drugs. Sulfadiazine as a positive control drug was purchased from Novartis (Beijing, China).

2.4 Effect of drugs on *T. gondii* infection in vivo

A total of 140 female mice were randomly divided into seven groups (A to G). Mice in groups B–G were i.p. infected with 1×10^3 tachyzoites of *T. gondii* RH strain. Different medical administrations were carried out after infection with *T. gondii* RH strain as follows: Group A, blank control; Group B, PBS control; Group C, mice were treated with 10 mg/d of sulfadiazine through oral gavage; mice in Groups D–G were treated with Chinese herbs through oral gavage in single dosages of 300, 400, 500, and 600 mg/d, respectively, according to the Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2010).

The animals were observed daily for mortality. A total of three mice in each group were euthanized at 3 and 7 days post infection (dpi), when whole blood was collected for a blood routine test; liver, spleen,

and lung tissues were collected for quantitative real-time polymerase chain reaction (PCR) and histological section assay.

2.5 Real-time PCR for detection of *T. gondii* DNA in tissues

The presence of *T. gondii* DNA was investigated by real-time PCR based on the SAG1 gene as described previously (Yu *et al.*, 2013). The genomic DNA of blood, liver, spleen, and lung tissues was extracted using a Universal Genomic DNA Extraction Kit (TaKaRa, China) according to the manufacturer's instructions. The forward and reverse primer sequences used in this study were 5'-CTGATGTCGTTCTTGCGATGTGGC-3' and 5'-GTGAAGTGGT TCTCCGTCGGTGT-3', respectively, designed by the Primer Express software (PE Applied Biosystem), which could amplify a 128-bp fragment under predetermined conditions. SYBR[®] Green was then used to detect fluorescence using the iQ5[™] Real-Time PCR System (Bio-Rad). Sterile water and genomic DNA of *T. gondii* tachyzoites were included as negative and positive controls in each run. In addition, a plasmid pMD-SAG1 was constructed based on pMD18-T vector using the same primers. To establish standard curves, plasmid pMD-SAG1 was tenfold serially diluted ranging from 2×10^4 to 2×10^9 copies per milliliter tested in each independent experiment for *T. gondii* quantification. Each sample had three parallel wells and was detected three times.

2.6 Blood routine test

At least 30 μ l of anticoagulated blood samples collected from mice of different groups at 3 and 7 dpi were applied to an IDEXX ProCyte Dx[®] hematology analyzer. Each blood sample was read three times. The data were analyzed using the software SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and statistical difference was accepted at the level of $P < 0.05$.

2.7 Histological section assay of tissues

Liver, spleen, and lung tissue specimens from different groups were first fixed in 10% (v/v) buffered formalin and then processed to paraffin for embedding. Tissue sections were obtained at approximately 5- μ m thickness and stained with haematoxylin and eosin (H & E) stains as described previously (Fischer *et al.*, 2008). Sections were examined under light microscopy.

3 Results

3.1 Protection of mice against challenge with *T. gondii* RH strain

In order to determine whether these combined Chinese herbs could provide effective protection against *T. gondii* RH infection, mortality was recorded daily following the *T. gondii* challenge until all mice were dead and survival curves of all groups were generated as shown in Fig. 1. The survival time of mice in the sulfadiazine group (13.5 ± 2.0 d) and 500 mg Chinese herb group (9.8 ± 0.69 d) were significantly longer than that in the PBS control group (6.9 ± 0.18 d) ($P < 0.05$). The average survival time of mice in the 300, 400, and 600 mg Chinese herb groups was all under 8 d which was slightly longer than that of PBS group, but the difference was not statistically significant ($P > 0.05$).

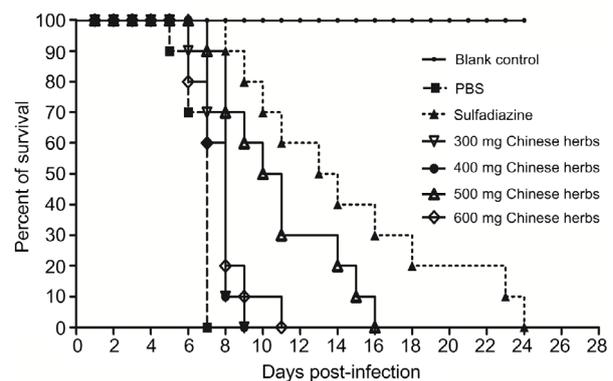


Fig. 1 Evaluation of survival curves against the lethal challenge of *Toxoplasma gondii*

Survival rate of mice treated with sulfadiazine and different dosages of Chinese herbs after challenge with 1×10^3 tachyzoites of *T. gondii* RH strain. Mice treated with PBS were included as negative control and mice infected with no *T. gondii* were included as blank control. Survival rate was monitored daily until all infected-mice died ($n=10$ mice per group)

3.2 Real-time PCR for detection of *T. gondii* DNA

Blood and tissue samples were collected from different experimental groups at 3 and 7 dpi with *T. gondii*, and then DNA was extracted as the template for SAG1-based real-time PCR to evaluate the parasite loads and DNA copy number in *T. gondii*-infected mice (Fig. 2). Every blood and tissue DNA sample was tested in triplicate and no amplification products were observed in wells from the blank control group. Results from the blood are shown in Fig. 2a. Parasite loads in all groups at 7 dpi were

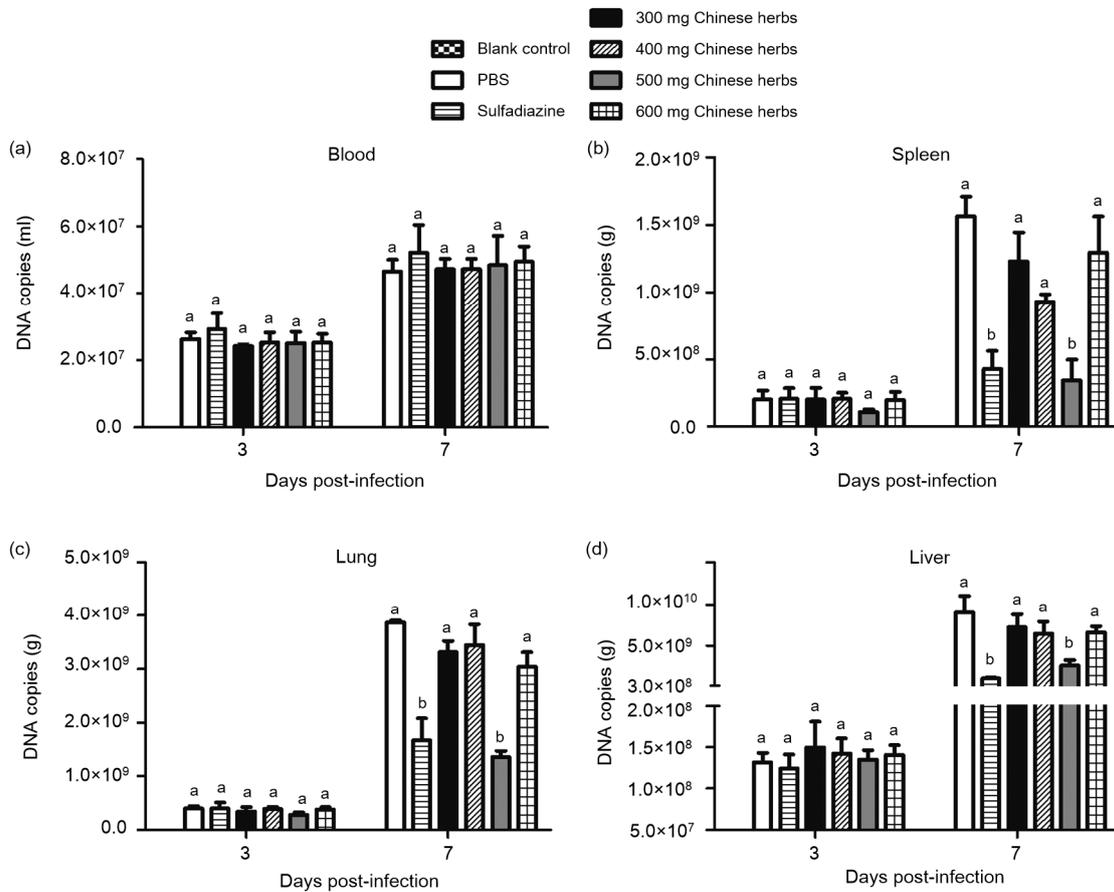


Fig. 2 Determination of the parasite load in blood, spleen, lung, and liver at 3 and 7 dpi

Blood (a), spleen (b), lung (c), and liver (d) tachyzoite burdens at 3 and 7 d after challenge. The DNA copies of *T. gondii* (per milliliter blood or per gram tissue) in blood and tissues of mice after challenge with 1×10^3 tachyzoites of *T. gondii* RH strain per mouse. The counting of DNA copies of *T. gondii* has been done in triplicate for each tissue and for three mice from each group. Values are expressed as mean \pm SD. Columns with different letters present statistical difference ($P < 0.05$)

almost double that at 3 dpi, but no significant difference was observed among these groups. Results of spleen, lung, and liver tissues presented a similar pattern (Figs. 2b–2d) in that parasite loads were all largely increased from 3 to 7 dpi, and mice of the PBS control group had statistically significantly higher parasite load compared with sulfadiazine and 500 mg Chinese herb groups ($P < 0.05$). However, the parasite loads of mice in 300, 400, and 600 mg Chinese herb groups were all fewer than those in the PBS control group but not at a statistically significant level.

3.3 Blood routine test

Blood samples were collected from different groups at 3 and 7 dpi directly into a 1-ml tube containing 0.3 mg dipotassium ethylenediamine tetraacetic

acid (K_2EDTA) and analyzed by an IDEXX ProCyt Dx[®] hematology analyzer. No significant increases were observed in eosinophil among the different *T. gondii*-infected groups (Fig. 3a). The proportion of monocyte of infected mice was remarkably increased compared to that of the blank group at 7 dpi (Fig. 3b; $P < 0.05$). However, lymphocytes of the infected groups were dramatically decreased compared with the blank control group (Fig. 3c) and those of the sulfadiazine and 500 mg Chinese herb groups were significantly low compared to the other four infected groups ($P < 0.05$). As shown in Fig. 3d, neutrophil in the infected groups was increased more than double compared to the blank control group at 7 dpi ($P < 0.05$), and the sulfadiazine and 500 mg Chinese herb groups also increased significantly higher among these groups.

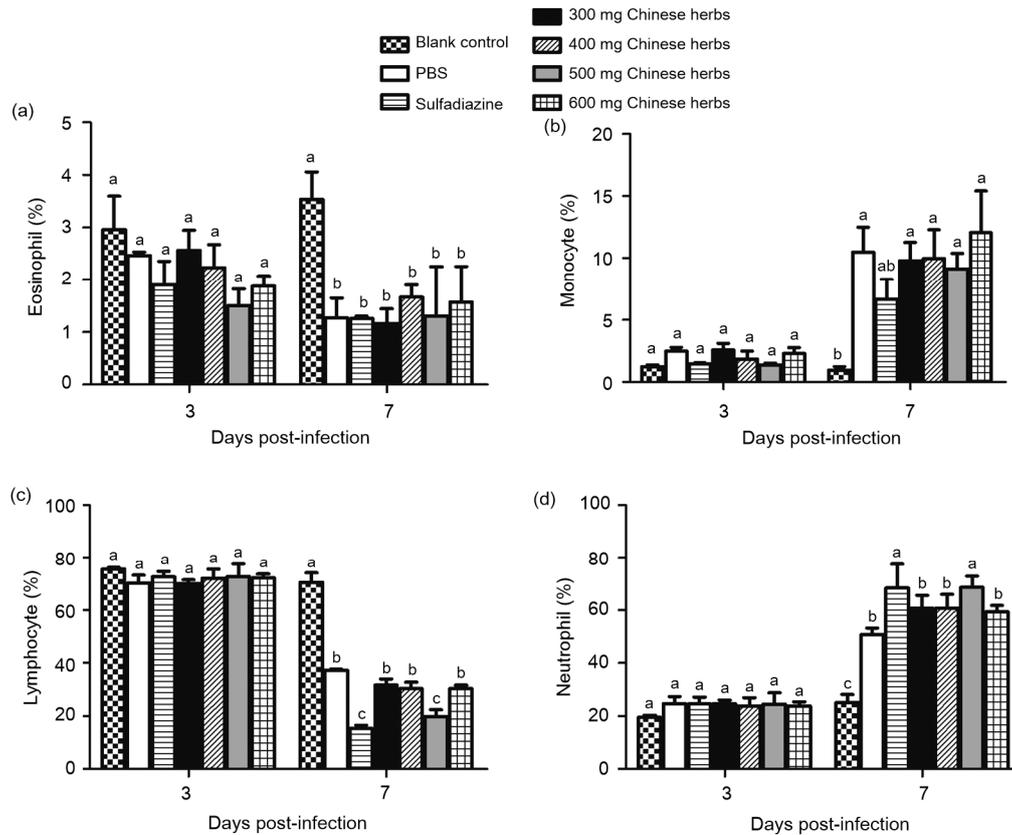


Fig. 3 Assessment of blood routine test at 3 and 7 d after the lethal challenge of *Toxoplasma gondii*

The results showed the percentage of cells in blood at 3 and 7 d after challenge with 1×10^3 tachyzoites of *T. gondii* RH strain per mouse. Eosinophil (a), monocyte (b), lymphocyte (c), and neutrophil (d) cell percentages were analyzed by IDEXX ProCyte Dx[®] hematology analyzer at 3 and 7 dpi. Blood samples were collected from three mice at each group at 3 and 7 dpi, respectively. Values are expressed as mean \pm SD. Columns with different letters present statistical difference ($P < 0.05$)

3.4 Pathologic changes detected by histological section assay of tissues

Pathological studies were performed through histological sections of liver, lung, and spleen tissues which were collected at 7 dpi from all groups. The livers of sulfadiazine and 500 mg Chinese herbs-treated mice displayed few changes compared to normal mice (Figs. 4c and 4f), but the livers of the PBS group mice showed a clear cellular separation, which revealed its morphologically incomplete and hepatocellular dysfunction (Fig. 4b), and the livers of 300, 400, and 600 mg Chinese herbs-treated mice showed slightly more cellular separation (Figs. 4d–4g). The lungs of all *T. gondii*-infected mice represented interstitial pneumonia at different degrees with much thicker alveolar walls compared to that of the blank control group (Figs. 4i–4n), and more severe cellular damage was observed in 300 and 600 mg

Chinese herbs-treated mice and the PBS control group. The spleen of the PBS group was extensively necrotic with few lymphoid cells and plenty of scattered cells observed. Lymphoid follicles were also absent (Fig. 4p). The spleens of other groups also had evidence of necrosis and degeneration with small scattered nodules of dead lymphoid cells (Figs. 4q–4u), but they were not as deteriorated as that of the PBS group shown in Fig. 4p.

4 Discussion

T. gondii can infect almost all warm-blooded animals, but no available drugs can eliminate this pathogen from its host without any clinical side effects. The discovery of artemisinin, isolated from *A. annua*, which can provide effective treatment worldwide against *P. falciparum* malaria, raises the

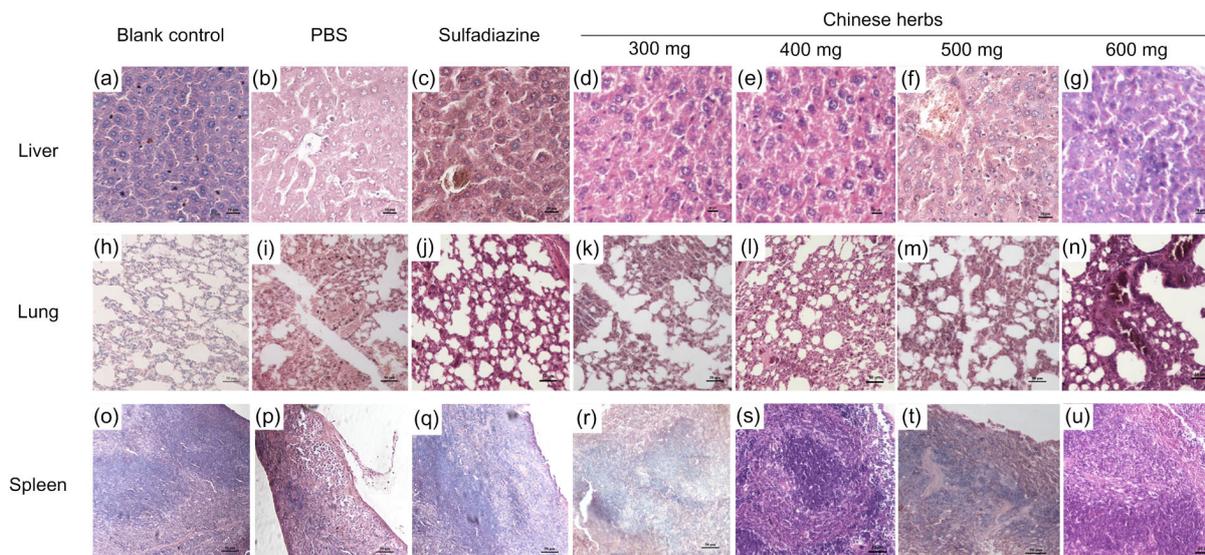


Fig. 4 Pathological examinations of spleen, lung, and liver tissues after the lethal challenge of *Toxoplasma gondii*

Pathological examinations were based on H & E-stained 5 μm -thick longitudinal sections of liver, lung, and spleen tissues in each group at 7 dpi. Liver (a–g), lung (h–n), and spleen (o–u) were collected from seven groups. Scale bars represent 20 μm (a–g) and 50 μm (h–u)

question as to whether Chinese traditional medicines could afford protection against *T. gondii*. Therefore, five herbal medicines that have different pharmacological effects were screened and combined into a prescription according to the traditional Chinese medicine compatibility principle and then the drug potential and protective efficacy were evaluated through a mouse model.

Molecular methods, particularly real-time PCR, have been treated as sensitive and reliable tools for determining the parasite loads of *T. gondii* (Bell and Ranford-Cartwright, 2002; Li *et al.*, 2012). Hence, we applied an SAG1-based real-time PCR method to determine the parasite loads in blood and tissues. We found *T. gondii* DNA could be detected in blood and tissues from all infected groups at 3 dpi with a relatively high level and was almost all doubled from 3 to 7 dpi while no significant difference was observed among these groups.

The level of the parasite load of the liver of sulfadiazine and 500 mg Chinese herbs-treated groups was lower than that of the PBS control group ($P < 0.05$). We also found the livers of sulfadiazine and 500 mg Chinese herbs-treated mice displayed few changes while a clear cellular separation could be observed in the liver of PBS group mice that presented a morphologically incomplete and hepatocellular

dysfunction. The lungs of sulfadiazine and Chinese herbs-treated mice possessed a significantly lower parasite load than that of the PBS control group ($P < 0.05$) and the histological result verified this from the morphological perspective. The lungs of all *T. gondii*-infected mice displayed interstitial pneumonia which had much thicker alveolar walls compared to those of the blank control group. However, the situations of the PBS group, the 300 and 600 mg Chinese herbs-treated groups were the worst such that no complete pulmonary alveoli structure could be observed. This revealed the fact that the lungs barely functioned and resulted in rapid death at 7 dpi as shown in the survival curve. As for the spleen, the parasite in sulfadiazine and 500 mg Chinese herbs-treated groups was at a significantly lower level than that in the PBS group at 7 dpi ($P < 0.05$). The spleens of all infected mice showed necrosis and degeneration. Lymphoid follicles of the PBS group were barely observed, and those of sulfadiazine and 500 mg Chinese herbs-treated groups also had evidence of necrosis and degeneration with small scattered nodules of dead lymphoid cells. These results were in accordance with the survival curve that the mice of sulfadiazine and 500 mg Chinese herb groups lived much longer than the controls. Mice treated with 500 mg Chinese herbs ((9.8 \pm 0.69) d) showed a

significantly longer survival time ($P < 0.05$) than the PBS-treated mice. In addition, the average survival time of 500 mg Chinese herbs-treated mice presented a similar level as many studies on DNA vaccines; the average survival time of rROP18-immunized ICR mice is 11.5 d (Qu *et al.*, 2013) and Zheng *et al.* (2013) found that after being immunized with rROP5+rSAG1, mice had prolonged survival time of 12.1 d. We assume that *T. gondii* was able to move to most tissues of mice through blood based on the fact that DNA copies of *T. gondii* had been detected in blood, liver, spleen, and lung. Actually, Dadimoghaddam *et al.* (2014) found *T. gondii* moved to various tissues within 24 h after i.p. injection and the largest number of parasites was observed in the heart, kidney, and liver. The movement trend of *T. gondii* and parasite load in tissues have been used mainly to evaluate the vaccine effect, anti-parasite and drug disease severity (Bell and Ranford-Cartwright, 2002; Romand *et al.*, 2004). The results gathered here suggested that these combined Chinese herbs could afford efficient protection against acute *T. gondii* infection.

Machado *et al.* (2014) demonstrated that the increased monocytes and lymphocytes could be treated as a relevant hematological biomarker of acute retinochoroidal lesions caused by *T. gondii*. Thus we used an IDEXX ProCyte Dx[®] hematology analyzer to determine the changes of cell proportions in blood at 3 and 7 dpi. We found that there was no significant difference among the *T. gondii* infected groups on eosinophil at 3 or 7 dpi. However, monocytes and neutrophils of infected mice were remarkably increased while lymphocytes were dramatically decreased compared to those of the blank control group at 7 dpi. On the other hand, the significantly lower lymphocyte and slighter lower monocyte of sulfadiazine and 500 mg Chinese herbs-treated mice than those of other Chinese herbs-treated mice suggests that effective drugs may decrease the cell proportions to hinder the cellular transmission of *T. gondii*. There are no differences between normal mice and infected mice at 3 dpi. We found *T. gondii* was capable of escaping the immune system in blood when combining the results of parasite load in blood at 3 dpi. In accordance with the results of Machado *et al.* (2014), the monocytes were increased drastically at 7 dpi. The recruitment of monocytes is essential in restricting the replication of the *T. gondii* murine

model (Mordue and Sibley, 2003; Robben *et al.*, 2005). On the other hand, monocytes as well as neutrophils and eosinophils are strong candidates for transport of *T. gondii* in blood in a way known as a "Trojan Horse" (Lachenmaier *et al.*, 2011), so the activity of monocyte must be carefully controlled. However, the trend of lymphocytes was opposite to that of Machado *et al.* (2014). The percentage of lymphocytes was decreased in infected mice mainly because ICR mice were sensitive to *T. gondii* and failed to eliminate the parasite, which was in accordance with the result that all infected mice eventually died.

In summary, mice treated with Chinese herbal medicines had been showed to elicit partial protection against acute *T. gondii* infection. Moreover, our study indicated that 500 mg Chinese herbs could afford more efficient protection than the other three dosages. The results also suggested that our 500 mg Chinese herbs could slow down the replication of *T. gondii* and prolong the survival time of mice. These results demonstrated this combination of Chinese herbs could be considered as possible candidate drugs against toxoplasmosis and also that the therapies of traditional Chinese herbal medicine can be developed for anti-*Toxoplasma* in the future.

Compliance with ethics guidelines

Xun-hui ZHUO, Hong-chao SUN, Bin HUANG, Hai-jie YU, Ying SHAN, and Ai-fang DU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

- 题目:** 小鼠模型中研究复方中草药抗弓形虫感染的作用
- 目的:** 评估复方中草药对小鼠抗弓形虫的保护力, 以期找到新型防治弓形虫病的药物。
- 创新点:** 首次在小鼠模型中研究威灵仙、茺菘、白术、刘寄奴和甘草组成的复方中草药的抗弓形虫功效, 并发现 500 mg 的药物能提供较好的保护力。
- 方法:** 将 ICR 雌性小鼠随机分为 7 组: 空白组不攻虫不给药; PBS 组攻虫; 药物对照组攻虫并口服磺胺嘧啶 10 mg/d; 实验组攻虫并分别灌胃 300、400、

500 和 600 mg/d 中药煎剂；攻虫组每只小鼠腹腔注射弓形虫 RH 株 1000 个。每日观察各组小鼠死亡情况，在感染后 3 和 7 d 采集抗凝血检测血常规，并采集血液、肝、脾和肺组织，提取基因组 DNA，通过绝对荧光定量 PCR 检测各组织样品中的虫荷量，采集感染后 7 d 的肝、肺和脾组织，制作组织切片，通过 H & E 染色观察组织病变情况。

结论：试验结果表明，500 mg 中药组及磺胺嘧啶药物对照的小鼠显著地延长了存活时间（图 1），且

这两组小鼠组织及血液中的虫荷数显著低于其他组（图 2），这与小鼠的肝、肺和脾组织病理染色结果一致（图 4）。另外，与 300、400 和 600 mg 中药处理组相比，500 mg 处理组小鼠的单核细胞和淋巴细胞比例均较低（图 3），这在一定程度上阻碍了弓形虫在血细胞中的传播。综上所述，500 mg 中草药处理组能降低弓形虫的胞内增殖速率，延长小鼠的存活时间，证明该浓度的复方中药具有一定的抗弓形虫效果。

关键词：弓形虫；中草药；荧光定量 PCR；存活率