Original Article

In Vivo Efficacy of Drugs against *Toxoplasma gondii* Combined with Immunomodulators

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**SUMMARY:** The aim of this study is to evaluate the effects of pyrimethamine (PYR) and sulfadiazine (SDZ) combined with levamisole and echinacea on the survival of mice infected with *Toxoplasma gondii*. For this, we used 99 specific pathogen-free BALB/c mice. All the mice were infected intraperitoneally with 10⁵ *T. gondii* tachyzoites and were divided into 11 groups, each including 9 mice. Except for the control group, oral treatment was initiated in all groups 24 h post infection and was continued for 10 days. The treatment regimen included dual combinations of PYR (dose, 6.25 and 12.5 mg/kg/day) and SDZ (dose, 100 and 200 mg/kg/day), triple combinations of PYR + SDZ, and levamisole (dose, 2.5 mg/kg/day) or echinacea (dose, 130 and 260 mg/kg/day) and echinacea alone (dose, 130 and 260 mg/kg/day). We observed that an effective dose of the combination of PYR + SDZ and levamisole resulted in a statistically significant increase in the survival rate from 33.3% to 88.9%. Similarly, half the dose of this combination resulted an increase in the survival rate from 33.3% to 44.4% (p < 0.05). Survival rate also increased in the groups treated with the combinations including echinacea; however, the difference did not reach statistical significance. The triple combination of PYR-SDZ-levamisole could be an alternative treatment option in case of infections caused by *T. gondii*.

**INTRODUCTION**

Toxoplasmosis caused by *Toxoplasma gondii* is commonly observed worldwide and its seroprevalence increases with age (1,2). *T. gondii*, generally, has a latent course in healthy individuals; however it may lead to complications in pregnant women, congenital abnormalities in fetuses and life-threatening severe disorders in patients with immune deficiency (3,4). With an increase in both the diseases causing immune system impairment and the respective medications, various clinical presentations are observed that may be fatal (2,5). In chronic cases, tissue cysts are mostly observed in different organs including the brain, eyes, striated muscles, and the adrenals; additionally, it has been reported that 99% of the untreated cases result in death (2).

The intracellular localization of the causative agent further complicates the treatment. There is no fully effective treatment against the tissue cysts in infected people; however, treatment is recommended against the tachyzoites that are released because of activation of these tissue cysts. Moreover, the currently used drugs are effective against the tachyzoites but not against the bradyzoites (2), and there is no ideal antimicrobial agent against toxoplasmosis (6,7). Alternative treatments are available as add-ons to standard therapies; however, an ideal treatment has not yet been achieved because of the development of resistance. Particularly in adults, less toxic and more effective novel drugs are required to treat *T. gondii*-associated chorioretinitis and congenital toxoplasmosis (3,4,8,9). Recently, in vitro resistance against pyrimethamine (PYR), clindamycin, spiramycin, and azithromycin has been demonstrated in *T. gondii* mutants, and this has increased the attention towards new antimicrobials (10,11). A treatment that is particularly effective against the bradyzoite forms of *T. gondii* in the tissues might eliminate the life-threatening potential of the infection. The efficacy of antimicrobial agents against *T. gondii* has been investigated in several in vivo studies; however, to our knowledge, no study has been performed so far to explore the efficacy of immunomodulatory agents on *T. gondii*. Levamisole and echinacea are immunomodulators that have been used in the treatment of other infectious diseases. Levamisole is a heterocyclic compound that is an effective anthelmintic agent, and it is also an immunoregulatory agent. Its probable immuno-regulatory mode of action is mimicry of the thymic hormone. Thymopoietin affects many components of the immune system, and its therapeutically important actions probably target the stimulation of phagocytosis and regulatory T cells to restore homeostasis in a perturbed immune system. Levamisole has also been used to treat a variety of dermatological conditions, including skin infections (12–16).

Echinacea is a genus or a group of herbaceous flowering plants. That affects various roles of the immune system including functions of macrophages and lymphocytes, increase in activity of natural killer (NK) cells and...
a probable increase in production of interferons (IFNs) and phagocytosis. It is one of the basic antimicrobial herbs, and its use has been documented for snakebite, anthrax, and for relief of pain. Recently, it has been used as a treatment for upper respiratory tract infections and other infections. Echinacea extract is safe in children and pregnant women (17–20).

The purpose of the present study is to evaluate the effects of PYR and sulfadiazine (SDZ) combined with immunomodulatory agents, levamisole and echinacea, on the survival of mice infected with *T. gondii*.

**MATERIALS AND METHODS**

**Animals:** In total, 99 specific pathogen-free BALB/c female mice, were used that weighed 18–22 grams and were aged 5–6 weeks. This study was approved by the animal research local ethics committee.

**Preparation of the *T. gondii* strain and infection model:** Standard RH strain tachyzoites of *T. gondii* were used. Maintenance of *T. gondii* RH strain was assured by intraperitoneal (IP) passage in BALB/c mice once every 4 days. The exudates obtained from the peritoneum of the mice on the 4th day using a Pasteur pipette were counted on a Thoma slide under a light microscope. The exudate was buffered with physiological serum to obtain 10^6 tachyzoites/mL. In order to induce acute infection, 0.1 mL (10^6 *T. gondii* tachyzoites) of this suspension was administered IP to each mouse. The mice were divided into 11 groups, containing 9 mice each, and were put into separate cages.

**Treatment protocol:** Except for the control group, treatment was initiated in all groups 24 h post-infection. The treatment included oral administration of a single dose using gavage and was continued for 10 days. Group 1 was administered a combination dose of PYR (Fluka, Milwaukee, WI, USA) at 12.5 mg/kg/day and 200 mg/kg/day, respectively (half of the effective treatment dose); Group 2 was administered a combination dose of PYR and echinacea (*Echinacea purpurea*, Hankintatukku oy, Helsinki, Finland) at 12.5, 200 mg/kg/day and 130 mg/kg/day, respectively; Group 3 was administered a combination dose of PYR + SDZ and echinacea (*Echinacea purpurea*, Hankintatukku oy, Helsinki, Finland) at 12.5, 200 mg/kg/day and 130 mg/kg/day, respectively; Group 4 was administered a combination dose of PYR + SDZ and levamisole at 6.25, 100 mg/kg/day and 2.5 mg/kg/day, respectively; Group 5 was administered a combination dose of PYR + SDZ and echinacea (*Echinacea purpurea*, Hankintatukku oy, Helsinki, Finland) at 12.5, 200 mg/kg/day and 130 mg/kg/day, respectively; Group 6 was administered in combination dose of PYR + SDZ and echinacea at 6.25, 100 mg/kg/day and 130 mg/kg/day, respectively; Group 7 was administered in combination dose of PYR + SDZ and echinacea at 6.25, 100 mg/kg/day and 260 mg/kg/day, respectively; Group 8 was administered in combination dose of PYR + SDZ and echinacea at 6.25, 100 mg/kg/day and 260 mg/kg/day, respectively; Group 9 was administered echinacea monotherapy at 130 mg/kg/day; and Group 10 was administered echinacea monotherapy at 260 mg/kg/day; Group 11 did not receive any therapy.

**Determining the treatment efficacy:** The mice were observed for 30 days after completion of the treatment, and their survival was recorded every day. Survival rate was assessed on days 1, 5, 10, 15, 20, 25, and 30.

**Statistical analysis:** Data analysis was performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Kaplan-Meier survival analysis was used to calculate survival probabilities. Log-rank test was used to compare the survival probabilities among the groups. If the p-value (p) < 0.05 is statistically significant. p ≥ 0.05 value is not significant.

**RESULTS**

On evaluation of the 30-day survival rate of the mice in the control group (Group 11), it was observed that 4 mice died on the 6th day, 3 died on the 7th day, and 1 died on each of the 8th and the 9th days, hence the survival rate was 0% on the 9th day. Survival rate was also 0% in Groups 2, 9, and 10. With a survival rate of 88.9%, Group 3 showed the highest survival probability, and this was followed by Group 5 with a survival rate of 66.7%. Survival rates in Groups 4, 6, 7, and 8 were 44.4%, and 33.3% in Group 1. Treatments administered to each group and their survival rates are shown in Table 1.

In the group receiving the effective treatment dose of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (mg/kg/day)</th>
<th>Observation day</th>
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<tbody>
<tr>
<td></td>
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<td>1st</td>
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<tr>
<td>1</td>
<td>PYR (12.5) + SDZ (200)</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>PYR (6.25) + SDZ (100)</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>PYR (12.5) + SDZ (200) + levamisole (2.5)</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>PYR (6.25) + SDZ (100) + levamisole (2.5)</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>PYR (12.5) + SDZ (200) + echinacea (130)</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>PYR (6.25) + SDZ (100) + echinacea (130)</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>PYR (12.5) + SDZ (200) + echinacea (260)</td>
<td>100</td>
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<tr>
<td>8</td>
<td>PYR (6.25) + SDZ (100) + echinacea (260)</td>
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<tr>
<td>9</td>
<td>echinacea (130)</td>
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<tr>
<td>10</td>
<td>echinacea (260)</td>
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<td>11</td>
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1: PYR, pyrimethamine; SDZ, sulfadiazine.
PYR + SDZ (Group 1), the survival rate at the end of the 30-day observation period was 33.3%; whereas, in the group treated with half of the effective treatment dose of PYR + SDZ (Group 2), the survival rate at the end of the 30-day observation period was 0%. In the group that was treated with a combination of full-dose PYR + SDZ and levamisole (Group 3), the survival rate at the end of the 30-day observation period had increased from 33.3% to 88.9%. This difference was statistically significant (p < 0.05). In the group that was treated with a combination of half-dose PYR + SDZ and levamisole (Group 4), the survival rate at the end of the 30-day observation period had increased from 0% to 44.4% (p < 0.05). In the group that was treated with a combination of full-dose PYR + SDZ and echinacea (130 mg/kg) (Group 5), the survival rate at the end of the 30-day observation period increased from 33.3% to 66.7%; however, the difference was not statistically significant (p ≥ 0.05). In the group that was treated with a combination of half-dose PYR + SDZ and echinacea (130 mg/kg) (Group 6), the survival rate at the end of the 30-day observation period increased from 0% to 44.4%; however, the difference was not statistically significant (p ≥ 0.05). In the group that was treated with a combination of full-dose PYR + SDZ and echinacea (260 mg/kg) (Group 7), the survival rate at the end of the 30-day observation period increased from 33.3% to 44.4% (p ≥ 0.05). In the group that was treated with a combination of half-dose PYR + SDZ and echinacea (260 mg/kg) (Group 8), the survival rate at the end of the 30-day observation period increased from 0% to 44.4%, however, the difference was not statistically significant (p ≥ 0.05). In the groups treated with echinacea alone (with doses of 130 mg/kg and 260 mg/kg in Group 9 and Group 10, respectively), the survival rates at the end of the 30-day observation period were 0%. There was no significant difference compared to the control group (Group 11) (p ≥ 0.05).

When the survival probabilities were compared among the groups, no significant difference was seen among Groups 2, 9, 10, and 11 which had survival rates of 0% (p ≥ 0.05). When the survival probabilities were compared between Group 1 and Group 3, and between Group 2 and Group 4, it was noted that Group 3 and Group 4 had statistically significant higher survival probability (p < 0.05). There was no significant difference between Group 1 and Group 5, between Group 1 and Group 7, between Group 2 and Group 6, and between Group 2 and Group 8 with respect to survival probabilities (p ≥ 0.05).

**DISCUSSION**

There is a need for developing treatment regimens with fewer side effects and that are effective against bradyzoites enclosed within tissue cysts to combat toxoplasmosis. To our knowledge, this study is the first to investigate the in vivo efficacy of established immunomodulatory agents, echinacea and levamisole, against *T. gondii* and the interaction of these agents with standard therapies. Previous studies have explored the effects of various combinations including PYR and SDZ against *T. gondii*. In experimental toxoplasmosis models like mice, the molecules such as trovafloxacin (21), gatifloxacin (22), roxithromycin (23), clarithromycin (24), azithromycin (25), atovaquone (26), and hydroxynaphthoquinones such as PHNQ6 (27), two 3-alkyl-substituted 2-hydroxy-1,4-naphthoquinones NSC52, and NSC55 (28) have been investigated either as monotherapies or in combination with PYR and SDZ. These studies have demonstrated the synergic effects of the combinations, and significant elongations of survival periods have been observed with combination therapies.

It is known that *E. Purpurea*, *Echinacea angustifolia*, and *Echinacea pallida* species of the echinacea plant have immunostimulatory effects (29). In vivo and in vitro studies have shown that *E. purpurea* activates the natural immune response by stimulating interleukin (IL)-6, IL-12, and nitric oxide (NO), and tumor necrosis factor (TNF) in the macrophages; it also demonstrates immunomodulatory effects by increasing macrophage-mediated phagocytosis (12-15).

In their study on the natural and acquired immune effects of *Echinacea* species, Zhai et al. have demonstrated that oral administration of *E. purpurea*, *E. angustifolia*, and *E. pallida* extracts to mice results in an increase in INF-α and a decrease in TNF-γ and IL-1 β secretion (16). They also noted that IL-4 and IL-10 production increased in mice treated with *E. angustifolia* and *E. pallida* extracts. These findings suggest that echinacea is a wide-spectrum immunomodulatory agent for both natural and acquired immune responses. In the present study, *E. purpurea* extract was administered to the respective groups as monotherapy and in combination with PYR + SDZ. When the infected mice were treated with 130 mg/kg and 260 mg/kg *E. purpurea* extract, no statistically significant difference compared to the control group was noted in the survival rate. We observed an increase in the survival rate when *E. purpurea* extract at doses of 130 mg/kg and at 260 mg/kg (a dose which we expected to be more effective) was combined with full-dose and half-dose PYR + SDZ; however the differences were not statistically significant. Based on these results, it can be concluded that the doses of echinacea administered in this study are not effective in the combination treatment against experimental toxoplasmosis.

To our knowledge, this study is the first to evaluate the efficacy of the immunomodulatory drug levamisole combined with PYR + SDZ against *T. gondii*. When combined with full-dose PYR + SDZ, levamisole increased the survival rate from 33.3% to 88.9% on Day 30, while its combination with half-dose PYR + SDZ resulted in an increase in the survival rate from 0% to 44.4% on Day 30. The statistically significant increases noted in the survival rates of the groups receiving treatment with levamisole suggest that this drug might increase the treatment efficacy when administered in combination with the other drugs. It seems possible that the already established regimen of the human dose of levamisole is clinically useful for the treatment of toxoplasmosis. The anthelmintic dose of levamisole in all living species is 7.5 mg/kg, while its immunomodulatory dose corresponds to the 1/4, 1/3, or 1/2 of the anthelminthic dose (17). In this study, levamisole was administered at the 1/3 of the anthelmintic dose, at 2.5 mg/kg. Along with its anthelmintic effects, levamisole also...
shows immunostimulatory activity (17). It has been reported that levamisole treatment improves defective leukocyte reactivity, and provides clinical recovery in chronic infections, malignancies, and inflammatory diseases (18–20).

Levamisole directly stimulates the lymphocytes, macrophages, and granulocytes. It is more effective on cellular immunity than on humoral immunity. This is because of its enhanced stimulatory effects on the T lymphocytes rather than B lymphocytes. In order to achieve apparent immunostimulatory activity, it should be administered along with a primary stimulus such as an antigen. Rather than being an immunostimulant, it is an immunopotentiator (30). In this study, we have observed that levamisole significantly increases the survival rate in the groups being treated with it in combination with PYR + SDZ. We believe that the T. gondii tachyzoites might have acted as primary stimulants and increased the efficacy of levamisole.

The cytokine IFN-γ plays a major role in the host defense against T. gondii. Macrophages, T lymphocytes, NK cells, and cytotoxic T cells are activated when T. gondii enters the body and the immune system controls the parasite by releasing various cytokines (IFN-γ, TNF-α, IL-2, and IL-12). IFN-γ, additionally, shows antiparasitic activity by aiding in the release of toxic oxygen radicals and NO (2). In the present study, we aimed to enhance the immune system against T. gondii by combining the available effective agents with immunomodulatory agents. We believe that such a combination might be beneficial particularly in the treatment of patients who are mildly-immunosuppressed.

PYR and SDZ combination is used in various clinical types of toxoplasmosis, especially in the treatment of toxoplasmic encephalitis seen in the patients with AIDS. Adverse effects are further enhanced when PYR and SDZ are used in combination. Considering the dose-related adverse effects of these drugs, they should be administered in the lowest possible doses (2). In this study, we have demonstrated that the survival rate is also increased when the half-dose PYR + SDZ (with fewer adverse effects) is combined with levamisole or echinacea.

Studies have reported that the antimicrobial agents with proven in vivo or in vitro efficacy against T. gondii might also be effective in the treatment of toxoplasmosis; however, these studies cannot be continued as there are cases of toxoplasmosis where the received treatment protocols become insufficient or the drugs cannot be tolerated because of adverse effects.

In conclusion, the present study shows that combination of the immunomodulatory agents, levamisole and Echinacea (anti-toxoplasmic activity for these had not yet established), and PYR + SDZ for the treatment of T. gondii increases the survival of mice. Particularly, the combination with levamisole, which resulted in statistically significant survival, can be considered as a treatment option. Advanced in vivo studies investigating the combinations with different doses and other effective drugs are required in order to establish more clearly the efficacy of immunomodulatory agent against T. gondii.

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Conflict of interest None to declare.

REFERENCES


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