SUSCEPTIBILITY OF TRICHINELLA SPIRALIS TO IVERMECTIN IN INFECTED MICE

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ABSTRACT

The objective of this study was to assess the susceptibility to ivermectin treatment of 300 Trichinella spiralis larvae in infected imprinting control region (ICR) mice. The mice received a single dose of ivermectin (200µg/kg) per subcutaneous route one day post-infection (DPI). Parasites were detected and counted using a stereomicroscope, and any inflammatory reaction was evaluated histologically with hematoxylin and eosin staining. Ivermectin reduced parasite yield from 6% after 1 DPI and 66% after 32 DPI (1 DPI, p-value = 0.00). However, the inflammatory reaction in various tissues and internal organs was not completely reduced (p-value > 0.05). These results suggest that the effectiveness of a single dose of ivermectin (200µg/kg) depends on the time of treatment post-infection. The optimum time for treatment should be within 6 DPI, to maximize efficacy and reduce the number of parasites by 54-66%.

Keywords: Trichinella spiralis, ivermectin, inflammatory reaction, parasite numbers

INTRODUCTION

Trichinellosis is a globally distributed zoonosis (Kaewpitoon et al, 2008) caused by the ingestion of uncooked meat infected with the encysted larval stage of Trichinella spiralis (Capo and Despommier, 1996). The parasite is digested by an enzyme in the stomach, which releases the larvae, which then enter the intestine and develop into the adult worm stage. After mating, the adult female worm sheds newborn larvae, which migrate to the internal organs by invading the lymphovascular system. Newborn larvae end up in the skeletal muscle, forming a nurse cell complex (Gottstein et al, 2009). The clinical pathology is characterized by an inflammatory reaction response in tissues and internal organs, which causes the signs and symptoms, such as high grade fever, diarrhea, myalgia, periorbital swelling, and serious complications, such as myocarditis. Death may result from inflammatory reactions in the heart, lungs, and central nervous system (Capo and Despommier, 1996). Trichinellosis treatments using antihelminthic drugs, such as dormectin, ivermectin, and levamisole, are not completely successful in eliminating T. spiralis (Soliman et al, 2011). Therefore, finding an effective treatment for trichinellosis will beneficial to patients – particularly a treatment that can prevent the encysted larvae from ending up in the muscle, or prevent serious complications, such as myocarditis, from developing. The research could be focused on therapeutic treatment for trichinellosis in various phases with ivermectin or other antihelminthic drugs.

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**MATERIALS AND METHODS**

**Experimental research design**

This study was performed on 9–12-week-old male ICR mice, obtained from the National Laboratory Animal Center, Mahidol University (ethical clearance number FTM-ACUC 007/2555, dated 15-03-2012, approved by the Faculty of Tropical Medicine Animal Care and Use Committee, Mahidol University). All mice were treated according to the standard protocol of the National Laboratory Animal Center, Mahidol University. The mice were divided into three groups: the experimental group contained mice infected with *T. spiralis*, which were treated with ivermectin; control group-A contained infected mice by *T. spiralis*, which were not treated with ivermectin; and control group-B contained uninfected mice treated with ivermectin. Four mice in each group were examined after 1, 6, 12, 18, 26 and 32 DPI.

**Sample size calculation**

The sample sizes were calculated according to the formula below [1]. The power of the test was set at 90%, significance level 5%; the appropriate standard deviation and mean were obtained from a review of the public literature (Soliman et al, 2011). In this study, the sample size per group was calculated as 3 mice. However, error-prevention techniques during the experimental period require the sample size to increase by 20% per group, resulting in 4 mice per group (Figure 1).

\[
n = \frac{(u+v)^2(\sigma_1 + \sigma_0)^2}{(\mu_1 - \mu_0)^2}
\]

\[
n = \text{total sample sizes per groups for each case and control}
\]

\[
u = \text{power} = 90\%
\]

\[v = \text{significance level} = 5\%
\]

\[(\mu_1 - \mu_0) = \text{difference between the means of review literature}
\]

\[(\sigma_1 - \sigma_0) = \text{difference between the standard deviation of review literature}
\]

**Preparation of parasite and infecting the mice**

*T. spiralis* larvae were isolated from 35 infected DPI mice in the Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University. A small piece of skeletal muscle tissue was cut excised (~2 cm), then digested with pepsin solution (Pepsin 1g / Hcl 1ml / distilled water to 100ml) for 12 hours (Siriyasatien et al, 2003). The freshly isolated *T. spiralis* larvae were counted under a stereomicroscope. After counting, each mouse in the experimental group and control group-A was infected with 300 *T. spiralis* live larvae per oral feeding gavage tube No. 18 (Siriyasatien et al, 2003).

**Treatment with ivermectin**

Mice in the experimental group and control group-B were treated with ivermectin 200µg/kg per subcutaneous tissue injection, at periods 1, 6, 12, 18, 26, and 32 DPI (Soliman et al, 2011).

**Detection and collection of *T. spiralis* post-treatment**

All dissections and gross examinations were performed at the same study periods (at 1, 6, 12, 18, 26, and 32 DPI) as follows: the experimental group and control group-B mice were anesthetized with carbon dioxide gas inhalation until death, 24 hours after receiving...
ivermectin treatment (based on peak plasma concentration) (Geyer et al., 2009). Dissection and gross examination was then performed on tissues and internal organs. Control group-A, which did not receive ivermectin treatment, was also anesthetized until death, and dissection and gross examination was performed at the same time periods. Parasites in the infected mice were detected by crushing technique (compression of the fresh muscle between glass slides and examination of parasites under a stereomicroscope) (Ko et al, 1994; Smith et al., 1976). The infected tissues were digested as previously described and parasites in the intestine were washed with saline solution into a petri dish. The total number of parasites were counted under a stereomicroscope.

**Histology (H&E staining) analysis**

The tissue and internal organs were cut into a small piece (~2 cm), fixed for 24 hours in 10% neutral buffered formalin, then underwent paraffin embedding and H&E staining at the Department of Pathology, Chulalongkorn University. Three pathologists independently evaluated, interpreted, and graded the inflammatory reactions. Inflammatory reactions were graded as follows: The criteria of inflammation grading in the intestine were modified based on disease activity index and histology activity index (Galitovskiy et al., 2011; Krenn et al., 2002) (table 1). Inflammation grading criteria in the heart were modified based on international public papers on myocarditis factors: inflammatory cells, necrosis, and fibrosis. This was assigned a histological grading, as seen below. Normal: no involvement; Mild: < 25% involvement; Moderate: 26-50% involvement; Severe: > 50% involvement (Leon et al., 2003; Quijano-Hernandez et al., 2013). The criteria for grading inflammation in the muscle were modified based on international public papers on myositis (Arnardottir et al., 2003; Pap et al., 2009; Sukura et al., 2002) (table 2).

**Statistical analysis**

The total number of T. spiralis in the experimental group and control group-A, detected by independent T-test, were considered significant at P-value < 0.05, and the inflammatory reaction in experiment groups and control groups were compared by Mann-Whitney U-Test.

**Table 1**  
Histological inflammatory reaction grading of the intestine

<table>
<thead>
<tr>
<th>Grading</th>
<th>Inflammatory cell infiltration.</th>
<th>Extent of inflammatory cell infiltrate.</th>
<th>Goblet cell mucin depletion.</th>
<th>Mucosal thickness</th>
<th>Length of bowel damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 5 cell per HPF</td>
<td>none</td>
<td>&gt; 28 goblet cells per HPF</td>
<td>normal</td>
<td>None</td>
</tr>
<tr>
<td>Mild</td>
<td>5 to 20 cell per HPF</td>
<td>mucosa</td>
<td>11 to 28 goblet cells per HPF</td>
<td>focal thickening</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Moderate</td>
<td>21 to 60 cell per HPF</td>
<td>mucosa and submucosa</td>
<td>1 to 10 goblet cells per HPF</td>
<td>multifocal thickening</td>
<td>21–40</td>
</tr>
<tr>
<td>Severe</td>
<td>crypt abscesses or more than 61 cell per HPF</td>
<td>transmural</td>
<td>1 goblet cell per HPF</td>
<td>extensive</td>
<td>&gt; 41</td>
</tr>
</tbody>
</table>
Table 2  Histological inflammatory reaction grading of skeletal muscle.

<table>
<thead>
<tr>
<th>Grading</th>
<th>number of inflammatory cells around the capsule.</th>
<th>number of nuclei in each nurse cell.</th>
<th>number of inflammatory cells around myocyte 10 HPF.</th>
<th>Histopathological and morphological change in muscle.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 5 cell</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Mild</td>
<td>5 to 20 cell</td>
<td>&lt; 3 cell</td>
<td>&lt; 25 cells</td>
<td>Increased variability in muscle fibre sizes, internal nucleation, slight degenerative and regenerative changes.</td>
</tr>
<tr>
<td>Moderate</td>
<td>20 to 30 cell</td>
<td>3-10 cell</td>
<td>26–50 cells</td>
<td>Prominent degenerative and regenerative changes.</td>
</tr>
<tr>
<td>Severe</td>
<td>&gt; 30 cell</td>
<td>&gt; 10 cell</td>
<td>&gt; 51 cells</td>
<td>No fibres of normal size, fibrosis and fatty infiltration.</td>
</tr>
</tbody>
</table>

RESULTS

Evaluation of the number of *T. spiralis* (adults and larvae)

The mean numbers (standard deviation) of adult and larvae *T. spiralis* at 1, 6, 12, 18, 26, and 32 DPI in the experimental group were: 49.75 (3.30), 40.75 (20.42), 13.25 (11.59), 239.70 (117.40), 2911.00 (295.20), and 2826.00 (376.90), respectively. In control group-A, these numbers were 143.75 (11.47), 87.75 (80.05), 18.00 (6.27), 3220.00 (284.90), and 2992.00 (330.10). Ivermectin treatment on 1, 6, 12, 18, 26, and 32 DPI decreased the percentage of *T. spiralis* (adult and larvae) yield by 66%, 54%, 26%, 31%, 10%, and 6%, respectively. The statistical analysis of the experimental group and control group-A by independent T-test was considered highly significant only after 1 DPI (*P*-value < 0.0001) (Table 3).

Inflammatory reactions: histopathological studies of infected tissues

The inflammatory reactions and morphology of the small intestine, heart, and skeletal muscle were observed by histological technique with H&E staining. The sections of small intestine in the experimental group and control group-A showed mild to moderately acute enteritis, characterized by predominant neutrophil infiltration in the mucosa to submucosa. In addition, the morphological changes of the villi and crypts ratio were decreased (2-3 crypts per villus). The sections of the small intestine in control group-B show normal morphological architecture of the villi and crypts, with normal villi: crypt ratio (3-4 crypts: 1 villus) (Figure 1). The statistical analysis of inflammatory reactions and morphological changes in the small intestine of the experimental group compared with control group-A were not statistically significant (*P*-value > 0.05) for 1 to 32 DPI. The changes in control group-B were statistically significant (*P*-value < 0.05) at 6, 12, and 18 DPI (Figure 4).

Sections of the heart in the experimental group and control group-A showed mild to moderately acute and chronic myocarditis, characterized by numerous neutrophils, some of mononuclear cells, and eosinophil infiltraton in the interstitial tissues. Morphological changes in the cardiac myocytes were degenerated by intact cell membrane, deep eosinophilic cytoplasm, and clumping hyperchromatic nuclei. Sections of the heart in control group-B showed unremarkable cardiac myocytes (Figure 2). Statistical analysis of the inflammatory reaction and morphological changes in the heart of the experimental group compared with
control group-A were not significantly different ($P$-value > 0.05) at 1 to 32 DPI. However, for control group-B, they were significant ($P$-value < 0.05) at 12, 18, and 26 DPI (Figure 4).

Sections of the skeletal muscle in the experimental group and control group-A showed severe acute and chronic myositis, characterized by mononuclear cells, lymphocytes, eosinophils and predominant neutrophils infiltrated into the interstitial tissues and surrounding nurse cell complex. The regeneration of myocytes with deep basophilic cytoplasm, and central multinucleated with prominent nucleolus, were observed. Control group-B was unremarkable (Figure 3).

Statistical analysis of the inflammatory reaction and morphological changes in the skeletal muscle in the experimental group compared with control group-A was not significant ($P$-value > 0.05) at 1 to 32 DPI, and for control group-B it was significant ($P$-value < 0.05) at 12, 18, 26, and 32 DPI (Figure 4).

**DISCUSSION**

Examination of the effectiveness of a single dose of ivermectin (200µg/kg) per subcutaneous injection showed that the overall efficacy of ivermectin treatment depends on the time of treatment post-infection.

**Table 3** Statistical analysis of the mean number of *T. spiralis* (SD), and percent percentage of ivermectin treatment on study date (n=4)

<table>
<thead>
<tr>
<th>Day post-infection</th>
<th>Experimental group $\bar{x}$ (SD)</th>
<th>Control group-A $\bar{x}$ (SD)</th>
<th>Experiment groups compare to control group-A P-value (95% CI)</th>
<th>Efficacy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.75 (3.30)</td>
<td>146.75 (11.78)</td>
<td>.000 (79.57, 114.42)*</td>
<td>66%</td>
</tr>
<tr>
<td>6</td>
<td>40.75 (20.42)</td>
<td>87.75 (80.05)</td>
<td>.329 (-76.33, 170.33)</td>
<td>54%</td>
</tr>
<tr>
<td>12</td>
<td>13.25 (11.59)</td>
<td>18.00 (6.27)</td>
<td>.506 (-12.61, 22.11)</td>
<td>26%</td>
</tr>
<tr>
<td>18</td>
<td>239.66 (117.40)</td>
<td>349.66 (146.13)</td>
<td>.369 (-196.04, 416.04)</td>
<td>31%</td>
</tr>
<tr>
<td>26</td>
<td>2913.50 (297.51)</td>
<td>3220.00 (284.93)</td>
<td>.187 (-197.73, 810.73)</td>
<td>10%</td>
</tr>
<tr>
<td>32</td>
<td>2826.25 (376.89)</td>
<td>2992.25 (330.09)</td>
<td>.533 (-449.6, 781.6)</td>
<td>06%</td>
</tr>
</tbody>
</table>

*Note:* Asterisks (*) denotes; significant $P$-value < 0.05 and n=3 at 18th DPI as death occurred in the experiment.

**Fig. 1-** (A, B, C). Photomicrographs (400X) of sections of the small intestine of *T. spiralis*-infected mice treated with ivermectin (A) and untreated (B) at 12 DPI shows mild to moderate enteritis characterized by inflammatory cells, predominantly neutrophils (arrow), infiltrated in the lamina propria, intraepithelium, and submucosa. Morphology shows villi and crypts were intact (villi: crypt ratio, 2-3 crypts: 1 villus). Small intestine of uninfected mice treated with ivermectin (C) shows normal architecture, villi and crypts intact (villi: crypt ratio, 3-4 crypts: 1 villus).
Fig. 2- (D, E, F). Photomicrographs (400X) of sections in heart of *T. spiralis*-infected mice treated with ivermectin (D) show mild acute myocarditis; untreated (E) show moderately acute myocarditis at 18 DPI, characterized by numerous neutrophils (arrow) infiltrated in the interstitial tissues and degenerative morphological changes to the cardiac myocytes (arrowhead). Hearts from uninfected mice treated with ivermectin (F) at 18 DPI were unremarkable (✱).

Fig. 3- (G, H, I). Photomicrographs (400X) of skeletal muscle sections from *T. spiralis*-infected mice treated with ivermectin (G); untreated (H) show severe acute and chronic myositis at 18 DPI; myocytes containing *T. spiralis* larvae (L) surrounded by eosinophilic material with predominant numerous of neutrophils (arrow) and mononuclear cell infiltration. Morphology shows regenerated myocyte (arrowhead). The skeletal muscle from uninfected mice treated with ivermectin (I) at 18th DPI was unremarkable (✱).

Fig. 4- The inflammatory reaction grading of small intestine, heart, and skeletal muscle from mice in the experimental group (■), control group-A (●) and control group-B (▲). An asterisk (✱) indicates *P*-value < 0.05, compared with experimental groups.
Highest efficacy in eliminating *T. spiralis* is achieved at the intestinal phase (at 1 to 6 DPI), because ivermectin is reacting with parasites in the lumina of the small intestine. There, ivermectin binds to bile and protein in the plasma of the circulated blood, thus blocking neurotransmitters in the somatic neuromuscular system of the parasites and interfering with the functioning of the locomotive apparatus, thereby paralyzing them (Gonzalez Canga et al, 2009). In contrast, the efficacy of ivermectin in the muscle phase, at 18 to 32 DPI, is low, because ivermectin cannot kill parasites in the nurse-cell complex. Probably, ivermectin cannot readily cross the capsule of the nurse-cell complex. Therefore, the optimal period for treatment of trichinellosis with ivermectin should be within 6 DPI, during the intestinal phase, when the efficacy of ivermectin can eliminate > 54% parasite yield. However, this study revealed that the efficacy of ivermectin treatment for trichinellosis could not reduce inflammatory reactions in various tissues and internal organs, because the main cause of the clinical features of trichinellosis were inflammations of various tissues and internal organs (such as myositis myocarditis, fever that results from the immune response between host and parasite) (Beiting et al, 2004; Zhang et al, 2013). In summary, this study shows that treatment for trichinellosis could be improved by designing appropriate dosage intervals for ivermectin, combined with other antihelmintics and anti-inflammatory drugs. Eliminating the parasites in the enteral phase could reduce complications of encysted larvae in the migratory and muscular phases, when it is difficult to eliminate them.

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REFERENCES


