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Original Article

Improvements of cognitive functions in mice heavily infected by *Angiostrongylus cantonensis* after treatment with albendazole, dexamethasone, or co-therapy

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Spatial memory and learning;
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Dexamethasone;
Co-therapy

Abstract *Angiostrongylus cantonensis*, the causative agent of human eosinophilic meningitis and eosinophilic meningoencepalitis, has been reported to cause cognitive impairments in the host. To determine whether drug treatment improves the cognitive functions, BALB/c mice infected with 50 third-stage larvae were treated with albendazole, dexamethasone, or co-therapy since day 7 or 14 post-infection for one or two weeks. Abilities of spatial memory and learning of these animals were assessed with the Morris water maze.

Our results showed that body weight was significant higher then infected group in the albendazole and combined therapy groups. Significantly lower worm recovery rates were found in mice treated with the same groups. The mice treated with dexamethasone since day 7 for 14 day had significant longer time in the remaining groups were found in forced swimming test. The animals treated with albendazole and combined therapy since day 7 for 14 days was demonstrated to have significantly shorter latencies to the platform in learning memory on day 3 and 4. Mice in these two groups were demonstrated to have significantly higher sores in spatial memory tests. These results indicate that treatment with albendazole or combined

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therapy may be more efficient in preventing brain damages and depression as well as preserving their capabilities in learning and memory. Therefore, administration of albendazole alone or combined with dexamethasone should have higher efficacies than dexamethasone alone in treatment of BALB/c mice infected with a heavy dose of 50 third-stage larvae of *A. cantonensis*.

Introduction

Angiostrongylus cantonensis is an important zoonotic parasite causing eosinophilic meningitis and/or eosinophilic meningoencepalitis in humans. The disease is acquired by eating raw or uncooked intermediate hosts (snails or slugs), paratenic hosts (crustaceans, frogs, fishes or planarians), or vegetables contaminated with the infective third-stage larvae (L3).[1,2] The L3 then penetrate the small intestine and reach the central nervous system via the bloodstream. The larvae molt twice, develop into the fifth-stage larvae or young adults, and cause a series of pathological and immunological changes in the brain.[3] Severe headaches, fever, nausea, vomiting, neck stiffness, and neurologic abnormalities may persist for weeks to months. The parasite may lead to irreversible neurological damages and even death.[4]

Although administration of albendazole alone was not very suitable for the treatment of cerebral angiostrongyliasis,[5] it has been evaluated co-therapy of albendazole and dexamethasone to be a more effective and safe chemotherapeutic strategy.[6–8] Recently, we employed histopathological and RNA-seq techniques to determine the effects of co-therapy of these two drugs in Th-1 and Th-2 dominant mice infected with *A. cantonensis*. The infected BALB/c and C57BL/6 mice had similar patterns in the pathological changes. Meningitis, hemorrhage, size of worms, and encephalitis in the cerebral parenchyma were slighter in the mice treated with co-therapy than the remaining groups. Mice treated from day 14 had more severe changes than those from day 7. The histopathological findings were found to be consistent to immune responses determined by RNA-seq analysis.[9]

In addition to the clinical manifestations, mice infected with *A. cantonensis* have been reported to have impairments in cognitive functions.[10–12] In our previous study, we have found eosinophilic meningitis occurred in mice infected with *A. cantonensis* from day 12 post-infection and the lesions were then mainly focused in the meninges, the third ventricle, and the hippocampus.[13] Moreover, depression-like behavior was found in the infected BALB/c mice but not in C57BL/6 mice by force swimming test. However, anxiety-like behavior was found to occur only in C57BL/6 mice by open field test. Impaired spatial learning and memory were also found in the two strains of mice which occurred since day 14 post-infection by Morris water maze test.[14] These findings indicate that injuries to the hippocampus should be the cause of impairments in cognitive functions.

The forced swimming test involves placing the test mouse in a transparent acrylic cylinder filled with water. The animal struggles in the water and tries to escape. The will of the mouse to survive becomes weaker with time. Finally it gives up struggling and floats still on the water surface. The non-swimming time is proportional to the depression level.[15] The Morris water maze test is an experimental method for studying learning and memory mechanisms. The principle is to force the mouse to swim and learn to find platforms hidden in the water. It is mainly used to test the learning and memory abilities of mice on spatial position and sense of direction (space and positioning). The place navigation test lasted for several days. The mouse is placed into the water from four entry points facing the pool wall on each day and the time to find the platform hidden under the water is recorded. The spatial probe test is to remove the platform after the positioning navigation test. A water entry point is then chosen to put the mouse in the pool and its swimming trajectory within a certain period of time is recorded to investigate the memory of the original platform.[16,17]

In this study, we employed the force swimming test and Morris water maze test to evaluate different chemotherapeutic strategies (albendazole, dexamethasone, and albendazole-dexamethasone co-therapy) for the treatment of mice infected with *A. cantonensis*. In addition, the effectiveness of administration time (day 7 or day 14 post-infection) and treatment duration (7 or 14 days) were also assessed.

Materials and methods

Parasite and laboratory animals

The parasite employed in the present study was a Taiwan strain of *A. cantonensis* maintained through *Biomphalaria glabrata* snails and Sprague–Dawley (SD) rats since 1980.[18] The rats were used for life-cycle maintenance and 7–8-week-old BALB/c mice were used for experimental studies. Animals were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). These animals were reared in the Laboratory Animal Center of Chang-Gung University. They were kept in plastic cages and provided with food and water ad libitum. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC Approval No.: CGU15-193).

Experimental infection and weight determination

On day 21 post-infection, tissues of the snail hosts were removed and homogenized after shell crushing. The tissue samples were digested with 0.6% (w/v) pepsin-HCl (pH 2–3) for at 37 °C for 1 h.[19] L3 of *A. cantonensis* were removed from the standing precipitations using a medical dropper and counted under a dissecting microscope. Each mouse was inoculated with 50 L3 by stomach intubation. Throughout the experimental studies, each mouse was weighed daily.

Drug administration

From day 7 or 14 post-infection, each mouse received albendazole (Sigma–Aldrich, St. Louis, MO, USA) (10 mg/kg/day) alone, dexamethasone (Xindong Biotech, Taiwan) (0.5 mg/kg/day) alone, or co-therapy of the two drugs for 7 or 14 days. The mice in the treatment groups included the early-short group (from day 7 for 7 days), early-long group (from day 7 for 14 days) and late-short group (from day 14 for 7 days). Albendazole was administered by stomach intubation and dexamethasone by intraperitoneal injection.

Forced swimming test

The level of depression was evaluated by the forced swim test.[20] The test was carried out on days 7, 14 and 21 post-infection. There were 10 mice in each of the control group, infection groups and treatment groups. A total of 130 mice were used in this experiment. The tested mouse is placed in a transparent cylindrical tank (45 cm height $\times$ 10 cm radius) and filled with water (25 $\pm$ 1 °C) at a level of 15 cm from the bottom for 6 min. The duration that the mouse does not swim in the last 4 min is recorded.

Morris water maze test

Spatial learning and memory was assessed by the Morris water maze test.[21] There were 10 mice in each of the control group, infection groups and treatment groups. A total of 130 mice were used in this experiment. The mice were trained on days 3–6, 10–13 and 17–20 post-infection, and tests performed on days 7, 14 and 21 post-infection. The custom-made maze was a circular pool 120 cm in diameter and 40 cm in height, filled with water at 25 $\pm$ 1 °C and made opaque by adding milk. In the acquisition session, each mouse was given four trials per day and four days of training in total to find a hidden platform located 1.5 cm below the water surface. Each mouse was placed into the pool, facing the wall, with a different starting point for each trial that the direct route to the platform differed each time. The time required by the mouse to find and stand on the platform was recorded for up to 90 s. The mouse was allowed to stay on the platform for 30 s. It was then removed from the maze and placed into its cage. For the mouse that found the platform within 90 s, the animal was placed on the platform for 30 s. The inter-trial interval was at least 30 min. In the probe session, on day 5, the platform was removed from the pool. The mouse was tested in a probe trial for 60 s. Mouse swimming tracts were recorded using a TopScan automated tracking system (Clever Sys Inc., Virginia, USA).

Determination of worm recovery

The infected mice were sacrificed by inhalation of 3% (v/v) isoflurane (Panion & BF Biotech Inc., Taipei, Taiwan). Brains were removed from the cranial cavity. Worm recovery rates were then determined by counting the number worms under a dissecting microscope.

Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SE). Differences between groups were analyzed by one-way ANOVA, two-way ANOVA, or two-way repeated measure ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Worm recovery rates

The recovery rate in the infected mice treated with albendazole from day 7 for 7 days (early-short) was not significantly different from that in the untreated group ($P > 0.05$) (Fig. 1a). The rates in the infected mice treated with the drug from day 7 for 14 days (early-long) or from day 14 for 7 days (late-short) were significantly lower than the untreated group ($P < 0.001$) ((Fig. 1b). However, there were no significant differences among the rates of the dexamethasone treated groups and the untreated mice ($P > 0.05$) (Fig. 1c and d). In the co-therapy groups, the recovery rate of early-short group was significantly lower than the untreated group ($P < 0.01$) (Fig. 1e). The rates in the co-therapy early-long group and late-short group were significantly lower than the untreated group ($P < 0.001$). However, the rate in the early-long group was significantly higher than that in the late-short group ($P < 0.05$) (Fig. 1f).

Changes in weight

No significant difference was found in the weight of albendazole early-short group and the untreated group ($P > 0.05$) (Fig. 2a). The weight in the early-long group became significantly higher than the untreated group on day 17 post-infection ($P < 0.05$) (Fig. 2b) and that in the albendazole late-short group became significantly higher than the untreated mice on day 20 ($P < 0.05$) (Fig. 2c). However, no significant differences in weight of mice treated with all dexamethasone treated groups and the untreated groups ($P > 0.05$) (Fig. 2d, e, and f). Although there was no significant difference between the weights in the co-therapy early-short group and the untreated group ($P > 0.05$) (Fig. 2g), those in the early-long and later-short groups was significantly higher than the untreated groups ($P < 0.05$) (Fig. 2h and i).

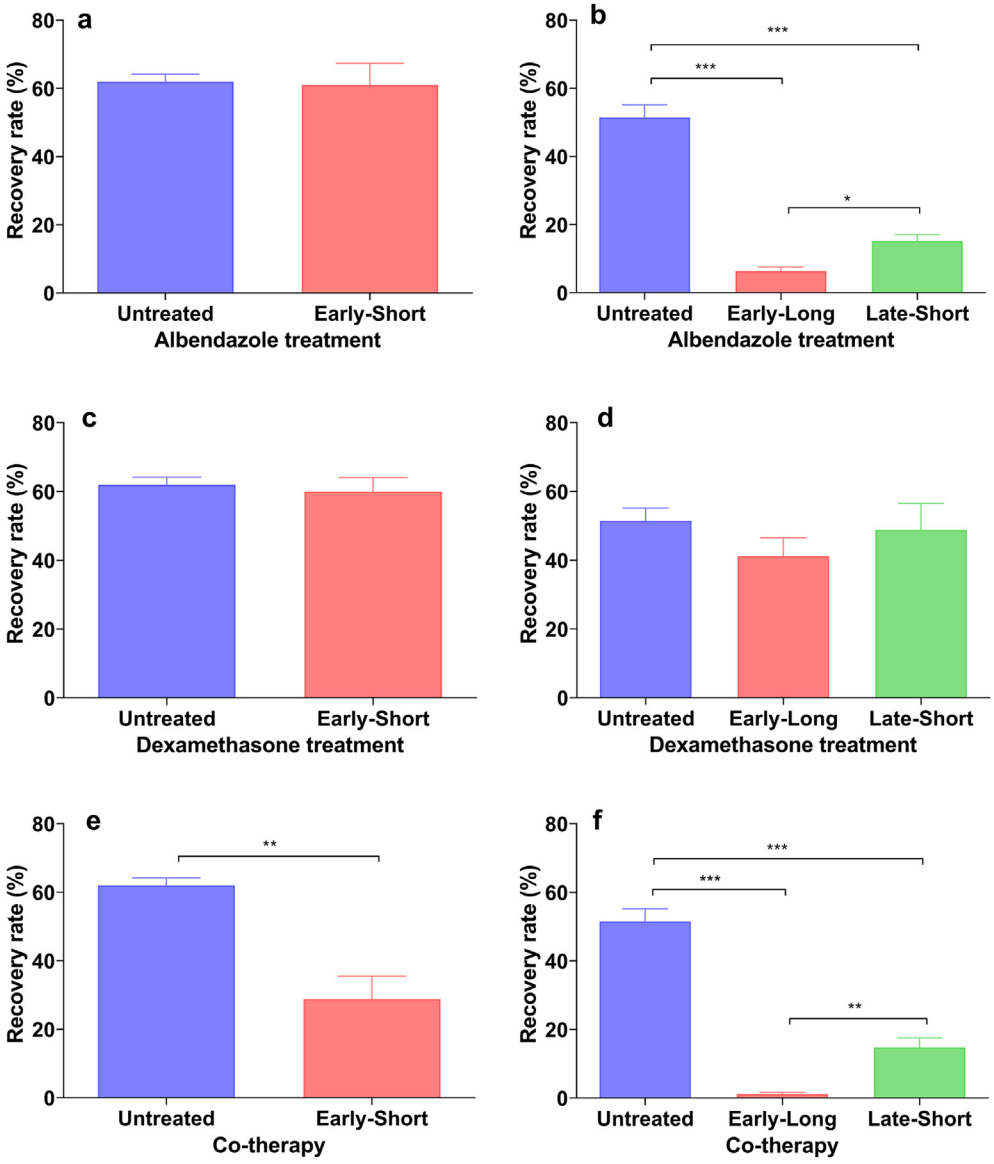

Figure 1. Worm recovery rates in BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, c, e) and from day 7 for 14 days (early-long) and from day 14 for 7 days (late-short) (b, d, f). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The data were analyzed by one-way ANOVA.

Level of depression

No depression was found in the groups treated with albendazole (Fig. 3a and b) and the co-therapy group (Fig. 3e and f) as well as dexamethasone early-short and late short groups (Fig. 3c and d). However, the non-swimming time was higher in the dexamethasone early-long group than the non-infected mice and the untreated group ($P < 0.05$) (Fig. 3h).

Changes in spatial learning

There was no significant difference in the escape latency to the platform between the albendazole early-short group and the untreated group ($P > 0.05$) (Fig. 4a). In the early-long or late-short groups, the escape latencies became significantly lower than those in the untreated group on day 3 of training ($P < 0.05$) (Fig. 4b and c). In the mice treated with dexamethasone, the early-short group had significantly longer escape latency than the untreated mice ($P < 0.05$) (Fig. 4d). The early-long group had longer escape latency on day 4 of training ($P < 0.05$) (Fig. 4e). There was no significant difference between the late-short group and the untreated mice ($P > 0.05$) (Fig. 4f). For the co-therapy mice, no significant difference was found in the escape latency between the early-short group and the untreated group ($P < 0.05$) (Fig. 4g). The early-long group had longer escape latency than the untreated mice after day 3 of training ($P < 0.001$) (Fig. 4h). The late-short group had significantly shorter escape latency than the untreated mice on day 4 of training ($P < 0.05$) (Fig. 4i).

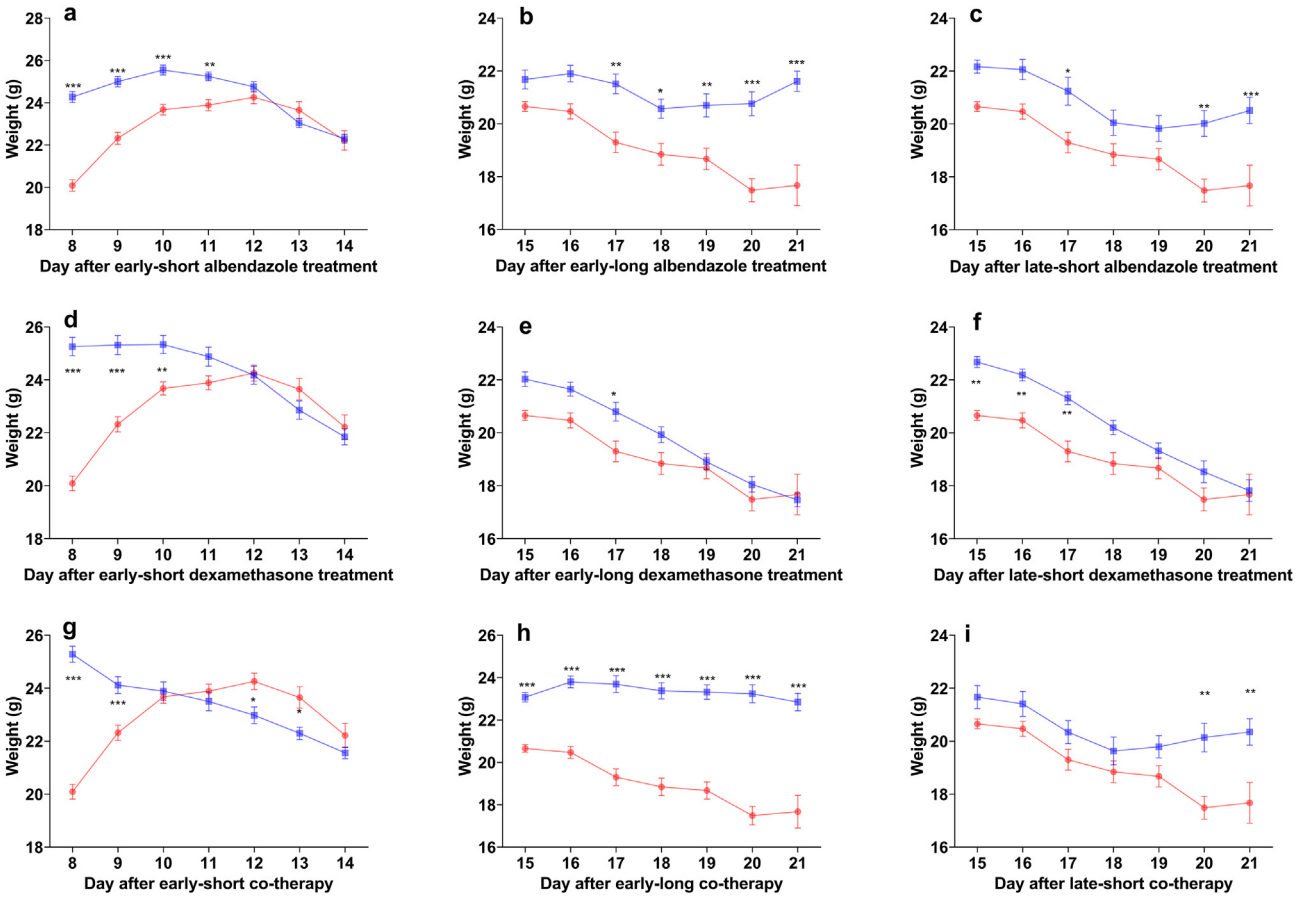

Figure 2. Weight in BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, d, g) and from day 7 for 14 days (early-long) (b, e, h) and from day 14 for 7 days (late-short) (c, f, i). Blue: mice treated, red: mice untreated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The data were analyzed by two-way repeated measure ANOVA.

Changes in spatial memory

The space exploration test is to remove the water maze platform 24 h after the positioning and navigation test. The first part is to observe the time required for the mouse to swim to the target quadrant, and to evaluate the spatial memory ability of the mice after infection with *A. cantonensis*. There was no significant difference in the time required for the mice to swim to the target quadrant between the albendazole early-short group and the untreated group ($P > 0.05$) (Fig. 5a). However, the early-long and late-short groups required significantly longer time to swim to the target quadrant ($P < 0.001$) (Fig. 5b). There was no significant difference in the time required to swim to the target quadrant between the dexamethasone treated mice and the infected group ($P > 0.05$) (Fig. 5c and d). In the co-therapy groups, there was no significant difference in the time required to swim to the target quadrant between the early-short group and infected group ($P < 0.05$) (Fig. 5e). In the early-long and late-short groups, the mice required significantly shorter time to swim to the target quadrant than the infected group ($P < 0.001$) (Fig. 5f).

The second part of the space exploration test is to remove the water maze platform 24 h after the positioning and navigation test and to observe the time that the mouse stays in the target quadrant and evaluate the spatial memory ability. No significant difference in the time of staying in the target quadrant was found between the albendazole early-short and the untreated groups ($P > 0.05$) (Fig. 6a) whereas the early-long and late-short groups stayed in the target quadrant were significantly longer than uninfected mice ($P < 0.001$) (Fig. 6b). In the dexamethasone treated groups, no significant difference was found in the time stayed in the target quadrant between the treated and untreated mice ($P > 0.05$) (Fig. 6c and d). Although no significant difference in the time of staying in the target quadrant was found between the co-therapy early-short and the untreated groups ($P > 0.05$) (Fig. 6e), the early-long and late-short groups stayed in the target quadrant were significantly longer than uninfected mice ($P < 0.001$) (Fig. 6b).

Discussion

Parasitic infections have been documented to have significant effects on the behavior of the host. These behavior changes may facilitate of the spread of the parasites and the maintenance of life history. Rats infected with *Toxoplasma gondii* lose their nature of hiding when they see

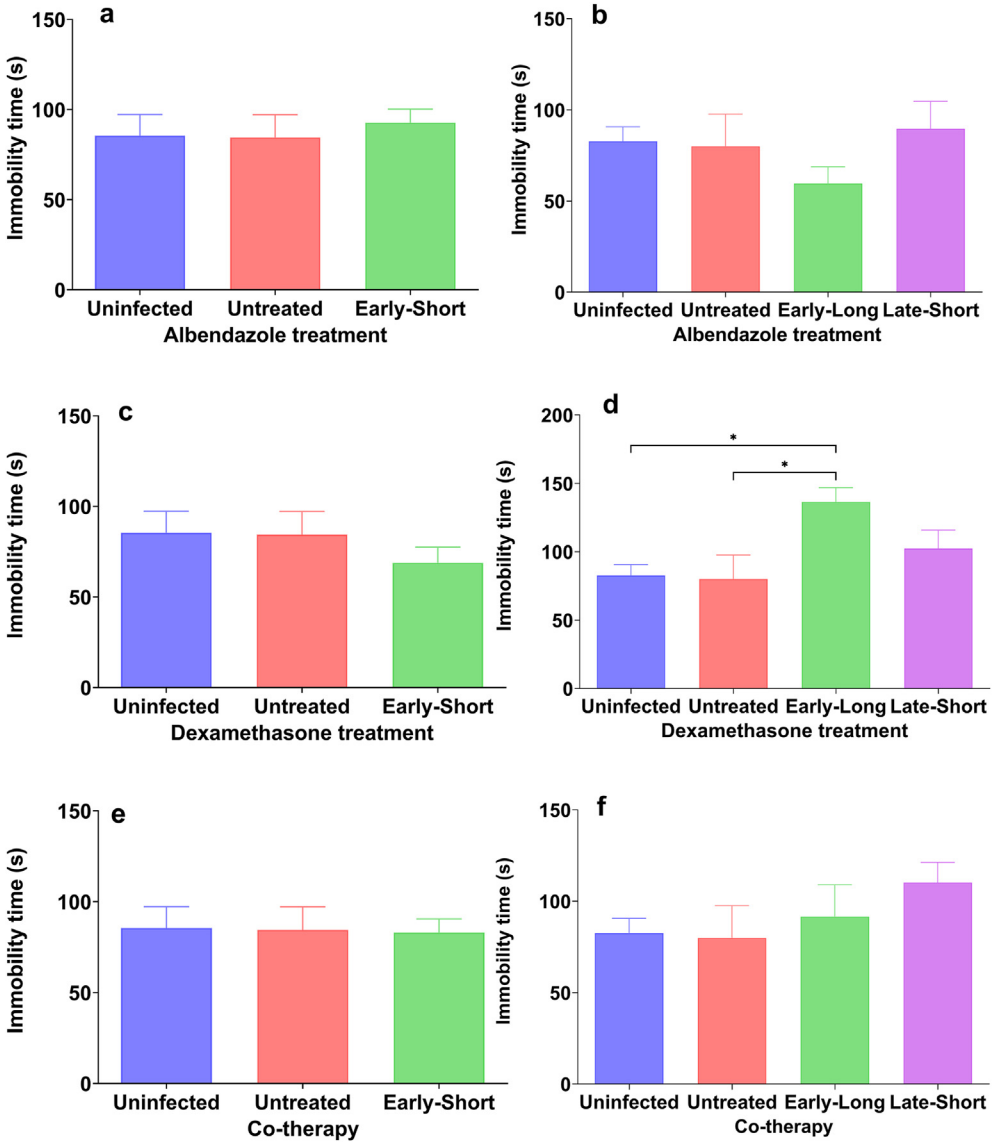

Figure 3. Level of depression in BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, c, e) and from day 7 for 14 days (early-long) and from day 14 for 7 days (late-short) (b, d, f). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data were analyzed by one-way ANOVA.

cats. Moreover, they are attracted by the smelling of cats and to be preyed. This phenomenon makes *T. gondii* more likely to be transferred between the final host (cats) and the intermediate hosts (rats).[22] Cockroaches (*Rutilus rutilus* L.) infected with the tapeworm *Ligula intestinalis* prefer to move on the shore. This behavior change increases the chance of being preyed by birds.[23] After infected with the tapeworm *Schistocephalus solidus* the three-spine fish *Gasterosteus aculeatus* lose the ability to escape from the attack of the birds. This behavioral change drives the entire fish school to move in the shallow danger zone vulnerable to bird attacks.[24]

In addition to changes in behavior in infected animals, memory of the hosts may also be affected. After SD rats infected with *T. gondii*, spatial memory and learning ability of the infected group was found to be significantly lower than the uninfected group by the Morris water maze.[25] Injection of herpes simplex virus type 1 (HSV1) into the olfactory bulb of rats to causes a negative correlation of spatial memory or learning ability to the damage of the olfactory bulb by the water maze test.[26] Spatial memory and learning ability of C57BL/6 mice infected with *Legionella pneumophila* were found to be significantly decreased with a water maze. However, significant improvement in memory and learning was recognized after administration of anti-IL-1β antibody to neutralize IL-1β in the body. These changes suggest inflammation may be a factor in the decline of spatial memory.[27] Significant decreases in spatial memory and learning ability have also been demonstrated in experimental meningitis in the right forebrain of C57BL/6 mice by *Streptococcus pneumoniae*.[28] Inoculation of H1N1 virus to the nasal cavity of BALB/c mice

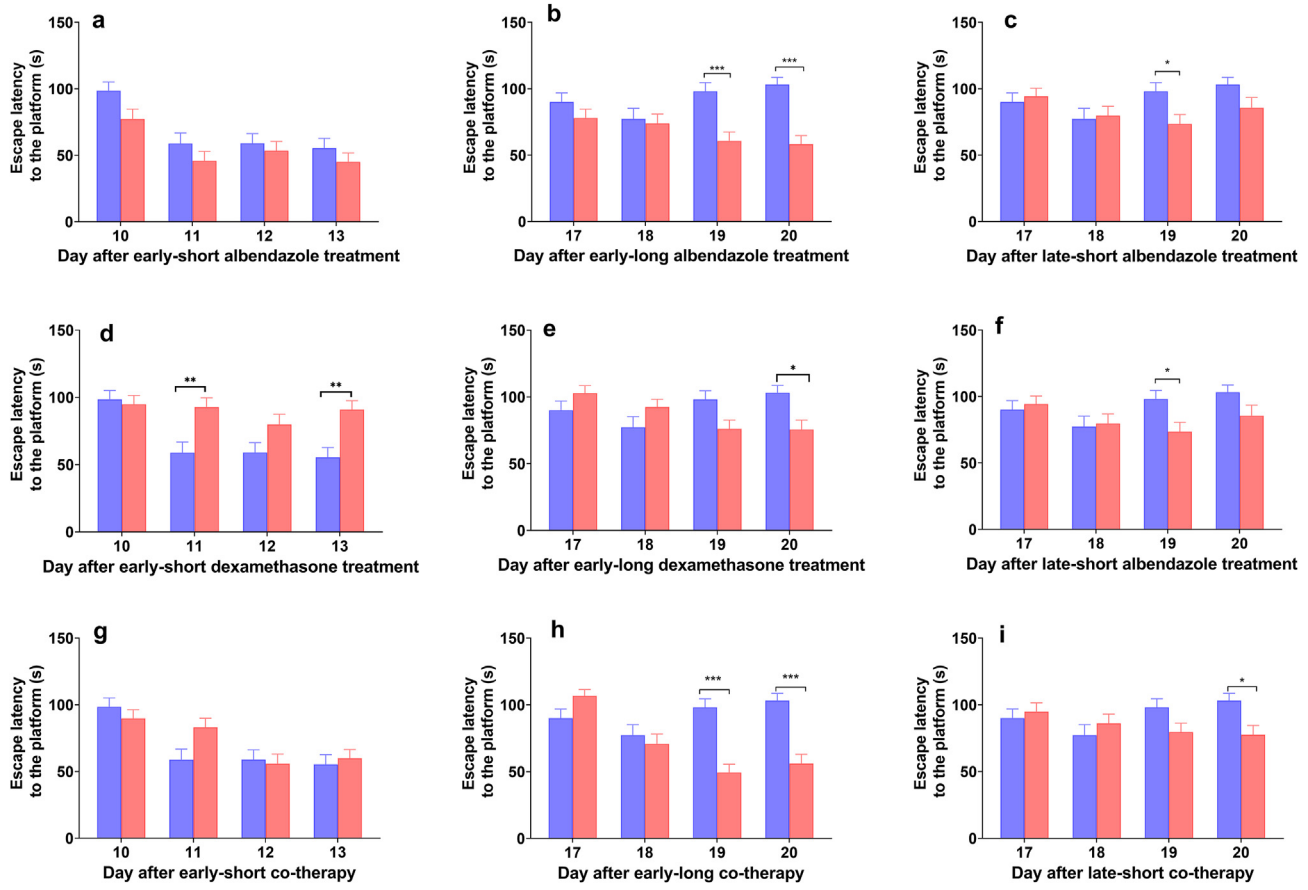

Figure 4. Changes in spatial learning in BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, d, g) and from day 7 for 14 days (early-long) (b, e, h) and from day 14 for 7 days (late-short) (c, f, i). Blue: mice treated, red: mice untreated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data were analyzed by two-way ANOVA.

not only causes neuritis and hippocampal gyrus inflammation but also impairments in the spatial memory and learning ability.[29]

BALB/c mice infected with *A. cantonensis* have been reported that larvae migrated to the brain and caused meningitis and other pathological changes.[13] The infection also causes cognitive impairments in BALB/c and C57BL/6 mice.[14] However, changes in immune status may also affect the cognitive functions learning. C57BL/6 mice with IL-2 deletion were found to have spatial memory and learning ability degradation.[30] Knocked out mice without the IL-1 receptor type I gene required longer time to find the platform in a water maze test.[31] C57BL/6 mice lacking CD4+ T cell in the hippocampal gyrus became stunted their spatial memory and learning abilities were significantly reduced.[32] Impairments of spatial memory and learning ability were also demonstrated in mice with IL-6 deletion.[33] However, knocking out the CD226 gene in C57BL/6 mice improves cognitive functions and ameliorates anxiety-like behaviors[34]

Brain damages may be reduced through some therapeutic manipulations. These treatments have been reported to improve the degraded spatial memory and learning. Feeding of *Lactobacillus helveticus* to rats with ammonium acetate induced chronic hyperammonemia and encephalitis was effective in elevating cognitive functions and reducing anxiety behavior.[34] Administering the emotional stabilizer LiCl to young rats inoculated with *S. pneumoniae* not only prevented apoptosis and reduced damages in hippocampus but also improve spatial memory and learning.[35] In addition, icariin has been reported to be effective in treating mice with traumatic brain injuries via enhancing hippocampal acetylation.[36]

After infection with *A. cantonensis*, the weight and appetite of BALB/c mice were found to be significantly decreased.[37] In this study, we also found the weight of the infected mice was significantly lower than that of the uninfected ones (Fig. 2), especially day 21 post-infection. In the early-long albendazole treated group (Fig. 2), the weight of the mice was higher than that of the untreated infection group and the differences was significant in the last 5 days. These findings were not observed in the late-short albendazole treated group (Fig. 2) showing that early administration of anthelmintics was more effective. The weight of mice in each group treated with the co-therapy groups showed similar results to those treated with albendazole alone. However, the weight improvement in the early-long co-therapy groups was significantly higher

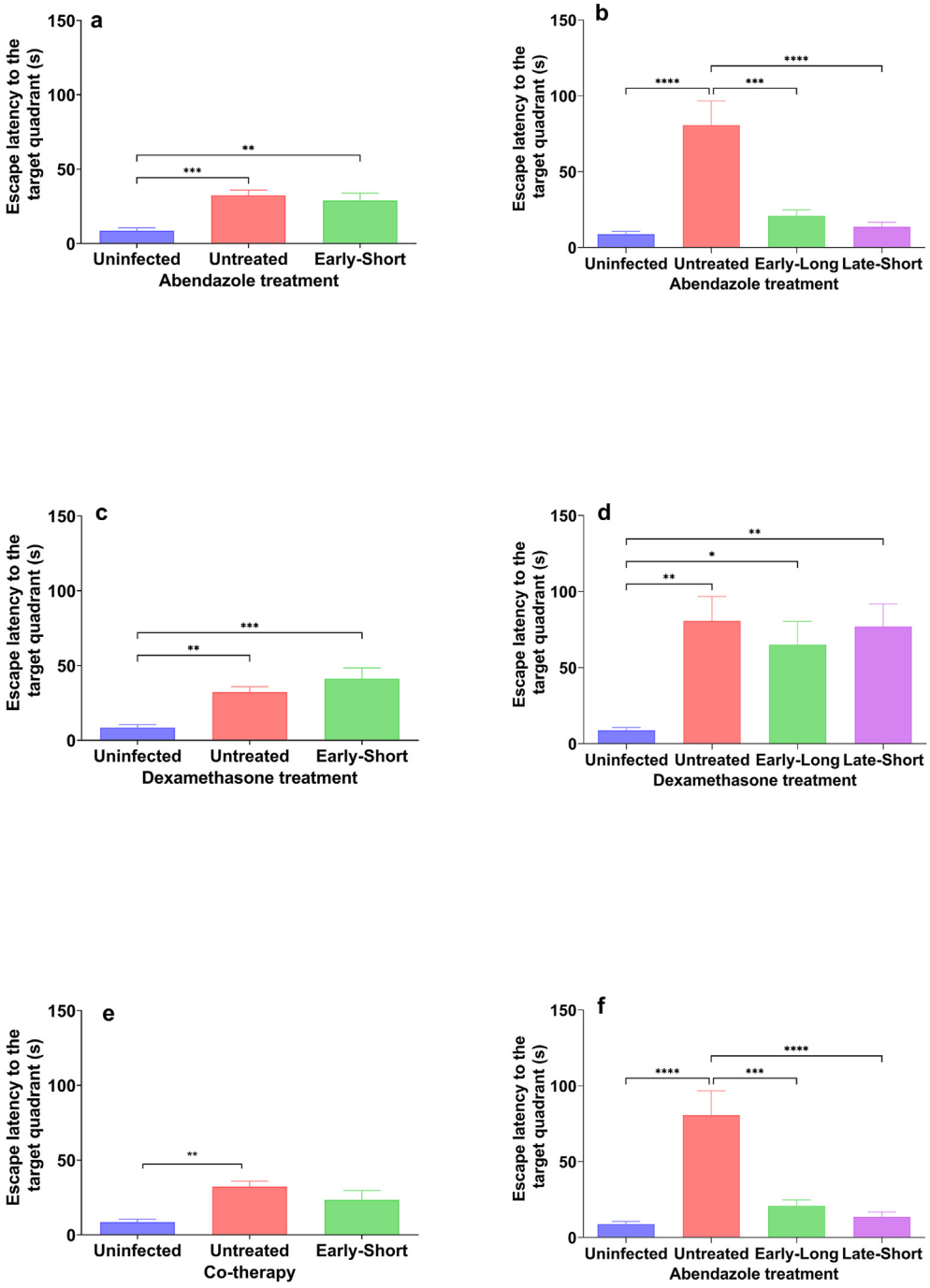

Figure 5. Escape latency to the target quadrant in the Morris water maze test among BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, c, e) and from day 7 for 14 days (early-long) and from day 14 for 7 days (late-short) (b, d, f). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data were analyzed by one-way ANOVA.

than the untreated mice. These results suggest that therapeutic effects of co-therapy are superior than albendazole alone in weight improvement.

The hippocampal gyrus has been report to be associated with spatial learning and memory. Rats with hippocampal damages required a prolong duration to find the test platform in the Morris water test.[38] Moreover, hippocampal gyrus injury has been related to the occurrence of depression[39] and the duration of depression is directly proportional to the volume of hippocampal gyrus injury.[40] Prior to the water maze test in the present study, the BALB/c mice were given a forced swimming test to assess whether the mice were depressed after infected with *A. cantonensis*. The results showed that depression was not revealed in the infected mice administered with the albendazole alone and co-therapy groups, no significant

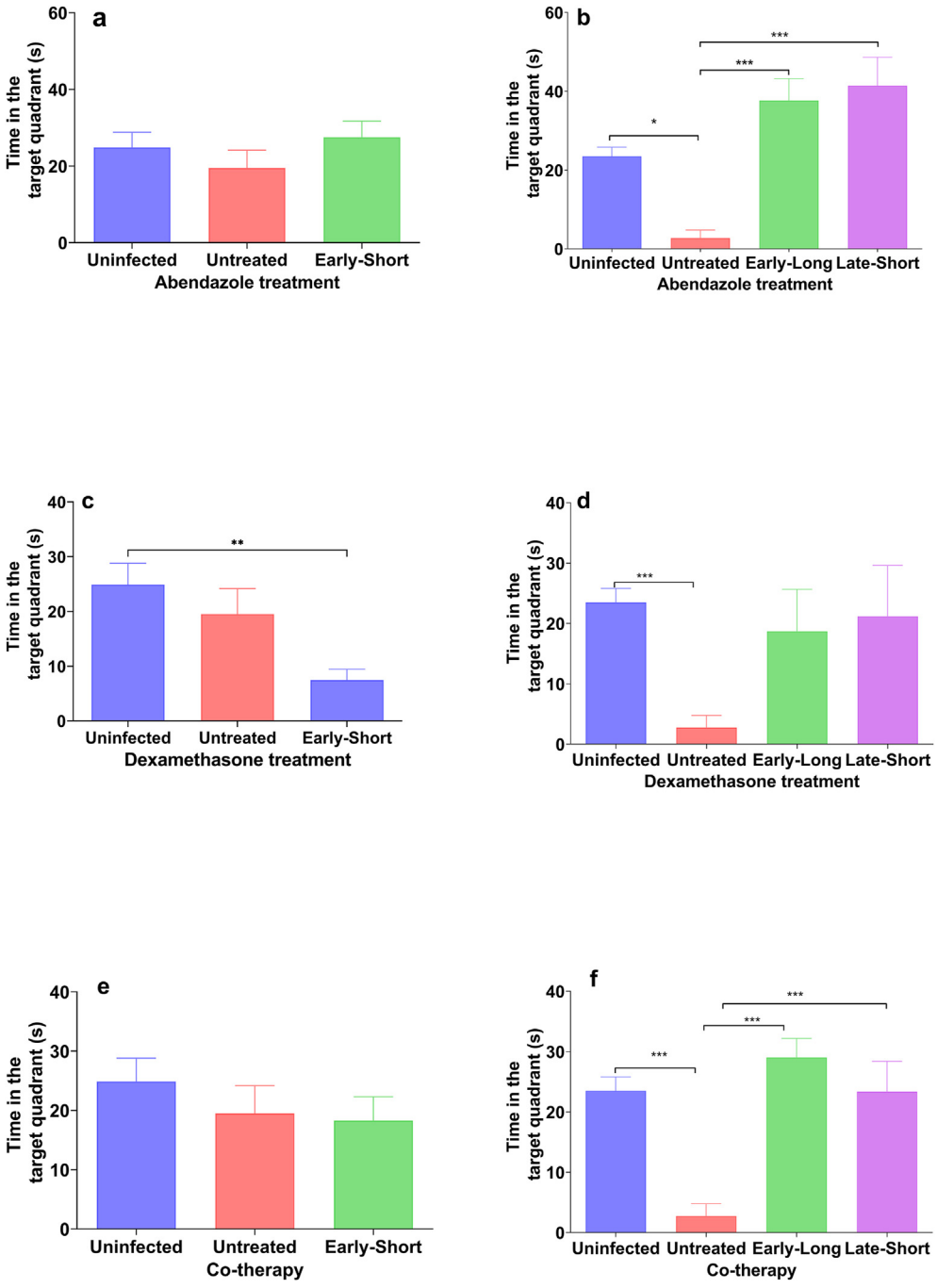

Figure 6. Time in the target quadrant in the Morris water maze test among BALB/c mice infected with *Angiostronglus cantonensis* and treated with albendazole, dexamethasone, and co-therapy of the two drugs from day 7 for 7 days (early-short) (a, c, e) and from day 7 for 14 days (early-long) and from day 14 for 7 days (late-short) (b, d, f). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data were analyzed by one-way ANOVA.

difference was found between the treated groups and the infected controls (Fig. 3). However, the non-swimming time in the dexamethasone early-long group was found to be higher than the non-infected mice and the untreated group indicating depression occurred (Fig. 3h). In humans, depression is often accompanied by dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis. The end products of this axis, glucocorticoids, are involved in the formation of many physiological and behavioral responses to stress.[41,42] Qin et al. indicated that chronic glucocorticoid exposure could disturb HPA axis reactivity, which eventually led monkeys to display depression-like phenotype.[43] The cause of depression may be due to the administration of the anti-inflammatory drug dexamethasone.

In the positioning navigation test, the infected mice with *A. cantonensis* spent more time on the platform than the untreated group, suggesting that the learning and memory ability of the mice was reduced after infection. The time for mice in each group of albendazole alone and co-therapy groups to reach the platform was significantly shortened. And the effect of the early-long groups was more obvious. Moreover, the mice in receiving these two therapeutic strategies require significantly less time to reach the platform after the day 3 of training than the untreated group, indicating that the learning and memory abilities of the mice were improved after treatment.

The space exploration experiment is divided into two parts, detecting the time required for the mice to reach the target quadrant and the time spent in the target quadrant. The results of this study showed that untreated mice after infection spend significantly longer time to reach the target quadrant than the untreated group, suggested that the spatial memory ability was significantly reduced. In the untreated mice, the time for staying in the target quadrant was significantly shorter than uninfected ones. This result indicates that the spatial memory ability of BALB/c mice was significantly reduced after infection. After receiving albendazole alone and co-therapy, the mice in the early-long and late-short groups stayed in the target quadrant significantly longer than the untreated group. These findings suggested the effectiveness of these therapeutic strategies in restoring the impaired spatial memory ability in the infected mice.

In our previous study, analysis of brain histopathology in infected mice found that albendazole alone and co-therapy can kill the larvae of *A. cantonensis* in the brain; dexamethasone alone cannot eliminate the larvae, but it can effectively reduce brain inflammation.[9] Based on the above, the treatment strategies of killing larvae may have better neuroprotective effect.

Conclusion

Co-therapy of corticosteroid and albendazole has been reported to be safe and may get rid of some symptoms such as headaches. Simultaneous administrations of albendazole and dexamethasone will increase the blood concentration of albendazole. Co-therapy of these two drugs can reduce the clinical symptoms of patients. Recently, we have reported that co-therapy of albendazole and dexamethasone significantly reduces pathological changes in the cerebral parenchyma of Th-1 and Th-2 dominant mice heavily infected with *A. cantonensis*. Based on the results of this study, co-therapy albendazole and dexamethasone should be a better strategy for the treatment of *A. cantonensis* in mice and to improve cognitive functions in mice heavily infected *Angiostrongylus cantonensis*.

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Declaration of competing interest

The author declares that there is no conflict of interest.

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Appendix A. Supplementary data

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