

ORIGINAL ARTICLE

EVALUATION OF THE THERAPEUTIC EFFICACY OF LEVAMISOLE HYDROCHLORIDE ON THIRD-STAGE LARVAE OF *Lagochilascaris minor* IN EXPERIMENTALLY INFECTED MICE

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SUMMARY

Lagochilascariosis, a disease caused by *Lagochilascaris minor*, affects the neck, sinuses, tonsils, lungs, the sacral region, dental alveoli, eyeballs and the central nervous system of humans. A cycle of autoinfection may occur in human host tissues characterized by the presence of eggs, larvae and adult worms. This peculiarity of the cycle hinders therapy, since there are no drugs that exhibit ovicidal, larvicidal and vermifugal activity. Given these facts, we studied the action of levamisole hydrochloride on third-stage larvae in the migration phase (G1) and on encysted larvae (G3) of *L. minor*. To this end, 87 inbred mice of the C57BL/6 strain were divided into test groups comprising 67 animals (G1-37; G3-30) and a control group (G2-10; G4-10) with 20 animals. Each animal was inoculated orally with 2,000 infective eggs of the parasite. The animals of the test groups were treated individually with a single oral dose of levamisole hydrochloride at a concentration of 0.075 mg. The drug was administered either 30 minutes prior to the parasite inoculation (G1 animals) or 120 days after the inoculation (G3 animals). The mice in the control groups were not treated with the drug. After the time required for the migration and the encysting of *L. minor* larvae, all the animals were euthanized and their tissues examined. The data were analyzed using the Student's unpaired *t*-test and the Levene test. The groups showed no statistically significant difference. Levamisole hydrochloride was ineffective on third-stage larvae of *L. minor*. These findings explain the massive expulsion of live adult worms, as well as the use of long treatment schemes, owing to the persistence of larvae and eggs in human parasitic lesions.

KEYWORDS: Levamisole; *Lagochilascaris*; Third-stage larvae; Infected mice.

INTRODUCTION

Human lagochilascariosis, whose etiological agent is *Lagochilascaris minor* (Leiper, 1909)¹ is a disease that occurs in the Neotropics. Among the five species of the genus that are described, *L. minor* is the only one associated with human infections^{2,3}.

Lagochilascariosis has been found in Brazil, Colombia, Venezuela, Mexico, Costa Rica, Trinidad and Tobago, Suriname, Bolivia and Paraguay^{1,4,5,6,7,8,9,10,11,12,13,14}. Human infections are caused by eating raw or undercooked meat of wild rodents which are naturally infected by the parasite^{15,16,17}.

The symptoms and severity of the disease depend on the affected organ, the parasite load, the parasite's reproductive capacity, the degree of tissue invasion, and the immune response of the human host^{4,5}. Lagochilascariosis causes a foreign body granulomatous reaction^{18,19}. The lesions consist of numerous abscesses interconnected by fistulous tracts, enveloped by a granulation tissue, giant multinucleated cells and areas

of dense fibrosis formation^{4,18,19}. The proteolytic enzymes of *L. minor* can facilitate its migration to the host's tissue by means of hydrolyzing collagens of the extracellular matrix²⁰.

The onset of the disease is usually insidious, presenting a chronic evolution with periods of remission and relapses, sometimes leading to death²¹. The parasite has been found in different parts of the body, e.g., the central nervous system, lungs, sacrum, dental alveoli, paranasal sinuses, eyeballs, cervical spine, and the parietal-temporal-occipital region^{1,4,5,21,22,23,24,25,26,27,28,29,30}. Clinical symptoms of tonsillitis, rhinitis, otitis and mastoiditis have been reported. In the central nervous system (CNS), there have been reports of sudden headache, neck stiffness, tetraparesis, meningeal inflammation and death, as well as seizures, paresis and mental confusion²¹. In cases of pulmonary involvement, fever, dyspnea, cyanosis, respiratory failure and death have been reported³⁰.

Various drugs have been used in the treatment of human lagochilascariosis, including methylpiperazine citrate, methylpiperazine hydrochloride, ivermectin, praziquantel, benzimidazole derivatives such

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as mebendazole, thiabendazole, levamisole, albendazole and cambendazole^{4,5,14,23,24,25,26,27,28,31}.

The parasite is able to multiply in the tissues through a cycle of self-infection^{4,5,15}, which is why adult worms, larvae, eggs at different stages of segmentation, including embryonated eggs, can be found in the sites of lesions¹⁵. This characteristic of the cycle complicates the treatment of the disease, since a drug would have to be effective against all the stages of the cycle^{4,5,15}.

Levamisole, which is indicated for the treatment of ascariasis, has been used to treat human lagochilascariosis in different schemes, with conflicting results. Cured cases have been reported²⁷ as well as the recurrence of disease after prolonged therapeutic schemes had been discontinued^{4,5,23,24}. The use of lengthy therapies does not mean that patients infected by human lagochilascariosis will be cured^{4,5,23,24}.

Although levamisole hydrochloride is widely used in the treatment of human lagochilascariosis, there are no experimental studies of this drug on *L. minor*. These facts motivated us to evaluate the action of this anthelmintic on third-stage larvae of *L. minor* in experimentally infected mice.

MATERIALS AND METHODS

Culture of parasite eggs

L. minor eggs were obtained from feces of experimentally infected cats. Feces samples were subjected to the spontaneous sedimentation method. The sediment was stored at room temperature in sedimentation tubes containing 1% formalin solution, and the material was oxygenated daily by stirring. This egg suspension was kept in these conditions for 40 days, which is the period of time required for the development of third-stage larvae^{4,15,16,17,19,20}. After 40 days, the suspension containing motile and viable larvae was centrifuged for 2 minutes, at 1,500 rotations per minute. This process was repeated three times, until a clean supernatant free of fecal debris was obtained.

Animals

The experiment involved 87 mice of the C57BL/6 isogenic strain, which were supplied by the Vivarium of the Department of Parasitology of the Institute of Tropical Pathology and Public Health, Federal University of Goiás and transferred to the Vivarium of the University Center of Anápolis. The animals were divided into two test groups (G1 - 37 animals; G3 - 30 animals) and two control groups (G2 - 10 animals; G4 - 10 animals). The sample size was defined based on Barbosa *et al.* 1998³².

Therapeutic scheme and inoculation of the mice

Each animal in groups G1, G2, G3 and G4 was inoculated orally, by gavage, with 2,000 infective eggs of *L. minor*.

To evaluate the efficacy of levamisole hydrochloride on migratory larvae, and in view of the plasma concentration of the anthelmintic, the animals of the G1 group (37) were treated individually with levamisole hydrochloride (Janssen-Cilag Farmacêutica Ltda)³³ at a concentration of 0.075 mg administered in a single oral dose 30 minutes prior to the inoculation of parasites.

Considering the data on the life cycle of *L. minor*, each animal in group G3 (30) was treated with levamisole hydrochloride for 120 days after inoculation to verify the drug's effect on encysted larvae.

The concentration of the drug was defined according to the established dose of 150 mg for humans³³ (considering the average of weight approximately 60 kg), and the weight of each mouse as 30 g.

The mice of the control groups (G2 and G4) were inoculated with the parasite but they were not treated with levamisole hydrochloride.

The animals were examined three times a week until necropsy.

Necropsy

The mice were euthanized by cervical dislocation. The abdominal, thoracic and oral cavities, as well as the head and neck were exposed^{4,15,16,17,19,20,32}. To search for larvae, the tissues were examined under an entomological microscope.

To evaluate the effect of the drug on migratory larvae, the animals of the G1 group were necropsied starting 60 days after inoculation and treatment with the anthelmintic, corresponding to the amount of time required by larvae to migrate and encyst^{4,5,6,7,13,15,29,33}. The animals of the G2 group were euthanized, starting 150 days after inoculation, or 30 days after treatment^{4,15,16}.

The animals of the control groups underwent a similar procedure.

Evaluation of drug efficacy

The data were analyzed using the Student's unpaired *t*-test and the Levene test, the former to check if there was any significant difference in the number of larvae between the controls and test groups, and the latter to confirm the assumption of homogeneity of variance³⁴.

Ethics committee

This project was examined and approved by the Ethics Committee for Animal Research of UFG, under the protocol nº 064/14.

RESULTS

The presence of third-stage larvae was observed inside nodules distributed in skeletal muscles and subcutaneous tissues of all the mice inoculated with infective eggs of *L. minor* (group G1), which were treated with a single dose of 0.075 mg of levamisole hydrochloride and necropsied 60 days after inoculation (DAI). The animals of the test group G3 and the control groups, G2 and G4, showed similar results. These data are described in Tables 1, 2 and 3.

No significant difference was found in the average number of nodules (larvae) recovered from the animals of the test and control groups to assess the effect of the drug on migratory and encysted larvae.

There was no mortality among the animals of this study.

The nodules containing larvae were opened with a scalpel and the

Table 1

Effect of levamisole hydrochloride on migratory larvae (G1) and encysted larvae (G3) of *Lagochilascaris minor*. Total and average number of nodules recovered from mice in the test groups and in the control groups inoculated with 2,000 infective eggs of the parasite

Group		n (mice)	Total number of nodules (larvae)	Average
Test	G1	37	10,772	291
	G3	30	9,240	308
Control	G2	10	2,198	220
	G4	10	2,100	210

G1 = migratory larvae; G3 = encysted larvae; G2 = control group; G4 = control group; n = number of mice.

larvae were alive, showing the serpentine motility that is characteristic of viable nematode larvae.

DISCUSSION

Neglected tropical diseases (NTDs) are a group of chronic debilitating diseases caused by viral, bacterial, fungal microorganisms and also by parasites associated with poor living conditions in developing countries³⁵. These diseases are known since ancient times¹¹. Their impact on human health and productivity has contributed in the past to build up a considerable body of knowledge about them. The transmission of these diseases has been significantly reduced by improvements to the living conditions of some populations. However, NTDs are still highly prevalent in social groups that live in poverty³⁶. It is estimated that one third of the 213 million inhabitants of Latin America and the Caribbean live in poor rural areas, surviving on subsistent farming or fishing, or living in slums on the outskirts of urban or peri-urban areas, without access to treated water, without adequate sanitation, and living with rodents and arthropod vectors³⁶.

Lagochilascariosis, an infection caused by a helminth, a nematode of the genus *Lagochilascaris*, is not listed among the neglected diseases, but it fits this description perfectly, affecting people that live in precarious conditions in wild environments, eating raw or undercooked wild rodents as food, and it is known that these rodents are naturally infected by the parasite^{4,15,16,17,19,20}. As with other neglected diseases, the drugs available to treat lagochilascariosis are very old.

Pharmaceutical companies have invested only scanty financial resources in this area¹¹. Several anti-helminthic drugs as diethylcarbamazine, praziquantel, and benzimidazole derivatives such as levamisole, cambendazole, mebendazole, albendazole, thiabendazole and ivermectin are used in the treatment of human lagochilascariosis^{4,5,14,23,24,25,26,27,28,31}.

Lagochilascariosis has reportedly been cured with a dose of 150 mg/day of levamisole for three consecutive days¹⁷. However, relapse of the disease has been reported in patients treated with levamisole using the same therapeutic scheme^{4,5,23,24}.

Good results were obtained by the association of cambendazole and levamisole⁵. Used alone or in combination, these drugs have a visible impact on the parasites, which quickly abandon the lesions in large numbers, above all on the first day of treatment, resulting in a rapid clinical improvement^{4,5,15}. However, notwithstanding the use of different dosage schemes, therapeutic combinations, and long-term treatment with levamisole hydrochloride, human lagochilascariosis is evidently difficult to cure with this drug. Hundreds of worms are expelled after administering the initial doses of the drug, followed by healing of the lesions, a phenomenon that seems to indicate that the disease has been cured. However, if patients continue to be monitored, one will find out that relapses after the apparent cure are frequent^{4,5,23}.

The use of levamisole associated with ivermectin and albendazole resulted in a marked improvement of the clinical status of lagochilascariosis, followed by recurrence of the disease, requiring the prescription of long-

Table 2

Effect of levamisole hydrochloride on migratory larvae (G1) of *Lagochilascaris minor*. Analysis of the results obtained by the T-test and the Levene test for the group treated with 0.075 mg of levamisole hydrochloride, 30 min prior to inoculation with 2,000 infective eggs of the parasite. The control group was composed of mice inoculated with the parasite but not treated with the anthelmintic

Group		n (mice)	σ	Difference	t	df	p
Test	G1	37	252	- 71	- 0.780	45	0.439
Control	G2	10	273				

G1 = migratory larvae; G2 = control group; n = number of mice; σ = standard deviation; t = t-test; df = degrees of freedom; p = statistical significance.

Table 3

Effect of levamisole hydrochloride on encysted larvae of *Lagochilascaris minor*. Analysis of the results obtained by the T-test and the Levene test for the group inoculated with 2,000 infective eggs of the parasite and treated with 0.075 mg of levamisole hydrochloride 120 days after inoculation. The control group was composed of mice inoculated with the parasite but not treated with the anthelmintic

Group		n (mice)	σ	Difference	t	df	p
Test	G3	30	198,258	118,247	- 0.806	36	0.425
Control	G4	10	316,533				

G3 = encysted larvae; G4 = control group; n = number of mice; σ = standard deviation; t = t-test; df = degrees of freedom; p = statistical significance.

term treatment with albendazole and ivermectin²⁸.

The association of ivermectin and benzimidazole derivatives produced positive long-term results²⁹. The occurrence of the auto-infecting cycle contributes to hinder therapy, since adult worms, larvae and eggs in different stages of segmentation, including embryonated eggs, can be found in the sites of lesions^{4,15,16,20}.

To ensure good therapeutic efficacy, an anthelmintic must have larvicidal, ovicidal and vermifugal activity to prevent egg embryogenesis^{4,15}. Therefore, more studies are needed on the anthelmintic activity in the different stages of the parasite. For example, albendazole has been tested *in vitro* in suspension at a dose of 400 mg/10 ml of 1% formalin solution on newly eliminated eggs and embryonated eggs³⁷. These authors found that, at this dosage, the drug prevents egg embryogenesis but has no effect on embryonated eggs, so its activity is restricted to embryogenesis, and there is no effect on larvae³⁷.

Ivermectin (*in vitro*) at a concentration of 200 µg per liter of 1% formalin, administered for 28 days, did not prevent embryogenesis or devitalize larvae inside the eggs of *L. minor*³⁸. However, *in vivo*, at a dosage of 200 mg/kg, it has devitalized fourth-stage larvae, blocking their development into adult worms in experimentally infected cats³⁹. At a dose of 200 µg/kg, the drug was ineffective on both third-stage migratory larvae and third-stage encysted larvae³². In mice infected with the parasite and treated with a dose of 1,000 mg/kg, ivermectin was 99.5% effective on third-stage migratory larvae but less than 5% effective on third-stage encysted larvae³². The recommended dosage for both humans and animals is 200 µg/kg; therefore, 1,000 µg is not advisable.

In ascariasis, the mechanism of action of levamisole hydrochloride occurs by the selective inhibition of enzyme activity in the worm's muscle, preventing the conversion of fumarate to succinate, causing paralysis of the helminth³³. This drug reaches its peak plasma level after two hours and the plasma half-life is approximately four hours³³.

In experimental lagochilascariasis in mice inoculated with infective eggs of the parasite, larvae hatch from the eggs in the intestine four to six hours after inoculation, migrating to the liver and lungs for about 24 to 48 hours, and then encysting in skeletal muscles and subcutaneous tissues around 7 to 8 days after inoculation (DAI)^{4,15,16,17,19,20}.

To evaluate the effects of levamisole on the first larval migration, the mice were treated with levamisole and immediately inoculated with infective eggs of the parasite. Despite that, the larvae of *L. minor* were not devitalized by levamisole hydrochloride during the first migration in infected mice, similarly to what occurred with the use of ivermectin according to by Barbosa *et al.* 1988³².

The larvae inside the nodules were likewise not devitalized, possibly because the wall that surrounds the granulomatous nodule acts as a barrier, preventing the drug from penetrating the encysted larvae. Barbosa *et al.* 1998³² reported a similar result when they used ivermectin on mice experimentally infected with *L. minor*. Therefore, ivermectin and levamisole hydrochloride proved ineffective on both migratory larvae and on larvae encysted in the experimental host.

To treat lagochilascariasis in humans, long treatment schemes are used to block the development of the stages of the helminth⁴. Such long-term treatments have toxic effects, often leading to the abandonment of treatment. However, a single dose, as recommended by the manufacturer, resulted in the failure described herein.

Among the drugs tested experimentally, only albendazole prevents egg embryogenesis³⁷. Ivermectin used at a higher than recommended dosage and levamisole hydrochloride have no activity against encysted larvae.

Although therapeutic combinations with levamisole often present satisfactory results in human lagochilascariasis, it is important to consider the safety and efficacy of these combinations because these parameters have not yet been well established. It is known that levamisole may reduce the bioavailability of albendazole and also increase the bioavailability of ivermectin. Thus, dosage adjustments are needed. Although it is used in humans to treat both ascariasis and lagochilascariasis, this is the first experimental study conducted to test the effects of levamisole hydrochloride on *L. minor* larvae.

In view of these results, we believe that further studies are needed to investigate the effects of this and other drugs on the other stages of the life cycle of this parasite, as well as new therapeutic schemes, aimed at inhibiting successive relapses and the chronicity of this helminthiasis. It is essential to provide the population with a safe treatment scheme with proven clinical efficacy.

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