



Article/Artigo

Ultrastructural study on the morphological changes to male worms of *Schistosoma mansoni* after *in vitro* exposure to allicin

Estudo ultraestrutural das alterações morfológicas de vermes machos de *Schistosoma mansoni* após exposição *in vitro* à alicina

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ABSTRACT

Introduction: Garlic has a wide range of actions, including antibacterial, antiviral, antifungal, antiprotozoal and anthelmintic actions. This antiparasitic activity has been attributed to allicin, which is the main constituent of garlic. The present study aimed to investigate the *in vitro* activity of allicin on the tegument of adult *Schistosoma mansoni* worms using scanning electron microscopy. **Methods:** Swiss Webster mice were infected with *S. mansoni* cercariae (100 per mouse) and sacrificed 50 days later to acquire the adult worms. These worms were collected by perfusion and placed in RPMI medium 1,640 at 37°C before transferring to RPMI media containing 0 (control), 5, 10, 15 and 20mg/mL of allicin, where they were incubated for 2h. The worms were fixed in 2.5% glutaraldehyde solution, washed twice, post-fixed in osmium tetroxide, washed twice and then dehydrated with ascending grades of ethanol. The samples were air-dried, mounted on stubs, gold coated in an ion sputtering unit and viewed using a scanning electron microscope. **Results:** A concentration of 5mg/mL caused wrinkling in the tegument; a concentration of 10mg/mL resulted in changes to tubercles and loss or modification of spines. With 15 and 20mg/mL increasing damage to the tegument could be seen, such as vesicle formation and the presence of ulcers. **Conclusions:** These findings demonstrate the effect of allicin on adult *S. mansoni* worms and indicate that most of the changes occur at concentrations greater than that normally indicated for treatment.

Keywords: Garlic. *Schistosoma mansoni*. Scanning electron microscopy.

RESUMO

Introdução: O alho apresenta uma ampla gama de ações, incluindo antibacteriana, antiviral, antifúngico, antiprotozoário e anti-helmíntico. Esta atividade antiparasitária tem sido atribuída à alicina, que é o principal constituinte do alho. O presente estudo teve como objetivo investigar a ação *in vitro* da alicina no tegumento de vermes adultos de *Schistosoma mansoni* utilizando a microscopia eletrônica de varredura. **Métodos.** Camundongos Swiss Webster foram infectados com cercárias de *S. mansoni* (100 por camundongo) e sacrificados 50 dias depois para aquisição de vermes adultos. Estes vermes foram coletados por perfusão e colocados em meio RPMI 1.640 a 37°C antes de transferir para o meio RPMI contendo 0 (controle), 5, 10, 15 e 20mg/mL de alicina, onde eles foram incubados por 2h. Os vermes foram fixados em uma solução de glutaraldeído a 2,5%, lavados duas vezes, pós-fixados em tetróxido de ósmio, lavados duas vezes e então desidratados em séries crescentes de etanol. As amostras foram secadas, montadas em *stubs*, metalizadas em ouro e visualizadas utilizando o microscópio eletrônico de varredura. **Resultados:** A concentração de 5mg/mL causou o enrugamento do tegumento; a concentração de 10mg/mL resultou em alterações nos tubérculos e perda ou modificações nos espinhos. Com 15 e 20mg/mL crescentes danos no tegumento pode ser visto, tais como formação de vesículas e presença de úlceras. **Conclusões:** Esses resultados demonstram os efeitos da alicina nos vermes adultos de *S. mansoni* e indicam que a maioria das alterações ocorrem numa concentração maior do que a normalmente indicada para o tratamento.

Palavras-chaves: Alho. *Schistosoma mansoni*. Microscopia eletrônica de varredura.

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INTRODUCTION

Schistosoma species include parasites of medical and veterinary importance that are responsible for schistosomiasis^{1,2}. This is one of the most prevalent parasitic infections and it constitutes a major public health problem with significant economic impact³⁻⁵. Praziquantel is used to treat infections caused by *Schistosoma* spp⁶ and is the drug of choice for human and veterinary parasites⁷. The effectiveness of this drug is well documented, despite evidence suggesting that resistance to this drug exists⁸. To develop alternative therapies for treating schistosomiasis, studies have been conducted to evaluate the power of medicinal plants⁹.

Allium sativum (garlic) is a bulbous herbaceous plant belonging to the Liliaceae family. It has been used since the beginning of humanity for treating many different diseases^{10,11}. Garlic has gained credit as a formidable prophylactic and therapeutic agent in many cultures over the centuries and attention has been drawn to its use within modern medicine¹².

Garlic is also known as an antiparasitic agent and this activity has been attributed to allicin, the main constituent of garlic¹³. Although it is recommended for treating intestinal parasites in humans, few studies exist regarding the action of *A. sativum* on parasites¹⁴. Although some studies have indicated that garlic is effective as an antibilharzial drug^{15,16}, evaluation of the action of *A. sativum* has only been rarely reported. Therefore, this study aimed to characterize the *in vitro* activity of garlic on the tegument of male worms of *S. mansoni*.

METHODS

Obtaining adult *Schistosoma mansoni* worms

Adult *S. mansoni* worms were obtained by perfusion of the hepatic portal system of Swiss Webster mice 45-50 days after they were subjected

to infection. The worms of the BH strain (Belo Horizonte-Brazil) were expelled and collected in Petri plates containing RPMI 1640 culture medium at 37°C.

Determination of *in vitro* susceptibility of *Schistosoma mansoni* to allicin

After removal of the definitive host, the adult *S. mansoni* worms were washed in RPMI 1640, and then transferred to 35mm diameter sterile tissue culture plates containing 2mL of culture medium. Each well received two worms, which were then incubated at 37°C. After allowing a period for the worms to adapt to the culture medium, allicin was added at concentrations of 0, 5, 10, 15 and 20mg/mL. These doses were equivalent to the plasma concentrations in individuals who respectively ingest 0, 1, 2, 3 and 4 garlic capsules. Each concentration was assayed in triplicate. The parasites were incubated for two hours and monitored during this time to evaluate motility, changes to the tegument and mortality rate.

Motility evaluation

The motility of the worms in the control group and the groups exposed to medication, were studied over a two-hour period by means of bright field microscopy. According to the motility criterion, the worms were considered to be dead when no movement was observed after three minutes of observation under a stereoscopic microscope.

Ultrastructural evaluation

Ultrastructural analysis was performed on worms at different concentrations recovered through *in vitro* analysis.

Scanning microscopy

The samples were fixed (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.2) for 12h at room temperature, washed in the same buffer and post-fixed in 1% OsO₄ in sodium cacodylate buffer 0.1M, pH 7.2, for one hour. After post-fixing, the material was washed in the same buffer, dehydrated

in an increasing ethanol series and then maintained at the critical point. After assembling and metallization, the material was observed by means of scanning electron microscopy (JEOL - JSM 5600LV).

RESULTS

Motility evaluation

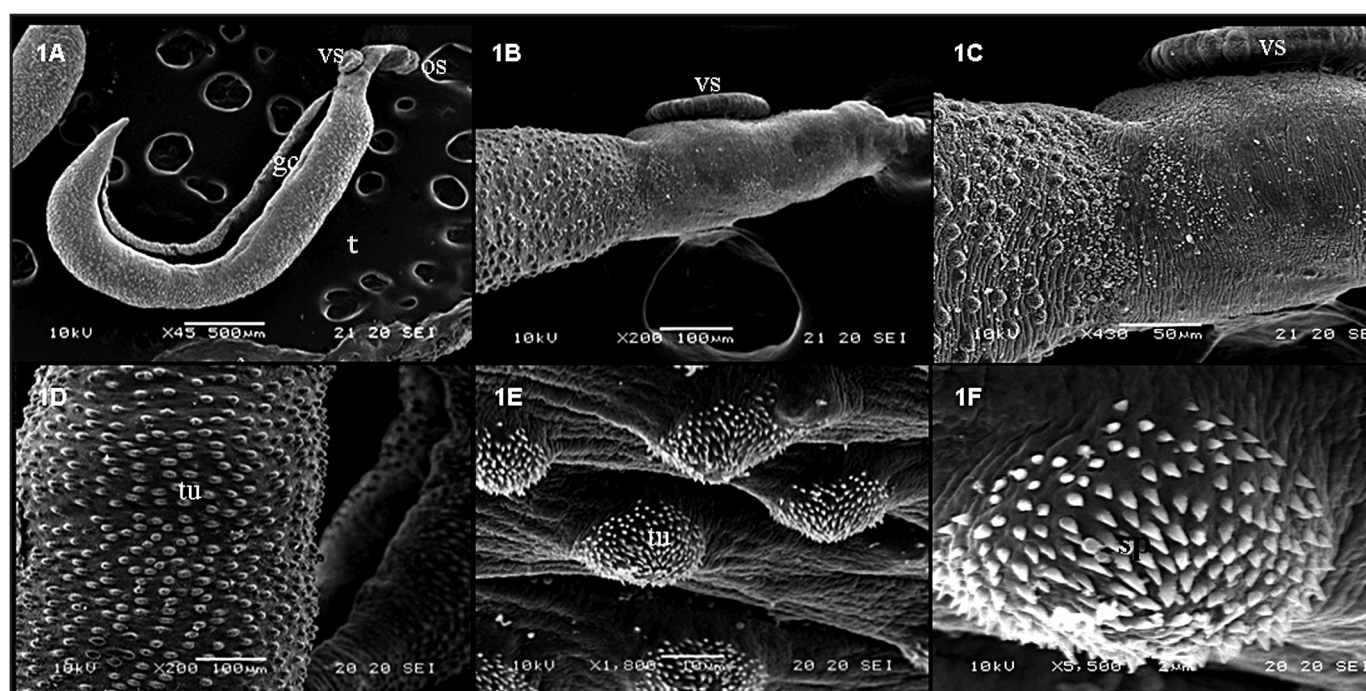
The worms were monitored at 30, 60, 90 and 120min and no mortality was observed at any of the concentrations studied. However, a change in motility between the groups was verified. At a concentration of 20mg/ml, the worms presented higher motility than at the other concentrations tested. While evaluating the adult worms, the females showed similar motility to the control group and the males presented lower motility.

General morphology

Using the routine procedure, the adult *S. mansoni* worms were analyzed by means of scanning electron microscopy (Figures 1A-F). The male worms exhibited two distinct portions: an anterior short, thin and cylindrical part containing the oral sucker (os) and the ventral sucker (vs). The ventral sucker was larger and more prominent than the oral sucker. The back was long and contained the gynecophoral canal (gc). The area between the oral and ventral suckers did not have any tubercles (tu), spines (sp) or sensory papillae. In the posterior region of the adult worms, there were tubercles with numerous spines randomly distributed throughout the body.

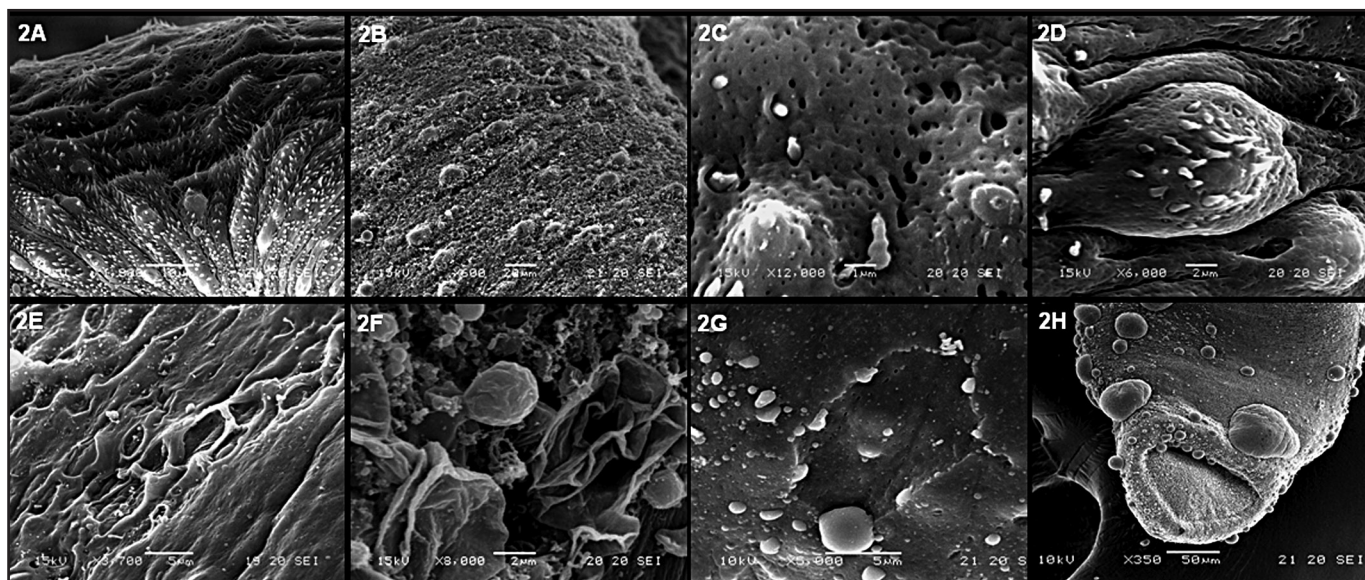
Ultrastructural evaluation

Treatment with allicin promoted damage to the structure of the tegument at all concentrations (Figures 2A-H). A concentration of 5mg/mL caused the formation of projections on the tegument, while a concentration of 10mg/mL resulted in damage to tubercles and the thorns became shorter and fewer. At 15 and 20mg/mL, increasing damage to the tubercles was observed, with the formation of vesicles and ulcers that exposed the musculature of the worm.



FIGURES 1 A-F: Worms of *Schistosoma mansoni* control processed to scan electron microscopy.

t: tegument, tu: tubercles, sp: spines, os: oral suckers, vs: ventral suckers, gc: gynecophoral canal.



FIGURES 2 - A-H: *Schistosoma mansoni* worms after treatment with 5, 10, 15 and 20 mg/mL of allicin processed for scanning electron microscopy.

A-B: 5mg/ml of allicin, observe wrinkling in the tegument, this observation is common in all concentrations.

C-D: 10mg/ml of allicin, drilling tegument with tubercle deformation and loss of the spines.

E-F: 15mg/ml of allicin, observe the ulceration in the tegument (2E) and tegumental projections that can rupture and expose the tissue below it (2F).

G-H: 20mg/ml of allicin, at this concentration, greater damage to the tegument is observed, with the formation of vesicles.

DISCUSSION

Schistosomiasis is treated through administration of two drugs that show good efficacy and low toxicity: oxamniquine and praziquantel¹⁷. Of these, praziquantel is the drug that is most often used. However, in recent years, *S. mansoni* has exhibited drug tolerance or resistance. It is therefore important to investigate and develop new drugs for treating this disease. Since the available drugs that are currently used for treating schistosomiasis are far from ideal, many scientists have sought substances originating from plants as alternative treatments for this helminthiasis^{15,18}.

The antiparasitic activity of garlic has been known since antiquity¹³ and its antischistosomal action has been reported^{16,19,20}. The present study aimed to investigate the antischistosomal activity levels of allicin by means of scanning electron microscopy. The investigations were based on analysis of ultrastructural changes, since such tests have been documented for the drugs currently used for treating schistosomiasis: oxamniquine²¹⁻²⁵ and praziquantel^{22,26-32}.

Many functions and features of the tegument of *S. mansoni* have raised the importance of studying it. It is involved in functions that are important for the worm, such as nutrient absorption, and it presents proteins that are responsible for maintenance of the host immune response or damage repair. Moreover, the tegument is the interface between the parasite and the host environment. Therefore, this structure of the parasite is an important target for drug action^{33,34}.

The present results demonstrated that treatment with garlic did not result in killing the *S. mansoni* worms; however, variations in the tegument between the groups treated with garlic were observed. Ultrastructural analysis *in vitro* on the adult *S. mansoni* worms revealed that the tegument was the principal target for allicin. Observations verified by means of scanning electron microscopy on *S. japonicum*, *S. mansoni* and *S. haematobium* at different stages of development, which were obtained from animals treated with Artemether, a new antischistosomal drug, revealed that its target of action was also the tegument³⁴.

The ultrastructural evaluation was performed on male specimens for two reasons: females are not in frequent contact with the host microenvironment and studies in the literature have shown that soft-tissue alterations are more pronounced in males than in females^{9,18}.

The main changes in the worms' tegument induced by allicin at different concentrations were characterized by: the formation of projections at a concentration of 5mg/mL; changes to the tubercles and reduction and modification of the thorns at a concentration of 10mg/mL; and blistering and the presence of ulcers at concentrations of 15 and 20mg/mL. These data corroborate the findings of Nahed et al, in which they investigated the characteristics of the tegument of adult worms obtained from mice infected with cercariae of *S. mansoni* and subjected to treatment with garlic juice at a dose of 50mg/kg. They found a variety of structural changes to the tegument of the worms, including destruction of the tubercles, edema and ulcers. In addition, there were changes to the numbers and volume of thorns and modification of tubercles.

Ulcer formation was observed in the worms of the present study at concentrations of 15 and 20mg/mL. Similar lesions in the tegument were also observed by Mostafa and Soliman¹⁸, in investigations involving antischistosomal agents. Ulcers in the cuticle of the worm foster greater exposure of antigens on the tegument of the worm, leaving them more vulnerable to the host immune system. In such situations, the worm is recognized as non-self and is destroyed by the parasitized body³⁵⁻³⁷. This demonstrates the relevance of the results obtained here and justifies further studies *in vivo*, since praziquantel, the drug of choice for treating schistosomiasis, presents an important mechanism of action of many changes to the tegument of the worm^{38,39}.

Analysis using scanning electron microscopy on adult *S. mansoni* worms treated with praziquantel showed that at a concentration of 0.8mg/mL, the dorsolateral tegument was found to be twisted, with shriveled tubercles and spines apparently deteriorated and numerically reduced in the gynecophoral channel. At a concentration

of 6.3mg/mL, the dorsolateral region showed tissue loss and exposure of subcutaneous tissue, and the tubercles also showed loss of spines³⁹. These findings demonstrate the same effect as garlic on adult worms of *S. mansoni* and indicate that most of the changes occur at concentrations greater than that normally indicated for the treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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