

Spirolactone: A promising anti-schistosomal drug as revealed by scanning electron microscopy of adult worms

Original
Article

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ABSTRACT

Background: Schistosomiasis *mansoni* is still a widespread parasitic infection especially among poor developing countries. Depending on praziquantel (PZQ) as a single medication for treatment and control, causes the emergence of isolates resistant to this drug. Continuous efforts are needed for the introduction of new effective therapies. Some diuretics including spironolactone, showed promising anti-parasitic effects.

Objectives: In this study, spironolactone, a potassium-sparing diuretic, was evaluated regarding its *in vitro* lethal effect on *Schistosoma mansoni* (*S. mansoni*) adult worms using scanning electron microscopy (SEM).

Material and Methods: A total of 144 worms; 72 males and 72 females were exposed to a concentration of 10 µg/ml of spironolactone for 5 days at 37±0.5°C in 5% CO₂ incubator. A pure medium and a medium containing 0.5% of dimethyl sulfoxide (DMSO) (vehicle) were used as negative controls, while PZQ at 10 µg/ml was used as a reference drug. The compound was then retested using the same technique by successive descending dilutions of the solution. Mortality rate of 50% (lethal concentration) (LC50) and 100% (lethal concentration) (LC100) were calculated after 72 h and 5 days exposure.

Results: Our results highlighted the potent anti-schistosome effect of spironolactone as it showed 100% mortality of worms at 6.4 µg/ml and 5 µg/ml after 72 h and 5 days exposure, respectively. While 50% mortality of worms was obtained at 4.5 µg/ml and 3 µg/ml after 72 h and 5 days exposure, respectively. Moreover, SEM of male and female *S. mansoni* worms exposed to spironolactone *in vitro*, showed disintegration and sloughing of the tubercles on the dorsal surface of males, erosion of the tegument with exposure and appearance of the sub-tegumental tissue in males and females.

Conclusion: Being a safe, oral, inexpensive, and well tolerated drug, spironolactone is capable of being a potent future anti-schistosome drug.

Keywords: praziquantel, scanning electron microscopy, schistosomiasis *mansoni*, spironolactone.

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INTRODUCTION

Schistosomiasis is still a serious public health problem that affects 230 million people worldwide, especially in tropical and subtropical areas with poor sanitary conditions^[1]. It is one of the most significant, neglected tropical diseases in the world due to infection by intravascular trematode flatworms of the genus *Schistosoma*^[2]. About 80 million infections by *S. mansoni* were reported in Africa, the Near East, and South America causing intestinal schistosomiasis^[3]. Presence of parasite eggs in the tissues is the main issue of the disease causing chronic inflammatory response to *Schistosoma* ova with granuloma formation leading to periportal fibrosis and portal hypertension manifestations^[1].

Praziquantel (PZQ) is the only recommended drug for schistosomiasis, but still, it has some disadvantages; the tablets are large, have an unpalatable taste, a large dose is needed^[4], and the

juvenile forms of the parasite are not affected^[5]. Increasing PZQ resistance against some isolates of *S. mansoni* has been recorded as a result of widespread use of the drug^[6]. Thus, the scientific community was concerned with the development of new, cheap and safe anti-schistosomiasis drugs^[7].

Diuretics are the most commonly indicated drugs for treatment of fluid retention, especially in cases of heart failure, renal failure and liver cirrhosis^[8]. They are generally well tolerated, cheap, orally administered, and safe^[9]. Spironolactone, a potassium-sparing diuretic, is a mineralocorticoid receptor antagonist, specifically an antagonist of aldosterone^[10]; and is a very effective agent in the treatment of heart failure^[11], myocardial infarction^[12] and resistant hypertension^[13]. Moreover, together with short-term course of corticosteroids, spironolactone was used in the treatment of resistant ascites complicating schistosome liver disease^[14]. In a case of myocarditis due to malarial infection, spironolactone was also

effective in treating respiratory distress^[15]. Recently, Guerra *et al.*^[16] showed that spironolactone is able to alter adult *S. mansoni* worm morphology and motor activity, leading to parasitic death. Moreover, it was found that oral treatment with spironolactone in mice harboring infections significantly reduced worm burden, egg production and hepatosplenomegaly^[16]. In this study, spironolactone was evaluated as regarding its *in vitro* lethal effect on *S. mansoni* adult worms, and hence, its use as a potential anti-schistosomal drug.

SUBJECTS AND METHODS

This experimental study was conducted in Theodor Bilharz Research Institute (TBRI) during the period from May-June 2019.

Adult worms: The *S. mansoni* worms as well as the materials used were provided by the Schistosome Biological Material Supply Centre of Theodore Bilharz Research Institute (TBRI), Giza, Egypt. Adult worms were obtained by the main procedure previously described by Yousif *et al.*^[17] and Ramirez *et al.*^[18] from hamsters (*Mesocricetus auratus*), percutaneously infected with 350-400 *S. mansoni* cercariae per hamster 6 weeks earlier. The worms were perfused by citrated saline, and the recovered worms were washed from blood in small sieves (20 µm mesh) by phosphate buffer. Worms were washed three times with the culture medium used for the assay under a sterilized laminar flow chamber. The culture medium was composed of Roswell Park Memorial Institute Medium (RPMI) 1640 + l-glutamine + 20% fetal calf serum + antibiotics (300 µg streptomycin + 300 IU penicillin + 160 µg gentamycin per ml)^[19].

Bioactivity testing: The bioassay was carried out according to Eissa *et al.*^[19] using 24 wells tissue culture plates. A stock solution 5 mg/ml of the compound was prepared in DMSO immediately before use. Six worms, three males and three females were introduced into each test well in 2 ml medium. Worms were exposed to a concentration of 10 µg/ml of spironolactone for 5 days at 37±0.5°C in 5% CO₂ incubator (2 replicates were prepared to test spironolactone). A pure medium and a medium containing 0.5% of DMSO (vehicle) were used as negative controls, while PZQ at 10 µg/ml was used as a reference drug. Worms were examined for their viability using a stereomicroscope, and those not showing motility for one minute were considered dead. The mortality rate of worms was calculated after 72 h and 5 days exposure. The compound was then retested using the same technique by successive descending dilutions of the solution.

SEM study: Adult worms of *S. mansoni* exposed to spironolactone and PZQ, and non-exposed samples were fixed in a 10% glutaraldehyde, then processed for examination by SEM^[20].

Statistical analysis: The statistical program SPSS version 20 was used for the calculation. Linear regression analysis was applied to determine the relationship between worm mortality and drug concentration. Probit regression graphing was used to determine LC50 and LC100.

Ethical approval: The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University, Cairo, Egypt, FWA 00006444, and hamsters were handled according to the NIH guidelines for animal experimentation. The animal experiments were performed according to the national regulations for the Animal Ethics rules, Ain-Shams University, Cairo, Egypt. Hamsters were maintained under conventional conditions, fed a standard commercial pellet diet, housed in 12 h dark cycle at 22 ± 2°C, humidity of 55% ± 10% and continuous air renovation, at the Schistosome Biological Material Supply Centre of Theodore Bilharz Research Institute (TBRI), Giza, Egypt.

RESULTS

In vitro schistosomicidal activity of spironolactone showed 100% mortality of worms at 6.4 µg/ml and 5 µg/ml after 72 h and 5 days exposure, respectively. While 50% mortality of worms was obtained at 4.5 µg/ml and 3 µg/ml after 72 h (3 males and 3 females were dead) and 5 days (2 males and 4 females were dead) exposure, respectively. However, this estimation of mortality proved to be lower than that of the reference drug (PZQ) which showed 100% mortality of worms at 0.4 µg/ml and 0.3 µg/ml after 72 h and 5 days exposure, respectively. While 50% mortality of worms was obtained at 0.3 µg/ml and 0.2 µg/ml after 72 h and 5 days exposure, respectively, under the same condition (Table 1).

Table 1. *In vitro* schistosomicidal activity of PZQ and spironolactone on *Schistosoma mansoni* adult worms (after 72 h and 5 days exposure).

	Praziquantel		Spironolactone	
	LC50 µg/ml	LC100 µg/ml	LC50 µg/ml	LC100 µg/ml
72 h	0.3	0.4	4.5	6.4
5 days	0.2	0.3	3.0	5.0

In the present work, SEM of adult male *S. mansoni* worms exposed to spironolactone *in vitro*, showed disintegration and sloughing of the tubercles on the dorsal surface; erosion of the tegument with exposure and appearance of the sub-tegumental tissue occurred in males and females (Figure 1). These findings were similar to those obtained when worms were exposed to PZQ (Figure 2). While SEM of *S. mansoni* worms from both DMSO and pure medium controls (negative controls) showed normal dorsal tubercles with intact tegument (Figure 3).

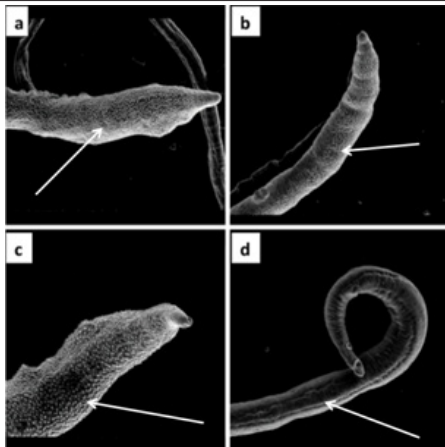


Fig. 1. Scanning electron microscopy (SEM) of adult *S. mansoni*: *in vitro* exposed to spironolactone showing disintegration and sloughing of the tubercles on the dorsal surface, erosion of the tegument with exposure and appearance of the subtegumental tissue of both male; (a) (x218), (b) (x218), (c) (x300) and female adult worms; (d) (x380).

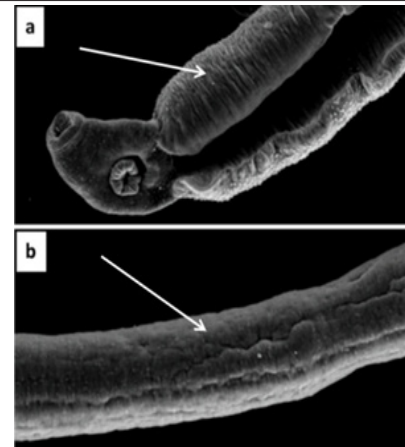


Fig. 2. Scanning electron microscopy (SEM) of adult *S. mansoni*: *in vitro* exposed to PZQ showing complete sloughing of the tubercles on the dorsal surface, erosion of the tegument and exposure of the subtegumental tissue of both male; (a) (x349) and female adult worms; (b) (x600).

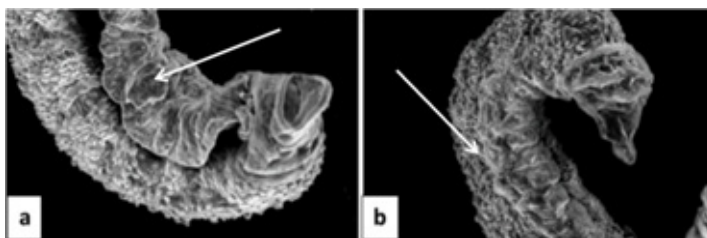


Fig. 3. Unexposed adult male *S. mansoni* worm showing normal tegument with intact dorsal tubercles at both anterior (a) and posterior ends (b) ($\times 440$).

DISCUSSION

Schistosomiasis is one of the most prevalent human parasitic infestations that records high rates of morbidity. Approximately 20 million persons are affected with mortality rate of about 280000 deaths per year, especially in tropical and subtropical regions^[21]. PZQ is the only commercially available anti-schistosomal drug for the treatment of schistosomiasis^[22]. However, it is inadvisable to rely on one drug for any infectious condition, especially a disease with high prevalence as schistosomiasis, because of the appearance of drug-resistant/ tolerant parasites^[23].

Research is now directed towards repurposing of known drugs and evaluation of new drug combinations, in order to reach novel anthelmintics^[24]. In a study conducted by Kamel and Bayaomy^[25], it was found that the antimalarial drug, primaquine, possesses moderate anti-schistosomal activity against juvenile and adult *S. mansoni* worms, demonstrated by high mortality and tegumental changes. In 2010, Manneck *et al.*^[26] evaluated the schistosomicidal activity of mefloquine, where SEM observations revealed extensive tegumental destruction, including blebbing, shrinking and sloughing following *in vitro* incubation of adult *S. mansoni* with the drug. Concerning diuretics, some drugs have shown an anti-parasitic effect, as furosemide, that was found to control intracellular

leishmanial growth, and hence, has a therapeutic effect against murine cutaneous leishmaniasis^[27]; and spironolactone that showed, in a study carried out by Guerra *et al.*^[16], a lethal effect on adult *S. mansoni* worm by altering its morphology and motor activity.

In our *in vitro* study, we assessed the ability of spironolactone, a potassium-sparing diuretic, as a potential drug for schistosomiasis in comparison with PZQ. The exact toxic effect of spironolactone is at this time not yet clear, except that it has detrimental effect on the worm tegument, resulting in 100% mortality of worms at drug concentrations of 6.4 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ after 72 h and 5 days exposure, respectively. While LC₅₀ of worms was obtained at 4.5 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$ concentrations after 72 h and 5 days exposure, respectively. In accordance Guerra *et al.*^[16] tested LC₅₀ of thirteen diuretics including spironolactone against *S. mansoni* worms, and observed that only spironolactone, displayed activity at 50 μM , with LC₅₀ value of 7.2 μM determined after 72 h. Also, after a series of three independent experiments, authors revealed that schistosomes incubated with spironolactone showed increased motor activity, including contractions, associated with a decrease in body length together with increase in the mortality in a concentration-dependent manner.

In our study using SEM, it was noticed that spironolactone causes disintegration and sloughing of the tubercles on the dorsal surface of *S. mansoni* worm; in addition to erosion of the tegument with exposure and appearance of the sub-tegumental tissue. This agrees with Adler^[28] who stated that schistosomes' tegument is an important target for drugs. These findings were also analogous with the report of Guerra *et al.*^[16] who observed alteration in tegumental structure in both male and female *S. mansoni* worms that intensified progressively when the concentrations of spironolactone increased. Generally the mentioned morphological alterations were similarly observed in previous studies with other antihelminthic drugs^[29,30], including PZQ which exerts a potent *in vitro* effect against schistosomes causing extensive tegumental alterations in a concentration-dependent manner^[31,32].

In conclusion, our study confirms the *in vitro* potent lethal effect of spironolactone on *S. mansoni* adult worms, supported by SEM findings on examination of dead worms. Thus, spironolactone can be used not only as a diuretic, but also as a schistosomicidal derivative. This work sets the base for further studies on the effect of spironolactone on other stages of *S. mansoni*, as well as *S. haematobium*; and *in vivo* on experimentally infected animals.

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