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THE SUSCEPTIBILITY OF RECENT ISOLATES OF *Schistosoma mansoni* TO PRAZIQUANTEL

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SUMMARY

Introduction: Schistosomiasis is a chronic disease caused by trematode flatworms of the genus *Schistosoma* and its control is dependent on a single drug, praziquantel (PZQ), but concerns over PZQ resistance have renewed interest in evaluating the *in vitro* susceptibility of recent isolates of *Schistosoma mansoni* to PZQ in comparison with well-established strains in the laboratory. **Material and methods:** The *in vitro* activity of PZQ (6.5-0.003 µg/mL) was evaluated in terms of mortality, reduced motor activity and ultrastructural alterations against *S. mansoni*. **Results:** After 3 h of incubation, PZQ, at 6.5 µg/mL, caused 100% mortality of all adult worms in the three types of recent isolates, while PZQ was inactive at concentrations of 0.08-0.003 µg/mL after 3 h of incubation. The results show that the SLM and *Sotave* isolates basically presented the same pattern of susceptibility, differing only in the concentration of 6.5 µg/mL, where deaths occurred from the range of 1.5 h in *Sotave* and just in the 3 h range of SLM. Additionally, this article presents ultrastructural evidence of rapid severe PZQ-induced surface membrane damage in *S. mansoni* after treatment with the drug, such as disintegration, sloughing, and erosion of the surface. **Conclusion:** According to these results, PZQ is very effective to induce tegument destruction of recent isolates of *S. mansoni*.

KEYWORDS: Praziquantel; *Schistosoma mansoni*; Ultrastructure.

INTRODUCTION

Schistosomiasis is a chronic and debilitating disease caused by trematode flatworms of the *Schistosoma* genus that continue to threaten millions of people, particularly the poor, rural populations in the developing world[1]. Praziquantel (PZQ) is used to treat the main schistosomiasis in humans, since it can eliminate adult worms during the chronic phase of infection[2]. Currently, over 200 million people are estimated to be infected, while close to 600-780 million are at risk of contracting the disease[3].

Despite the many existing forms of schistosomiasis control, antiparasitic chemotherapy is now the only immediate resource for minimizing the prevalence and incidence of schistosomiasis in the world[4]. Since the discovery of PZQ in the 1970s, no other therapeutic advance has been achieved, and it is now the drug of choice, mainly due to treatment consisting of a single oral dose, the absence of severe side effects and its low cost[10]. It should be noted, however, that relying on only a drug is certainly a dangerous situation, particularly with regard to its strength[4]. However, *Schistosoma mansoni* isolates with reduced susceptibility to PZQ have already been identified[5].

Chemotherapy for many helminth infections is complicated by the incidence of resistance or tolerance to certain anthelmintic drugs. In addition, the appearance of drug-resistant strains of *Schistosoma* is a constant concern for public health authorities[1]. Research into the biological activity of PZQ on recent isolates of *S. mansoni* is an imperative and urgent matter[6].

Once resistance to a drug becomes clinically relevant, it becomes a difficult problem to solve. Therefore, vigilant monitoring aimed at preventing clinical drugs becoming resistant is critical for the treatment and control of infectious diseases[7].

In this study, the *in vitro* effects of PZQ were investigated on different isolates of *S. mansoni*: a secluded wild one, taken directly from the field and recovered after initial infection in a laboratory mouse; who was secluded yet in a stabilization phase in a laboratory; and another one in a well-stabilized laboratory, in mice maintained since the late 1970s. The susceptibility of the worms to the PZQ was evaluated for its motor activity, survival rate and it was demonstrated that the PZQ-induced membrane damage efficiently killed adult worms.

MATERIALS AND METHODS

Ethics: This study was approved by the Ethical Committee of the *Aggeu Magalhães* Research Center/CPqAM – FIOCRUZ/PE (protocol 06/2010).

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Obtaining isolates of *S. mansoni*: Isolated wild (SOT) was obtained from *Biomphalaria glabrata* collected from water in *Sotave*, *Jaboatão dos Guararapes*/PE (8°6'46"S and 35°0'54"W). The collected snails (n = 80) were sent to the Schistosomiasis laboratory in the *Aggeu Magalhães* Research Center/CPqAM - FIOCRUZ, in order to analyze the positivity of infection by *S. mansoni*. The snails were screened, the diameter of the shell measured and individualized in small plastic containers with a capacity of 10 mL, containing 5 mL of dechlorinated water. The snails were exposed to artificial light for a period up to two hours, to verify the release of cercariae, after which it was found that 63 snails were infected.

Isolated in a laboratory setting: This SLM isolate came from *São Lourenço da Mata*/PE (08º00'08"S and 35°01'06"W), from patients infected with *S. mansoni*. The isolate is in the stabilization phase in colonies of *Swiss Webster* mice.

Isolated established in laboratory: This BH isolate was from *Belo Horizonte*/MG (19°49'01"S and 43°57'21"W) and has been kept stable in colonies of *S. Webster* mice, in the *Aggeu Magalhães* Research Center/CPqAM - FIOCRUZ/PE since the 1970s. None of the three isolates had prior exposure to PZQ.

***In vitro* studies with *S. mansoni*:** After eight weeks, *S. mansoni* adult worms (male and female) were recovered under aseptic conditions from mice previously infected with 120 cercariae by perfusion of the liver and mesenteric veins. The worms were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), kept at pH 7.5 with 20 mM HEPES, and supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), and 10% heat-inactivated fetal calf serum. After washing, one pair of adult worms was transferred to each well of a 24-well Falcon plate containing 2 mL of the same medium and incubated at 37 ºC in a humid atmosphere containing 5% CO_2 prior to use. Twenty-four hours after incubation, PZQ was dissolved in 1.6% DMSO and used at concentrations of 0.003, 0.005, 0.008, 0.3, 0.5, 0.8, 3.0 and 6.5 µg/mL. The control worms were assayed in RPMI 1640 medium with 1.6% DMSO as a negative control group and in 10 µg/mL with PZQ as a positive control group. The experiment was carried out in triplicate and repeated at least three times. Observations of worm motor activity, tegumental alterations and mortality[8], were made at 30 minute intervals for 1.5 to 3 hours and were monitored using an inverted microscope and a stereomicroscope (Nikon). The motor activity was evaluated according to the following classification criteria: (+) low motility, (++) average motility, (+++) normal motility, (-) absence of motility. Parasites that showed no motility during 2 minutes of observation were considered dead. The control groups were observed concomitantly.

Groups of treatment: Adult worms of *S. mansoni* were divided into three groups: SOT: adult worms isolated from the wild (*Sotave*/PE); SLM: adult worms isolated in the established phase in the laboratory (*São Lourenço da Mata*/PE); BH: adult worms isolated at the already established laboratory (*Belo Horizonte*/MG).

Scanning Electron Microscopy (SEM): After incubating and washing adult worms, they were fixed with 2.5% glutaraldehyde, formaldehyde, and 4.0% sodium cacodylate buffer 0.1 M, pH 7.2, washed in the same buffer and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for 1 hour. After washings in cacodylate buffer 0.1 M pH 7.2, the samples were dehydrated in ethanol baths in increasing order of concentration (50%, 70%, 90% and 3 x 100%) for 30 min. The worms were dried in a Hitachi HCP-2 critical point dryer machine using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus, FINE-COAT 1100-JEOL (Ion Sputter JFC-1100; Tokyo, Japan), at 1.2 kV, 10 mA for 1 min, and examined under a JEOL 5600LV microscope[10].

RESULTS

In all the experiments, the negative control groups remained viable throughout the observation period. On the other hand, PZQ caused 100% parasite death after 30 minutes of incubation in the positive control group, whereas no mortality was observed in the worms of the negative (RPMI medium) and solvent control (RPMI medium plus 1.6% DMSO) groups.

After 3 h of incubation, PZQ at 6.5 µg/mL, caused mortality of all adult worms (females and males) in the three types of recent isolates, while PZQ was inactive at concentrations of 0.008-0.003 µg/mL after 3 h of incubation. The results show that the SLM and *Sotave* isolates basically present the same pattern of susceptibility, differing only in the concentration of 6.5 µg/mL, in which deaths occurred from the range of 1.5 h in *Sotave* and just in the 3h range in SLM (Table 2).

In the BH isolate, although deaths were evident within the first 30 minutes of incubation at concentrations of 3.0 and 6.5 µg/mL, it was observed that males and females were still alive 3 hours after treatment at a concentration of 3.0 µg/mL.

All flukes (including both males and females) in the negative control group exhibited normal motility (+++), and were alive throughout the experimental period. In the group treated with 0.003 µg/mL PZQ, at 3 h after treatment all the flukes exhibited active movement and appeared normal as in the negative control group (+++). At 1.5 h, with 6.5-0.3 µg/mL PZQ, the flukes exhibited low motility (+). At 3 h, with 6.5 µg/mL PZQ, all the flukes exhibited absence of motility (-) (Table 1).

Using the routine procedure, the adult *S. mansoni* worms were analyzed by means of scanning electron microscopy. In the control group without treatment, the male and female worms exhibited two distinct portions: an anterior short, thin and cylindrical part containing the oral sucker and the ventral sucker. The ventral sucker was larger and more prominent than the oral sucker. The ventral surface of the male worm contains the gynecophoric canal. The area between the oral and ventral suckers did not have any tubercles, spines or sensory papillae. In the posterior region of the male adult worms, there were tubercles with numerous spines randomly distributed throughout the body, while the female worms had a smooth body without tubercles, only with little spines randomly distributed. (Figures 1A-F).

In addition to the exposure time, the data obtained in this study show that the damage caused by PZQ on the integument of adult worms of *S. mansoni* is also dose-dependent, because the intensity of the changes increases with concentration. Further damage was found in male and female worms undergoing concentration of 6.5 µg/mL; this damage was observed on the entire surface of their bodies. In general, males and females (SOT, BH and SLM strains) showed winding of the body, most often taking the form of a spiral (Figures 2A-B, 3A-B and 4A-B).

Table 1
Evaluation of the motility of adult worms of *S. mansoni* after incubation of the parasites *in vitro* containing different concentrations of PZQ*.

Isolates	T**	Concentrations of PZQ (μg/mL)								Control
		6.5	3.0	0.8	0.5	0.3	0.008	0.005	0.003	
SOT	0.30’	+	+	+	+	+	+++	++	+++	+++
	1.5	+	+	+	+	+	++	++	+++	+++
	3.0	-	-	-	+	+	++	++	+++	+++
SLM	0.30’	+	+	+	+	+	+++	++	+++	+++
	1.5	+	+	+	+	+	++	++	+++	+++
	3.0	-	-	-	+	+	++	++	+++	+++
BH	0.30’	+	+	+	+	+	+++	++	+++	+++
	1.5	+	+	+	+	+	++	++	+++	+++
	3.0	-	+	+	+	+	++	++	+++	+++

(-) Absence of motility (+) low motility (+ +) medium motility, (+ + +) normal motility. *Praziquantel; ** Hours.

Table 2
Percent survival of adult worms of *S. mansoni* determined by observing motility at different time intervals after incubation of the parasites *in vitro* in RPMI medium containing different concentrations of PZQ *.

Isolates	T**	Concentrations of PZQ (μg/mL)							
		6.5	3.0	0.8	0.5	0.3	0.008	0.005	0.003
SOT									
Females	0.30’	100	100	100	100	100	100	100	100
	1.5	75	100	100	100	100	100	100	100
	3	0	0	0	12.5	37.5	100	100	100
Males	0.30’	100	100	100	100	100	100	100	100
	1.5	62.5	100	100	100	100	100	100	100
	3	0	0	0	0	12.5	100	100	100
SLM									
Females	0.30’	100	100	100	100	100	100	100	100
	1.5h	100	100	100	100	100	100	100	100
	3h	0	0	0	25	50	100	100	100
Males	0.30’	100	100	100	100	100	100	100	100
	1.5h	100	100	100	100	100	100	100	100
	3h	0	0	0	0	37.5	100	100	100
BH									
Females	0.30’	100	100	100	100	100	100	100	100
	1.5h	75	87.5	100	100	100	100	100	100
	3h	0	75	87.5	100	100	100	100	100
Males	0.30’	75	87.5	100	100	100	100	100	100
	1.5h	50	75	87.5	100	100	100	100	100
	3h	0	50	62.5	87.5	100	100	100	100

*Praziquantel; ** Hours.

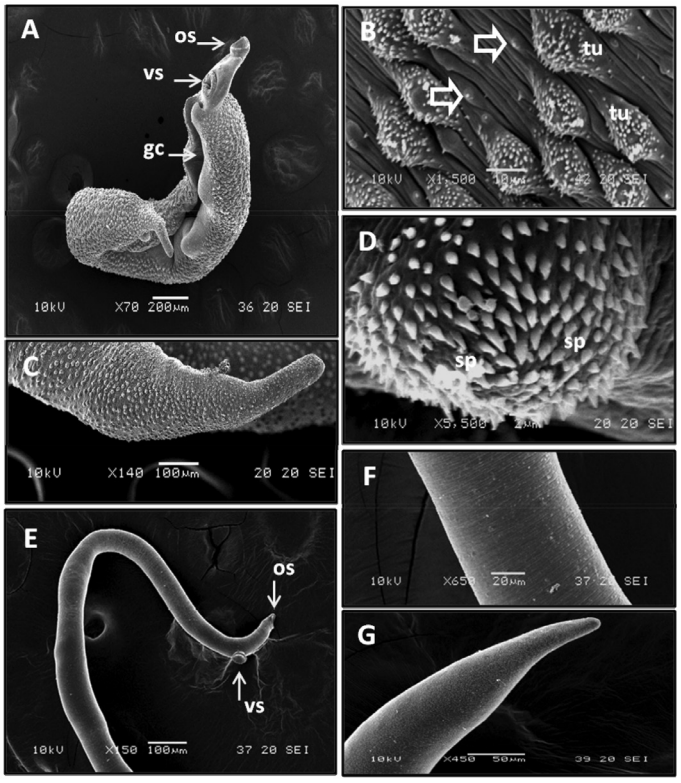

Fig. 1A-G - Male and female adult worms of *Schistosoma mansoni* (BH strain) control processed to scanning electron microscopy. A) Male with presence of oral sucker (os), ventral sucker (vs) and gynecophoral canal (gc). B) Distribution of the tubercles (tu) along the body and sensory papillae (open arrows). C) Caudal portion of the male worm. D) Tubercle detail with spines (sp). E) Female with presence of oral sucker (os) and ventral sucker (vs). F) Smooth body of female without tubercles. G) Caudal portion of the female worm.

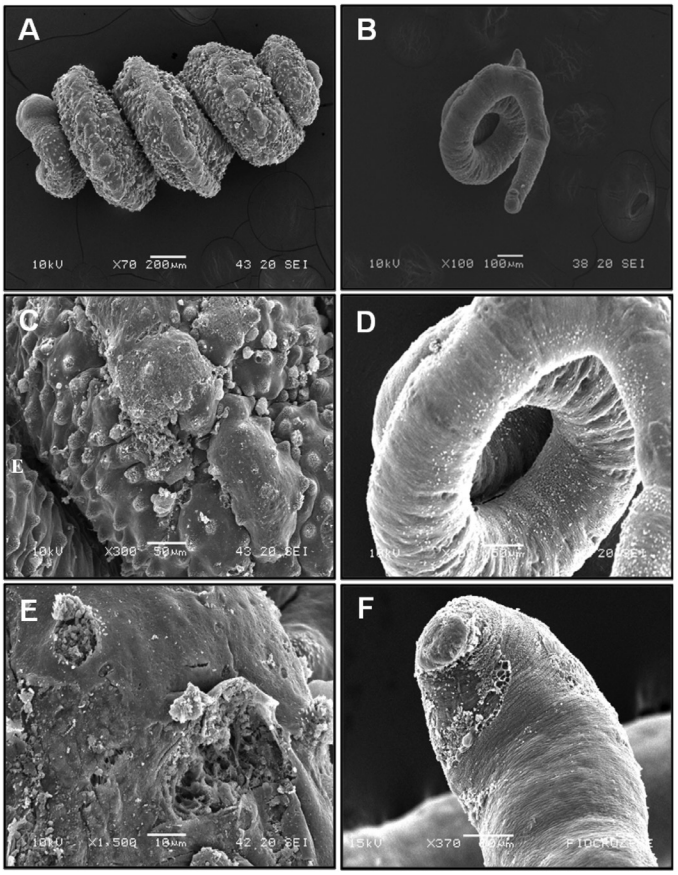

Fig. 2A-F - Male and female adult worms of *S. mansoni* (SOT strain) treated with 6.5 µg/mL PZQ after 3h incubation. **Male**: A) Coiled worm. C) Severe vacuolization of the tegument. E) Collapse of the tubercles, erosion and exposure of internal muscles. **Female**: B) Wrapped worm. D) Female worm, destruction and peeling of the tegument surface. F) Exposure of internal muscles.

In males, the collapse of the tubercle was observed as there were loss of spicules and extensive vacuolization of the integument, as evidenced by the formation of bubbles throughout the length of the worm (Figures 2C, 3C and 4C). In addition, large foci of erosion and exposure of internal muscles were observed (Figures 2E, 3E and 4E). In females, extensive and severe erosion were evidenced, exposing the muscle layer presents just beneath the integument (Figures 2E-F, 3E-F and 4E-F). Linking these results to the survival test, the conclusion is drawn that the modifications induced by PZQ in this concentration are responsible for the death of all worms in three isolates after 3 hours of incubation.

DISCUSSION

This study compared the *in vitro* anthelmintic effect of PZQ against recent *S. mansoni* isolates with existing/known PZQ concentrations, by estimating the relative motility, survival indices and tegumental surface alterations in the parasites treated[11].

Currently, the majority of field surveys of PZQ resistance focus on *S. mansoni*. Although few PZQ resistant isolates are detected in the field, a reduced susceptibility of the drug in *S. mansoni* has been widely found in many endemic foci, notably in African countries such as Egypt and Senegal[5]. Nowadays, there is no direct evidence of *S. haematobium* developing resistance to praziquantel; however, it has been reported that repeated standard treatment fails in schistosomiasis cases caused by *S. haematobium* infection[12-15].

LIANG *et al*.[4] concluded that the intensity of response of *S. mansoni* adult worms to PZQ is dependent on the concentration and exposure time, where immediate contraction after exposure to concentrations of 3.2 x 10^{-4} 8 x 10^{-4} to 1.6 x 10^{-3} were observed. These observations were also recorded by PICA-MATTOCCIA *et al.*[16], when they did *in vitro* culture of male and female worms of *S. mansoni* in medium containing PZQ concentrations from 0.1 to 80 µg/mL. In the same study, in a specific test to observe the effect of PZQ on the appearance of worms, where PZQ concentrations of 0.05 to 1.0 µg/mL were used, the authors found contraction and shrinkage among worms subjected to concentrations between 0.1 and 1.0 µg/mL, but no changes were observed in worms subjected to the concentration of 0.05 µg/mL.

The study also shows, in general, greater sensitivity to PZQ in male compared to female worms, especially in the less susceptible isolate (BH). Female *S. mansoni* also proved to be less sensitive than male ones in *in vitro* experiments conducted by PICA-MATTOCCIA and CIOLI[16] after recovering worms from mice subjected to bisexual infection. These data corroborate those found by LIANG *et al*.[4], who also showed greater

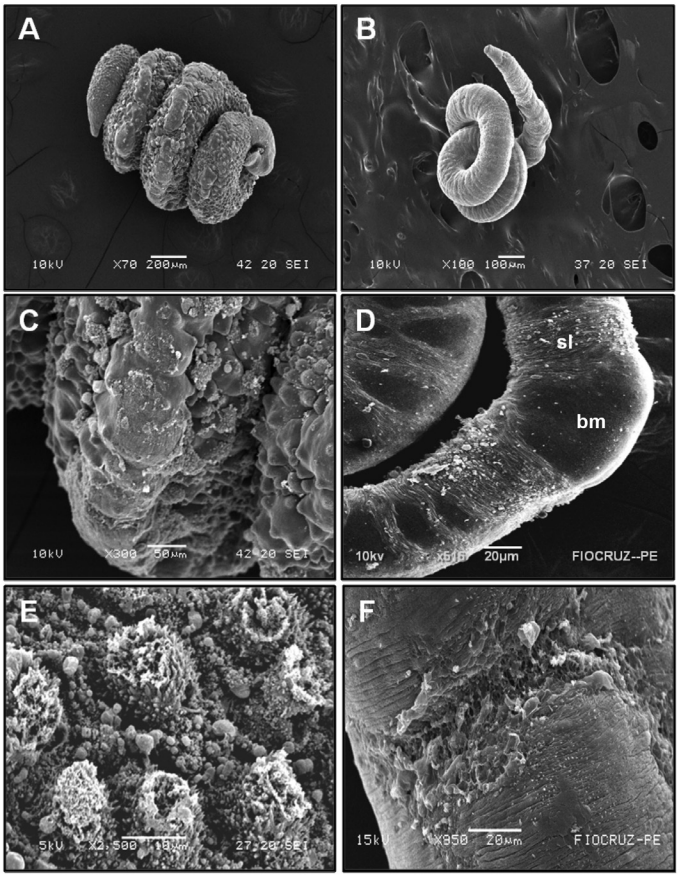

Fig. 3A-F - Male and female adult worms of *S. mansoni* (BH strain) treated with 6.5 µg/mL PZQ after 3h incubation. **Male**: A) Coiled worm. C) Severe vacuolization of the tegument. E) Collapse of the tubercles, erosion and exposure of internal muscles. **Female**: B) Wrapped worm. D) Extensive sloughing (sl) exposing to view the basal membrane (bm). F) Exposure of internal muscles.

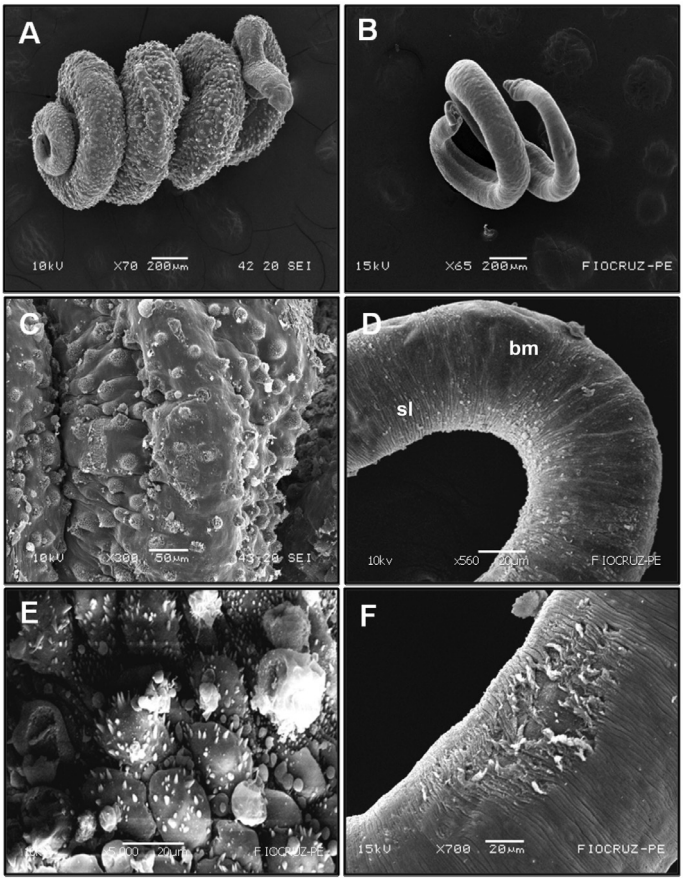

Fig. 4A-F - Male and female adult worms of *S. mansoni* (SLM strain) treated with 6.5 µg/mL PZQ after 3h incubation. **Male**: A) Coiled worm. C) Severe vacuolization of the tegument. E) Collapse of the tubercles, erosion and exposure of internal muscles. **Female**: B) Wrapped worm. D) Extensive sloughing (sl) exposing to view the basal membrane (bm). F) Exposure of internal muscles.

sensitivity in adult male compared to female *S. mansoni* in both isolates and in which there are PZQ resistant isolates, as did previous studies, in experiments using *in vivo* and *in vitro S. mansoni* resistant to PZQ[6].

The fact observed in the range of peak plasma concentration of the therapeutic dose, after 3 hours of incubation (100% of death in males and females, and in SLM and *Sotave* isolates sharp changes in motility, intense contraction, integument and severe bodily harm (shown below) indicative of imminent death in worms of the BH isolate, shows the susceptibility of three isolates to PZQ. Like the contractility of the body, the mortality of the worms was also dependent on the concentration of the drug and time of incubation. These data were also highlighted by LIANG *et al*.[4] after incubation of adult worms of *S. mansoni* resistant to PZQ.

Damage caused by PZQ in different concentrations on the tegument of the worms was identical in the three strains, but in SLM and *Sotave*, changes at the range of peak plasma concentration of the therapeutic dose of PZQ (6.5 and 3.0 µg/mL) were observed only 1.5 h after the onset of incubation while, in the BH isolate, the onset of the changes was observed within the first 30 minutes. This shows that the changes caused by the drug on the integument of the worms are also dependent on the exposure time and that these may vary with each isolate.

Considering the degree of injury produced in the seed coat at different drug concentrations, death is evidently directly related to the intensity of the injury. The seed coat of *S. mansoni* is a structure of great importance to the survival of the worms, since it is involved in nutrient absorption, secretion of metabolites in the osmotic balance of the worms and the defense against the host immune system[17].

PZQ is considered a Ca^{2+} channel agonist that may disrupt the interaction of the $\alpha 1$ and β subunits, which would allow more Ca^{2+} channels to open leading to the disruption of normal Ca^{2+} homeostasis[2].

KOHN *et al*.[17] in their *in vivo* study also showed the rapid action of PZQ on the tegument of the worms, which was already observed within 1 hour after treatment with PZQ. Similar changes were observed in *S. mansoni* in other studies using Oxamniquine[15-17].

CONCLUSIONS

The present findings provide a sound basis for further in-depth

studies of the schistosomicidal activity properties of PZQ, both in the recent isolates of *S. mansoni* in comparison with well-established strains held in laboratory. Furthermore, there is increasing concern about the development of the resistance of this parasite to PZQ. Consequently, vaccine strategies represent an essential component for the future control of schistosomiasis as an adjunct to chemotherapy. In addition, once resistant strains have been detected, some interventions should be quickly carried out to prevent the spread of drug-resistant genes in schistosome-endemic regions.

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