Chemotherapeutic effects on larval stages of *Schistosoma mansoni* during infection and re-infection of mice

Efeitos da quimioterapia nos estágios larvais do *Schistosoma mansoni* durante infecção e re-infecção de camundongos

Luciana M. Silva[1], Rejane M. C. Menezes[1], Sheilla Andrade de Oliveira[2] and Zilton A. Andrade[1]

Abstract *The sensitivity of the larval stages of Schistosoma mansoni to chemotherapy with praziquantel and oxamniquine was tested in mice during primary and secondary infections and after different intervals from cercarial exposure. Worm recovery by perfusion of the porto-mesenteric system, followed by counting and a morphometric study of the parasite, allowed the conclusion that the relative resistance of the larval stages of S. mansoni to schistosomicide drugs, demonstrated in primary infections, also persists when the host is already infected. This indicates that a therapeutic failure may result when an infected host is treated some time after being re-infected, because of the presence of migrating, drug-resistant, immature forms of the parasite.*

Key-words: *Schistosoma mansoni*. Larval stages. Praziquantel. Oxamniquine.

Resumo *A susceptibilidade dos estágios larvais do Schistosoma mansoni aos esquistossomicidas praziquantel e oxamniquine foi testada em camundongos durante infecção primária ou secundária, e após diferentes intervalos de tempo após a exposição cercariana. A avaliação foi feita pela contagem dos vermes após recuperação destes por perfusão do sistema porto-mesentérico e pelo estudo morfométrico dos mesmos. O estudo revelou que a relativa resistência das formas larvais aos esquistossomicidas, já demonstrada em infecção primária, persiste no caso de hospedeiros já infectados. Este fato indica que uma falha terapêutica pode resultar quando o tratamento é feito em hospedeiros re-infectados recentemente, em virtude dos mesmos apresentarem formas migrantes e imaturas do parasita, as quais são particularmente resistentes aos esquistossomicidas.*

Palavras-chaves: *Schistosoma mansoni*. Estágio Larval. Praziquantel. Oxamniquine.

Oxamniquine and praziquantel are the two drugs used in Brazil for the treatment of schistosomiasis. Large scale treatment programs using either drug have been carried out in several Brazilian endemic areas[10 11 13]. Treatment efficacy is considered satisfactory, but failures have been registered with variable frequency. Such therapeutic failures are often attributed to the development of drug resistance by the worms and to early post treatment re-infection[2 6 7]. However, in areas of high transmission, another possibility may occur. Treated individuals, even though apparently cured, may re-establish the infection in consequence of the existence of migrating larval forms of *Schistosoma mansoni*, which are usually resistant to chemotherapy, as experimentally demonstrated[16 21].

Sabath et al[16] have shown that schistosomula are far less susceptible than adult worms to six schistosomicides at curative doses, including praziquantel and oxamniquine. However, these studies were performed in mice with experimental primary infections treated at different points of time after cercarial exposure. Since the effect of chemotherapy is influenced by the immune state of the host[3 4 15], the response of the larval stages of *S. mansoni* to schistosomicides should also be tested during re-infection.

The present investigation studied the effects of oxamniquine or praziquantel on different larval stages of *S. mansoni* in mice with primary infection compared to mice submitted to re-infection.

MATERIAL AND METHODS

Two main experiments were performed. The first experiment involved 185 outbred Swiss mice of both sexes, weighing 18-22g, that were infected transcutaneously with 100 *S. mansoni* cercariae of the Feira de Santana strain[1]. This strain has been maintained through successive passages in laboratory-raised *Bimphalaria glabrata.* The infected animals were randomly separated into three groups:

Group 1: consisted of 25 mice that were not subjected to drug treatment, and served as untreated controls. Animals were sacrificed 40 days after cercarial exposure, when the worms were recovered from the portal-mesenteric venous system by perfusion, according to Duvall and DeWitt[8].

Group 2: included 80 mice treated with oxamniquine. The drug was suspended in distilled water and administered by gavage in a single dose of 100mg/kg/bw. at 5, 10, 20 or 30 days following infection, 20 different animals being treated at each time point. Thirty days after treatment (35-60 days after infection), the animals were anesthetized and sacrificed by severing the abdominal aorta. Worms were recovered by perfusion of the portal system.

Group 3: also with 80 mice, received praziquantel, administered by gavage in a dose of 400 mg/kg/w, otherwise following the same steps as for the previous Group. Another approach was made with 153 Swiss mice, which were at first submitted to transcutaneous infection with 30 *S. mansoni* cercariae. Thirty to forty-five days afterwards, the animals were exposed to re-infection with 50 cercariae. Only mice passing viable eggs in the stools were used. Similarly to the first experiment, re-infected animals were separated into three groups, as follows: a) untreated re-infected controls (n = 13); b) treated with oxamniquine (100mg/kg, n = 64); and c) treated with praziquantel (400mg/kg, n =

64). Chemotherapies were administered by gavage one time only, 10, 20 or 30 days following re-infection, for sub-groups of animals, according to the intervals after re-infection. Twenty days after treatment, re-infected animals were anesthetized and sacrificed for recovery of worms. Untreated controls were sacrificed 30 days after re-infection.

An additional group was also included in this experiment, consisting of an extra non-treated control group with primary infection for comparing the effect of the cumulative infections (n = 12, 30 cercariae).

Drug efficacy was determined through the percentage of worm load reduction, by using the formula by Sabah et al[15] (R*=100 – (t x 100/c), where t = median of worms recovered from the treatment group and c = median of worms recovered from the untreated group, and comparing treated and non-treated animals in their respective groups and sub-groups. Median numbers of worms were compared by means of the Mann Whitney test.

In an attempt to separate the *young* and *old* worms from infections made at different times, a morphometric analysis was performed. Male and female worms were recovered from mice with one single infection, and from those infected and re-infected, including treated and non-treated controls. The worms were fixed in neutral 10% formalin, stained with carmine chloride, dehydrated in alcohol, cleared in creosote and mounted on glass slides with a 1:1 mixture of Canadian balsam and creosote for microscopic examination. Considering sexual dimorphism, morphometric evaluation was made separately for male and female worms. A Leica Quantimet 500C (Leica Cambridge, U.K.) system, with a Sigma-Scan Measurement semi automatic morphometric device (Jandel Scientific, SF, USA) was used. The variables considered were: corporeal length; number of testicular masses; and the presence of eggs in uterus.

RESULTS

The first experiment revealed that the sensitivity of *S. mansoni* larvae to both oxamniquine and praziquantel varied according to their developmental stage. Oxamniquine yielded poor results during the first 20 days of infection, the percentage reduction of worm-load was 28-34% in treated as compared to non-treated mice (Table 1). However, 30 days after exposure, the percentage reduction was 86 to 100%, which was statistically significant when compared to pre-treatment. The *p* value varied from 0.001 to < 0.0001. For the other periods the effect of chemotherapy was the same. Regarding praziquantel, efficacy was 48 to 52% for the animals treated until 10 days after cercarial exposure, dropping to 19% on the 30th day. For the two first experimental points, praziquantel did not improve the efficacy in further reducing worm load (*p* = 0.9440)**.** However, between the 10th to 30th days, reduction in worm load was significant. Compared to oxamniquine,

the efficacy of praziquantel did not essentially differ on the 10th day period (*p* = 0.07), although the latter seemed more effective on the 20th day of infection (*p* <0.0001), while better effects occurred with the former by the 30th day, *p* <0.0001. (Table 1).

During the second experiment the percentage reduction of worm load was greater for both treatment groups when compared to the re-infected controls. (Figures 1b, c, d, e, f and g; and Table 2). Oxamniquine was seen to be very effective by the 10th day following re-infection, presenting a reduction of 64%. These figures decreased subsequently by the 20th day post infection (p=0.0409), while still varying with time, a few mice were totally cured among those treated later on. Thus**,** no variation in the chemotherapy action was demonstrated between 20 and 30 days following cercarial exposure (*p* = 0.1031). In fact, praziquantel

Table 1 - Mice exposed to 100 Schistosoma mansoni *cercariae and treated with either oxamniquine or praziquantel after different periods of pre-patent infection.*

Groups	No. of animals per group	Day of treatment following cercarial exposure	Mean no. of worms recovered	Variation	%R*	*p*
Untreated controls	22	0	26.7	14-52	-	-
Oxamniquine	20	5	18.0	6-30	33.0	0.0004
	20	10	19.4	1-39	28.0	<0.014
	18	20	17.8	3-37	34.0	0.0016
	20	30	3.6	0-13	86.0	<0.0001
Praziquantel	19	5	14.0	6-43	48.0	<0.0001
	20	10	13.5	2-30	52.0	<0.0001
	20	20	5.4	0-18	80.0	<0.0001
	20	30	21.9	1-40	19.0	0.08

R* Percent of worm-load reduction.

Table 2 - Mice exposed to 30 Schistosoma mansoni *cercariae, re-infected with 50 cercariae, and then treated with either oxamniquine or praziquantel after different periods of pre-patent re-infection.*

Groups	Day of treatment followingre-infection	No. of animals per group	Mean number of worms	Variation	%R*	*p*
ControlInfection with 30c	-	12	12.2	3-26	-	-
Re-infection	-	13	20.6	10-31	-	-
Oxamniquine	10	23	7.4	0-16	64.0	0.005
	20	21	14	0-41	32.0	0.03
	30	20	9.0	0-27	57.0	0.035
Praziquantel	10	19	6.0	0-16	76.0	0.027
	20	22	7.7	0-18	74.0	0.034
	30	23	5.4	0-16	76.0	0.004

R* Percent of worm-load reduction.

reduction of worm-load for the animals re-infected and treated reached 75%. Praziquantel was more effective than oxamniquine in reducing worm load during re-infection, although not at a statistically significant level. A 76% reduction occurred when treatment was given at the 10th, 20th (74%) and 30th day following re-infection (76%). All animals treated after re-infection showed statistically significant differences in worm-load reduction when compared with the re-infected but untreated control group.

The mean male worm length for the control group of single infection was 7,958 x 10^{-3}mm, with a confidence interval between 7,644.5 and 8,272.2 x10^{-3}. The figures for females were: 10,457.4 x 10^{-3} with confidence interval of 9,536.1 to 11,378.7 x 10^{-3}. To evaluate worm length from a second infection, the values obtained were distributed in quartiles. In quartile 25 it was noted that the length of male and female worms was 6,300 x 10^{-3} and 7,356 x 10^{-3}, respectively. It was considered that worms of both sex with body length equal or inferior to these figures represented worms from the second infection. Figure 2 is based on such data.

DISCUSSION

The present study shows that the effect of the schistosomicide drugs, praziquantel and oxamniquine, upon migrating schistosomula in mice differs according to the drug used and the time of infection. Furthermore, several authors have reported that the larval stages of *S. mansoni* are less susceptible to chemotherapy than young and adult worms[3 16 21]. These differences were now seen to be accentuated when the treatment was made after several different periods following re-infection.

It has been demonstrated that the drug-induced killing of adult worms is mediated by the host immune system[3 4 15]. The schistosomicides administered cause focal areas of damage to the worm tegument[12 18], exposing target antigens to host antibodies, which ultimately provoke the worm death[3 9 17]. Unfortunately,

the role played by the immune system cannot be enhanced by means of artificial antigenic stimulation[19].

There are few and contradictory experimental data concerning the participation of the immune response during the drug killing of immature schistosomes. Studies made with immunofluorescence labeling after recovering of lung-stage larvae, soon after treatment with praziquantel, revealed mild tegumental lesions with exposure of parasite antigens[9]. However, transfer of the immune serum from 6 to 7-week-old infections to intact mice carrying 25-day-old schistosome infections did not enhance praziquantel chemotherapy of these infections[3].

Some reduction in worm load in re-infected animals is to be expected since infected mice are not only immunologically protected from a new cercarial
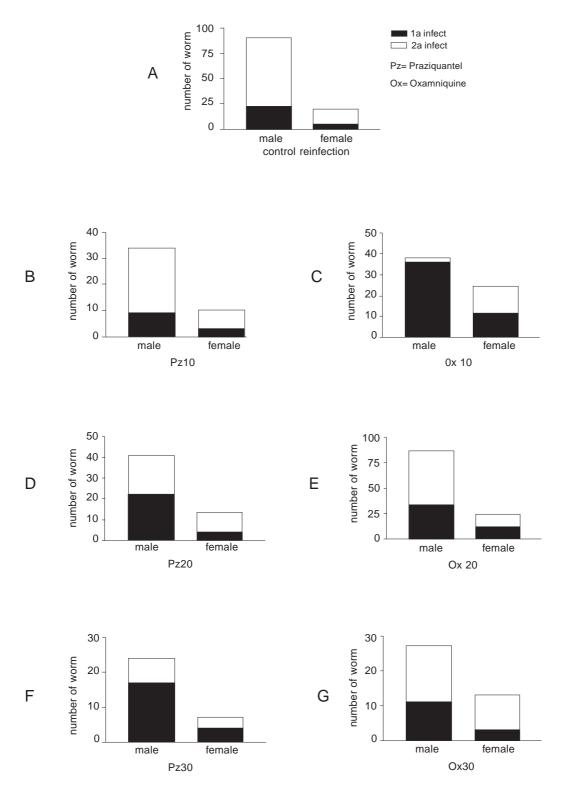
337

Figure 1 - Worms recovered (males and females) after single infection and re-infection with Schistosoma mansoni and following treatment with either praziquantel or oxamniquine at different periods after re-infection.
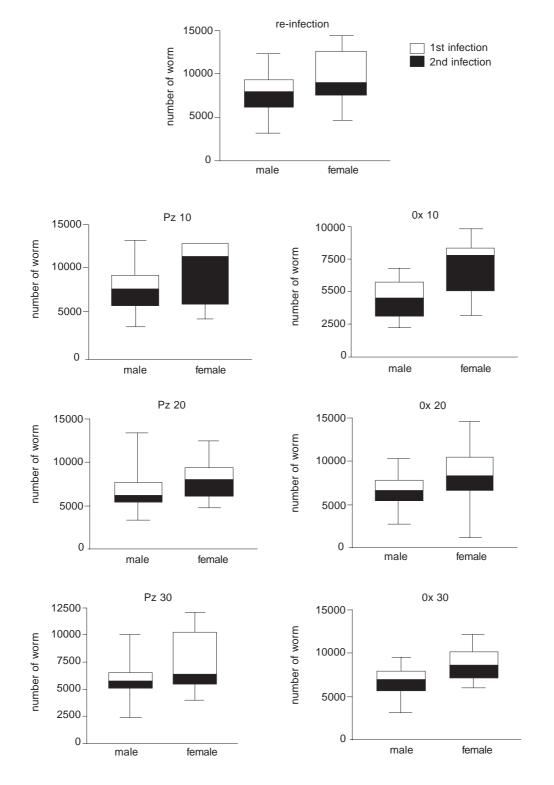
Figure 2 - Length (mm) of recovered Schistosoma mansoni after treatment with praziquantel (Pz) or oxamniquine (Ox), at 10, 20 and 30 days following re-infection of mice.

infection[5], but also by other non-immunological factors. Some studies have demonstrated that the migrating schistosomula of a re-infection can be diverted into portal collateral vessels toward peripheral tissues, where they may die as a result of being out of their natural habitat[14 20]. However, although these factors are important, they alone do not explain the present results.

A significant worm load reduction occurred after treatment in all treated and re-infected groups. In addition, there were also marked differences in relation to the period after re-infection when the treatment was made. In this regard, the 64% reduction in worm load observed with oxamniquine treatment starting on day 10 after re-infection, can be confronted with that of 28% in controls with single infections. This latter result also contrasts with the general observation, derived from the treatment of single infections, which revealed that the more immature the schistosomula, the more resistant they are to schistosomicides.

In animals exposed to a second infection, praziquantel exhibited greater homogeneity of positive results, with a percentage of worm reduction in the order of 75%, in comparison to the 32 to 64% obtained with oxamniquine (Table 1). The number of testicular masses evaluated in the different groups, did not show significant differences in regard to re-infection or treatment, although oxamniquine revealed a greater reduction in egg production.

In spite of the observation that schistosomicide drugs were effective against migrating *S. mansoni* larvae during re-infection, the effect was always partial.

Therefore, these data support the possibility that a therapeutic failure may be due to the presence of migrating forms of *S. mansoni,* which escaped being killed by drugs within the organism of a treated individual.

ACKNOWLEDGEMENT

REFERENCES

1. Andrade ZA, Sadigursky M. Um estudo comparativo das cepas Feira de Santana (Bahia) e Porto Rico do *Schistosoma mansoni* na infecção experimental do camundongo. Memórias do Instituto Oswaldo Cruz 80:37-40, 1985.

2. Araújo N, Souza CP, Passos LKJ, Simpson AJG, Dias Neto E, Pereira TR, Curutti Jr C, Alencar FEC, Dietze R, Katz N. Susceptibilidade aos agentes quimioterápicos de isolados de *Schistosoma mansoni* oriundos de pacientes tratados com oxamniquine e praziquantel e não curados. Revista da Sociedade Brasileira de Medicina Tropical 29:467-476, 1996

3. Brindley PJ. Relationship between chemotherapy and immunity in schistosomiasis. Advances in Parasitology 34:133-161, 1994.

4. Brindley PJ, Sher A. The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. The Journal of Immunology 139:215-220, 1987.

5. Coelho PMZ, Mello RT, Pollom TEV. *Schistosoma mansoni*: Re-infections and concomitant immunity in mice. Importance of perfusion time after challenge infection for evaluation of immunoprotection. Memórias do Instituto Oswaldo Cruz 90:507-509, 1995.

6. Conceição MJ, Agento CA, Correa A. Study of *Schistosoma mansoni* isolates from patients with failure of treatment with oxamniquine. Memórias do Instituto Oswaldo Cruz 95: 375-380, 2000.

7. Costa MFFL, Katz N, Dias JCP. Reinfecção de pacientes em áreas endêmicas de esquistossomose mansoni após tratamento específico. Revista do Instituto de Medicina Tropical de São Paulo 22:97-107, 1980.

8. Duvall RH, DeWitt WB. An improved perfusion technique for recovering adult schistosomes from laboratory animals. The American Journal of Tropical Medicine and Hygiene 16:483-486, 1967.

9. Flisser A, McLaren DJ. Effect of praziquantel treatment on lung-stage larvae of *Schistosoma mansoni in vivo*. Parasitology 98:203-211, 1989.

10. Katz N. Experiência com quimioterapia em grande escala no controle da esquistossomose no Brasil. Revista do Instituto de Medicina Tropical de São Paulo 22:40-51, 1980.

11. Kloetzel K, Schuster NH. Repeated mass treatment of schistosomiasis mansoni: experience in hyperendemic area of Brazil. I. Parasitological effects and morbidity. Transactions of the Royal Society of Tropical Medicine and Hygiene 81:365-370, 1987.

12. Kohn A, Serapião CJ, Katz N, Dias EP. Ação da oxamniquine sobre o *Schistosoma mansoni* em camundongos experimentalmente infectados. Revista do Instituto de Medicina Tropical de São Paulo 21:217-227, 1979.

13. Machado PA. The Brazilian program for schistosomiasis control, 1975-1979. The American Journal of Tropical Medicine and Hygiene 31:76-86, 1982.

14. Medeiros MVMJ, Andrade ZA. The importance of collateral portal veins for resistance to schistosomiasis in mice. Brazilian Journal of Medical and Biological Research 19:85-88, 1986.

15. Sabah AA, Fletcher C, Webbe G, Doenhoff MJ. *Schistosoma mansoni*: Reduced efficacy of chemotherapy in infected T-cell-deprived mice. Experimental Parasitology 60:348-354, 1985.

16. Sabah AA, Fletcher C, Webbe G, Doenhoff MJ. *Schistosoma mansoni:* Chemotherapy of infections of different ages. Experimental Parasitology 61:294-303, 1986.

17. Shaw MK. *Schistosoma mansoni*: stage-dependent damage after *in vitro* treatment with praziquantel. Parasitology 100:65-72,1990.

18. Shaw MK, Eramus DA. *Schistosoma mansoni*: structural damage and tegumental repair after *in vivo* treatment with praziquantel. Parasitology 94:243-254, 1987.

19. Silva LM, Andrade ZA. Immunostimulation as adjuvant for the chemotherapy of experimental schistosomiasis. Revista do Instituto de Medicina Tropical de São Paulo 39:11-14,1997.

20. Wilson RA, Coulson PS, McHugh SM. A significant part of the "concomitant immunity" of mice to *Schistosoma mansoni* is the consequence of a leaky hepatic portal system, not immune killing. Parasite Immunology 6: 595-601, 1983.

21. Xiao SH, Catto BA, Webster LT Jr. Effect of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo.* Journal of Infectious Diseases 151:1130-1137, 1985.