

COMPARATIVE STUDIES ON THE PATHOLOGICAL FINDINGS AND MORTALITY IN *SCHISTOSOMA MANSONI* INFECTED MICE AFTER TREATMENT WITH ARTESUNATE AND THE CURRENT ANTISCHISTOSOMAL DRUGS

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Abstract. The effect of artesunate (ART) on the pathology and mortality rate of in *Schistosoma mansoni* infected mice was comparatively studied with the current drugs of choice for the treatment of schistosomiasis mansoni: praziquantel (PZQ) and oxamniquine (OX). *S. mansoni* experimentally infected mice were treated at 9th week of infection with ART, PZQ or OX at an oral dosage of 300 mg kg⁻¹, 600 mg kg⁻¹ and 100 mg kg⁻¹, respectively. Untreated, infected mice and non-infected mice were added as controls. Samples of mice were sacrificed and examined for the pathological findings at 1 week, 1 month, and 3 months after treatment. At 1 week after treatment, both gross and microscopic lesions were observed. No significant differences were noted among the infected groups. Differences were observed at 1 month after treatment. The lesions decreased more rapidly in groups treated with PZQ and OX. At 3 months after treatment, there were significant differences in the pathological findings among groups. In the groups treated with PZQ and OX, the lesions were markedly reduced and rarely found, but they were clearly observed in the group treated with ART and in the untreated, infected group. High mortality was also recorded in the group treated with ART and in the untreated, infected group. Therefore, the treatment of *S. mansoni* infected mice at 9 weeks of infection with ART did not reduce the pathological findings or the mortality rate compared to treatment with the current recommended schistosomicides, PZQ and OX.

INTRODUCTION

Praziquantel (PZQ) and oxamniquine (OX) have been accepted as drugs of choice for the treatment of *Schistosoma mansoni* infection for a long time (Cioli, 1998; Stanley, 2004). However, some drug tolerance and a high incidence of reinfection have been observed (Wilkins, 1989; Giboda *et al*, 1994; Cioli, 1998). Therefore, the effect of other drugs on schistosome infections should be considered and studied.

The derivatives of artemisinin, artemether, arteether, artesunate (ART) and dihydroartemisinin, not only show antimalarial activity but also

antischistosomal activity (Li *et al*, 1996; Xiao *et al*, 1996, 2000; De Clercq *et al*, 2000; Utzinger *et al*, 2002). They reduced the number of recovered worms, especially female worms, and also reduced the egg excretion. One interesting antischistosomal activity of artemether and arteether is their effect on the reproductive organs of adult worms, which are considered as a target for the reduction of egg-induced pathology (Xiao and Catto, 1989; Giboda and Smith, 1994). Previous studies on the effect of artemisinin derivatives against schistosomiasis mostly emphasized the prophylaxis effects of the drugs, particularly artemether, and the definitive hosts were treated at an early stage of infection (Xiao *et al*, 1994a,b, 2000; Li *et al*, 1996). In addition, the effects of the drugs were generally assessed based on parasitological findings (Xiao and Catto, 1989; Li *et al*, 1996; Xiao *et al*, 1996; Utzinger *et al*, 2002).

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This study was designed to investigate the effect of artesunate on the pathological findings and mortality rate of *S. mansoni* infected mice after treatment at a late stage of infections and compared with the current most effective antischistosomal drugs at the present time.

MATERIALS AND METHODS

Parasite

The P.R. strain of *S. mansoni* was experimentally maintained in *Biomphalaria glabrata* snails and ICR mice at the Applied Malacology Center, Faculty of Tropical Medicine, Mahidol University, Thailand.

Mice

Female, 5-week old outbred ICR mice were obtained from the National Laboratory Animal Center, Thailand and used throughout the experiment. A total of 60 mice were percutaneously infected with 75 *S. mansoni* cercariae using the tail immersion method under anesthesia with sodium pentobarbital. A group of 15 normal mice was added as non-infected controls. All the mice were kept under same conditions and fed *ad libitum*.

Drugs and drug administration

A 600 mg praziquantel tablet (Praziquantel® Atlantic Pharmaceutical, Thailand), 250 mg oxamniquine capsule (Mansil® Laboratorios Pfizer, Brazil) or 50 mg artesunate tablet (Guilin Pharmaceutical Works, People's Republic of China) were suspended in distilled water at the concentration of 30 mg ml⁻¹, 10 mg ml⁻¹ and 30 mg ml⁻¹, respectively. At 9 weeks after infection, all infected mice were randomly divided into 4 equal groups. Three groups were treated with an antiparasitic drug. PZQ was given orally to the first group of mice at a total dosage of 600 mg kg⁻¹ (divided into 2 equal doses of 300 mg kg⁻¹ given 8 hours apart). The second and third group of mice were treated orally with OX and ART at a single dosage of 100 mg kg⁻¹ and 300 mg kg⁻¹, respectively. The fourth group of mice were not treated, but kept as untreated, infected controls.

Mortality

The numbers of mice that died with lesions of schistosomiasis in each group were counted

to calculate the percentage of mortality. A final number of 15 mice in each group was achieved by the substitution of dead mice with equal numbers of infected or treated mice.

Mouse necropsy, gross lesion examination and specimen collection

At 1 week, 1 month, and 3 months after treatment, the number of 5 mice in each group were randomly selected and then sacrificed by cervical dislocation. Gross lesions in visceral organs were observed. Lesions of hepatomegaly and splenomegaly were determined by comparing the weights of liver and spleen as a percentage of body weight. The liver samples were usually obtained from the right lateral lobe and caudate lobe of the liver, and the whole intestines were collected, weighed and frozen at -20°C. Pieces of tissues that consisted of lesions were collected and fixed in 10% neutral buffered formalin.

Tissue egg count

Frozen liver and intestines samples were separately digested in 40 ml of 4% potassium hydroxide at 37°C for 18 hours. The numbers of eggs in the digest were counted and then calculated to determine the number of eggs per gram of tissue.

Histopathological examination

The formalin-fixed tissue was processed, embedded in paraffin, sectioned in 4 mm sections and stained with hematoxylin and eosin. Some slides were studied in more detail by staining using Masson's trichrome method. The size of the hepatic periovular granuloma was determined by measuring the diameter. The vertical and horizontal diameters of the granuloma with a visible centrally placed schistosome egg or egg debris were measured using an ocular micrometer. The average of vertical and horizontal diameters were taken to be the diameter of the granuloma. The average diameter of 10-70 granulomas was counted for each group.

Statistical analysis

Quantitative data differences between each of the group were analyzed using Mann-Whitney tests; quantitative data differences within each group were analyzed using Kruskal-Wallis tests and ANOVA. The percentage of mortality was

analyzed using the chi-square test. P-values less than 0.05 were considered to be statistically significant. SPSS 12.0 for Windows® was used in the data analysis.

RESULTS

Mortality

The number of mice that died after treatment and the percentages of mortality in each group are summarized in Table 1. Statistical analysis showed significant differences in the percentages of mortality in the groups of the experiment ($p=0.031$).

Gross lesions

Whitish spots distributed in the liver and intestines were numerous and commonly observed in the untreated mice and in the group treated with ART at each necropsy, while numbers of whitish spots in the liver and the gut were significantly reduced according to the time after treatment with PZQ and OX.

Hepatomegaly was distinctly observed in all the treated groups at 1 week after treatment, similar to the untreated control group, with no significant differences among the groups ($p=0.684$) (Table 2). At 1 month after treatment, the size of the liver was reduced in all the treated groups, significantly different from the untreated control group ($p=0.022$). However, the size of the livers in the group treated with ART were not significantly reduced in the groups treated with PZQ and OX at 3 months after treatment. Statistical analysis showed no significant difference

in the size of the livers between the ART treated mice and the untreated mice ($p=0.841$). In contrast, the size of the livers in the groups treated with PZQ and OX were decreased to normal size, and not significantly different from the non-infected control mice ($p=0.491$).

The size of the spleen in all the treated groups was decreased according to time after treatment, however, not as significantly reduced as the size of the livers. At 3 months after treatment, there was no statistical difference in the size of spleens among all the treated groups and the untreated groups ($p=0.052$).

Microscopic lesions

Histopathological findings were similarly observed in all the treated groups and untreated groups at 1 week after treatment. Most of the periovular granulomas in the livers and intestines were exudative phase granulomas that consisted of numerous eosinophils, lymphocytes and a few scattered collagen fibers surrounding a core of viable schistosome eggs (Fig 1). However, non-viable eggs were predominant in the group treated with PZQ. Inflammatory cell infiltrations in the livers and intestinal walls, coagulative necrosis of hepatocytes, hepatic and splenic congestion, and the presence of schistosome pigments in macrophages and Kupffer's cells were generally observed in the treated groups and the untreated group. Granulomas surrounding dead worms were found predominantly in the livers of mice treated with PZQ.

Microscopic lesions of mice sacrificed at 1 month after treatment showed significant differences among the groups. For the groups treated with PZQ and OX, most of the granulomas were non-exudative phase granulomas that consisted of macrophages, fibroblasts, epithelioid cells and dense concentric arranged collagen fibers surrounding non-viable eggs. This type of granuloma was found in the group treated with ART as well as the untreated group. However, exudative phase granulomas were still commonly observed in these two groups. Inflammatory cell infiltration and coagulative necrosis of hepatocytes were rarely observed in the groups treated with PZQ and OX, but frequently found in the group treated with ART and in the untreated group.

Table 1
Mortality of mice in the various groups.

Group (n=15)	No. of died mice	Mortality (%) ^a
Praziquantel	0	0
Oxamniquine	1	6.7
Artesunate	4	26.7
Untreated, infected control	4	26.7
Non-infected control	0	0

^aMortality (%) = $\frac{\text{Number of mice died after treatment}}{\text{Number of treated mice}} \times 100$

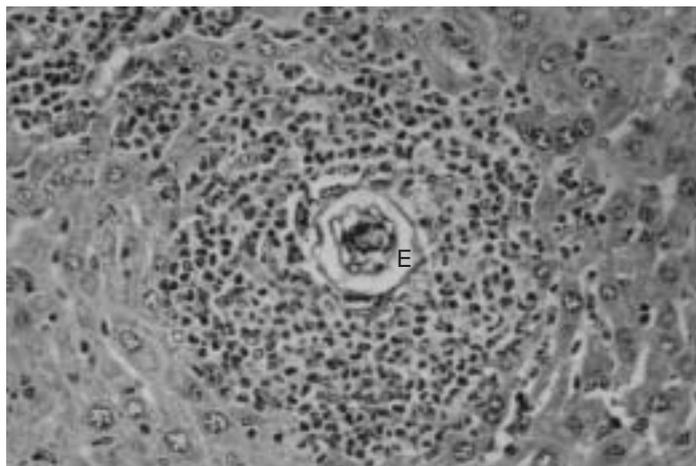


Fig 1—Hepatic periovular granulomas in mouse at 1 week after treatment with ART, shows a core of viable schistososome eggs (E) surrounded with numerous eosinophils and lymphocytes (H&E, 200 x).

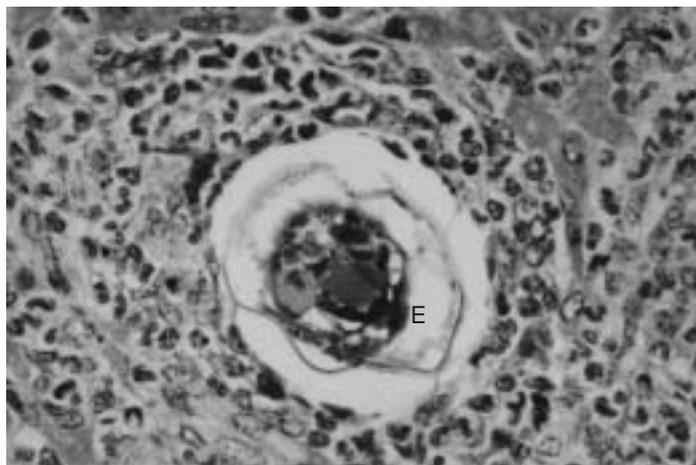


Fig 2—Viable schistososome eggs (E) surrounded with eosinophils in the liver of a mouse at 3 months after treatment with ART (H&E, 400 x).

At 3 months after treatment, microscopic lesions were different and clearly distinguishable among the experimental groups. For the groups treated with PZQ and OX, the hepatic periovular granulomas were rarely found and all were small granulomas with non-viable eggs and few macrophages. Fibrous scars were often observed. Numerous periovular granulomas were still commonly observed in the group treated with ART, similar to the untreated control group. Most of them were non-exudative granulomas. However, a small number of exudative granulomas with vi-

able eggs were occasionally observed (Fig 2). Inflammatory cell infiltration and coagulative necrosis of hepatocytes were still found in these 2 groups.

Numbers of eggs in tissues

The mean number of eggs per gram of liver and intestines is shown in Table 2. The numbers of eggs in all the treated groups were reduced according to time after treatment. For the group treated with PZQ, the number of eggs was significantly reduced beginning at 1 week after treatment, and was statistically different from the other treated groups and the untreated group ($p=0.016$). At 3 months after treatment, tissue-deposited eggs were rarely observed in the livers of mice treated with PZQ. For the group treated with OX, the number of eggs in the liver showed statistical difference from the untreated group beginning at 1 month after treatment ($p=0.016$). The number of eggs per gram of liver in the group treated with ART reduced slowly and was not significantly different from the untreated group at 1 week and 1 month after treatment. At 3 months after treatment, the number of eggs in the group treated with ART was lower than the untreated group, however, it was still much higher than the groups treated with PZQ ($p=0.007$) and OX ($p=0.014$).

The numbers of eggs in the intestines in all the treated groups was not reduced as rapidly as the number of eggs in the liver. At 1 week after treatment, there was no significant difference in the number of eggs per gram among all the groups ($p=0.7$). However, the number of eggs in the intestines was later reduced in the same pattern as the number of eggs in the liver.

Diameter of hepatic periovular granulomas

The diameters of the hepatic periovular granulomas were more rapidly reduced in the

Table 2
Weight of the liver and spleen as a percentage of body weight, number of eggs in the liver and guts, and diameter of hepatic periovular granulomas.

Time of necropsy	Group (n=5)	Weight of liver as % BWT ^{a,b} (mean ± SD)	Weight of spleen as % BWT ^b (mean ± SD)	Number of eggs per gram of liver (mean ± SD)	Number of eggs per gram of guts (mean ± SD)	Diameter of granuloma, μm (mean ± SD)
1 week post-treatment	Praziquantel	7.67 ± 0.69	1.26 ± 0.37	3,683 ± 638.46	5,546 ± 980.43	315.07 ± 67.46
	Oxamniquine	7.73 ± 0.97	1.07 ± 0.26	4,996 ± 1,168.78	5,837 ± 1,447.23	315.94 ± 64.16
	Artesunate	8.07 ± 1.04	1.21 ± 0.30	6,186 ± 1,058.13	6,524 ± 945.08	300.11 ± 56.32
	Untreated, infected control	7.44 ± 0.47	1.02 ± 0.04	6,635 ± 908.60	7,088 ± 811.77	314.32 ± 70.21
1 month post-treatment	Non-infected control	4.78 ± 0.29	0.26 ± 0.05	-	-	-
	Praziquantel	6.51 ± 0.43	0.71 ± 0.11	3,483 ± 691.17	5,404 ± 217.71	238.76 ± 65.61
	Oxamniquine	7.03 ± 1.37	0.78 ± 0.35	4,714 ± 873.79	5,420 ± 785.41	253.30 ± 56.20
	Artesunate	6.51 ± 0.54	0.88 ± 0.11	5,632 ± 1,287.64	7,141 ± 1,222.88	248.05 ± 58.28
3 months post-treatment	Untreated, infected control	7.91 ± 0.64	1.14 ± 0.09	7,012 ± 1,290.61	8,793 ± 1,263.58	254.56 ± 48.25
	Non-infected control	4.55 ± 0.21	0.32 ± 0.08	-	-	-
	Praziquantel	4.86 ± 0.31	0.47 ± 0.14	11 ± 25.04	93 ± 90.97	165.92 ± 40.40
	Oxamniquine	4.79 ± 0.38	0.62 ± 0.15	397 ± 282.25	985 ± 449.48	179.67 ± 46.43
3 months post-treatment	Artesunate	5.57 ± 0.42	0.92 ± 0.56	3,881 ± 935.24	4,633 ± 1,232.05	207.48 ± 61.80
	Untreated, infected control	5.90 ± 1.08	0.74 ± 0.18	6,216 ± 1,268.54	8,208 ± 1,417.92	217.43 ± 45.77
	Non-infected control	4.82 ± 0.37	0.28 ± 0.05	-	-	-
	Non-infected control	4.82 ± 0.37	0.28 ± 0.05	-	-	-

^a BWT = body weight; ^b Weight of liver or spleen as % BWT = $\frac{\text{Weight of liver or spleen}}{\text{body weight}} \times 100$

groups treated with PZQ and OX (Table 2). At 3 months after treatment, there were significant differences from the untreated group ($p=0.006$). For the group treated with ART, no statistical difference in the diameter of hepatic periovular granulomas was seen with the untreated group at 1 week and 1 month post-treatment ($p=0.569$ and $p=0.448$, respectively). However, there was a significant difference in the diameter of granulomas between the group treated with ART and the untreated group at 3 months post-treatment ($p=0.003$).

DISCUSSION

ART showed minimal effects on reducing mortality and the pathological findings of *S. mansoni* infected mice when compared with PZQ and OX after treatment at 9-week infection. The lesions of schistosomiasis mansoni were similar in all the groups in the experiment. However, in the treated groups, the number of tissue-deposited eggs at 3 months after treatment was the one exception where there was a significant difference from the untreated group.

The effect of artemisinin derivatives on the pathology of schistosome infection has been previously studied in *S. japonicum* infected mice, rabbits and dogs (Xiao *et al*, 1994a,b). Artemether was found to be effective in preventing pathology if treated at an early stage of infection. Since the schistosomulae and

immature worms are susceptible to the drug, pathology was improved early on. However, the treatment of *S. japonicum* infected rabbits at 7 weeks after infection did not reduce the pathological findings in the liver, because the oviposition of adult worms had already happened, and the adult worms were less susceptible to the drug compared with the schistosomulae and immature worms (Xiao *et al*, 1995). Recently, the effect of ART on different stages of *S. mansoni* worms was similarly observed (Utzing *et al*, 2002). Undoubtedly, treatment of *S. mansoni* infected mice with artesunate at the 9 weeks stage of infection in the present experiment showed a minimal effect on reducing pathology because of the stage-dependent effect of ART. In contrast, adult worms were rapidly damaged with PZQ and OX (Megalhaes *et al*, 1989; Fallon *et al*, 1996). Thus, the pathological findings were significantly reduced.

Apart from the effect of the antischistosomal drugs on worms, the effect of the drugs on the tissue-deposited eggs and egg excretions of adult worms are also important, because the egg is generally accepted as the major cause of pathogenesis in schistosomiasis (Giboda and Smith, 1994). Periovarian granuloma formation is activated with antigen released from fully developed larvae in mature eggs (Giboda *et al*, 1994). Previous studies on the effect of PZQ on tissue-deposited eggs showed that the hatching of mature eggs was immediately induced after drug administration (Matsuda *et al*, 1983; Giboda and Smith, 1994). The immunogenic enzyme, leucine aminopeptidase, was suddenly released and non-pathogenic empty eggshells were left (Cheever *et al*, 1992). Hepatic periovarian granulomas were subsequently reduced in size, and the number of inflammatory cells, as well as the disappearance of inflammatory cells in the liver parenchyma were found (Andrade *et al*, 1993). The direct effect of OX on tissue-deposited eggs has not been previously documented. A reduction in the numbers of eggs in the tissue should be caused by the cessation of new egg production in the adult worms. Thus, the involution in size and number of inflammatory cells in hepatic periovarian granulomas was the result of immunological modulation, not the drug (Coelho *et al*, 1994; Silva *et al*, 2000). The reduction in the

pathological findings was therefore slower than in the PZQ treated mice and the death of the mouse was recorded. Few studies on the effect of artemisinin derivatives on tissue-deposited eggs or egg production from adult worms have been previously documented. Reversible damage to the reproductive organs of adult *S. mansoni* and also the reversible alteration of the oogram in the liver of infected mice was observed after treatment with artemether (Xiao and Catto, 1989; Araujo *et al*, 1991). Treatment of *S. japonicum* infected rabbits with artemether at 7 weeks after infection caused a reduction in the numbers of periovarian granulomas and the numbers of fresh eggs in the liver (Xiao *et al*, 1995). For the present experiment, significantly reducing the numbers of tissue-deposited eggs in *S. mansoni* infected mice was observed at 3 months after treatment with ART. However, viable eggs and numerous inflammatory cells were commonly found in the hepatic periovarian granulomas. Therefore, this alteration in the number of tissue-deposited eggs may be caused by the partial diminution of egg excretion from adult worms, not the direct destruction of tissue-deposited eggs due to treatment with PZQ. In addition, it may be a temporary effect, because viable eggs were generally observed through 3 months after treatment with ART while *S. mansoni* eggs could survive for only 3-4 weeks after maturation in non-medicated mice (Cheever *et al*, 1992). Therefore, the detected viable eggs in the periovarian granulomas were newly excreted eggs from adult worms, similar to those found in the untreated control group.

The mortality of the mice was similarly to the pathological findings in all the groups and confirmed the treatment of *S. mansoni* infected mice with ART at the dosage regimen used caused a minimal effect on reducing the pathology and mortality. However, the treatment of *S. mansoni* infection with ART should not be rejected, since the antischistosomal activity of artemisinin derivatives is dose-dependent. The higher the dosage of ART, the better the efficiency (Xiao *et al*, 1994b; Utzing *et al*, 2002). Additionally, different developmental stages of worms are naturally found in definitive hosts at the same time. Although adult worms are less susceptible to the drug, treat-

ment with artemisinin derivatives can reduce egg-associated pathology due to the destruction of schistosomulae and immature worms, the stages which are not susceptible to PZQ and OX (Utzingler *et al*, 2002). In conclusion, artemisinin derivatives should be considered jointly with PZQ or OX for the complete elimination of parasites.

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