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In vitro* Effect of Mefloquine on Adult *Schistosoma mansoni

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ABSTRACT

Schistosomiasis is a chronic disease that infects over 200 million people worldwide. The treatment and control of schistosomiasis largely depends on a single drug, praziquantel that might result in emergence of drug resistant parasites. Consequently, developing new drugs is a true need. The anti-malarial drug mefloquine has shown schistosomicidal activity. The aim of this study was to assess the effect of mefloquine against adult *S. mansoni* using *in vitro* approach. Ten laboratory bred mice were infected with *S. mansoni* cercariae. After 56 days, mice were sacrificed and adult *Schistosoma* were collected by perfusion. The *in vitro* approach consisted of placing adult *Schistosoma* worms in culture plates containing 100, 10 and 1 $\mu\text{g mL}^{-1}$ mefloquine and incubating the plates at 37°C for 24 h. The length and maximum width of adult *Schistosoma* were measured and LC₅₀ and LC₉₀ of mefloquine and praziquantel were calculated. The results showed that the LC₅₀ for mefloquine and praziquantel were 3.961 and 6.675 $\mu\text{g mL}^{-1}$, respectively. The LC₉₀ for mefloquine was 7.332 $\mu\text{g mL}^{-1}$ while that of praziquantel was 8.695 $\mu\text{g mL}^{-1}$. A statistically significant reduction in length and maximum width in adult worms treated with mefloquine was observed. Mefloquine exerted promising *in vitro* effects on adult *S. mansoni* worms.

Key words: *Schistosoma mansoni*, *in vitro*, mefloquine

INTRODUCTION

Schistosomiasis is one of the most widespread parasitic infections in the world. Three major *Schistosoma* species are known to infect humans including; *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum* (Allen *et al.*, 2002). Schistosomiasis is estimated to cause 280,000 deaths annually (King *et al.*, 2005).

For schistosomiasis treatment three drugs have been used, which differ in their effects on *Schistosoma* species: metrifonate (targeting *S. haematobium*), oxamniquine (targeting *S. mansoni*) and praziquantel (for all human species). Due to its broader spectrum, praziquantel has finally become the first-line medicine (Botros *et al.*, 2005). Praziquantel has poor efficacy against immature *Schistosoma* stages and retreatment is required to kill these parasitic stages as they become mature (Pica-Mattoccia and Cioli, 2004). Production of new anti-schistosomal drugs that are active against all parasitic stages should be encouraged (Keiser *et al.*, 2014). Reliance on a single drug carries the risk of development of drug resistance. Lower cure rates following administration of praziquantel have already been observed in different regions with high prevalence (Doenhoff *et al.*, 2008).

Therefore, drug discovery for treatment of schistosomiasis remains an important task. Development of new drugs with anti-schistosomal properties has been accomplished by many research works as synthetic trioxolanes (Xiao *et al.*, 2007) and oxadiazoles (Sayed *et al.*, 2008). Another strategy for treatment and control of schistosomiasis is the redirection of already used drugs against other parasites to be used against *Schistosomes*. This strategy has a promising future being more rapid and less costly than developing new drugs (Debnath *et al.*, 2012). Over the last few years, the anti-malarial drug mefloquine was investigated for its schistosoicidal effect and evidenced by the reduction in egg burden in *S. mansoni* infected mice following administration of a single dose of 150 mg kg⁻¹ of body weight (Van Nassauw *et al.*, 2008). Keiser *et al.* (2009) reported that a single oral dose of 200 mg kg⁻¹ mefloquine caused a worm burden reduction of 72% in mice harboring *S. mansoni* infection. The authors also documented the effectiveness of mefloquine against the juvenile immature stage. Mefloquine also showed high activity against *S. japonicum* (Xiao *et al.*, 2011) and *S. hematobium* (Ingram *et al.*, 2012), which highlight a promising broad spectrum anti-schistosomal effect. This study aimed to assess the *in vitro* effect of mefloquine on adult *Schistosoma mansoni*.

MATERIALS AND METHODS

The study was carried out in Medical Parasitology and Pathology Departments in Faculty of Medicine, Cairo University and Parasitology Department in Theodor Bilharz Research Institute (TBRI).

The present study was carried out on ten laboratory bred male CD1 Swiss albino mice, aged 6-7 weeks and weighing 14-15 g each. Animals were fed a standard commercial pellet diet. Cercariae of *S. mansoni* were harvested from infected intermediate host snails (*Biomphalaria alexandrina*). Snails were left under ceiling illumination for 3-5 h at 25-27°C. At the end of the exposure time, cercariae from *B. alexandrina* snails were used to infect the mice. The fluid volume was adjusted to contain the desired number of cercariae; 60±10 cercariae/0.2 mL of dechlorinated water.

The cercarial suspension was thoroughly mixed, 0.2 mL of the suspension was withdrawn through a 22 gauge needle into an insulin syringe and injected subcutaneously into the loose skin of the back of the mouse according to the method of Peters and Warren (1969).

Mice in the study were sacrificed by cervical dislocation eight weeks post-infection. Sacrificed mice were decapitated and subjected to porto-mesenteric perfusion to collect adult *Schistosomes* from hepatic portal and mesenteric veins.

Flukes were washed several times with normal saline followed by selection of the healthy worms with normal macroscopic structure and good motility (Smithers and Terry, 1965). The worms were kept in small sterilized sieve and washed three times by phosphate buffer (pH 7.4).

Mefloquine (Mephaquin) tablets were purchased from Mepha Ltd. (Aesch- Basel, Switzerland) and praziquantel tablets were purchased from EIPICO (El-Asher Men Ramadan, Egypt). Drugs were dissolved in Dimethyl Sulfoxide (DMSO) to obtain stock solutions of 10 mg mL⁻¹. Stock solutions of the drugs were diluted in 24 well plates and the following descending drug concentrations were studied: 100, 10 and 1 µg mL⁻¹.

Worms were washed three times and were placed in each well containing RPMI-1640 medium (Sigma-Aldrich, US) containing L-glutamine, 300 mg streptomycin, 300 units penicillin,

160 µg gentamycin and 20% fetal calf serum (Atlanta Biologicals Inc., US). Adult *S. mansoni* worms were exposed to 100 µg mL⁻¹ and 10 fold dilutions were carried out to obtain a concentration of 10 and 1 µg mL⁻¹ of mefloquine. Adult *Schistosomes* were incubated in praziquantel at a concentration of 0.1 µg mL⁻¹ and served as positive control. *Schistosomes* incubated in medium containing 1% DMSO served as negative control. All cultures and assays were conducted at 37±0.5°C and 5% CO₂ for 24 h (10 worms per drug concentration).

Examination for worm viability was done after 24 h using a microscope (10-40 fold magnification). Worms showing no signs of motility for two minutes, associated with worm deformity such as blackening, twisting and contracting were considered dead. The activity of mefloquine was measured by calculating the number of dead worms relative to the total number of worms. Praziquantel was used as the reference drug. Descending concentrations of mefloquine were similarly assessed and the mortality of worms was recorded at each concentration.

The results were used to calculate the Lethal Concentration, 50% (LC₅₀) and the Lethal Concentration, 90% (LC₉₀) of praziquantel and mefloquine.

Adult worms were stained with carmine and mounted on microscopic slides with Permount (Fisher Scientific) and a cover slip. Worms' length and maximum width were measured with a light microscope equipped with an ocular micrometer.

Statistical analysis: Results were collected, tabulated and statistically analyzed using the statistical package SASS version 12. Data were tabulated as mean and Standard Deviation (SD) for quantitative variables. The means of groups were compared with the use of Students t-test. Data were considered significant at a p<0.001.

RESULTS

Mefloquine was tested for its *in vitro* effect on adult *S. mansoni* worms after 24 h of exposure using praziquantel as a reference drug. The earliest observed effects of tested drugs on adults *Schistosoma* were in the form of tight coiling with noticeable contraction and shortening of all worms with separation of the coupled worms that became evident within minutes after addition of each drug individually. After 24 h of exposure of adult *S. mansoni* worms to praziquantel, all worms acquired a curled or coiled appearance. *S. mansoni* worms exposed to mefloquine developed more obvious coiling and tegumental degeneration than those occurring in worms exposed to praziquantel. The worms were removed from incubation in praziquantel and mefloquine and were observed for signs of viability. No apparent motility or reverse of morphological changes were observed in worms exposed to either praziquantel or mefloquine.

The lethal concentration 50% (LC₅₀) for mefloquine was 3.96 µg mL⁻¹ while that of praziquantel was 6.67 µg mL⁻¹. The lethal concentrations 90% (LC₉₀) were 7.33 µg mL⁻¹ for mefloquine and 8.69 µg mL⁻¹ for praziquantel.

There was a statistically significant reduction in length and maximum width in mefloquine treated *Schistosoma* males and females (Fig. 1) in relation to the untreated negative control group (p<0.001) (Fig. 2).

The reduction in sizes of male *Schistosoma* treated with praziquantel (Fig. 3) was statistically insignificant compared to untreated negative control group (p>0.001).

The reduction in sizes of adult female *Schistosomes* in this group was statistically significant (Table 1) (p<0.001).

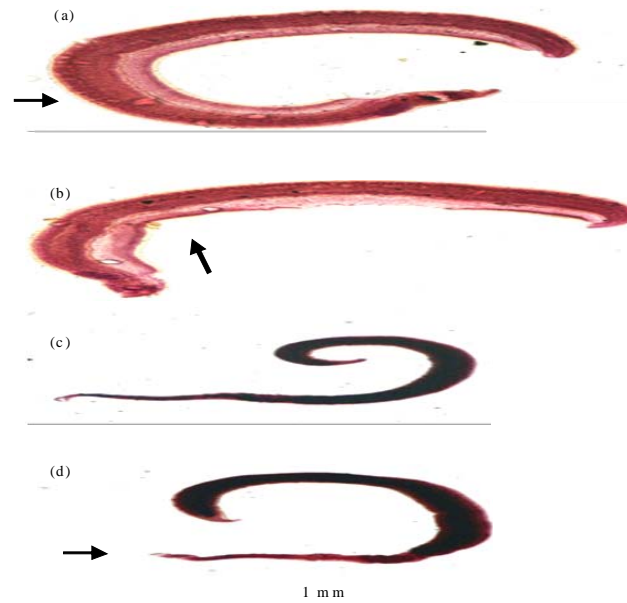


Fig. 1(a-d): Adult *Schistosoma mansoni* (x20) stained with acid carmine stain after incubation with mefloquine showing contraction and coiling (black arrows), (a, b) Male *Schistosoma mansoni* worms and (c, d) Female *Schistosoma mansoni* worms)



Fig. 2(a-c): Normal untreated *Schistosoma mansoni* worms (x20) stained with acid carmine stain, (a) Normal male and female *Schistosoma mansoni* worms in copula, (b) Normal female *Schistosoma mansoni* worm and (c) Normal male *Schistosoma mansoni* worm

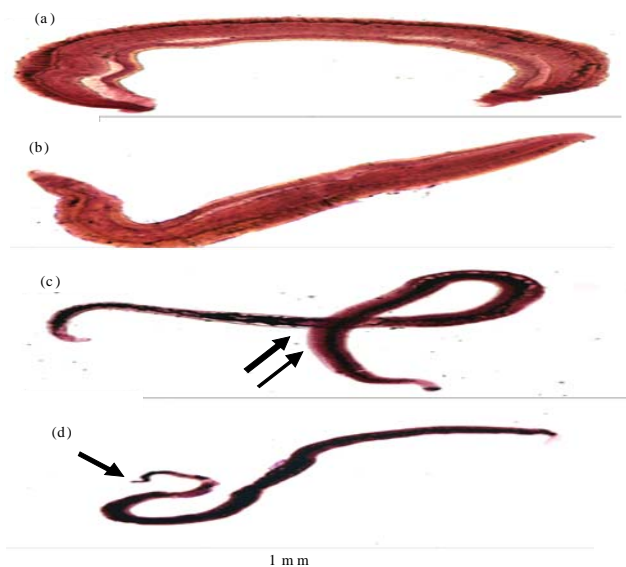


Fig. 3(a-d): Adult *Schistosoma mansoni* worms (x20) stained with acid carmine stain after incubation with praziquantel showing shortening and contraction (black arrows), (a, b) *Schistosoma mansoni* male worms and (c, d) *Schistosoma mansoni* female worms

Table 1: *In vitro* effect of mefloquine and praziquantel on the sizes of adult *Schistosoma mansoni* worms

Parameters	Mean	SD
Untreated worms		
Male (mm)		
Length	5.28	0.926
Maximum width	0.45	0.041
Female (mm)		
Length	7.03	0.970
Maximum width	0.13	0.013
Mefloquine treated worms		
Male (mm)		
Length	2.62	0.346
Maximum width	0.248	0.119
Female (mm)		
Length	2.88	0.176
Maximum width	0.2	0.007
Praziquantel treated worms		
Male (mm)		
Length	3.78	0.518
Maximum width	0.40	0.040
Female (mm)		
Length	4.71	0.769
Maximum width	0.15	0.019

The differences in length and maximum width between mefloquine treated adult *S. mansoni* and praziquantel treated worms were statistically significant with $p < 0.001$ for adult males and females. Mefloquine caused more reduction in length and maximum width of *S. mansoni* worms than the reduction in length and maximum widths of praziquantel treated *S. mansoni* worms as demonstrated in Table 1.

DISCUSSION

Schistosomiasis is a chronic and debilitating disease that occurs in tropical and subtropical areas. More than 230 million people in the tropics and subtropics are infected and the global

burden of schistosomiasis is estimated at 3.3 million disability-adjusted life years (Murray *et al.*, 2013). The treatment of this chronic and debilitating disease relies on the use of praziquantel, a broad-spectrum schistosomicide drug that combines safety and low price (Fenwick *et al.*, 2003). Consequently, this drug pressure could favor the emergence of praziquantel-resistant parasites (Doenhoff *et al.*, 2008). The *in vitro* anti-schistosomal effects of mefloquine have been investigated and the results suggested a promising schistosomicidal effect of this anti-malarial drug (Holtfreter *et al.*, 2011). There is a large geographical overlap between the distribution of malaria and schistosomiasis in sub Saharan Africa, hence the use of mefloquine for treatment of malaria would result in an additional therapeutic effect on schistosomiasis (Kabatereine *et al.*, 2011).

In the present study, exposure of adults *Schistosoma* to mefloquine and praziquantel resulted in a statistically significant reduction in sizes of adult worms, which developed shortening and contraction. These results were in accordance with those reported by Pica-Mattocchia and Cioli (2004), who examined the sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. They reported the occurrence of morphological changes in the form of contraction even after exposure to sub-lethal concentration of the drug.

Similarly, Xiao *et al.* (2009a) found that mefloquine exhibited a direct killing effect against adult *S. japonicum* *in vitro* and reported that the worm motor activity was first stimulated, then decreased significantly, followed by bleb formation, focal swelling and elongation of the worm body, cessation of gut peristalsis and death of 56.3% of worms within 24-72 h. Higher mefloquine concentrations of 20 and 30 $\mu\text{g mL}^{-1}$ caused death of all worms within 4-24 h. They added that the *in vitro* effect of mefloquine against adults *Schistosoma* is irreversible, while that of praziquantel is reversible.

Keiser *et al.* (2009) mentioned that mefloquine exerted a rapid action on *Schistosoma* in the form of marked alterations of the digestive tract and reproductive system of the worms.

Decoupling of the worms could be explained on the basis of morphological changes exerted by mefloquine and reported by Xiao *et al.* (2010) and Manneck *et al.* (2011) who reported various alterations in the tegument of the male *Schistosoma* including, shrinking and sloughing resulting in extensive tegumental destruction. Also, there were abnormalities in the sub-tegumental tissues and focal lyses of musculature, which can lead to decrease the efficiency of holding the female worms in the gynecophoric canal.

The observed morphological changes of adults *Schistosoma* upon exposure to mefloquine in the present study highlight a promising anti-schistosomal effect of mefloquine. Similarly, Holtfreter *et al.* (2001) reported that mefloquine showed time and dose-dependent schistosomicidal effects on the four life stages of *S. mansoni* *in vitro*. In their study, Ingram *et al.* (2012) confirmed the high antischistosomal effect of mefloquine with comparable activity against *S. hematobium* and *S. mansoni*. Recently, mefloquine, used as intermittent preventive therapy against malaria in pregnancy, showed high egg reduction rates in women with a concomitant *S. haematobium* infection (Basra *et al.*, 2013).

On the other hand, Keiser *et al.* (2014) suggested that combining mefloquine with praziquantel gave no better results than using praziquantel alone regarding percentage of egg reduction and caused more adverse effects in children having chronic *S. hematobium* infection. Accordingly, they recommended more studies to be performed to evaluate effects of mefloquine and mefloquine combinations on acute schistosomiasis.

In the present study, LC_{50} and LC_{90} values of mefloquine were 3.96 and 7.33 $\mu\text{g mL}^{-1}$, respectively. While the LC_{50} and LC_{90} values of praziquantel were 6.67 and 8.69 $\mu\text{g mL}^{-1}$,

respectively. On the contrary, Keiser *et al.* (2011) calculated *in vitro* LC₅₀ value for mefloquine against adult *S. mansoni* worms and found that it was 1.9 mg mL⁻¹ and this may be attributed to different strain of *S. mansoni* used in their study.

On the other hand, Xiao *et al.* (2012) studied the *in vitro* effect of mefloquine against *S. japonicum* and found that the LC₅₀ and LC₉₅ of mefloquine was 6.17 µg mL⁻¹ which is about two folds higher than our results in this study.

Regarding the size of the worms measured after staining with aceto carmine stain, there was a significant reduction of the sizes of male and female worms treated with mefloquine in comparison to the untreated worms and to that treated with praziquantel.

Xiao *et al.* (2009b) documented significant reduction in the dimensions of adult *S. japonicum* worms after 24 h of mefloquine treatment which became severe later on. Similarly, Manneck *et al.* (2010) reported distinct morphological characteristics of adults *Schistosoma* incubated with mefloquine in the form of convulsion. Manneck *et al.* (2011) added that when adult *Schistosomes* were exposed to 10 µg mL⁻¹ of mefloquine, they developed extensive tegumental alterations, decrease in metabolic activity and viability and death.

Evaluation of the combined treatment of mefloquine and praziquantel on *S. japonicum in vitro* was done by Xiao *et al.* (2012). They found that the drugs induced moderate to strong spasmodic contractions of the worm body and vesiculation along the worm surface.

In conclusion, exposure of adults *Schistosoma* to mefloquine *in vitro* caused a statistically significant reduction in their lengths and maximum widths. The LC₅₀ and LC₉₀ for mefloquine were 3.96 and 7.33 µg mL⁻¹, respectively. Therefore, mefloquine was found to have good *in vitro* schistosomicidal effect. The present work may open fresh avenues for development of alternative drug therapy to augment the standard chemotherapeutic agents currently employed in the treatment of schistosomiasis mansoni.

Ethical approval: All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The experimental animal studies were managed in accordance with international valid guidelines and animals were maintained under convenient conditions at the animal house in TBRI.

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