

ULTRASTRUCTURAL AND MUSCULAR MODIFICATIONS OF ADULT *SCHISTOSOMA MANSONI* WORMS INDUCED BY ARTEMETHER (ART) *IN VITRO*

By

MAHMOUD A. ELSHAFFEY¹, SAMIA E. ETEWA², MOHAMED H. SARHAN^{2*},
SALWA Z. A. ARAFA³, DALIA A. ABO EL-MAATY²
AND ABD-ALLAH A. A. ALHOOT³

Departments of Clinical Pathology¹, Medical Parasitology², Faculty of Medicine and
Department of Zoology³, Faculty of Science, Zagazig University, Egypt

(*Correspondence: drsarhan@gmail.com)

Abstract

Schistosomiasis is one of the most important public health problems worldwide. In spite of two decades of safe and effective widespread chemotherapy with praziquantel, the number of individuals with schistosomiasis remains high with the appearance of drug resistance. So, it diverts the attention to other anti-parasitic drugs like artemisinin derivatives e.g. artemether (ART).

This study was done to assess the effects of ART on the tegument and musculature of adult *Schistosoma mansoni* worms *in vitro*.

Five groups of *S. mansoni* coupled adult worms, each of 10 couples, were used. Four groups were tested *in vitro* using different concentrations of ART (20, 40, 80 and 100µg/ml) for 24 hours of exposure. These adult worms were obtained by hepatic perfusion technique seven weeks post-infection by 100±10 *S. mansoni* cercariae in male white albino mice using tail immersion method. Ultrastructural and muscular modifications of adult *S. mansoni* worms were assessed using Scanning Electron Microscopy (SEM) and a special device respectively.

SEM examination revealed that after 24 hours of exposure to 40µg/ml ART, the tegument showed vesicles and loss of spines. On exposure to 80µg/ml ART, the tegument showed cracking and more loss of spines. After exposure to 100µg/ml ART, tegument appeared mostly distorted with cracking, peeling, bleb formation and the spines covering the tubercles appeared to be partially lost.

ART elicited muscle contraction and reached the highest response with 100µg/ml ART. The maximal increase in muscle tension (% shortening of worms) was 44.1 ± 1.79 % shortening induced by the highest concentration (100µg/ml) of ART.

Keywords: *Schistosoma mansoni*, coupled adult worms, artemether, *in vitro*, scanning electron microscope, tegument, muscle tension.

Introduction

Schistosomiasis was reported from 78 countries, of which 52 endemic countries with moderate-to-high transmission. About 218 million persons living in rural and agricultural areas are estimated of requiring preventive treatment (WHO, 2017). In spite of decades of widespread chemotherapy, the number of individuals with schistosomiasis remains high (King, 2007). So, it diverts the attention to other parasitic drugs like artemisinin.

Artemisinin and its derivatives are effective anti-malarial drugs (McIntosh and Olliaro, 2000). The active principle is obtained from the stems of the leaves of *Artemisia annua*, a plant used in Chinese traditional

medicine (Klayman, 1985). In 1982, Chinese parasitologists discovered that administration of ART (methyl ether derivative of dihydroartemesinin) to mice infected with *S. japonicum* resulted in significant reductions of *Schistosoma* worm burden (Le *et al*, 1982). ART showed *in vivo* activity against adult schistosomes characterized by hepatic shift, degeneration of reproductive organs and hence inhibiting egg formation, and decrease of worm body size, which eventually led to death (Hamza *et al*, 2012).

The ultra-structural study of adult worms clarified the effects of some drugs on the adult worms such as the tegumental changes like swelling, fusion of tegumental ridges, formation of vesicles, peeling and even the

collapse of the tegument (Xiao *et al*, 2010; Silva *et al*, 2014). These ultra-structural observations on the tegumental surface of *Schistosoma mansoni* were observed by using antischistosomal drugs such as astiban (Otubanjo, 1981), niridazole (Popiel and Erasmus, 1984), oxamniquine (Fallon *et al*, 1996), praziquantel (Mohamed *et al*, 2006), thioxo-imidazolidine derivatives (Neves *et al*, 2011, Silva *et al*, 2014) and artemether (ART) (Xiao *et al*, 2002; Hamza *et al*, 2012). Moreover, Bennet *et al* (1997) developed a special device to monitor the *in vitro* effect of praziquantel on adult *Schistosoma* worm muscle contraction.

From the previous studies on anti-schistosomal compounds observed so far and due to the great need for more studies on the effective drugs such as ART, the present work was done to investigate the effect of ART on adult *S. mansoni* worms *in vitro* by ultrastructural and muscular tension study.

Materials and Methods

The study was conducted from October 2016 to March 2017 in the laboratory of Medical Parasitology Department, Faculty of Medicine, Zagazig University and Electron Microscope Laboratory of Faculty of Science, Zagazig University.

Animals: Male Swiss albino mice (6-8 weeks age 20-25gm weight) were obtained from the animal house, Theodor Bilharz Research Institute (TBRI), Imbaba, Egypt. Mice were fed with stock commercial pellet diet and were given water *ad libitum*.

The experimental animal study was carried out according to the international valid guidelines of experimental animal studies. All efforts were made to minimize the animal's suffering.

Parasite: *Schistosoma mansoni* cercariae (Egyptian strain) were collected from infected *Biomphalaria alexandrina* snails obtained from *Schistosoma* Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI), after exposure to artificial light for at least 45 minutes.

Drug: ART tablets (50mg, Kunming Pharmaceutical Corporation, Kunming, China) were used in this study. 5mg of ART was dissolved in 3ml of polyethylene glycol (PEG) 400 and then diluted to 5ml with Hanks balanced salt solution (HBSS; M.A. Bioproducts, Walkersville, Md.). Drug solution (50 to 200µl) was added to 1.8 to 1.95 ml of medium in each assay dish to yield a final volume of 2ml for *in vitro* study. The used concentrations in this study were obtained by calculation of the added ART different weights in µg to the used reagents. ART was applied in concentrations of 20, 40, 80 & 100µg/ml over 24 hours of *in vitro* exposure of *S. mansoni* coupled adult worms to test its effect on the worms' tegument and muscles.

Study design: 30 male white Albino mice were infected with 100±10 cercariae for each mouse by tail immersion method (Liang *et al*, 1987). Seven weeks post-infection, the mice were perfused and adult worms (couples) were collected in RPMI-1640 medium and divided into five groups, each in 35 by 10mm plastic petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). The worms were kept in an incubator at 37°C in an atmosphere of 95% air-5% CO₂. At 1 to 2 hours after incubation, ART solution (60% PEG 400, 40% HBSS) was added to the medium, resulting in final drug concentrations of 20, 40, 80 & 100µg/ml. The 1st group served as the control group of adult *S. mansoni* worms. The other four groups were investigated by four ascending concentrations of ART (20, 40, 80 & 100 µg/ml) for 24 hours as follows: 2nd Group: adults tested by 20µg/ml ART, 3rd Group: adults tested by 40µg/ml ART, 4th Group: adults tested by 80µg/ml ART and 5th Group: adults tested by 100µg/ml ART.

Ultrastructural study: The worms from the tested groups of mice were collected and examined by scanning electron microscopy (SEM). The tested worms were fixed in 25% glutaraldehyde-phosphate buffer (0.2ml, pH = 7.4) and kept at 4°C overnight. Specimens

were dehydrated in graded ethanol before embedding. Worms were mounted on stainless steel holders and put in a drier for about 24 hours and subjected to sputter coat of gold. Ultrastructure features of *Schistosoma* worms were the examined using Jeol JEM-1200 EXII scanning electron microscope.

Muscular tension study: Muscle tension of worm (% shortening of worm) was assayed in response to ART in rising concentrations of 20, 40, 80 & 100 µg/ml *in vitro*. The effect was determined by a special device, originally developed by Bennet *et al*, (1997), consists of a photo-optic transducer with an amplifier capable to measure the worm's muscle contraction (Fig. 1).

Changes in baseline tension after adding ART were measured as: a - Change in worm length = Change in baseline tension × device calibration (400µm), b- Percentage of change in worm length (% shortening) = (Change in worm length/length of worm between two electrodes) ×100. So, if the change in the baseline tension was 8mm, the change in the worm length = 16 % shortening.

Results

The surface topography of *S. mansoni* was studied by SEM. Study on the control group revealed no apparent damage. There were numerous large tubercles with apically directed spines (Fig. 2). After exposure of *S. mansoni* to ART in ascending concentra-

tions, all worms showed focal damage of the tegument dorsal surface (Figs. 3-6).

SEM revealed that after 24 hours exposure to ART in concentration of 20µg/ml, the tegument appears oedematous with loss of spines of few tubercles (Fig. 3). After exposure to ART in concentration of 40 µg/ml, the tegument showed swelling of the tubercles with vesicle formation and shortening or even loss of the tubercular spines. Finally, tegument appeared with more disruption and more shrinkage of tubercles (Fig. 4). While after exposure to ART in concentration of 80µg/ml, tegument appeared disrupted with cracking and loss of apical tubercular spines (Fig. 5). After exposure to ART in concentration of 100µg/ml, the tegument showed marked cracks and superficial peeling. Tubercles showed distortion and loss of spines with some blebs and absence of inter-tubercular ridges (Fig. 6).

In the present study, schistosome muscle tension (% shortening of worms) was assayed in response to ascending concentrations of ART. The drug led to muscle contraction and reached the highest response with 100µg/ml ART. The maximal increase in muscle tension was 44.1±1.79 % shortening induced by highest concentration (100µg/ml) of ART. *In vitro* effect of increasing doses of ART on muscle tension (% shortening) of *S. mansoni* adults (Tab. 1, Fig. 7, 8).

Table 1: *In vitro* effect of increasing doses of ART on muscle tension (% shortening of worms) of *S. mansoni* adult

Dose Conc.	% shortening of worms
20 µg/ml	9.7 ± 1.16 %
40 µg/ml	19.3 ± 1.89 %
80 µg/ml	33.6 ± 1.78 %
100 µg/ml	44.1 ± 1.79 %
P-value	<0.001 (Significant)

Discussion

Schistosomiasis is considered one of the most important human helminthiasis in terms of morbidity and mortality. Chemotherapy (like praziquantel) is the most widely advocated method of schistosomiasis control (WHO, 1993). ART, the anti-malarial to ART (Xiao *et al*, 1995; 2000a, b). The highest susceptibility was directed to the

drug, may become an alternative therapy to praziquantel as it elicited anti-schistosomal effects (Xiao *et al*, 2002; Hamza *et al*, 2012).

There was obvious evidence from laboratory investigations that *S. japonicum*, *S. mansoni* and *S. haematobium* are susceptible larval migratory stages i.e. immature stages. However, ART also showed *in vivo* activity

against adult schistosomes in the form of hepatic shift, degeneration of reproductive organs leading to inhibition of egg formation and reduction of worm body size, which eventually led to death (You *et al.*, 1992; Xiao *et al.*, 1995). So, ART in this study was used in different ascending concentrations in a trial to assess its *in vitro* anti-schistosomal effects. The maximum dose of ART used in this work is 100µg/ml as *in vitro* toxic effects of ART on *S. mansoni* were not seen at concentrations less than 100µg/ml (Xiao and Catto, 1989).

The tegument of schistosomes and other trematodes has secretory function and is involved in nutrient absorption, protects schistosomes from immune attack by the host and is an important target for antischistosomal drugs (Pearce and Sher, 1987). In common, all drugs were found to induce variable degrees of destruction to the tegument, subtegumental structures and the reproductive system of the adult schistosomes. ART displayed a considerably slower action than praziquantel (Xiao *et al.*, 2000b). So, tegumental changes are very important to assess the effect of ART on adult schistosomes.

Ultrastructural study for examination of the tegument was done using SEM. The 20 µg/ml concentration of ART produced some changes in the worm surface. These changes included slight disruption of the tegument and loss of spines of few tubercles. After 24 hours of exposure to 40µg/ml ART, the tegument showed formation of vesicles and more loss of apical tubercular spines. After exposure to 80µg/ml ART, the tegument showed more damage, more disruption and loss of some tubercular spines. After exposure to 100µg/ml ART, peeling, cracking and blebs appeared on the tegument with absence of intertubercular ridges and more loss of spines. These findings were consistent with Xiao *et al.* (2002), Jiraungkoo-rshul *et al.* (2005) and Hamza *et al.* (2012). The present results were compared to Bricker *et al.* (1983) work on the *in vitro* effects of praziquantel monitored by the SEM and

showed good achievement.

ART induced cracking of the tegument with variable degrees of distortion of the tegumental matrix. The spines covering the tubercles on the tegument appeared to be lost. Tubercles on the tegument showed damage. These findings agreed with Xiao *et al.*, 2002.

Moreover, Xiao *et al.* (2002) found that that ART showed marked affection to the gut epithelial cells and vitelline cells, which might further affect the physiological functions of adult worms as functions of the intestine and the production of the eggs by *S. mansoni* females by using different techniques in assessment of different criteria.

In vitro studies have shown that the very early noticeable effect of any drug on the adult worms was the spastic paralysis of the musculature of schistosomes. They showed that the process began as a transient stimulation of muscle tone which eventually leads to shortening of the worm through tetanic contraction (Pax *et al.*, 1978). So, muscle contraction was recorded in this work using a special device to assess muscular tension effects of ART on adult schistosomes.

In the present work, schistosome muscle tension (% shortening of worm) was assayed in response to different concentrations of ART. It elicited somatic muscle contraction and reached the highest response ($44.1 \pm 1.79\%$ shortening) with 100µg/ml ART. This result was highlighted by Ismail *et al.* (1999) concerning the *in vitro* effect of anti-schistosomal drug (praziquantel) in susceptible and unsusceptible *S. mansoni*.

Conclusion

The results showed that the *in vitro* use of ART with different concentrations induced important effects on *S. mansoni* adults with disruption of the worm tegument as monitored by SEM and muscle contraction with worm shortening as recorded by a special device. Thus, ART could be served as a promising anti-schistosomal drug with the need of more work on ART to highlight its other therapeutic effects, mechanism(s) of

action and toxic effects on immature and mature *S. mansoni* worms.

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Legend of figures

Fig. 1: A special device used to determine change in worm length (% shortening).

Fig. 2: Ultramicrograph of control adult *S. mansoni* showing large tubercles (T) with numerous spines (S).

Fig. 3: Ultramicrograph of worms tested with 20 µg/ml ART showing loss of spines of few tubercles (arrows).

Fig. 4: Ultramicrograph of worms tested with 40 µg/ml ART showing oedematous tegument with formation of vesicles (V) and more loss of spines (arrows).

Fig. 5: Ultramicrograph of worms tested with 80 µg/ml ART showing tegumental alterations of *S. mansoni* with cracks (C) and partial loss of spines (arrows).

Fig. 6: Ultramicrograph of worms tested with 100 µg/ml ART showing the distortion with cracks (C), peeling (P), bleb formation (B), loss of spines (arrows) and absence of intertubercular ridges.

Fig. 7: *In vitro* ART effect in different concentrations on muscle tension of adult *S. mansoni* worms (% shortening).

Fig. 8: Recording of *in vitro* effect of ascending ART doses on muscle tension (% shortening) of *S. mansoni* adults.



