Effects of Artemether Treatment on Prepatent and Patent Schistosoma mansoni Infection in Experimentally Infected Mice

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ABSTRACT

Background: Schistosomiasis is a chronic debilitating parasitic disease. Long-term repeated use of praziquantel (PZQ) for control of the disease morbidity may lead to resistance or reduced susceptibility to the drug. Artemether (ART), the anti-malarial drug may become an alternative therapy to PZQ as it also displays anti-schistosomal properties.

Objective: To assess the effects of artemether experimentally on prepatent (juvenile worms) and patent (adult worms) Schistosoma mansoni infection.

Methodology: Three groups each composed of twenty mice were used in the study. The first group (prepatent infection) was treated with double dose of ART (each 400 mg/kg) three and four weeks post infection (PI), the second group (patent infection) was treated with the same dose at six and seven weeks PI and the third group represented the infected control group. The effect of the drug was assessed by parasitological, histopathological and ultrastructural studies.

Results: It was found that ART significantly decreased the number of worms and their body sizes in treated mice groups. It also decreased the tissue egg load and decreased the granuloma size in the liver of mice in the second group. It had a more potent effect on juvenile worms (group 1) as no or few fibrous granulomas appeared in the tissues. Scanning electron microscope revealed swelling and erosion of the tubercles with breaks in some areas of the tegument in both groups of treated mice groups. The oral sucker showed breaks and focal lysis of the underlying muscle fibers.

Conclusion: Artemether is an effective drug against Schistosoma mansoni infection. It has schistosomicidal and ovicidal effects and hence anti-pathologic activities especially in those treated early after infection (3 and 4 weeks PI).

Recommendations: Additional clinical studies using artemether in different epidemiologic settings with higher doses as well as future trials to study its ability to prevent reinfection and against S. hematobium are also recommended.

Keywords: S. mansoni, Artemether, Schistosomicidal, Histopathology, Scanning Electron Microscope.

INTRODUCTION

Schistosomiasis is a parasitic disease which is endemic in 74 tropical developing countries. It is estimated that 200 million people are already infected and 600 million people are at risk of becoming infected with 200,000 deaths occurring annually[4]. The disease is usually chronic and debilitating, with severe damage to the liver and intestine when S. mansoni or S. japonicum is involved. The disease morbidity is mainly caused by eggs; hence the fundamental aim of morbidity control is to reduce intensity of infection. Because a vaccine is not yet available, chemotherapy is the only method of schistosomiasis control which is dependent mainly on the use of PZQ[2,3]. However, with the extensive, long-term repeated use of the drug for morbidity control, there is a growing concern that PZQ resistance or reduced susceptibility may emerge[5]. In addition, the drug is less sensitive to young developmental stages (schistosomula); hence, retreatment is necessary to kill parasites that have since matured[6,7]. Screening and development of new anti-schistosomal agents as an alternative to PZQ, is therefore given a high priority[8,9].
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Artemisinin and related derivatives are effective anti-malarial drugs (10). The active principle stems from the leaves of Artemisia annua, the herbal plant used for centuries in Chinese traditional medicine (11). Artemether (ART), the β-methyl ether derivative of artemisinin, is used as an anti-malarial drug and it has proved to be a good prophylactic agent against schistosomiasis japonica in China (12). It selectively targets the schistosomulae migratory stages of the parasite, and it blocks the development of oviposition by adult schistosome worm pairs in the vasculature (13). Furthermore, ART exerts helminthotoxic effects which may be through synergy with hemin or related heme-containing compounds (14). Another advantage of artemether over PZQ is its ability to prevent reinfection. It proved to be very efficacious in inducing high-level resistance against S. mansoni in mice as well as in a randomized controlled trial (15).

In the present report we studied the effect of ART on the Egyptian strain of Schistosoma mansoni (juvenile and adult) in experimentally infected Swiss albino mice by parasitological, histopathological and ultrastructural studies.

MATERIALS AND METHODS

Study type: Experimental case control study.
The study was conducted in the period from June to December, 2011 in Parasitology Department, Faculty of Medicine, Zagazig University.

Mice infection: Swiss albino female mice (aging 3 weeks, weighing 10–20 g) were obtained from the Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute, Giza, Egypt. Mice were kept in groups of 10 in cages in environmentally controlled conditions (temperature: ~25°C and 12 h light and 12 h dark cycle) and acclimatized for 1 week. They had free access to water and food (stock commercial pellet diet ad libitum obtained from Elkahara Company for Oil and Soap). S. mansoni cercariae (Egyptian strain) were obtained from infected Biomphalaria alexandrina snails (obtained from Theodor Bilharz Research Institute) and used for mice infection using tail immersion technique by 100 cercariae/mouse (16).

ART treatment: Artemether was obtained from Ipca Laboratories Ltd., India. ART was suspended in a solution of 3% ethanol and 7% Tween 80 and water just before treatment. ART treatment was given to each mouse by gavage tube (17).

Study design: Three groups each composed of twenty mice were used in the study. The first group (prepatent infection) was treated with double dose of ART (each 400 mg/kg) three and four weeks post infection (PI), the second group (patent infection) was treated with the same dose at six and seven weeks PI and the third group represented the infected control group. Six mice from each group were sacrificed by cervical dislocation at 24 h; 3 days and 14 days after the second dose of treatment (two mice/group/each time) for electron microscope study. The remaining mice were sacrificed at the 10th week PI: two mice/group for histopathological study and 12 mice/group for parasitological study. Perfusion of adult worms from infected mice was done (18) by sectioning the portal vein of the mice and saline was gently injected into the base of the left ventricle and worms were recovered, classified according to sex and counted.

Ova count (tissue egg load): The number of ova/gm intestinal or hepatic tissue was determined after digestion overnight in 5% KOH and counted (19), where number of ova in 1 g of liver was estimated as number of ova in 5 ml KOH divided by weight of liver in grams recorded before digestion. The induced percentage reduction in worm number and egg number was calculated (20).

P=C-V/Cx100 (Where P: percentage; C: mean number of parasites or eggs recovered from control infected mice; V: mean number of parasites or eggs recovered from infected treated mice).

Ogram pattern: The percentage of eggs at various developmental stages in the small intestine was determined (21).

Histopathology (22): The liver was removed from each one of the sacrificed mice, rinsed with phosphate buffered saline and fixed in Bouin fluid for 4 h, then transferred to 70% alcohol for several days. After dehydration in absolute alcohol and clearing in xylool, it was embedded in paraffin wax, sectioned at thickness of 4 micron. Sections were stained with hematoxylin and eosin (H&E) stain. Sections were examined for periovular granulomas. The diameter of granulomas was measured using the ocular micrometer. This was done only for granulomas containing ova in their centers and not confluent ones. The mean diameter for each group was calculated.

Electron microscopy (23): Worms were collected from mice from treated group and untreated infected control group and immediately fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, and post-fixed with 1% osmium tetroxide. Worms were dehydrated in graded concentrations of acetone and embedded for 12 h in Poly-bed 812 (Embedding Media Polysciences, IVC). Selected ultrathin sections (50-70 nm) were made with a Reichert (Ultratome Supernova Leica) ultramicrotome and mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate. Specimens were examined in a transmission Zeiss EM-9 electron microscope, operated at an acceleration voltage of 50 Kv.

Data analysis: Results of studies are reported as mean ± standard deviation (SD). Data were entered and analyzed using SPSS version 19 for Windows. The statistical analyses were done by Student's t test. P values of less than 0.05, 0.01 or 0.001 were used to indicate statistical significance.
**Ethical considerations:** Ethical animal practices were followed that were under the standard regulations dictated by the animal care committee of Faculty of Medicine, Zagazig University.

**RESULTS**

**Parasitological results:** Table (1) summarizes the mean number of worms which were collected from mice harboring juvenile (group 1) and adult *S. mansoni* infections (group 2) after ART treatment and those collected from infected control group (group 3). The mean total worm burden showed a high significant decrease (*P*<0.001) in group (1) and group (2) as compared to infected control group (group 3). ART achieved total and female worm burden reductions of 97.4% and 99.1% (*P*<0.001) respectively in group (1), and 78.8% and 91.6% (*P*<0.001) respectively in group (2). Few number of *S. mansoni* couples were detected in group (2), while group (1) showed only separated male and female worms. In addition, ART decreased the size of worms as shown in table (2). Worms from group (1) were highly significantly smaller (shorter and thinner) (*P*<0.001) as compared to infected control group (3) while worms from group (2) were significantly smaller (shorter and thinner) (*P*<0.01) as compared to infected control group (3). Table (3) represents the number of eggs in liver and intestine (tissue egg load) of ART treated mice groups and infected control group and the oogram pattern. There was a complete absence of eggs in the hepatic and intestinal tissues with a complete disappearance of all egg developmental stages (*P*<0.001) in group (1) as compared to infected control group (3). There was a highly significant decrease (*P*<0.001) in eggs of hepatic and intestinal tissues in group (2) with percentage reduction of 89.6% and 86.8% respectively as compared to infected control group (3). As regards the oogram pattern of group (2), the percentage of total immature stages were negligible at 0.7% (*P*<0.001) as compared to infected control group (3) and mature eggs showed a high significant reduction to 2.4% (*P*<0.001). In contrast, a massive increase of dead eggs was observed with a high significant increase to 96.9% (*P*<0.001).

**Histopathological results:** The surface of the liver in ART treated group (1) showed few or no dispersed miliary egg tubercles. The structure of the hepatic lobules was normal with normal arrangement of the liver bundles; few or no eggs appeared in the portal vein area and there was apparent diminution of total egg granuloma. No eggs were detected in the centres of the granulomas whether inflammatory or fibrous. Meanwhile, there was a high significant reduction (*P*<0.001) in the mean granuloma diameter from 265.5 μm in infected control group 3 (Figure 2) to 135.8 μm in ART treated group (2) (Figure 1).

**Electron microscopic results:** Twenty four hours post-treatment, the tegument of male worms showed extensive swelling. Tubercles showed marked swelling and most of the spines were completely encompassed by the tegument (Figures 3 and 4). The lesions were more extensive in group (1) than in group (2). Three days post-treatment, worms showed severe damage and extensive lysis in the tegument. The tubercles still exhibited swelling and extensive erosions (Figures 5 and 6) with breaks in some areas of the tegument (Figures 6 and 8). The lesions were more extensive in group (1) than in group (2). The oral sucker showed collapse and large break and focal lysis of the underlying muscle fibres (Figure 7). Fourteen days after artemether administration, most of worms still showed degeneration in the cuticle and on the other hand, some worms showed different degrees of regenerations (Figures 9 and 10).

| Table (1): *S. mansoni* worms burden collected from ART treated mice (Groups 1 and 2) and from infected control (Group 3) (mean number ± SD, and %). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Mice groups** | **Liver** | **Mesenteric vein** | **Worm burden** | **Worm burden reduction(%)** |
| **12 mice/group** | **Males** | **Females** | **Total** | **Female** | **Total** |
| **Group (1)** | 0.7±0.24 | 0.0±0.0 | 0.6±0.11 | 0.1±0.04 | 0.7±0.24*** |
| **Group (2)** | 4.6±0.82 | 1.2±0.21 | 4.9±1.23 | 0.9±0.22 | 5.8±1.42*** |
| **Group (3)** | 7.4±1.34 | 19.9±2.42 | 16.6±2.71 | 10.7±1.94 | 27.3±3.83 |

***Highly significant (*P*<0.001).

| Table (2): The size of *S. mansoni* worms collected from ART treated mice (Groups 1 and 2) and from infected control group (Group 3) (mean ± SD). |
|-----------------|-----------------|-----------------|
| **Mice groups** | **Length (mm)** | **Width (mm)** |
| **12 mice/group** | **Male** | **Female** | **Male** | **Female** |
| **Group (1)** | 5.88±0.38*** | 0.48±0.03*** | 7.56±0.49*** | 0.08±0.01*** |
| **Group (2)** | 7.72±0.43** | 0.69±0.04** | 10.37±0.75** | 0.14±0.03** |
| **Group (3)** | 8.85±0.58 | 0.92±0.13 | 13.0±0.82 | 0.18±0.09 |

**Significant (*P* < 0.001). ** Highly significant (*P*<0.001).
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**Table (3):** Egg count (eggs/gram) in tissues (liver and intestine) and oogram pattern in ART-treated mice (Groups 1 and 2) and infected control (Group 3).

<table>
<thead>
<tr>
<th>Mice groups /group</th>
<th>Tissue egg load</th>
<th>Oogram</th>
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<tbody>
<tr>
<td></td>
<td>Liver eggs</td>
<td>Intestinal eggs</td>
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<td></td>
<td>Mean±SD</td>
<td>P%</td>
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<tr>
<td>Group (1)</td>
<td>0.0±0.0***</td>
<td>100</td>
</tr>
<tr>
<td>Group (2)</td>
<td>1082±176.5***</td>
<td>89.6</td>
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<tr>
<td>Group (3)</td>
<td>10355±811.2</td>
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***Highly significant (P< 0.001).***

**Figure (1):** H&E stained egg granuloma in the liver of *S. mansoni* infected mice after treatment with ART showing small cellular granuloma with degenerated ova (Group 2) (×100).

**Figure (2):** H&E stained egg granuloma in the liver of *S. mansoni* of infected untreated mice showing well-circumscribed cellular granuloma with central ova (Group 3) (×100).

**Figure (3):** Scanning electron microscope of *S. mansoni* male 24 hours after the second dose of ART (group 1). It shows marked swelling of the tubercles with disappearance of the spines (Arrow) (×1000).

**Figure (4):** Scanning electron microscope of *S. mansoni* male 24 hours after the second dose of ART (Group 2). It shows marked swelling of the tubercles (Arrow) (×1000).

**Figure (5):** Scanning electron microscope of *S. mansoni* male 3 days after the second dose of ART (Group 1). The arrows point to extensively eroded tubercles (×1000).

**Figure (6):** Scanning electron microscope of *S. mansoni* male 3 days after the second dose of ART (Group 2). It shows erosion of the tubercles and breaks in the tegument (Arrows) (×800).
DISCUSSION

Hemoglobin metabolism is a common feature of schistosomiasis and malaria. Host hemoglobin is ingested by Schistosoma worms and degraded to amino acids in the caecum of parasites, and the generated free heme is eliminated as hemozoin. Hemozoin is a disposal product shared by Plasmodium and Schistosoma (24). In 1982, Chinese Parasitologists discovered that administration of artemether, which is a heme-alkylating agent, to mice infected with S. japonicum resulted in significant reductions of the schistosome worm burden (25). Recent studies have shown that anti-malarial artemisinin derivatives displayed significant activity against several Schistosoma species (26-28).

In our study, ART caused a highly significant decrease in the mean total worm burden which, as previously reported (29), may be attributed to hepatic shift and growth retardation and hence death of worms. In addition, other reports on in vitro experiments suggested that artemether interacts with heme when activated by hematin, cleaving its endoperoxide bridge to generate free radicals (30,31). The authors concluded that this might be the ultimate cause of schistosome death. Our recorded higher percent worm reductions in juveniles (group 1) compared to adults (group 2), under the same dose level of artemether agrees with the suggestion that the anti-oxidant system in adult worms is stronger than in immature worms (32).

Exposure of S. mansoni worms to artemether early in its life as in group 1 caused a highly significant reduction in their sizes. This decrease may be explained by the destructive effect of the drug on gut epithelial cells and shortening of microvilli (33). In addition, it was found that artemether increased glycogen phosphorylase activity, decreased glucose uptake and decreased glycolysis activity (13). Furthermore, schistosomes kept in media containing artemether together with heme showed marked intestinal changes, including extension, distortion and depigmentation (31,34). All these changes are clear indications of disturbance of the digestion process and hence the altered parasite growth.

Our findings showed that juvenile S. mansoni worms, that were treated 3 and 4 weeks PI, failed to produce eggs which, as previously reported, may be due to the inhibitory effect of ART on sexual maturation causing atrophy of testis and ovaries (34,36). On the other hand, Botros et al. (37) reported a reduction of 99% in the tissue egg loads in infected mice, treated with ART (400 mg/kg) at 4 and 6 weeks PI. These findings indicate that the early treatment is more effective.
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As regards adult worms, ART treated worms showed a highly significant decrease in egg count. This, as explained by Sano et al.,[29], may be due to the effect of ART causing separation of worms (divorce) and then hepatic shift from their usual vascular beds towards the liver. In another opinion the separation of worms causes degeneration of their reproductive systems[38]. The authors reported that the different stimulatory pathways for growth and for reproductive maturation in S. mansoni female require physical contact with the male. The female vitelline glands occupy about one-half of the posterior portions of the worm body, providing vitelline cells that are necessary for the development of eggs. It was postulated that damage of these cells by ART usually impacts the formation and production of eggs[31].

The failure or the highly significant reduction in egg production in groups 1 and 2 resulted in apparent diminution of total egg granuloma, whether inflammatory or fibrous, without eggs in the centre of the granuloma (group 1) and a significant reduction in the mean granuloma diameter (group 2). The juvenile stages of S. mansoni were found to be significantly more susceptible to artemether than the adult worms. These observations are in agreement with those of other studies[14,34].

The tegument of schistosomes has been described as a living, anucleate, and cytoplasmic structure[39]. It has secretory functions, is involved in nutrient absorption, shields schistosomes from the immune response by the infected host and is a key target for anti-schistosomal drugs[40-2]. In our study, ART produced severe damage and extensive lysis in the tegument in both treated groups (1 and 2) proved by scanning electron microscopic observations. These findings are in agreement with those of several studies[14,31,43] reporting that the tegument is a key target in S. mansoni of different ages recovered from ART treated host animals. The tegumental damage might lead to disappearance of the immunological ‘disguise’ of the worm. This is believed to be of prime importance in causing the death of the worms[44]. It was suggested that focal damage induced by an anti-schistosomal drug in the tegument of either juvenile or adult S. mansoni might be repaired effectively over the course of 7-14 days after cessation of the drug while in case of severe tegumental damage, the host immune response might impact this repair process[45]

In conclusion, artemether is an effective drug against Schistosoma mansoni infection. It has schistosomicidal and ovicidal effects and hence anti-pathologic activities especially in those treated early after infection (3 and 4 weeks PI). Its underlying mechanism(s) may be due to a direct assault on the tegument and the genital systems of male and female worms, by worm separation (divorce) or through production of free radicals through its activation by hematin. These results encourage us and emphasize the need to conduct additional clinical studies of this drug in different epidemiologic settings and at higher doses. Future trials are needed to study ART ability to prevent reinfection and its effect on Schistosoma hematobium.

Author contribution: RS Hamza shared DA Abdel Khalik in study design, assisted in performing the parasitological and histopathologic studies and wrote the manuscript. D Abdel Khalik assisted in performing the parasitological study, interpreted the results and revised the manuscript. AS Salah assisted in the parasitological studies and conducted the scanning electron microscopic studies.

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تأثير العلاج بالأرتيميثير على عدوى البلهارسيا المنسونية السابقة للظهور والظاهرة في الفئران المضادة.

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مقدمة: تعد الأصابات بالبلهارسيا من الامراض الطفيلية المزمنة والموهنة. وربما يؤدي الاستخدام المتكرر لعقار البرازيكوانتين (من أجل السيطرة على المرض) على المدى الطويل إلى مقاومة الطفيل للعقار أو انخفاض حساسيته له. وبعد ظهور خصائص مضادة للبلهارسيا لعقار الأرتيميثير (العلاج المضاد للملاركيا)، فقد يصبح الأرتيميثير والعلاج البديل للبرازيكوانتين.

هدف البحث: تقييم تأثير العلاج بالأرتيميثير على عدوى البلهارسيا المنسونية السابقة للظهور والظاهرة.

خطوات البحث: استخدمت الدراسة 3 مجموعات تتألف كل منها من 20 فأرًا، وقد عولجت المجموعة الأولى بجرعة مزدوجة من عقار الأرتيميثير (0.04 ملجم/كجم) في الأسبوع الثالث والرابع (العدوى السابقة للظهور) بعد العدوى بالبلهارسيا، وعولجت المجموعة الثالثة بنفس الجرعة في الأسبوع السادس والسابع (العدوى الظاهرة)، ومثلت المجموعة الثالثة المجموعة الضابطة المضادة. وقد تم تقييم تأثير العقار عن طريق الدراسات الطفيلية والهستوباثولوجية والمجهر الإلكتروني.

نتائج: أظهرت النتائج انخفاض عدد ديان البلهارسيا وكذلك أحجام أجسام انتفاخها في مجموعتين الفئران التي تم معالجتها بالأرتيميثير. كما انخفض عدد البويضات في أنسجة الكبد والأمعاء وتقلص قطر الورم الحبيبي في كبد الفئران في المجموعة الثانية. وكان للعقار تأثيرا أكثر فعالية على الدماغ غير مكتنزة النضج (المجموعة الأولى) حيث لم توجد اورام حبيبية في الأنسجة إلا قليل من الامراض الليبية. وكشف المسح الضوئي بالمجهر الإلكتروني تورم وتآكل للدرنات الموجودة على غشاء الدماغ مع وجود تمزق بالغشاء في بعض المناطق، كما ظهر تهتك بالمصب الفماني للديدان.

الاستنتاج: عقار الأرتيميثير فعال ضد عدوى البلهارسيا الممزقة حيث إن له آثار مضادة للبلهارسيا لا سيما إذا استخدم في وقت مبكر (3 أسابيع) بعد العدوى.