Effect of artemether on cytokine profile and egg induced pathology in murine schistosomiasis mansoni

Neveen A. Madbouly a, Ibraheem R. Shalash b, Somaya O. El Deeb a, Azza M. El Amir a,*

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**ABSTRACT**

Artemether (ART), the methylated derivative of artemisinin, is an efficacious antimalarial drug that also displays antischistosomal properties. This study was designed to evaluate the immunomodulatory action of a single intramuscular dose (50 mg/kg body weight) of ART in comparison with PZQ treatment (42 days PI). ART administration was 7, 14, 21 and 45 days PI. ART effect was studied parasitologically, histopathologically and immunologically. It was found that maximum effect was reached when ART treatment interfered with 14 or 21 days old schistosomula. ART treatment 14 or 21 days PI was associated with shift from Th2 to Th1 predominancy (decrease in IL-4 and upgrading of serum IFN-γ levels). In conclusion, ART is a promising drug in control of schistosomiasis mansoni due to its reductive effect on worm burden and its role in improvement of hepatic granulomatous lesions.

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**Introduction**

Schistosomiasis is a common intravascular trematode infection. It is one of the most prevalent parasitic diseases in the world, after Malaria [1]. Currently; praziquantel (PZQ) is the drug of choice for mass treatment of schistosomiasis. PZQ is active against all five human Schistosoma species [2]. To be left with only one drug for schistosomiasis treatment is a very dangerous situation; especially that PZQ does not prevent reinfection, mainly in high transmission areas. Furthermore, there is increasing concern about the possible development of parasite resistance and tolerance against PZQ. Recent attempts are directed toward natural products to design novel drugs that avoid the side effects of the synthetic medications. These may be from plant extracts and even camel milk [3]. One of these plant extracts is artemisinin, derived from the herb, Artemisia annua. Artemether (ART) is the methylated
derivative of artemisinin. In addition to the amazing antimalarial effect of ART, it showed anti-parasitic properties toward many protozoan parasites such as *Leishmania*, *Toxoplasma gondii* and *Trypanosoma* spp. [4].

Metazoa parasites as, *Schistosoma* spp., *Echinostoma caproni*, the liver fluke *Opisthorchis viverrini*, *Clonorchis sinensis* [5] and *Fasciola hepatica* [6] are also greatly susceptible to ART.

One of the great advantages of ART therapy is its prophylactic action. The prophylactic effect of ART is defined by its ability to eradicate the developing stages of schistosomula, so that the egg-laying mature female worms are not allowed to develop in the vasculature [7].

The aim of the present study was to evaluate the immunomodulatory effect of a single intramuscular ART dose (50 mg/kg) on the cytokine profile in experimental *Schistosoma mansoni* infection.

**Material and methods**

**Animals**

Laboratory bred female, Swiss albino mice *Mus musculus* (CD-1 strain), each weighing 18–20 g, were used in this study. Experimental animals were obtained from Schistosome Biological Supply Center (SBSP) at Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Mice were kept for 8 weeks (experiment duration) in air-conditioned animal house at 20–22 °C and maintained on food containing 24% protein. Mice were also given carrot, lettuce and milk as source of vitamins. *Animal experiments were carried out according to the internationally valid guidelines and in an institution responsible for animal ethics.*

**Parasites and infection**

The Egyptian strain of *S. mansoni* cercariae was obtained from SBSP at TBRI. Infection was performed by subcutaneous injection (s.c.) of *S. mansoni* cercariae (80 ± 10/mouse) [8].

**Drugs**

**PZQ**, was obtained in the form of Tablets (600 mg/Tablet) (Distocide, Epico, Corporation, Cairo). The drug was freshly prepared and administered orally as a suspension in 2% Cremophor (Sigma) in a dose of 500 mg/kg/b.wt. for two consecutive days, 42 days postinfection (PI). ART, suspended in ground-nut oil, in the form of intramuscular (i.m.) ampoules (80 mg/ampoule) with documented purity of 99.6% was purchased from Kunming Pharmaceutical Corporation (Kunming, China). This preparation is stable at room temperature for 4 years [18]. The drug was administrated i.m. as a single dose of 50 mg/kg/b.wt. according to the experimental design [9].

**Experimental design**

The experimental groups are illustrated in Table 1. Mice were euthanized 8 weeks postinfection (PI) by decapitation. Then, the blood was collected individually in plastic tubes without anticoagulant. Blood was allowed to stand for 1 h at 37 °C, then overnight at 4 °C and centrifuged at 2500 rpm for 15 min. The serum was obtained and kept in aliquots at −20 °C for cytokine assessment.

**Worm burden**

Individual worm burdens were examined after perfusing the hepatic and portomesenteric vessels of each animal. Infected mice were perfused.

**Histopathology and granuloma measurement**

Liver sections were microscopically studied to evaluate the pathological changes including portal tracts and schistosomal granulomatous reactions. Pieces of mice livers were fixed in 10% phosphate-buffered formalin, pH 7.2, processed into Paraffin blocks. Transverse sections (5 μm in thickness) were taken, 5 sections from each liver, using a microtome (Bright 5030, UK). Each section was at a distance of at least 300 μm from the proceeding one. Sections were mounted on glass slides. Deparaffinization was performed by dipping slides in 100% xylene and descending ethanol series (100%, 95%, 80% and 70%) for rehydration. Sections were stained with Hematoxylin and Eosin (H&E) and Masson Trichrome.

**Mean hepatic granuloma number (MGN) and diameter (MGD)**

Measurements were taken only for granuloma containing single egg in the center using an ocular micrometer. The number of granuloma in 5 successive low power fields (10×10) was counted and recorded for MGN [10]. The MGD of each granuloma was calculated by measuring two diameters of the lesion at right angles to each other [11].

**Cytokine assay**

Levels of the cytokines IL-4, IL-10 and IFN-γ were measured in serum using sandwich ELISA. Briefly, plates (Nunc, Roskilde, Denmark) were coated with capture antibodies with 100 μl of serum sample or recombinant cytokine. Following addition of the biotinylated detection antibody and streptavidin–alkaline phosphatase conjugate, the reaction was developed with p-nitrophenyl phosphate (PNPP) (Sigma). Absorbance at 405 nm was measured with a Benchmark reader (Bio-Rad Laboratories Inc., Hercules, Calif.). Assays were performed in duplicates. The cytokine concentration was obtained from a regression curve prepared with the help of Microplate Manager Software (Bio-Rad). The results were expressed as pg/ml.

**Statistical analysis**

The data were presented as mean ± standard error of mean (mean ± S.E). Statistical analysis of results was carried out using one-way analysis of variance (ANOVA). Comparison between two groups was done by the Student’s *t*-test. All statistical analysis was performed with the aid of the SPSS computer program (version 13.0 Windows). The data were considered significant if (*P* < 0.05), highly significant if (*P* < 0.01).
reduction (PR) in all parameters was calculated according to Fonseca et al. [12] using the following equation:

$$PR = \frac{1 - \text{Mean value of IT group}}{\text{Mean value of IU control group}} \times 100$$

where IT is the infected treated group and IU is the infected untreated group.

**Results**

The effect of the treatment with PZQ or ART on worm burden and distribution

Table 2 shows the worm burden and distribution for all infected groups. PZQ-treated mice showed a very highly significant reduction ($P < 0.001$) in the mean worm burden for males, females and couples with PR: 72.15%, 75.26% and 60%, respectively. The total worm burden was reduced by 92.67% in comparison with infected-untreated mice. Moreover, ART injection 7, 14 and 21 days PI induced a very highly significant decrease ($P < 0.001$) of both total and mean worm burden in comparison with infected-treated group, and the percentage of total worm reduction was 73.10%, 77.43% and 87.15%, respectively. On the other hand, infected mice treated with ART 45 day PI showed only 25.35% of total worm reduction. It was clear from Table 2 that pre-treatment has a very highly significant reductive ($P < 0.001$) effect on total worm burden of 68.75% compared with infected-untreated group.

**Histopathological features**

Liver sections of uninfected-untreated, infected-untreated and different treated groups were studied for MGN, MGD and cellular profiles (Fig. 1)

The effect of the treatment with PZQ or ART on mean granuloma number (MGN)

The MGN in 5 successive low power fields ($10 \times 10$) was determined in the different infected groups and tabulated in Table 3. Hepatic tissues of infected-untreated mice showed the maximum MGN, while mice treated with PZQ showed a very highly significant decrease ($P < 0.001$) in MGN with PR of 78.33%. The schistosomicidal effect of ART injection on the developing schistosomules was reflected as a decrease in MGN with time from the 7 days old schistosomula to 21 days old S. mansoni larvae. This decrease in MGN was even lower than that recorded after the PZQ treatment. When infected mice were injected with ART 45 days PI MGN showed highly significant reduction ($P < 0.01$) with PR: 44.64%. In the ART pretreated-infected mice, there was a very highly significant decrease ($P < 0.001$) in the MGN with PR: 69.44%, compared with the infected-untreated mice.

### Table 1
Experimental layout, indicating times of infection, PZQ administration, ART injections and perfusion of mice.

| Animal group                  | n   | Time (days) |
|-------------------------------|-----|-------------|
| Uninfected-untreated          | 10  | -           |
| Infected-untreated            | 15  | (80 ± 10) cercariae |
| Infected, PZQ                 | 15  | (80 ± 10) cercariae |
| Infected, ART 7 day PI        | 15  | (80 ± 10) cercariae |
| Infected, ART 14 day PI       | 15  | (80 ± 10) cercariae |
| Infected, ART 21 day PI       | 15  | (80 ± 10) cercariae |
| Infected, ART 45 day PI       | 15  | (80 ± 10) cercariae |
| ART pretreated-infected       | 15  | ART (80 ± 10) cercariae |

| a  | ART injection 2 days preinfection. |
| b  | Time of infection. |

### Table 2
The effect of single intramuscular dose (50 mg/kg) of artemether (ART) in comparison to praziquantel (PZQ) treatment on worm distribution and worm burden in *Schistosoma mansoni*-infected mice.

| Animal groups            | Mean worm burden ± S.E. (PR) | Total worm burden ± S.E. (PR) |
|--------------------------|-----------------------------|-----------------------------|
|                          | Males          | Females        | Couples       | Males        | Females        | Couples       |
| Infected-untreated       | 11.28 ± 1.20    | 7.52 ± 0.4     | 5.0 ± 0.7     | 28.8 ± 1.1   | 12.5 ± 2.0    | 7.25 ± 0.4    |
| Infected, PZQ            | 1.4 ± 0.53***   | 0.67 ± 0.29*** | 0 ± 0***     | 2.11 ± 0.79*** | 0.75 ± 0.28*** |
| Infected, ART 7 day PI   | 3.88 ± 0.67***  | 1.88 ± 0.40*** | 1 ± 0.38***  | 7.75 ± 1.46*** | 3.5 ± 0.58*** |
| Infected, ART 14 day PI  | 4.3 ± 0.83***   | 1.2 ± 0.33***  | 0.5 ± 0.22*** | 6.5 ± 0.93*** | 2.3 ± 0.55*** |
| Infected, ART 21 day PI  | 1.8 ± 0.51***   | 1.1 ± 0.23***  | 0.4 ± 0.22*** | 3.7 ± 0.95*** | 1.4 ± 0.63*** |
| Infected, ART 45 day PI  | 9.25 ± 1.44***  | 3.25 ± 0.84*** | 4.5 ± 0.50*** | 21.5 ± 1.44*** | 8.2 ± 0.87*** |
| ART pretreated-infected  | 3.14 ± 0.59***  | 1.86 ± 0.34*** | 2 ± 0.22***  | 9 ± 1.25***  | 3.6 ± 0.58*** |

Significance from infected-untreated group: *Significant ($P < 0.05$); **Highly significant ($P < 0.01$); ***Very highly significant ($P < 0.001$).
Fig. 1 Masson trichrome stained liver sections (400x) of (a) uninfected-untreated mouse showing normal hepatic architecture surrounding central vein (C.V.). (b) Infected-untreated mouse with massive fibrocellular granuloma consisting of collagen fibers and inflammatory cells surrounding living-intact egg. (c) Infected, PZQ-treated mice 42 days post infection (PI) showing medium sized fibrocellular granuloma with degenerated egg (arrow). (d) Infected, ART-treated 7 days PI showing medium sized granuloma with starting ovum degeneration. (e) Infected, ART-treated 14 days PI showing small sized granuloma with less collagenous fibers and inflammatory cells surrounding degenerated ova. (f) Infected, ART-treated 21 days PI showing reduced granuloma with little collagen fibers and inflammatory cells surrounding degenerated ovum. (g) Infected, ART-treated 45 days PI showing large fibrocellular granuloma with living ovum. (h) ART-treated 2 days preinfection showing small sized granuloma with low collagenous fibers and low inflammatory cell infiltration.
The effect of the treatment with PZQ or ART on mean granuloma diameter (MGD)

In comparison with infected-untreated group, almost the same MGD reduction was obtained when infected mice received either PZQ treatment 42 days PI or ART treatment 14 days PI. The maximal reduction of MGD was found in mice treated with ART 21 days PI or 2 days pre-infection. These results go beyond that revealed from that in the case of PZQ treatment on MGD (Table 3).

Effect of PZQ or ART on cytokine profile

IFN-γ: In comparison with the infected-untreated mice the infected, PZQ-treated group showed a very highly significant increase (P < 0.001) in serum level of IFN-γ. ART treatment affected the serum level of IFN-γ in a deferential manner depending on the time of injection. This increase was very highly significant (P < 0.001) 14 or 21 days PI. The early (7 days PI) or late ART treatment (45 days PI) induced non-significant effect on the IFN-γ level (P > 0.05). ART injection 2 days pre-infection showed a very highly significant increase (P < 0.001) in IFN-γ level (Table 4).

IL-4: In comparison with the infected-untreated control the infected, PZQ-treated mice showed a very high significant decrease (P < 0.001) in serum IL-4 level (Table 4).

Discussion

Schistosomiasis mansoni occupies the first place in the list of endemic diseases in Egypt [13]. Although PZQ is the only drug available for the treatment of human schistosomiasis, many scientific views worry about the complete dependence on it as a sole antischistosomal drug. This is because of evidences of decreased susceptibility. ART, the methylated derivative of the naturally occurring artemisinin, is efficient toward 3–21 days old schistosomulae [14]. In the present study, mice treated with a low single i.m. ART dose (50 mg/kg) at different timings pre- and post-S. mansoni infection found to modulate the course of schistosomiasis infection.

The present study confirmed earlier observations which concluded that ART prominently affect the early stages of Schistosoma. Reduction in worm burden was improved by the treatment at 7 days PI and reached 73.1%. On the other

### Table 3

| Animal groups                  | MGN (mean ± S.E.) | PR (%) | MGD (μm) (mean ± S.E.) | PR (%) |
|--------------------------------|-------------------|--------|------------------------|--------|
| Infected-untreated             | 9.23 ± 1.92       | –      | 179.98 ± 28.99         | –      |
| Infected, PZQ                  | 2.00 ± 0.86***    | 78.33  | 144.14 ± 0.87*         | 19.90  |
| Infected, ART 7 day PI         | 2.50 ± 0.81***    | 72.91  | 168.87 ± 30.02         | 6.61   |
| Infected, ART 14 day PI        | 1.30 ± 0.65***    | 85.92  | 146.33 ± 42.51         | 18.69  |
| Infected, ART 21 day PI        | 1.64 ± 0.80       | 82.23  | 125.05 ± 33.88***      | 30.51  |
| Infected, ART 45 day PI        | 5.11 ± 1.48       | 44.64  | 179.66 ± 24.58         | 0.02   |
| ART pretreated-infected       | 2.82 ± 1.44***    | 69.44  | 132.81 ± 30.35***      | 26.20  |

** Significance from infected-untreated group: Highly significant.
*** Significance from infected-untreated group: Very highly significant.

### Table 4

| Animal group              | IFN-γ (pg/ml) | IL-4 (pg/ml) | IL-10 (pg/ml) |
|---------------------------|--------------|--------------|---------------|
| Uninfected-untreated      | 238.60 ± 6.96| 227.00 ± 5.13| 322.17 ± 4.54 |
| Infected-untreated        | 273.33 ± 13.99| 587.20 ± 22.92***| 888.00 ± 6.93***|
| Infected, PZQ             | 638.10 ± 6.99*** | 307.80 ± 15.30*** | 617.90 ± 14.38*** |
| Infected, ART 7 day PI    | 283.29 ± 12.50* | 688.40 ± 35.84*** | 719.56 ± 24.47*** |
| Infected, ART 14 day PI   | 531 ± 24.56*** | 464.20 ± 29.55*** | 637.88 ± 45.65*** |
| Infected, ART 21 day PI   | 664.63 ± 19.03*** | 274.00 ± 10.55*** | 621.30 ± 19.23*** |
| Infected, ART 45 day PI   | 351.75 ± 27.34*** | 623.75 ± 24.04*** | 844.57 ± 9.85*** |
| ART pretreated-infected   | 477 ± 7.73*** | 425.43 ± 23.28*** | 767.6 ± 26.17*** |

* Significance from normal group, * Significance from infected-untreated group: Significant (P < 0.05), ** Significance from infected-untreated group: Highly significant, *** Significance from infected-untreated group: Very highly significant.
hand, maximal PR was recorded when the treatment was interfering with the 14–21 day old schistosomula stage. This was in consistency with the data recorded by El-Beshbishy et al. [15]. The susceptibility of juvenile worms to ART may be due to the fact that they have a lower capacity of detoxification than the adult worms. ART was found to cause inhibition of glutathion-s-transferase and to some extent superoxide dismutase enzymes. These enzymes are involved in passive detoxification of antischistosomal drugs and elimination of superoxide radicals produced during metabolism. Such inhibition aims finally to the protection of the free radicals that formed by ART from interfering with the action of these enzymes [16].

By comparing the PR in total worm burden after PZQ treatment 42 days PI to that obtained after ART treatment 45 days PI, data showed that the PR was 92.67% and 25.35%, respectively. These results confirm suggestions of many earlier studies e.g. Araújo et al. [17] that ART and PZQ act against different developmental stages of the parasite and can act well together. As the anti-oxidant system in adult worms is stronger than that in immature worms [18], less susceptibility of adults compared to juveniles, under the same dose level of ART, is clarified.

Xiao et al. [19] suggested that adult female worms are more susceptible to ART treatment rather than adult male worms. The present study confirmed this suggestion as ART treatment caused dramatic decrease in female worm burden even with such low dose.

In addition, results recorded a striking reduction in worm pairs in all ART treatment regimens before sexual maturity. The reduction rate reached its top value for 14–21 day old worms with 90–92% PR. This may be attributed to the ultra-structural toxic effect of ART on schistosomula as ART was proved to induce tegumental damage, muscular paralysis and even sustained shrinkage, atrophy and degeneration of the worm’s reproductive glands (the testis in males and ovary as well as vitelline gland in females) [20].

Infection with S. mansoni induces chronic disease as a result of the ongoing host immune response toward the tissue trapped eggs. Granuloma is the main pathology of such chronic disease which is induced by CD4+ T-cell programmed inflammations under stimulation of soluble egg antigens (SEA). Granulomas contain eosinophils, macrophages, lymphocytes and are also characterized by collagen deposition. MGN and MGD were reduced by 78.33% and 19.9%, respectively with PZQ treatment. Similar observations were recorded by Andrade and Grimaud [21] who found that granulomas around eggs decreased 2–3 folds in volume 3 weeks after PZQ treatment and in the subsequent weeks.

Records in the present work revealed dramatic reduction in MGN especially in groups which received ART treatment at 14 or 21 days PI (PR: 82.23% and 85.92%, respectively). An interesting observation was that the PR in MGN for these two groups exceeded that recorded in PZQ treated group which was 78.33%. This reduction in tissue granuloma may be attributed to the recorded reduction in total worm burden and female worm burden. The correlation between worm burden, egg load and granuloma formation was studied and was proved by Botros et al. [22].

The present results demonstrated that MGD diminished significantly when ART was administrated 14–21 days PI this was parallel to the histopathological reduction in collagen deposition surrounding eggs. The correlation between the reduction in MGD and the reduction of type III procollagen was suggested by Badawy et al. [23].

In murine schistosomiasis, Th1 reaction (with a predominant secretion of IFN-γ, minimal level of IL-4 and IL-5) occurs during prepaternity and then shifts to a Th2-based profile which develops after the onset of oviposition and persists throughout the acute phase of infection (with high IL-4 and IL-5, but low IFN-γ) [24].

The shift from Th2- to Th1-like immune response is essential for the down modulation of granuloma reaction and disease control. The relation between Th1 cytokine profile and the development of smaller granulomas was also reviewed by Brunet and his colleagues [25].

The antischistosomal action of PZQ was proved to be dependent on T cell mediated immunity [26]. In contrast, the role of T-cell immunity in ART action is not clearly understood. While some studies suggesting immunosuppressive action of ART by inhibition of T cell progression [27], other studies recorded T-cell independent action of ART [28].

The current study revealed that ART treatment may cause a switching over effect from Th2 to Th1 predominancy (decrease in IL-10, IL-4 and upgrading of serum IFN-γ levels). We suggest two probable hypotheses explaining Th1 predominancy. The first is in light of Wang et al. [27] study which proved the immunosuppressive properties of ART. So it prevents the ongoing of TH2 response which is responsible for granuloma progression.

The second hypothesis is that ART helminthotoxic to schistosomulae itself, regardless to immune response of the host, preventing their development to egg-laying adult worm pairs. The reduction in worm burden and fecundity in turn down regulates the egg-induced Th2 response and maintains Th1 predominant cytokine profile characterized by high levels of IFN-γ and low IL-4 and reduction in liver pathology.

The involvement of IFN-γ in protective immunity to schistosomiasis is well documented in the murine model [29].

In conclusion, after i.m. administration of ART to S. mansoni infected mice, it was shown that i.m. ART has promising prophylactic properties on acute schistosomiasis. ART administration is associated with shift to Th1 response and reduction in liver pathology. But ART use in schistosomiasis control still facing many challenges in both basic and applied research. Further research as well as clinical studies will determine the successes of ART as antischistosomal drug.

**Conflict of Interest**

The authors have declared no conflict of interest.

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