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ORIGINAL RESEARCH ARTICLE

Evaluation of mefloquine-praziquantel combination therapy in pre-patent and patent *Schistosoma mansoni* infection in mice

Nashwa S. Abdel-Fattah[1]✉, Naglaa S. Ahmed[2]

1 – Ain-Shams University, Faculty of Medicine, Department of Parasitology, 25b Omar Lotfy St., 8th district, Nasr City, 8th floor, Cairo, Egypt.

2 – Ain-Shams University, Faculty of Medicine, Department of Pathology, Abbassia, Cairo, Egypt.

Correspondence: Tel. 0222701299, 0124265848, Fax (202)6837673, E-mail n_marakby@hotmail.com

Abstract. The reliance on a single drug to treat a population of over 200 million people infected and over 700 million people at risk over three continents seems particularly perilous when considering the threat of drug resistance. An important shortcoming of praziquantel is its relative inactivity against migratory juvenile and sub-adult worms. Antischistosomal properties of the antimalarial drug, mefloquine were previously noticed and it was found potent against the young developing stages of the parasite. Moreover, the difference in the mechanism of action between the two drugs was encouraging to assess the antischistosomal effect of their combined curative doses (400 mg/kg mefloquine and 500 mg/kg praziquantel) or half-curative doses (200 mg/kg mefloquine and 250 mg/kg praziquantel) in both pre-patent and patent schistosome infections. Therapeutic efficacies of the drugs were assessed using the worm count technique, ova count technique in intestine and liver, oogram pattern, liver function tests and histopatholoical examination of liver sections. With the exception of mild additive reduction of ova count in the liver, mefloquine monotherapy was as potent as the combined full dose regimen. The combined treatment was much potent than praziquantel monotherapy even in its low dose regimen.

Keywords: *Mefloquine; Praziquantel; Schistosoma*.

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Introduction

Schistosomiasis remains a major neglected, poverty-related health problem in many tropical areas (Hotez et al., 2007). The possible availability of vaccines appears remote in near future. The global strategy for schistosomiasis control is morbidity control, relying on a single drug, praziquantel (PZQ). Since the first clinical trials in the late 1970's (Katz et al., 1979), it has proven safe and effective. The mechanism of action of PZQ is directly or indirectly associated with Ca2+ redistributions between worm tissues and the surrounding environments (Andrews, 1985; Cioli et al., 1995). Its low cost and its efficacy against adult worms of all schistosome species that infect humans has led to its very widespread use (Fenwick et al., 2006). However, while PZQ effectively kills adult schistosomes and the very young stages

shortly after skin penetration, its efficacy against schistosomula is minimal with only a 25-30% reduction in worm burdens (Sabah et al., 1986; Xiao et al., 1987). This might explain the low cure rates and rapid re-infection rates in endemic areas where patients are likely to be infected with juvenile and adult parasites concurrently (Dabo et al., 2000; N'Goran et al., 2003), meaning that, for effective treatment and sustainable control, PZQ must be given on a regular basis which raises the potential for drug resistance.

Recent *in vivo* and *in vitro* studies found that the antimalarial drug mefloquine-an arylaminoalcohol compound (Ohnmacht et al., 1971) possesses antischistosomal properties (Keiser et al., 2009). Importantly, they observed high worm burden reductions for young developing stages of *Schistosoma mansoni* harbored in mice.

Both *Plasmodium* and *Schistosoma* parasites degrade hemoglobin. Corrêa Soares et al. (2009) found that interference with haemozoin formation in *S. mansoni* represents an important mechanism of schistosomicidal action of the antimalarial compounds and points out the heme crystallization process as a valid chemotherapeutic target to treat schistosomiasis.

The rationale for the use of combination chemotherapy was first developed in the treatment of bacterial infections and has subsequently been adapted for chemotherapy for cancer, AIDS and malaria primarily to delay the emergence of drug resistance (Nosten and Brasseur, 2002). In fact, PZQ resistant isolates of *S. haematobium* and *S. mansoni* have been identified (Herwaldt et al., 1995; Ismail et al., 1999; William et al., 2001).

Considering that PZQ and mefloquine display broad-spectrum antischistosomal activities by two different mechanisms, and target different developmental parasite stages, this work was planned to investigate the possible role of this combination in enhancing cure in pre-patent and patent *S. mansoni* infection. And to study if a similar or comparable efficacy might be achieved using smaller doses of both drugs, which might result in fewer or milder side effects.

Materials and methods

Experimental animals

Seventy female CD-1 Swiss albino mice (weight, 20 ± 2 g), bred and maintained under conventional conditions at the experimental animal research unit of the Biological Supply Unit at Theodor Bilharz Research Institute (Giza, Egypt), were used. They were fed a standard commercial pelleted diet and were kept in an air–conditioned room at 21°C. All animal experiments were conducted in accordance with valid international guidelines for animal experimentation.

Schistosome Infection

S. mansoni cercariae were provided by the Malacology Lab of the Schistosomiasis Unit (SBSP), Theodor Bilharz Research Institute (TBRI), where laboratory-bred *Biomphalaria alexandrina* are maintained. Infection was subcutaneously performed using freshly shed 80 *S. mansoni* cercariae to each mouse.

Drugs

Mefloquine (Larium, 250 mg tablets) was provided by F. Hoffmann-La Roche (Basel, Switzerland). PZQ (Biltricide, 600 mg tablets) was provided by Alexandria Co. for pharmaceuticals (Alexandria, Egypt). Each drug was suspended in 7% Tween-80 and 3% alcohol at a concentration of 40 g/L. Mefloquine was administered to mice at a single full dose of 400 mg/kg (Keiser et al., 2009; Xiao et al., 2009a) or a single half dose of 200 mg/kg. PZQ was administered to mice at a single full dose of 500 mg/kg (El-Hoseiny, 1999) or a single half dose of 250 mg/kg. Drugs were given to mice intragastrically.

Treatment regimens and experimental groups

The mice were divided into an infected untreated control group (C) which consists of 14 mice, subgroup which consists of 28 mice that were treated 3 weeks post infection (p.i.) and subgroup II which consists of 28 mice that were treated 6 weeks p.i. Each of subgroups I and II were further divided in to 4 groups of 7 mice each according to the treatment regimen

received. In subgroup I: P3, M3, MP3 & M0.5P0.5(3) received PZQ, mefloquine, full dose combination and half dose combination respectively. In subgroup II: P6, M6, MP6 & M0.5P0.5(6) received PZQ, mefloquine, full dose combination and half dose combination respectively. All mice of control group, subgroups I and II were sacrified 8 weeks p.i.

Assessment of the therapeutic effect

The effect of the drugs used was evaluated on the basis of the following criteria:

1. Parasitological parameters

Worm load and distribution were performed by perfusion of portomesenteric veins using the technique described previously (Duval and Dewitt, 1967). Percentage reduction in worm burden was calculated by the following equation: %reduction = mean worm burden in (control) minus mean worm burden in (treated) × 100 over mean worm burden in (control).

Tissue egg loads in liver and intestine were counted as described previously (Cheever, 1968) and oogram pattern was performed according to the method described before (Pellegrino et al., 1962).

2. Liver function tests

Blood samples obtained from mice during sacrifaction were centrifuged. Serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), total protein and albumin levels were assayed by conventional methods.

3. Histopathological study

Liver tissues of mice in each group were preserved in 10% formalin for preparing paraffin-embedded sections, stained by haematoxylin and eosin and examined by light microscopy. The mean number of granulomas per 10 low power fields were counted and the mean size of 10 granulomas/mouse were measured using Olympus software method (Tokyo-Japan) in which the microscope was connected to the computer using (x200) power of magnification (figure 3E).

Statistical methods

IBM SPSS statistics (V. 19.0, IBM Corp., USA, 2010) was used for data analysis. Data were expressed as mean±SD for quantitative parametric measures in addition to median percentiles for quantitative non-parametric measures. The following tests were done: comparison between two independent mean groups for parametric data using Student t test and comparison between two independent groups for non-parametric data using Wilcoxon Rank Sum test.

Results

A descriptive statistical comparison concerning the parasitological parameters and liver functions tests in subgroup I subgroup II are shown in table 1.

In subgroup I of mice, mefloquine monotherapy eradicated the juvenile worms while in combination with PZQ, the worm burden reduction was 93.6% and 87.1% in their full and half doses respectively, such percentages (%) are significantly higher ($p<0.05$) than that obtained by PZQ monotherapy (50.3%) (figures 1A and 1B, table 3). The presence of mefloquine in the treatment regimen of subgroup I was found to cease oviposition. PZQ monotherapy affected also the tissue egg load. However the liver ova % reduction (95.7%) was significantly less ($p<0.05$) than in the groups treated by mefloquine-containing regimens (figure 1C, table 3). Also, PZQ monotherapy had no effect on the oogram pattern of subgroup I with even a higher % of mature ova than that in the untreated control group (figure 1D, table 3).

Although the combined treatment in subgroup I didn't increase the total worm reduction rates than mefloquine monotherapy, the liver function tests appeared better in the group treated by the combined half dose regimen, that's to say less serum levels of AST and ALT (a non statistically significant difference: $p>0.5$) and higher serum levels of total proteins and albumin (a statistically significant difference: $p<0.5$) (figures 1E and 1F, table 3).

Table 1. Descriptive results of the control group, subgroup I & II concerning worm burden, ova count in liver and intestine, oogram, and liver function tests (mean+ SD)

	Control	P3	M3	MP3	M½P½-3	P6	M6	MP6	M½P½-6
W-T	15.5±4.17	7.71±3.9	0	1±0	2±0	2.6±1.67	0	0	2
W-M	6.93±1.59	3.86±2.67	0	1±0	1±0	1.2±0.45	0	0	1
W-F	3.83±1.75	1.86±0.38	0	0	1±0		0	0	1
W-MF	5.21±1.6	3±2	0	0	0	2.3±0.58	0	0	0
OC-L	14298±9221	609±848	0	0	0	2254±598	664±100.8	200±335	463±645
OC-I	16589±5054	329±518	0	0	0	0	0	0	0
O-D	4.57±1.2	4.25±0.96				97.6±2.1	100±0	100±0	100±0
O-I	58.4±3.3	54±2.71				0	0	0	0
O-M	37.7±3.2	41.8±2.36				3.4±1.52	0	0	0
AST	388±151	469±104	482±89.0	487±55.2	413±104	223±41.8	189±51.4	239±104	305±101
ALT	84.1±18.1	100±25.1	70.1±16.7	65±4.3	54.86±6.2	42.4±5.8	51.3±16.7	56.4±10.3	42.3±7.2
TP	5.22±0.52	7.06±0.90	6.56±0.85	6.43±0.63	7.56±0.83	6.54±0.88	6.9±0.37	6.34±0.76	5.93±0.86
A	1.97±0.34	3.1±0.42	3.03±0.22	3.11±0.29	3.43±0.28	3.07±0.49	3.43±0.21	2.86±0.69	2.84±0.40

W-T: WormCount-Total; W-M: Worm Count-M; W-F: Worm Count-F; W-MF: Worm Count-MF; OC-L: Ova Count-Liver; OC-I: Ove Count-Intestine; O-D: Oogram-Dead; O-I: Oogram-Immature; O-M: Oogram-Mature; TP: Total Protein; A: Albumin; SD: standard deviation; M: male; F: female; MF: copula.

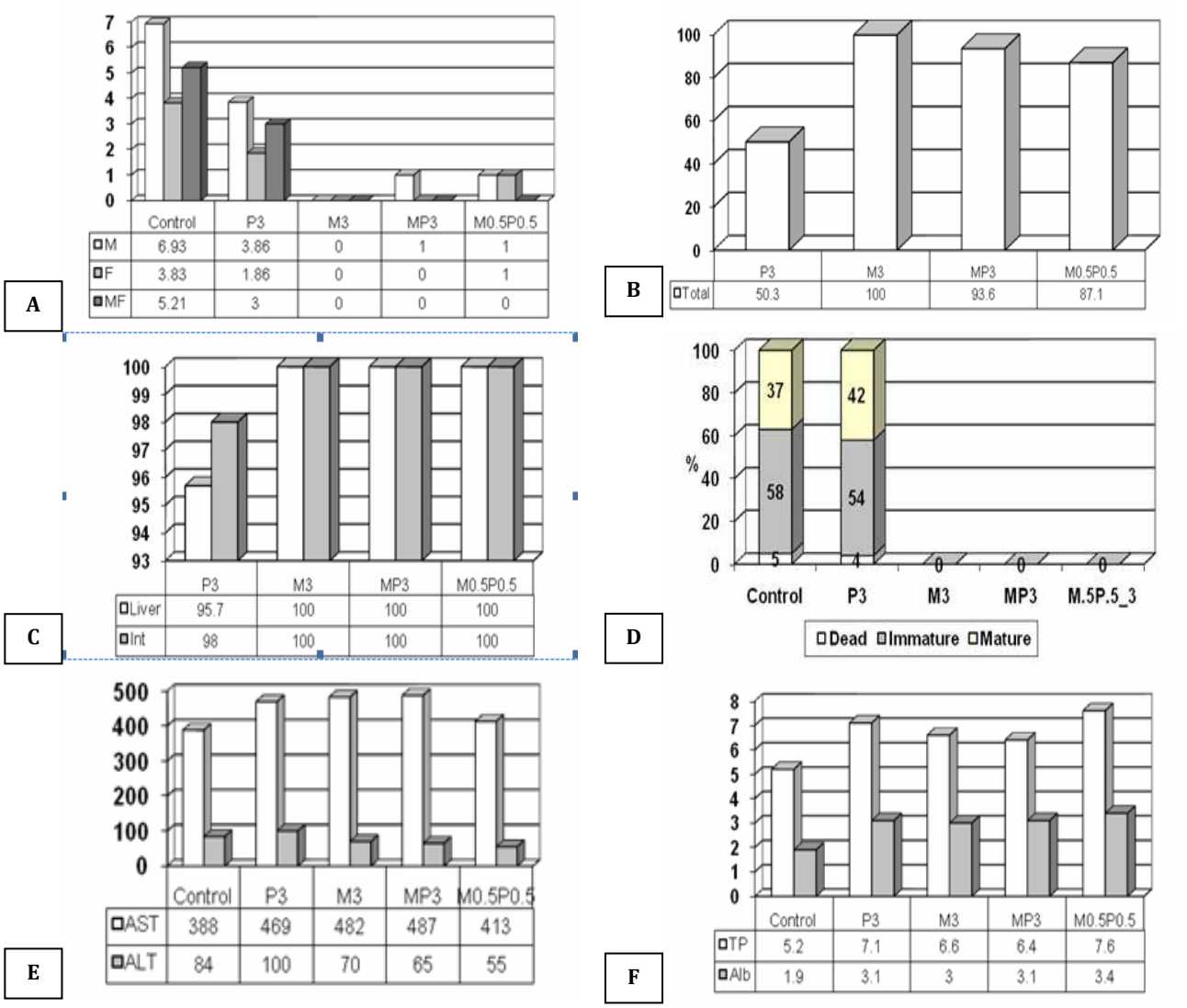

Figure 1. Comparison between all studied subgroup I as regards: A – Mean values of worm count [males (M), females (F) & copulae (MF)]. B – Percentage reduction in total worm count. C – Percentage reduction in ova count in liver & intestine (int.). D – Oogram pattern. E – Mean values of AST & ALT. F – Mean values of total proteins (TP) & albumin (Alb.).

In subgroup II of mice, mefloquine monotherapy and its full dose combination with PZQ succeeded in worm eradication while the half dose combination and PZQ monotherapy caused a % reduction of 87.1% and 83.2% respectively (figures 2A and 2B). All the treatment regimens in subgroup II succeeded in eradication of the ova from intestine and decreased ova count in liver in a highly significant values from that of the untreated control ($p<0.001$) (figures 2A and 2B, table 4). However, the treatment regimens that contain mefloquine showed a % reduction in liver ova count that is significantly higher than that obtained by PZQ monotherapy ($p<0.001$) (figure 2C, table 4). The % reduction in liver ova count is highest in the group treated by a full dose combination and the difference from that obtained by mefloquine monotherapy is statistically significant ($p<0.05$) (figure 2C, table 4). PZQ which didn't affect the developmental stages of the ova in subgroup I, altered the oogram pattern and 97% of the ova were found dead while in the groups received mefloquine-containing regimens, all ova were found dead (figure 2D).

The best liver function pattern in subgroup II was shown in the group treated by mefloquine monotherapy (figures 2E and 2F). Such values differ significantly ($p<0.001$) from the untreated control, from the group received the half dose combination ($p<0.05$) but don't differ significantly from the group received the full dose combination or PZQ monotherapy ($p>0.5$) (table 4).

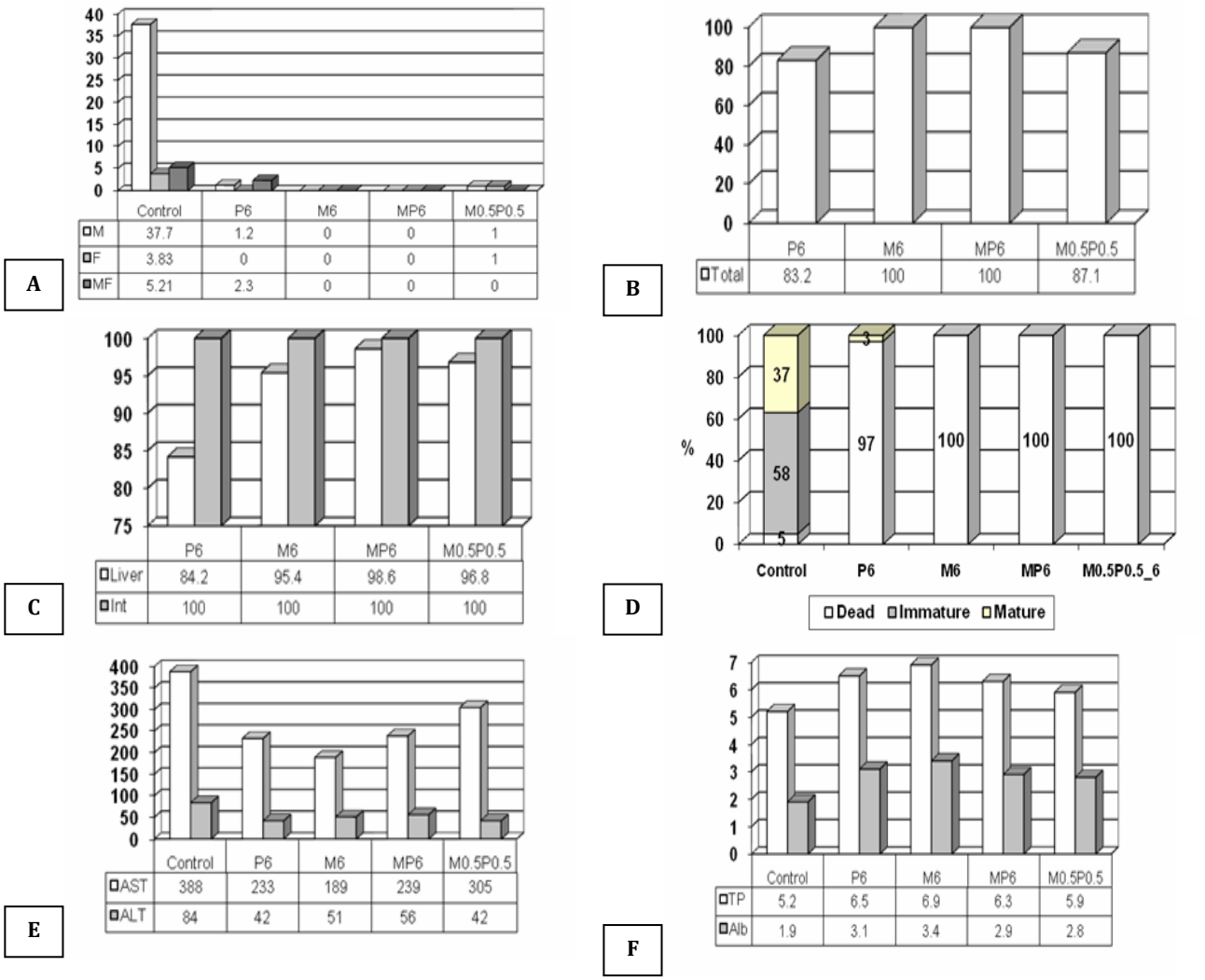

Figure 2. Comparison between all studied subgroup II as regards: A – Mean values of worm count [males (M), females (F) & copulae (MF)]. B – Percentage reduction in total worm count. C – Percentage reduction in ova count in liver & intestine (int.). D – Oogram pattern. E – Mean values of AST& ALT. F – Mean values of total proteins (TP) & albumin (Alb.)

Histopathological results. In subgroup I: The livers of mice treated with mefloquine-containing regimens showed no schistosomal granulomas and only mild portal tract infiltration was seen. Mefloquine monotherapy treatment led to intact liver parynchema. While in its full dose combination, liver parynchema showed mild degeneration and necrosis and in its half dose combination, moderate degeneration and necrosis was seen (table 2). The liver granulomas seen in PZQ monotherapy treated mice were of cellular nature (figure 3A) and were significantly less in number than in the control group ($p<0.001$). However, there was no significant difference in granuloma size between the two groups ($p>0.05$) (tables 2 and 3).

Table 2. Descriptive results of histopathological study of control group,subgroup I and II

	Mean no. ± SD of granulomas/10 low power feilds	Mean size ± SD of 10 granulomas	Type of cellular reaction	Portal tract infiltration	Liver parenchema
Control	6.83 ± 1.17	358.72 ± 79.20	Fibrocellular	Severe	Degeneration (+++) & necrosis
M_3	No	No	No	Mild	Intact
P_3	1.67 ± 0.82	286.79 ± 61.45	Cellular with mild fibrosis	Moderate	Degeneration (+) & necrosis
MP_3	No	No	No	Mild	Degeneration (+) &necrosis
$M_{0.5}P_{0.5}$(3)	No	No	No	Mild	Degeneration (++) &necrosis
M_6	0.67 ± 0.82	159.95 ± 30.28	Fibrocellular	Severe	Degeneration (++) &necrosis
P_6	6.17 ± 0.75	192.48 ± 28.73	Fibrocellular	Severe	Degeneration (+++) & necrosis
MP_6	4.5 ± 1.05	190.08 ± 34.91	Fibrocellular	Severe	Degeneration (+++) & necrosis
$M_{0.5}P_{0.5}$(6)	3.83 ± 0.75	240.80 ± 46.14	Fibrocellular	Severe	Degeneration (+++) & necrosis

SD: standard deviation.

In subgroup II: The number of liver granulomas was significantly less ($p<0.001$) than in the control in the groups of mice treated with mefloquine-containing regimens, while it is not significantly different ($p>0.05$) between the PZQ treated and the control group. The livers of mice treated with mefloquine monotherapy showed significantly less number of liver granulomas ($p<0.001$) than in mice treated with combination therapy (tables 2 and 4), while the size of granulomas decreased significantly in all treated groups than in the control group ($p<0.05$ in half dose combination regimen group and $p<0.001$in other treatment groups). All the livers of mice treated in subgroup II showed fibrocellular granulomatous reaction (figure 3D) and severe portal tract infiltration (figure 3B). However the livers of mice treated with mefloquine monotherapy showed less degeneration and necrosis of liver parynchema than in livers of mice that received other treatment regimens (figure 3C).

Table 3. Comparison between the control group, different groups of subgroup I concerning the statistical significance in worm burden, ova count in liver and intestine, oogram, liver function tests, number and size of granulomas

	Control Vs				P3 Vs			M3 Vs		MP3 Vs
	P3	**M3**	**MP3**	**M½P½-3**	**M3**	**MP3**	**M½P½-3**	**MP3**	**M½P½-3**	**M½P½-3**
wormCount-Total*	S		HS	HS		S	S			NS
wormCount-M*	S		S	S		NS	NS			NS
wormCount-F	HS			HS						
wormCount-MF*	S						HS			
Ova Count-Liver*	HS	HS	HS	HS	S	S	S	NS	NS	NS
Ove Count-Intestine*	HS	HS	HS	HS	NS	NS	NS	NS	NS	NS
Oogram-Dead	NS									
Oogram-Immature	S									
Oogram-Mature*	S									
AST	NS	NS	S	NS	NS	NS	NS	NS	NS	NS
ALT	NS	NS	HS	HS	S	S	HS	NS	NS	HS
Total Protein	HS	HS	HS	HS	NS	NS	NS	NS	S	S
Albumin	HS	HS	HS	HS	NS	NS	NS	NS	S	NS
No. of granulomas	HS									
Size of granulomas	NS									

Student t test was used to compare between 2 groups of parametric data except for (*) where Wilcoxon test was used to compare between the 2 groups of non-parametric data: p>0.05=NS, p<0.05=S and p<0.01 or 0.001=HS.
Vs: versus. HS: highly significant. S: significant. NS: non-significant, M: male, F: female, MF: copula.

Table 4. Comparison between the control group, different groups of subgroup II concerning the statistical significance in worm burden, ova count in liver and intestine, oogram, liver function tests number and size of granulomas

	Control Vs				P6 Vs			M6 Vs		MP6 Vs
	P6	**M6**	**MP6**	**M½P½-6**	**M6**	**MP6**	**M½P½-6**	**MP6**	**M½P½-6**	**M½P½-6**
Count-Total*	HS			HS			NS			
Count-M*	HS			NS			NS			
Count-F				NS						
Count-MF*	S									
Ova Count-Liver*	HS	HS	HS	HS	HS	HS	HS	S	NS	NS
Ove Count-Intestine*	HS	HS	HS	HS	NS	NS	NS	NS	NS	NS
Oogram-Dead	HS	HS	HS	HS	S	S	S			
Oogram-Immature										
Oogram-Mature*	HS									
AST	HS	HS	S	NS	NS	NS	NS	NS	S	NS
ALT	HS	HS	HS	HS	NS	S	NS	NS	NS	S
Total Protein	HS	HS	HS	NS	NS	NS	NS	NS	S	NS
Albumin	HS	HS	S	HS	NS	NS	NS	NS	HS	NS
No. of granulomas	NS	HS	HS	HS	HS	S	HS	HS	HS	NS
Size of granulomas	HS	HS	HS	S	NS	NS	NS	NS	HS	NS

Student t test was used to compare between 2 groups of parametric data except for (*) where Wilcoxon test was used to compare between the 2 groups of non-parametric data: p>0.05=NS, p<0.05=S and p<0.01 or 0.001=HS.
Vs: versus. HS: highly significant. S: significant. NS: non-significant, M: male, F: female, MF: copula.

Discussion

In combination chemotherapy for schistosomiasis, the partner drugs should have different mechanisms of action to reduce the likelihood of resistance development and/or target different developmental stages of the parasite to enhance cure and egg reduction rates (Utzinger et al., 2003).

The expected effect of mefloquine-PZQ combination had not been revealed in the present experiment. With the exception of mild additive reduction of ova count in liver, mefloquine monotherapy was as potent as the combined full dose regimen. The combined treatment was much potent than PZQ monotherapy even in its low dose regimen.

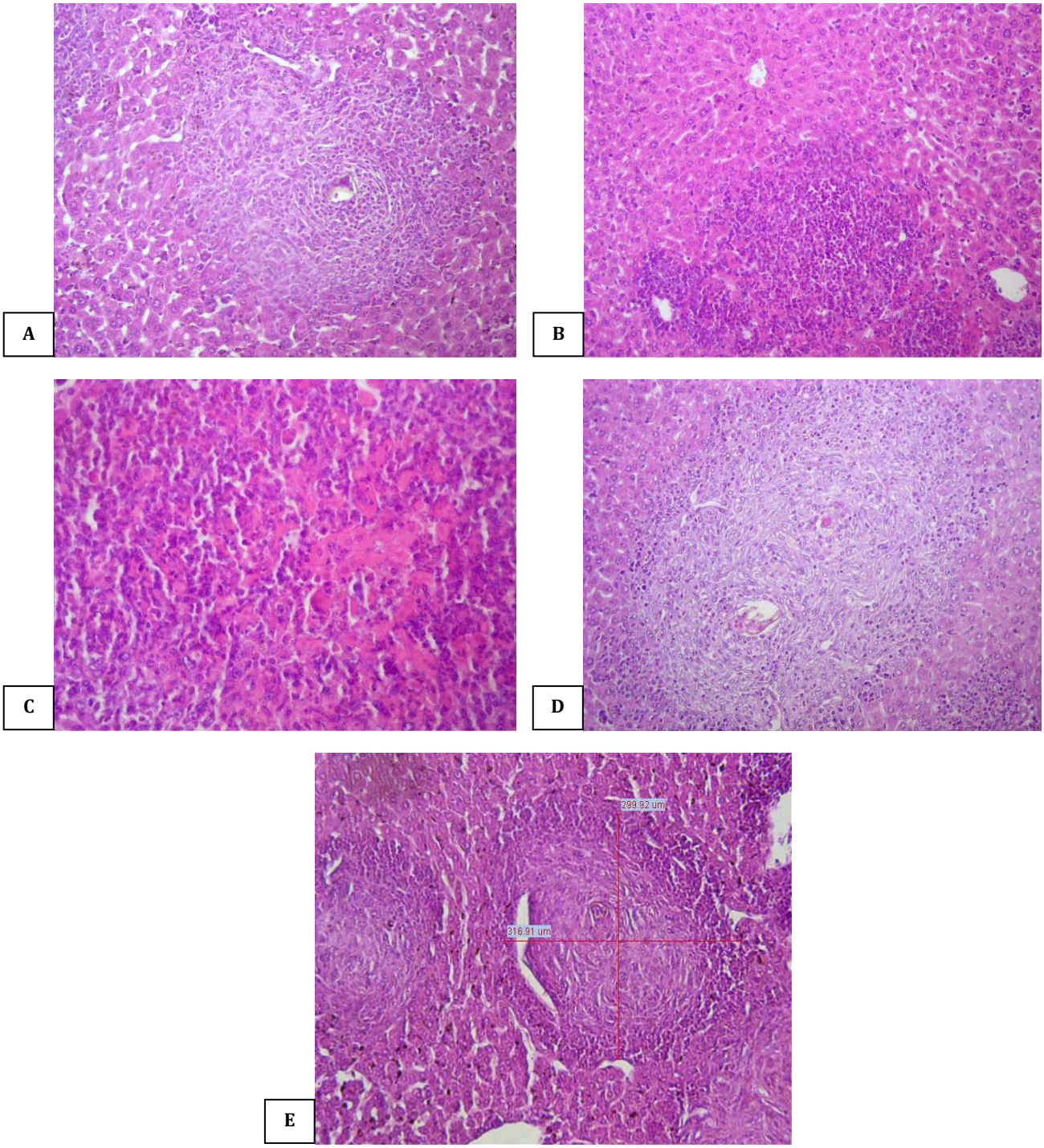

Figure 3. Histopathological findings of the studied groups: A – Ovular cellular granuloma formed of chronic inflammatory cellular infiltrate (x200); B – Widened portal tract which is severely infiltrated by chronic inflammatory cells (x200); C – Necrosed hepatocytes with deep eosinophilic cytoplasm and pyknotic nuclei (x400); D – Fibrocellular granuloma with scattered chronic inflammatory cells (x400); E – Measuring the size of granuloma using Olympus soft ware method

Previous studies evaluated the combined use of PZQ and other antimalarial drugs which are known to possess antischistosomal properties. There is a discrepancy in their results regarding the efficacy of these combinations in schistosomiasis. While some authors reported no apparent increase in efficacy by using the combination in treatment of *S. japonicum* (You et al., 1994) and *S. mansoni* (Mahmoud and Botros, 2005), others found higher worm burden reductions in treatment of both species compared to those achieved using the

monotherapies (Xiao et al., 2000; Utzinger et al., 2001). Both studies infected the experimental animals on three occasions: days 0, 28 and 35. Thus the additive effect of the drugs used may be attributed to the simultaneous presence of juvenile and adult parasites in the same animal host, a difference which might explain the failure to achieve an obvious additive effect in the present work. Thus we recommend to extend the experiment to assess mefloquine-PZQ combination on mixed infection of *S. mansoni* parasites of different ages.

Recently, Xiao et al. (2011) explored the efficacy of mefloquine in combination with PZQ in mouse-*S. japonicum* model after 35 days of infection. Mefloquine combined with PZQ at single dose of 50 mg/kg resulted in no apparent improvement in efficacy. Administration of mefloquine 100 mg/kg combined with PZQ 100 mg/kg resulted only in a significant decrease in female worm burdens between PZQ group and combined treatment group.

In the present work, mefloquine monotherapy was found to be superior to PZQ both in juvenile or mature worm infection and resulted in worm eradication, and its presence in the treatment regimen in combination with PZQ, weather in its full or half dose regimens, much improved the course of infection, a finding that confirms earlier observations concerning its potent antischistosomal effect (Van Nassauw et al., 2008; Keiser et al., 2009; Xiao et al., 2009b). However, the worm reduction rates observed by using doses of mefloquine lower than that in the present work didn't result in worm eradication. Keiser et al. (2009) reported worm burden reduction of 45% and 72% on a dose of 100 and 200 mg/kg of mefloquine respectively. Similarly, Xiao et al. (2009b) tested a single mefloquine dose of 200 mg/kg, their results showed total worm burden reductions of 56.3% on juvenile and 89.1% on adult schistosomes. When they increased the mefloquine dose to 400 mg/kg, worm reduction rates were 81.1-100% on juvenile and adult worms respectively.

Xiao et al. (2009a) assessed the effect of mefloquine 400 mg/kg single dose – the same dose used in the present experiment – on the morphology of adult *Schistosoma japonicum* under a light microscope. They found severely dilated guts and the entire worm body was swollen. Moreover, reproductive glands showed signs of degeneration, which resulted in disturbance of ova formation and cessation of oviposition.

Mefloquine administration was thought to be a cause of silent schistosomiasis in travelers using mefloquine for malaria chemoprophylaxis (Van Nassauw et al., 2008). We also assume that the excellent abortive effect of mefloquine in prepatent infection observed in the current study may result in the generation of a protective immune response and resistance to re-infection, an effect which was proved earlier in artemether treatment of pre-patent *S. japonicum* infection (Bartley et al., 2008).

In view of these observations, and in the presence of an intolerable burden of schistosomiasis, there is clearly a need, beside developing novel antischistosomal drugs, to make more effective use of existing ones.

Assessment of mefloquine-PZQ combination on mixed juvenile and mature *S. mansoni* infection remains necessary.

It's worth investigating if mefloquine treatment could be of value in patients who have mixed malaria and *Schistosoma* infections, such co-infection is not rare in endemic countries. Prevalence of *S. mansoni* – *P. falciparum* co-infections was 22.6% among schoolchildren in rural northwest Tanzania (Mazigo et al., 2010).

More studies on the potential mefloquine-induced toxicity are needed as a basis for possible wide use for schistosomiasis in humans. Mefloquine is generally well tolerated by adults and children. However, recent data suggest that mefloquine may result in harmful events in the gastrointestinal (Keiser et al., 2010) and central nervous systems (Van Essen et al., 2010).

Moreover, the proved potency of mefloquine in treatment of all schistosome species should be taken with caution before its dose adjustment

and wide use in human, because of concern that this strategy might select for *Plasmodium*-resistant parasites, such expectation is of argument by the malaria community.

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