Effect of Treatment with Antifibrotic Drugs in Combination with PZQ in Immunized *Schistosoma mansoni* Infected Murine Model

Ibrahim Rabia[1], Faten Nagy[2] 2, Eman Aly[1], Amina Mohamed[3] Fayza EL-Assal[3] and Azaa El-Amir[3]

1 Parasitiology, 2 Immunology Departments, Theodor Bilharz Research Institute, (TBRI) Giza, Egypt and 3 Faculty of Science, Cairo Univeresity.

ibrahimshalash@yahoo.com

Abstract : The main problem in schistosomal hepatic morbidity is fibrosis and extensive scarring induced by living eggs. In this study, we tried to study the effect of treatment using antihelminthic drug (PZQ) and/or antifibrotic drugs (PTX and silymarin) in combination with immunization. The parasitological parameters, the dynamics of serum-specific immunoglobulins and splenic cytokines associated with changes in granuloma diameter were assessed. Naïve mice were immunized intravenously with 10 ug of SEA in three doses at 2 days intervals 6 weeks before infection. Animals were infected by tail immersion with 100 cercariae and divided into several groups. Three groups were treated with PZQ, PTX or silymarin administered alone. Another two groups were treated with PZQ combined with PTX or silymarin. All treated animals and respective controls were sacrificed 12 weeks post infection. Immunization did not affect worm reduction , but slight decrease in granuloma diameter, increase in immunoglobulins and cytokines was observed . Reduction in worm burden was associated with reduction in ova count and changes in oogram pattern which were mainly due to PZQ treatment. Increasing reduction in granuloma diameter, elevation of immunogloulins and cytokines levels were observed in the groups treated with PZQ alone or cmbined with PTX or silymarin. In conclusion, in this study, treatment with PZQ complemented with immunization resulted in significant reduction of parasitological parameters and rise of specific Igs. Addition of antifibrotic drugs PTX or silymarin to PZQ, potentiated an antipathology effect which minimized and ameliorated liver fibrosis by inhibition of HSC activation and accentuation of the effect of suppressor Treg cells. [Journal of American Science 2010; 6(5):208-216]. (ISSN: 1545-1003).

Key word: *Schistosoma mansoni*, Praziquantel, Pentoxifyllin, silymarin.

1- Introduction

Schistosomal pathology is a direct consequence of the immunological response to ovideposition in host tissue especially liver. Liver injury is typically associated with infiltration of inflammatory cells leading to fibrosis (Friedman, 2003).

Liver fibrosis results from chronic damage of the liver and activation of hepatic stellate cells (HSC) which leads to excess production of extracellular matrix (ECM) components (Friedman and Arthur, 2002 ; Friedman, 2003 ; Bartley et al., 2006). Various investigators have focused on the protective immunization against schistoromiasis using several soluble egg antigen (SEA) fractions which were identified and tested in experimental models with the induction of variable levels of protection against infection (Tendler et al., 1996). Immunization of mice stimulates specific immunity which causes reduction in worm burden, intestinal egg load and liver pathology (Romeih et al., 2008 ; Garcia et al., 2008). Until recently, non of immunizing fractions was able to induce more than 67% protection, but the existence of at least partially protective immunity would make a logical complement to drug therapy (Bergquist et al., 2008 ; Maher et al., 2003 , Zovain et al., 2001). Praziquental (PZQ) is the drug of choice for all species of *Schistosoma* as an effective antischistosomal drug (Utzinger and Keiser, 2004). Although treatment with this drug is effective, but frequent schistosome reinfection occurs after treatment due to relative resistance to schistosomicide drugs (Silva et al., 2003). At the same time, it is stated that it is preferable to develop combination of PZQ and anti-fibrotic drugs in the treatment of murine schistosomiasis which could minimize liver fibrosis simultaneously with worm elimination (Mahmoud et al., 2002 ; Doenhoff et al., 2002). Pentoxyfilline (PTX) has been identified as an antifibrotic drug which can interfere on a large spectrum of cytokines with proinflammatory action and causes inhibition of ECM synthesis (Bienvenu et al., 1995 ; Reis et al., 2001). Antioxidants such as silymarin have received attention as potential antifibrotics which inhibit HSC activation and protect hepatocytes from undergoing apoptosis (Leiber et al.,

2003). In this work, parasitological parameters and the dynamics of serum-specific immnoglobulins and splenic cytokines associated with changes in hepatic pathogenesis and granuloma diameter, were assessed in an attempt to study the effect of treatment with PZQ alone and in combination with PTX or silymarin in immunized infected mice model.

2- Material and Methods:

Animals: C57 BL/6 mice (6-8 weeks old), (18-20g) were bred and maintained at Schistosome Biology Supply Center (SBSC) of Theodor Bilharz Research Institute (TBRI) and kept under standard housing conditions. The animal experiments have been carried out according to the internationally valid guidelines in an institution responsible for animal ethics (TBRI) (Nessim et al., 2000).

Preparation of *S. mansoni* soluble egg antigen (SEA):

SEA was prepared (Boros and Warren, 1970, Carter and Colley, 1978) and purified from host antigen by affinity chromatography using cyanogen bromide activated sepharose-4B beads (Nordon & Strand, 1984). SEA was sterilized by filtration and protein content was estimated using Bio-Rad kit (Bradford, 1976).

Drug and doses:

a) Praziquental (PZQ) (Distocide ®, Epico Pharma Cairo, Egypt) was orally administered 7 wks p.i. at a dose of 500 mg/kg body weight for 2 consecutive days. It was freshly prepared before use as a 2% suspension in Cremophor-El (Sigma chemicals Co. St. Louis, Missouri).

b) Pentoxifylline (PTX) (Trental ®, Aventis Pharma, Cairo, Egypt), was orally administered 4 wks post infection (PI) 5 days/wk at dose of 400 mg/Kg body weight. The treatment was continued until the date of sacrifice.

c) Silymarin: (SEDICO Pharmasetuical-co) was orally administered starting from the day of infection at dose of 140 mg / kg three times / week until the day of sacrifice.

Experimental design: 140 mice were immunized with SEA (10 ug X 3). Six weeks later, they were infected by tail immersion with 100 cercariae of an Egyptian strain of *S. mansoni* supplied from SBSP, TBRI and were divided into 6 groups. Three groups were treated with PZQ, PTX or silymarin as described before. Another two groups were treated with PZQ combined with PTX or Silymarin. The sixth group- immunized, infected untreated mice were used as immunized infected control. Infected not immunized, untreated animals were used as infected control. Clean uninfected, untreated animals were used as normal control. All animals were sacrificed 12 weeks post infection.

Parasitological Parameters:

1- Worm burden: Infected animals were perfused to recover hepatic and portomesenteric worms for subsequent counting (Duvall and DeWitt, 1967).

2- Tissue egg load: The number of eggs per gram tissue (liver and intestine) was studied according to the procedure described by Cheever (1968).

3- Oogram pattern: The percentages of immature, mature and dead eggs in the small intestines were computed from a total of 100 eggs per intestinal segment and classified according to categories previously defined by Pellegrino et al. (1962).

Immunological Study:-

Determination of anti-SEA immunoglobulin subclasses IgG1, IgG2 and IgG4 were measured using indirect ELISA, based on the method of Engvall and Perlman (1971). ELISA microtiter plates were coated with 100 ul / well of 30 ug/ml of SEA. Sera were diluted 1:20 and anti-mouse IgG subclasses (Binding site, Birmingham, UK) were used at a dilution of 1:500. Absorbance at 492 nm was measured.

Cytokine assay: Serum IFN-γ, IL-4 and IL-10 levels were measured by a sandwich ELISA technique. Briefly, plates were coated with capture antibodies and 100 ul of serum samples or recombinant cytokines were added. Following addition of the biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate, the reaction was developed with paranitrophenyl phosphate (Sigma) and absorbance was measured at 405 nm.

Granuloma measurement: Hepatic granuloma diameter was measured according to Von Lichtenberg (1962). The percent reduction in granuloma diameter relative to infected control was calculated as follows:

% reduction of granuloma diameter = mean diameter of controls - mean diameter of test groups / mean diameter of control group x 100.

Statistical analysis: The data were presented as mean ± standard error of the mean (X ± SE). The means of the different groups were compared globally using the analysis of variance ANOVA. Data were considered significant if p values were less than 0.05.

3- Results:

Parasitological parameters:

The total number of worms and the percent reduction of worm burden showed no significant difference between infected control and the immunized infected control. On the other hand, the groups treated with PZQ alone or combined with PTX or silymarin showed highly significant decrease ($P<0.001$)

compared to immunized infected control. It showed no significant or slight decrease (p<0.05) in groups treated with PTX or silymarin respectively compared to immunized infected control. The mean ova count in intestine and liver showed significant reduction (P<0.01) in immunized infected control compared to infected control, while all treated groups showed highly significant reduction (P<0.001) compared to immunized infected control (Table (1)). As regards oogram pattern, there was no significant change between the infected control and immunized infected control. On the other hand, highly significant decrease was shown only in the groups treated with PZQ alone or combined with PTX or silymarine (P< 0.001) compared to immunized infected control (Table (2).

Granuloma diameter:

Granuloma diameter showed slight decrease in immunized infected control compared to infected control (P<0.05), while in all treated groups, it showed highly significant decrease (P<0.001) except the group treated with PZQ alone which showed no significant change compared to immunized infected control (Table (3)).

Immunological Parameters:

Serum-specific immunoglobulin isotypes:

In infected control group, there was no significant change in IgG isotypes compared to normal control. However in immunized infected control there is significant increase in IgG1 (P< 0.01) and IgG4 (P<0.05) compared to the infected control. The level of IgG1 showed no significant change in the treated groups except in the groups treated with PTX combined with PZQ or silymarin alone which showed slight decrease (P<0.05) compared to immunized infected control. On the other hand , there was highly significant increase in IgG2 level in all treated groups (P<0.001), while the increase in IgG4 level was shown only in the groups treated with PZQ alone or combined with PTX or silymarin (P<0.05, P<0.05 and P<0.01 respectively) compared to immunized infected control (Table (4).

Serum cytokines level:

The profile of Th-1 related cytokine IFN-γ showed significant increase in infected control (P < 0.001) compared to normal control. On the other hand it showed slightly significant decrease in immunized infected control compared to infected control (P<0.05). In treated groups, the groups treated with PZQ alone or combined with PTX or silymarin showed significant increase (P < 0.05) compared to immunized infected control. On the other hand, significant decrease in IFN-γ level was observed in groups treated with silymarin or PTX alone (P<0.01 – P< 0.001 respectively) compared to immunized infected control. The Th-2-related cytokines IL-4 showed highly significant increase in the infected control compared to normal control (P< 0.001). At the same time, it showed significant decrease in the immunized infected control (P<0.01) compared to infected control. Also, it showed no significant change in all treated groups except groups treated with PTX or PZQ alone which showed slight decrease (P<0.05) compared to immunized infected control. On the other hand, the Treg-related cytokine IL-10 level showed significant highly increase in infected control (P<0.001) compared to normal control and slight increase in immunized infected control (P<0.05) compared to infected control. In the treated groups, it showed slightly significant increase in the groups treated with PZQ, silymarin or PTX alone (P<0.05) compared to immunized infected control (Table 5).

Table 1: Worm burden and tissue load in mice immunized with SEA (10 µg X3) 6 wks before infection and treated with different types of drugs then sacrificed 12 wks post infection.

Animal group	Mean no. of worms ± SEM	% reduction	Mean no. of ova count ± SEM / g tissue			
			Intestine	% reduction	Liver	% reduction
Infected control	31.6 ± 0.33		17234 ± 1291		3011 ± 374	
Immunized infected control	29.5 ± 0.17	6.65%	7450 ± 114 **	56.8%	1420 ± 210 **	53%
Treated groups						
PTX	27.1 ± 0.29	14.2%	#4158 ± 234	75.9%	##988 ± 277	67.3%
PTX + PZQ	###1.3 ± 0.30	95.9%	###612 ± 133	96.4%	###158 ± 33	94.8%
Sily	# 21.6 ± 0.21	31.6%	##3889 ± 303	77.4%	###812 ± 90	73.1%
Sily + PZQ	###1.1 ± 0.21	96.5%	###500 ± 125	97.1%	###99 ± 11	97.1%
PZQ	###1.2 ± 0.3	96.2%	###638 ± 131	96.3%	###112 ± 10	96.3%

 # # # P<0.001, # # *** P < 0.001 , ** P < 0.01, * P <0.05 relative to infected control.
P<0.01, # P<0.05 compared to immunized infected control.

Table 2: Oogram pattern in mice immunized with SEA (10 µg x3). 6 wks before infection and treated with different types of drugs then sacrificed 12 wks post infection.

Animal Group	Oogram pattern (% ova)		
	Immature	Mature	Dead
Infected control	67.2 ± 5	33.1 ± 2.6	1.7 ± 0.3
Immunized infected control	62.3 ± 2.4	31.8 ± 3.5	21.6 ± 0.7*
Treated groups			
PTX	# 47.9 ± 3.9	29.9 ± 3.1	22.2 ±1.1
PTX + PZQ	### 4.9 ± 5.4	###3.3 ± 1.4	###91.8 ± 7.4
Sily	## 22.1 ± 3.8	40.8 ± 3	37.1 ± 2
Sily + PZQ	### 9.5 ± 1.2	###8.8 ± 1.1	## 81.7 ± 4.1
PZQ	### 2.0 ± 0.3	###1.7 ± 0.2	## 96.3 ±4.9

***** P < 0.001 , ** P < 0.01, * P <0.05 relative to infected control.**
 # # # P<0.001, # # P<0.01, # P<0.05 compared to immunized infected control.

Table 3: Hepatic granuloma diameter and % reduction in mice immunized with SEA (10 µg x3). 6 wks before infection and treated with different types of drugs then sacrificed 12 wks post infection

Animal Group	Hepatic granuloma diameter(a) Mean µm ± SEM	% Reduction (b)
Infected control	299.8 ± 12.5	
Immunized infected control	254 ± 14.3*	15.3%
Treated groups		
PTX	## 139.9 ± 20.1	53.3%
PTX + PZQ	### 103.4 ± 17.5	65.4%
Sily	## 164.4 ± 13.2	45.2%
Sily + PZQ	### 128.6 ± 20.7	57.1%
PZQ	225.1 ± 19.3	24.9%

(a) The mean granuloma diameter per group was calculated from the mean values (10-15 granulomas per mouse).
(b) Percentage reduction was calculated relative to infected control.
***** P < 0.001 , ** P < 0.01, * P <0.05 relative to infected control.**
 # # # P<0.001, # # P<0.01, # P<0.05 compared to immunized infected control.

Table 4: Serum anti-SEA IgG subclasses levels in mice infected with SEA (10 µg x3). 6 wks before infection and treated with different types of drugs then sacrificed 12 wks post infection

Animal Group	X` O.D ± SEM Ig G1	X` O.D ± SEM Ig G2	X` O.D ± SEM Ig G4
Normal control	0.202 ± 0.178	0.294 ± 0.21	0.321 ± 0.02
Infected control	0.462 ± 0.103	0.606 ± 0.207	0.99 ± 0.126
Immunized infected control	0.954 ± 0.34 **	0.521 ± 0.383	1.12 ± 0.135*
Treated groups			
PTX	0.815 ± 0.251	###0.939 ± 0.178	1.08 ± 0.204
PTX + PZQ	# 0.783 ± 0.187	### 0.998 ±0.231	# 1.58 ± 0.179
Sily	# 0.791 ± 0.245	###1.049 ± 0.159	1.21 ± 0.321
Sily + PZQ	0.833 ± 0.213	###1.180 ± 0.256	## 1.69 ± 0.155
PZQ	0.802 ± 0.421	###0.897 ± 0.534	# 1.43 ± 0.254

***** P < 0.001 , ** P < 0.01, * P <0.05 relative to infected control.**
 # # # P<0.001, # # P<0.01, # P<0.05 compared to immunized infected control.

Table 5: Serum cytokine level in mice immunized with SEA (10 µg x3) 6 wks before infection and treated with different types of drugs the sacrificed 12 wks post infection

Animal Group	IFN – γ Pg/ml ± SEM	IL – 4 Pg/ml ± SEM	IL – 10 Pg/ml ± SEM
Normal control	235 ± 26.5	15.6 ± 0.57	90 ± 14.5
Infected control	611 ± 34 ♣	69.6 ± 12 ♣	510 ± 29.1 ♣
Immunized infected control	420 ± 22.4 *	35.4 ± 9.77 **	625 ± 16.5*
Treated groups			
PTX	###140 ± 51	# 28.8 ± 5.1	#733 ± 21.9
PTX + PZQ	# 587 ± 94	40.2 ± 11.7	655 ± 40
Sily	## 221 ± 59	# 25.5 ± 14.4	# 719 ± 46.5
Sily + PZQ	# 613 ± 72.2	45.1 ± 14.2	685 ± 50.3
PZQ	# 633 ± 53.4	39.8 ± 13.3	# 723 ± 45.5

♣ P<0.001 relative to normal control
***** P < 0.001 , ** P < 0.01, * P <0.05 relative to infected control.**
 # # # P<0.001, # # P<0.01, # P<0.05 compared to immunized infected control.

4- Discussion:

Schistosomal infection is cryptic as it frequently goes undetected and developed significant pathology before chemotherapy administration (Wilson et al., 2006). The combination of protection using SEA fractions and treatment was recommended in several studies as it provided many complementary goals, a reduction of egg- induced pathology, minimal parenchymal changes and the eradication of worms. Therefore, the assessment of the effect of treatment of immunized infected mice is important by studying several criteria related to the parasitic intensity, stages and distribution through the tissues of the host for the evaluation of the magnitude of infection and efficacy of the treatment (Abdel-Ghaffar et al., 2005).

The present study revealed that the immunization schedule used did not cause any significant change in worm burden but slightl significant reduction in tissue egg load which agreed with Botros et al. (1996). The treatment of PZQ alone or combined with PTX or silymarin in immunized infected animals caused almost similar high percentage of eradication of worms and tissue egg load which also agree with the work of Suleiman et al. (2004). The death of the worms due to the treatment with antischistosomal drugs was attributed to metabolic disorders, mechanical destruction and muscular contraction of the treated worms (Doenhoff et al., 2002). At the same time, percent reduction in the egg count in both immunized infected and treated groups was found to be higher in the intestinal tissue than in hepatic tissue. This variation was attributed to excretion of some ova from the intestine prior to digestion and to hepatic shift of worms after treatment (Abdel-Ghaffar and Qurtam, 2001, Abdel –Ghaffar, 2004). On the other hand, the treatment with PZQ alone or combined with PTX or silymarin caused decrease in immature egg stages and number of mature eggs with high increase in number of dead eggs which agree with the findings of Botros et al.(1996). The parasitological improvement is due to antiparasitic drug (PZQ) which causes direct or indirect toxic effect in combination with the effect of immunization with SEA which lead to reduction in tissue egg load. This may be attributed to marked decrease in the worm number or fecundity due to hindering the process of oviposition (Guirguis, 2003).

The manifestations of schistosomiasis are mainly attributed to granulomatous inflammation around parasite eggs (Abath et al., 2006). The formation of granulomas depends predominantly on CD_4^+ T cell specific for egg antigen and represents a delayed – type hypersensitivity (Stadecker, et al., 2004 ; Pearce, 2005; Garcia et al., 2008). At the same time, hepatic stellate cells (HSCs) comprise 10-15% of all hepatic cells and they are recruited to areas of hepatic injury and become activated (Cassiman et al., 2002). They adopted a myofibroblast–like phenotype, secreting extracellular matrix components (Iredale, 2003; Mann et al., 2009).

In this work, although all treated groups revealed significant diminution of granuloma diameter, but the groups treated with PTX or silymarin alone or combined to PZQ revealed more diminution in granuloma diameter. This is because PTX reduces transdifferentiation of HSC to myofibroblasts and inhibits HSC proliferation that

leads to inhibition of extracellular matrix synthesis, beside the effect of PZQ which reduces CD4 T cells and increase CD8 cells (Raetsch et al., 2002; El-Ahwany et al., 2006; El-Lakkany and Nosseir, 2007). At the same time, the groups treated with silymarin or PTX alone revealed lower pattern than the other treated groups and this many be due to the effect of previous immunization of the infected animals before treatment. This effect is considered complementary to the immunization effect which modulate the immune response by limiting the immunopathological reactions against schistosome eggs trapped in the liver. In this study, immunization before infection increased the levels of production of IgG1 and IgG4 . All treated groups had increased levels of IgG2 , but slight increase in the level of IgG4 was observed in the groups treated with PZQ alone or combined with PTX or silymarin. This increase in the production of immunoglobulins have an important role in the improvement of the pathology and the reduction in the ova count and worm burden (Soren et al., 2009; El-Ahwany et al., 2006).

Cytokines which act on lymphocytes are of special interest because of their role in regulating cells of the immune response (Kim et al., 1997). During schistosomal infection, both Th1 and Th2 responses directed against egg antigen and produce IFN- γ , IL-4, IL-5 and IL-13 (Hoffman et al., 2002; Sadler et al.,2003; Stadecker et al., 2004). In this study, the diminished production of Th1-cytokine IFN-γ and Th2-cytokine IL-4 in the immunized group may be implicated in the down modulation of the granulomatous response due to immunization (Chensue et al., 1992). Groups treated with PTX or silymarin alone showed significant decrease in IFN- γ and IL-4 . On the other hand, groups treated with PZQ alone or combined with PTX or silymarin showed increase in Treg cell cytokine IL-10. Recent studies suggest that Treg cells play a pivotal role in suppressing Th1 cell development as well as limiting the magnitude of Th2 response directed against egg antigen by a process dependent upon IL-10 (Hori et al., 2003; Wynn, 2004; Stadecker et al., 2004). The increasing level of IL-10 is probably implicated in the down regulation of granuloma formation as it reduces the intrahepatic inflammatory response and hence it has an antifibrotic effect (Nelson et al., 2003; Thompson et al., 1998). These results indicate the importance of the effect of PTX as it has a potent antifibrogenic role. Also using silymarin as an antioxidant drug can inhibit HSC activation and slow down the progression of liver fibrosis (Afdhal and Nunes, 2004; Jhy-wen et al., 2009). Recent studies recommended using PTX in early stage of infection and in a long-term treatment. Also, it can be used as an adjuvant therapeutic tool when combined with PZQ in treatment of schistosomiasis (El-lakkany and Nosseir, 2007). In conclusion, in this study, treatment with PZQ complemented with immunization resulted in significant reduction of parasitological parameters and rise of specific Igs. Addition of antifibrotic drugs PTX or silymarin to PZQ, potentiated an antipathology effect which minimized and ameliorated liver fibrosis by inhibition of HSC activation and accentuation of the effect of suppressor Treg cells.

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Corresponding auther

Dr. Ibrahim Rabia Bauiomy Aly
Associate professor
Immunoparasitology Dep.
Theodor Bilharz Research Institute, (TBRI) Giza, Egypt.
e- mail :- ibrahimshalash@yahoo.com

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