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IMMUNOMODULATORY EFFECTS OF PAEONIFLORIN COMBINED TO SOLUBLE EGG ANTIGENS IN SCHISTOSOMIASIS *MANSONI* MURINE MODEL

By

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Abstract

Praziquantel, the main schistosomiasis chemotherapy; cannot fully counteract the infection associated morbidity including hepatic fibrosis and its sequelae. A novel therapeutic strategy is mandatory. The current study is a novel trial to assess the immunomodulatory effect of Paeoniflorin (PAE) in combination to soluble egg antigen (SEA) in schistosomiasis *mansoni* murine model through; parasitological, histopathological, immunohistochemical and serological studies aiming to synergistic effect that not only eliminate the parasite but also ameliorate morbidity associated to infection. Forty laboratory bred Swiss albino male CD-1 mice were used. The mice were classified into five groups (8 mice each), control healthy, control infected, PAE treated (50 mg/kg/d), SEA vaccinated (50µ protein) and combined (PAE+SEA) groups. All mice groups were sacrificed 10 weeks post infection. Our results showed marked decrease in egg count/ gm stool, worm burden, granuloma diameter, tissue transforming growth factor-beta1 (TGF-β1) and serum interleukin-13 (IL-13) associated with marked increase in the serum interferon-gamma (INF-γ) level in (PAE+SEA) combined group compared to all groups.

Keywords: *Schistosoma mansoni*, Fibrosis, Cirrhosis, PZQ, Cytokines, IL-13, INF-γ, TGF-β1.

Introduction

Schistosomiasis is still a complicated long standing chronic disease with serious morbidity worldwide (Gouveia *et al,* 2019). Praziquantel (PZQ) monotherapy still has certain precautions in schistosomiasis treatment, not only due to lack of its effectiveness against immature stages but also, cannot fully counteract the infection associated morbidity and its sequelae including periportal fibrosis, esophageal varices. Several studies depend on the combinations of other chemotherapies with PZQ to overcome limitations of PZQ monotherapy and for discovery of recent alternatives were already done (Abd El-Aal *et al*, 2017a; Hegazy *et al,* 2018; Gouveia *et al,* 2018). Abd El-Aal *et al*. (2017b) reported that combination of soluble egg antigen (SEA) and PZQ had a hopeful prophylactic anti-schistosomiasis *mansoni* especially during re-infection in endemic areas.

Paeoniflorin (PAE) is a powerful anti-inflammatory and immune regulatory Chinase herbal therapy (Tu *et al,* 2019). Abd El-Aal *et al.* (2017c) reported that PAE could be a potential chemotherapy against *schistosoma mansoni* and exceeded PZQ in targeting apoptosis and improving hepatic fibrosis.

During treatment of infectious disease, the immune modulation might be accompanied with shifts in some cytokine profiles balance; therefore help in control morbidity (Powell and Sonnenfeld, 2006). The search for alternative control measures to decrease schistosomiasis morbidity is mandatory. Hence, there were in need for new policies, targeting not only the parasite but also the long standing pathogenesis and chronicity.

This study is a novel trial to assess the immunomodulatory effect of PAE in combination to SEA on schistosomiasis *mansoni* murine model through; parasitological, histopathological, immunohistochemical and serological studies aiming to achieve better bioavailability and higher anti-inflammatory immune regulatory effects to counteract the schistosomiasis morbidity.

Materials and Methods

The current experimental study was performed from July 2018 to March 2019.

Experimental mice: Forty Swiss Albino male laboratory bred CD-1 mice 6-8 weeks (ws) old (18-20g) were obtained from Schistosome Biological Supply Program (SBSP), TBRI. The mice were preserved on a standard commercial pelleted diet, available water and housing in an air conditioned room (22-26°C) till the study end. They were infected subcutaneously with Egyptian strain of *S. mansoni* ±100 cercariae (Peters and Warren, 1969) that was purchased from TBRI infected *Biomphalaria alexandrina* snails.

Chemotherapy and vaccine: Paeoniflorin (PAE) (purity >98%, P0038#, Sigma-Aldrich, St. Louis, USA) was dissolved in 1% carboxymethyl cellulose and given orally (50 mg/kg/ day) 6 ws post infection (pi) for one month (Abd El-Aal *et al*, 2017c).

Soluble egg antigen (SEA) was prepared at TBRI. SEA total protein content was determined according to Bradford (1976). Vaccination schedule was achieved (Nabih and Soliman, 1986). 200µl SEA initial dose was given to mice with total antigen concentration contained 30µg proteins, 200µl boos-ter dose containing 20µg proteins was given two weeks after; thus, each mouse received 50µg as a total antigen dose of protein (Etewa *et al*, 2014).

Experimental design and treatment protocol: Forty Swiss albino male laboratory bred CD-1 mice were divided into five groups (8 mice each): Control healthy (CH) non infected, non-treated, non-vaccinated mice; Control infected (CI): infected non treated, non-vaccinated mice; PAE treated: infected mice then treated with PAE; SEA vaccinated: vaccinated mice with SEA (50ug) then infected; PAE+SEA combined: vaccinated, infected then treated.

Treatment schedule was started 6ws/pi for a month, 1% carboxymethyl cellulose transporter orally for both CH and CI groups for one month. SEA was given initial dose then booster dose of SEA was given two weeks after. All mice groups were sacrificed by rapid decapitation under anesthesia 10ws/pi.

The results were evaluated as follows:

Parasitological study: A- Egg count per gram stool was done for each mouse by Kato thick smear method (Katz *et al*, 1972) started 6ws/pi to ensure the infection and as a follow up. B- Worm burden were recovered by liver and intestinal perfusion (Duvall and Dewitt, 1967). Reduction percentage of worm burden (R%) was calculated (Tendler *et al*, 1986).

Histopathological study: The liver tissues were removed from all sacrificed mice, fixed in 10% formalin solution immediately then paraffin wax. Sections were stained with Hematoxylin and Eosin (H&E) and with Masson's Trichrome stain to detect the histopathological changes and measure the mean granuloma diameter (µm). The percent reduction in granuloma diameter was calculated (Bancroft and Stevens, 1982).

Immunohistochemical study: 5µm thick paraffin embedded hepatic sections from all studied groups were stained using Avidin-Biotin Peroxidase technique for TGF-β1 staining [antibody (2Ar2) ab64715]; Abcam, Cambridge, MA, USA. For scoring, 10 random fields of intralobular and periportal areas were evaluated under microscope at 40 X magnification. The integral light density was determined by multimedia color pathographic (Chen *et al*, 2008).

Serological study: Blood samples from all studied mice groups were collected then centrifuged at 3000rpm for 15min, sera were separated and stored at -80°C for assessment of serum levels of INF-γ (ng/ml) and IL-13 (pg/ml) using an enzyme linked immunosorbent assays (ELISA) kit (RAB0477 & RAB0257 Sigma-Aldrich, St. Louis, USA, respectively) according to the manufacturer's instructions.

Ethical consideration: This experimental study was performed according to International guidelines approved by Research Ethics Committee, Faculty of Medicine, Zagazig University.

Statistical analysis: Data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Sci-

ence) version 18.0. Quantitative data were expressed as M±SD (Standard deviation). Groups were compared using ANOVA test or Student's t-test. P value <0.05 indicates statistical significant, P value <0.001 indicates statistical high significant.

Results

There was a statistical significant reduction in egg count/gm stool 96.7%, 84.2% 41.7% in PAE+SEA, PAE and SEA groups compared to control infected non treated group (CI). There was a statistical significant reduction in hepatic worm burden in PAE+SEA group (96.4%) that exceeded PAE (82.7%) and SEA (47.6%) groups compared to CI.

Regarding granuloma diameter, there was statistical significant reduction 69.5%, 30.1% 85.5% in PAE, SEA and PAE+SEA groups respectively compared to CI (Tab. 1). Hepatic sections stained with H&E showed granulomas in CI mice group were large hepatic fibrocellular granulomas with eosinophils, neutrophils, and lymphocytes and with central living ova. While, in the PAE & PAE+SEA groups fibrocellular granulomas formed of degenerated ova surrounded by giant cells, pigmented macrophages, lymphocytes, plasma cells and fibrous tissue (Fig. 1A).

Hepatic sections stained with Masson's Trichrome showed decrease in the severity of hepatic fibrosis in PAE and PAE+SEA groups compared to SEA and CI groups (Fig. 1B).

Regarding TGF-β1 expression, there was significant reduction 66.7%, 26.6 & 89.5% in PAE (0.314±0.03), SEA (0.691±0.02) & PAE+SEA (0.099±0.04) groups compared to CI group (0.942±0.08) (Fig. 1C)

Serologically serum INF-γ level, there were 32.4, 270.3, 103.8 & 483.9 folds increase in CI (326.8±11.3), PAE treated (564.7±10.9), (398.2±13.8), SEA vaccinated (398.2±13.8) & PAE+SEA combined (778.3±14.2) groups respectively compared to CH group (294.4±12.7). On the other hand, regarding serum IL-13 level there was statistical significant increase 603.7 & 562.5 folds in CI & SEA groups respectively, while, there was statistically insignificant increase 59.9 & 36.6 folds in PAE & PAE+SEA groups respectively compared to CH group (Tab. 2).

Table 1: Parasitological results in all groups

Parameters / Groups	Egg count/gm stool		Worm burden		Hepatic granuloma diameter (µm)	
	M±SD	%R	M±SD	%R	M±SD	%R
CI	714.3±10.6	-	33.6±1.8	-	342.7±0.96	-
PAE	114.8±5.4*	84.2%	5.8±0.64*	82.7%	104.6±0.87*	69.5%
SEA	416.3±6.7	41.7%	17.6±0.82	47.6%	239.4±0.099	30.1%
PAE+SEA	23.4±4.8**#	96.7%	1.2±0.71**	96.4%	49.8±0.52**#	85.5%

ANOVA test #(p<0.001) & Student t-test, *(P <0.05), ** (p<0.001) compared with CI; CI: control infected; PAE: Paeoniflorin; SEA: Soluble egg antigen.

Table 2: Serum INF-γ (ng/ml) and IL-13 (pg/mL) in all studied groups

Variable	INF-γ (ng/ml)	IL-13 (pg/ml)
CH	294.4±12.7	29.7±15.3
CI	326.8±11.3	633.4±12.8**#
PAE	564.7±10.9*	89.6±13.2*
SEA	398.2±13.8	592.2±14.7**#
PAE+SEA	778.3±14.2**#	66.3±10.6

ANOVA test #(p<0.001) & Student t-test, *(P <0.05), ** (p<0.001) compared with compared with CH; CH: control healthy.

Discussion

The main complain in *S. mansoni* endemic areas is the recurrent infection with long standing chronicity, new alternatives should be discovered to control schistosomiasis morbidity and prevent re-infection. In the current study, a novel therapeutic strategy aiming to achieve synergistic and/or additive effect that not only eliminate the parasite but also ameliorate morbidity associated to infection. Murine model was more suitable for this study as they have a similar growth

as human schistosomiasis with high homologization (Abdul-Ghani and Hassan, 2010).

In the current study, the treatment protocol 6/ws/pi by administration of PAE orally (50 mg/kg/ day) for one month (Ji *et al,* 2016) as previously verified with respectable results (Abd El-Aal *et al,* 2017c). Also, PAE was used in combination with SEA. SEA was previously proved with potential results and vaccination schedule started one week before infection and booster dose 2/ws/pi (Etewa *et al,* 2014).

In this study, Kato-Katz method was used 6/ws/pi to confirm mice infection and follow up the study. Kato-Katz method is the diagnostic keystone, simple inexpensive technique, commonly used in endemic areas of *S. mansoni* (Teixeira *et al,* 2007).

Regarding the anti-parasitic efficiency of PAE and SEA, there was significant reduction in egg count/ g stool in PEA treated group that exceeded SEA group compared to CI group, whereas PAE+SEA combined group had the highest effect in this aspect. This result highlights the expectation of worm burden in different studied groups.

Regarding PAE+SEA group, there was mar-ked reduction in worm burden which exceeded PAE group compared to CI group. However, reduction of worm burden in SEA group was the lowest. This is nearby to El-Ahwany *et al.* (2012) who detected marked decrease in worm burden following to administration of SEA previous *S. mansoni* infection. This anti-parasitic efficacy of the PAE+SEA combined group may be attributed to hepatic shift and death of adult worms by PAE and potentiation of its action by SEA through obstructing the oviposition process via increasing the dead eggs. Furthermore, SEA contains a variety of antigens that cross react with cercariae antigens, schistosomules and adult worm antigens. Taken together, Woolhouse (1994) and Curwen *et al.* (2004) detected the same suggestion. Moreover, Abdel-Ghaffar *et al.* (2005) and Abd El-Aal *et al.* (2017b) reported that combination of SEA and PZQ was recommended as it provided various complementary consequences; reduction in egg induced pathology, minimal parenchymal changes and eradication of worms. Although, Li *et al.* (2009) recorded that PAE has no anti-parasitic effect on worm burden in mice infected with *S. japonicum*. On the other hand, Abd El-Aal *et al.* (2017c) attributed PAE anti-parasitic efficacy to the differences in schistosome species, dosage and timing of PAE treatment that could disturb the adult worm tegument.

The granuloma formation and fibrosis is the cornerstone of morbidity and mortality in schistosomiasis *mansoni*. The present results recorded significant reduction in granuloma diameter and fibrotic areas in PAE+ SEA group which was superior to PAE and SEA groups compared to CI group; this is attributed to the combination of PAE+SEA achieve synergistic or additive effect for local inhibition of the inflammatory mediators released at the granulomatous reaction or decrease in fibrotic markers expressions via improvement of immune response and cytokines modulation. This agreed with Abd El-Aal *et al.* (2017c). Moreover, Abd El-Aal and Abdelbary (2019) detected that PAE is predicted to be a potential therapy for chronic hepatic diseases associated with fibrosis and angiogenesis, hopeful in protecting from advanced long standing serious complications. Coming with, Chen *et al.* (2015) who reported that PAE reduced the size of *S. japonicum* egg granulomas and fibrosis scores.

Cytokines are important regulators of immune-inflammatory responses and play effective role in regulation of granuloma formation and fibrosis. Hoffmann *et al.* (2002) reported the alteration in T helper cytokine profiles during the formation of granulomatous reaction. Transforming growth factor-beta1 (TGF-β1) is a fibrosis potentiation Th2 cytokine, also, a good indicator for hepatic fibrosis and evaluation, via activation and differentiation of Hepatic stellate cells (HSCs) that stimulates fibroblast proliferation (Lan and Chung, 2012). Besides,

Liu *et al.* (2012) reported that interleukin-13 (IL-13) endorses TGF-β1 receptors activation.

The current results showed marked decrease in the fibrotic marker expression; TGF-β1 in PAE+SEA combined group that was superior to PAE and SEA groups compared to CI group. The results are coming along with Ji *et al.* (2016) who reported that PAE produce suppression of TGF-β1 resulted in diminution of pulmonary fibrosis. Besides, Abd El-Aal *et al,* (2017c) recorded that PAE monotherapy has anti-fibrotic effect which exceed PZQ through down-regulation of TGF-β1 fibrotic marker.

Caldas (2008) detected that in early infection phase, expression of Th1 cytokines was predominant and Th2 cytokines appearance started with chronicity. IFN-γ is Th1 cytokine, detected early at the starting of hepatic granulomatous reaction, by the time granulomas size becomes maximal and cytokines production declined (de Jesus *et al,* 2004). IL-13 is Th2 cytokine, starts to appear 6-8 ws/pi and responsible for fibrosis and chronicity (Pearce and MacDonald, 2002).

The present results detected marked increase in serum level of INF-γ in PAE+SEA group that exceeded PAE and SEA groups compared to CI group. On the other hand, there was marked decrease in serum IL-13 level in PAE+SEA group that exceeded PAE and SEA groups compared to CI group. This agreed with Stadecker *et al.* (2004) who detected that mice infected with a single large dose of cercariae and then treated with PZQ established Th1 dominant response with elevated SEA induced IFN-γ.

On the other hand, infection with repeated small doses of cercariae developed Th2 IL-5 response (Farah *et al,* 2000). This may highlight our attention for the important role of SEA administration in endemic areas as a prophylaxis against recurrent infection.

Interleukin-13 (IL-13) is the important Th2 cytokine responsible for fibrosis (Fallon *et al,* 2000). Some Th1 mediators such as interferon-gamma (INF-γ), IL-12, tumor necrosis factor (TNF-α) prevent production of excess IL-13 during *S. mansoni* infection (Miranda *et al,* 2013).

Taken together, Wan *et al.* (2017) detected that egg induced hepatic granuloma and fibrosis regression were due to decreased IL-13 expressions. Similar to results of the previous studies, Chu *et al.* (2007) reported that IL-13 activated macrophages to secrete TGF-β1 that initiated granuloma formation, HSC proliferation, collagen production and fibrosis. Also, Abd EL-Aal *et al.* (2017c) detected a close positive correlation between serum IL-13 and TGF-β1fibrotic marker expression. Also, Montesano *et al.* (1997) reported that IFN-γ has important role during schistosoiasis *mansoni*, prevent the release of *Schistosoma* eggs by adult worms and thus reduces hepatic fibrosis during schistosomiasis chronic phase. Moreover, IFN-γ has potential role linked to the balance of IL-13 mediated fibrosis (Hesse *et al,* 2001). Miranda *et al,* (2013) recorded the potent anti-parasitic activity of IFN-γ via release of nitric oxide.

From the present results, it can gain insight into the anti-helminthic, antifibrotic and protective properties of PAE combined to SEA that leads to death of viable eggs and prevents more deposition, which is considered to be the activating stimulus for several pathways that altered Th1/ Th2 cytokines expression. Liang *et al.* (2011) and Attia *et al.* (2013) reported that the anti-fibrotic effect of PAE therapy inhibited the gene expression of both Th1 and Th2, which alternated the Th1/Th2 cytokines; decreased TGF-β1 & IL-13 and increased INF-γ; finally led to hepatic fibrosis regression.

SEA as prophylaxis or adjuvant aggravates and potentiates the effect of PAE and is considered the key factor in improvement of immune response or could be a sort of primary infection that prevents the challenge one.

Conclusions

The results confirmed that combination of PAE and SEA showed marked improves in

parasitological, histopathological, immunological parameters and has immunomodulatory effects associated with shift in the balance of some cytokine profiles. Consequently, this approach might be considered for prophylaxis or for use in regions with intense reinfection levels since the combination might block or retard parasite infection and morbidity development.

Conflict of interest: The authors declared neither have conflict of interest nor received fund.

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Explanation of figures

Fig.1: Hepatic sections: A- H & E, x100; B- Masson's Trichrome x100 & C- TGF-β1immunohistochemistry x100. CI: control infected; PAE: Paeoniflorin; SEA: Soluble egg antigen.