Original Article

Immunological evaluation of the prophylactic impact of soluble egg antigen (SEA) combined with schistosomula lung antigen preparation (SLAP) on murine model of schistosomiasis *mansoni*

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ABSTRACT

Background: Vaccination against schistosomiasis might be an excellent approach for its control and elimination. Despite the recent advances in identification of several potential vaccine candidates, no vaccine was validated for clinical trials.
Objective: This research aims to evaluate the preventive effects of soluble egg antigen (SEA) in combination with schistosomula lung antigen preparation (SLAP) on a murine model of schistosomiasis *mansoni*.
Material and Methods: In this study, 35 Swiss albino male mice (aged 6 to 8 weeks) were divided into five groups. Each group contained seven mice, with G1 serving as the normal control, G2 the infected control, G3 the infected and immunized by SEA, G4 the infected and immunized by SLAP, and G5 the infected and immunized by combination antigens (SLAP and SEA). All mice were infected two weeks after receiving their final dose of immunization. Seven weeks following infection, mice were decapitated in order to determine the impact of the injected antigens using determination of liver enzymes, IL10, TNF-α and schistosomal IgG, with immunohistochemical assessment.
Results: Reduced liver enzyme levels were detected in various vaccinated groups, with the combined group (SLAP and SEA) experiencing the greatest reduction. The combination of SLAP and SEA resulted in the highest significant increase in IL-10 and IgG antibody and the highest significant reduction in TNF-α level among all groups.
Conclusion: The combination of SLAP and SEA increased the protective immunity. This combination represents an exciting direction in vaccine research.

Keywords: immunohistochemical; IgG; IL-10; schistosomiasis; TNF-α; vaccine candidate.

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INTRODUCTION

Schistosomiasis is a chronic illness connected with poverty. The infection is due to *Schistosoma* spp. flukes[1]. Ninety percent of the 190 million actively infected people worldwide live in Africa[2]. The urinary and gastrointestinal tracts are affected by the two primary types of African schistosomiasis namely S. *haematobium* and *S. mansoni*, respectively. The World Health Organization (WHO) set an ambitious target in the previous ten years to control schistosomiasis by 2020 and eradicate it as a public health burden by 2025[3].

Initially, it was believed that expanding the reach of mass drug administration (MDA) with praziquantel, the sole medication now used to treat schistosomiasis[4], would be sufficient to accomplish its eradication. Unfortunately, higher MDA coverage was effective in reducing infection intensity and resultant morbidity but did not stop re-infection. As a result, six to eight months after using praziquantel, prevalence was regained. Additionally, the risk of drug resistance apparently increased with total reliance on a single medication[5]. This renders vaccination a crucial strategy for the management and eventual eradication of schistosomiasis. A successful vaccination could help prevent infections and provide protection from re-infection[6]. Current accepted criteria for successful preventive vaccination include induction of 75% reduction in worm load and egg excretion in infected individuals[7].

Suggested approaches for schistosome vaccination can be divided into three categories: (1) a preventive vaccine designed to stop or lessen the spread of infection, (2) a vaccine designed to decrease reinfection or transmission by preventing adult female survival or egg production and (3) a curative vaccine designed to lower the risk of illness [8]. One of the reasons for the sluggish development of an efficient schistosome vaccine is thought to

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be the capacity of schistosome parasites to evade the host's protection. This may be attributed to the parasite's intricate structure and capacity to diversify both its genetic structure and its antigens across its several life stages. Hence, a powerful immune system that targets both humoral and cellular responses directed at various stages of the parasite's life cycle is necessary to defend the host from schistosomiasis[9].

The immune evasiveness of mature schistosomes and their induced host immune modulation provide scientific obstacles for designing a novel effective vaccine. Most notably, the multi-stage nature of schistosomes entails intricate antigenic changes that can be present at all life stages as well as being stage-specific. Schistosomiasis vaccine development is challenging due to the generation of IgE responses, threat of allergic reactions, and possibility of exacerbating granulomas and fibrosis via egg-mediated responses[6]. Additionally, there is a lack of knowledge regarding how prior treatments, repetitive exposure to infection, polyparasitism, and past schistosome infections expressly shape the immune system in individuals, both within and between various nations. These elements probably cause greater variations in vaccination reactions. Besides, an integrated strategy for Praziquantel administration and vaccinations should comply with the present MDA control programs[10].

Strong Th1 responses with elevated IFN-γ, TNF-α productions during the acute stages of *S. mansoni* were in charge of controlling the immune response. In this context, IL-4, IL-5, and IL-10 levels are high while IFN-γ levels is decreased during the chronic stage of the disease, indicating a predominating Th2 response. The SEA- driven synthesis of IL-10 guides the regulation[11]. Accordingly, IL-10 has potent suppressor effects on both Th l-cytokines and macrophage activation. It could play a major role in down regulation of granuloma formation as well as host cell-mediated responses to established schistosomiasis[12], being also a significant factor in the spontaneous regulation of granuloma formation seen in chronic schistosomiasis[13].

Mountford and Harrop[14] identified proteins from *S. mansoni* lung-stage schistosomulum (SLAP) that stimulate protective Th1 cell-mediated immune responses. Consequently, a purified SLAP preparation was found to induce marked decrease in numbers of worm burden, egg load, granuloma diameter and its collagen contents, in association with an increase in percentage of degenerated ova. This was accompanied by improvement of pathological changes in pulmonary and hepatic tissues[15]. On the other hand, *Schistosoma* miracidia inside the ovum secrete glycoprotein antigens that pass through microscopic pores within the eggshell, so are called SEA. These antigens elicit a vigorous immune response that encapsulates the ova in pre-granuloma collagen fibers and immune cells, predominantly eosinophils and macrophages. The granuloma formation presents a barrier to sequester egg toxicity and antigenicity. Fibrosis of granulation tissues leads to disturbance of hepatic parenchymal architecture including its vasculature[16]. Crude SEA was more heterogenous, being composed of twelve antigens that include multiple proteins, glycoproteins, and carbohydrates with glycolipids[17]. Regarding antigenic efficacy, SEA was reportedly superior to soluble worm antigen preparation (SWAP); Etewa *et al.*[18] emphasized this superiority. A crucial stage in the development and use of effective anti-schistosome vaccines is the selection of an adequate adjuvant to aid in the induction of the proper immune response[19]. The present study aims to evaluate the preventive effects of combined *S. mansoni* SEA and SLAP on experimentally infected mice.

MATERIAL AND METHODS

This case control study was conducted at the Theodor Bilharz Research Institute (TBRI), Giza, Egypt, from September to November of 2022.

Experimental design: Five groups were assigned as: normal control, infected control, infected and immunized by SEA, infected and immunized by SLAP, and infected and immunized by combined antigens (SLAP and SEA). For serological and immunohistochemical evaluation of the injected antigens, mice were sacrificed 7 weeks after infection.

Animals: We utilized 35 male Swiss albino mice, 20-25 g weight and 4-6 weeks old. They were bought from TBRI Animal House and housed in clean, breathable plastic cages with timber bedding. The TBRI biological unit was the location where animal experiments took place.

Mice infection and schistosomula extraction: *S. mansoni* infected laboratory-bred *B. alexandrina* snails were obtained from the Schistosome Biological Supply Unit of TBRI[20]. After exposure of snails to light for at least 4 hours, emerged *S. mansoni* cercariae were utilized to infect mice subcutaneously[21]. After an infection of 18 d, schistosomula were extracted from the lungs. To maximize the quantity of schistosomula retrieved from mouse lungs, the perfusion process was slightly modified where lung pieces were re-incubated for a second time in RPMI 1640 medium with 2% fetal calf serum and 3% penicillin-streptomycin as mentioned by Lewis and Colley[22].

Antigens and adjuvant preparation: The schistosomulae were collected from lungs of mice and the antigen (SLA) was prepared according to Mountford *et al.*[23]; followed by determination of protein content using bicinchoninic acid according to Smith *et al.*[24]. Purified egg collection was used to prepare (SEA)

antigen according to Boros and Warren[25] and the protein content of the produced (SEA) was assessed according to Bradford[26]. Complete Freund's adjuvant (CFA) purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) was emulsified in phosphate-buffered saline (2:1 ratio).

Vaccination protocol: Two subcutaneous injections of 50 g SLAP[27] and 100 g SEA[28] with 15 d interval, were used to sensitize each mouse. All mice were infected with 80±10 *S. mansoni* cercariae subcutaneously two weeks following their last immunization dose.

Evaluation of the vaccine effect

Serological assessment: Blood samples (about 1 ml) were withdrawn from the neck veins of mice after scarification, centrifuged at 3,000 x g for 10 minutes. Serum was divided into two parts, one was used to measure liver enzymes and the other was stored at -20°C for determination of serum TNF- α and IL-10 levels. Both factors were determined using Quantikine®, Abcam®. Utilizing monoclonal antibodies, ELISA technique was used to determine serum TNF- α and IL-10 at 450 nm optical density (OD)[29]. Concentrations were determined by available standard curves in the manufacturer's instructions. *Schistosoma* IgG was also measured by ELISA method with some modifications, i.e., decreased incubation period of both the serum and conjugate (one hour instead of two hours for the serum, and half hour instead of one hour for conjugate), and using 3 ng/ml antigen instead of 1 ng/ml.

Immunohistochemical (IHC) assessment: Immunohistochemical staining was carried out using the EnVision (USA) method[30]. Deparaffinization and rehydration were performed on 5 µm thick paraffin sections. The sections were microwaved in citrate buffer (pH 6.0) to retrieve the antigen. Methanol containing 3% hydrogen peroxide (Sigma-Aldrich, Germany) was used to suppress endogenous peroxidase. Primary antibodies against TNF (Abcam, Cambridge, UK) were applied to cover the sections, and incubated overnight at 4°C in a humid environment with Envision detecting system application (DAKO). Substrate chromogen solution (3,3'-diaminobenzidine tetrahydrochloride, DAB), purchased from Universal Detection Kit (Dako Envision, Denmark) was added to localize the antigen. Sections were dehydrated, counterstained with hematoxylin, and mounted in the synthetic mounting medium (AEC, 3-Amino-9-ethylcarbazole, 3AC, Sigma-Aldrich, Germany). Slides for both the positive and negative controls were primed. As a negative control, liver tissue was treated using the aforementioned steps, but non-immune immunoglobulin G (DAKO, Glostrup, Copenhagen, Denmark) was used in place of the primary antibodies. Brown tinging of the cells' cytoplasm was taken as a sign of TNF-α positivity. The percentage of positive cells was determined using X5 HPF, and the color intensity was scaled from 1+ to 3+.

Statistical analysis: Version 26 of SPSS application was employed. Mean and standard deviation were used to assess quantitative data, whereas frequency and percentage were used to analyze qualitative data. Analysis of variance (ANOVA) with post hoc test (LSD) were used to compare means. Chi square test and Fischer exact test were employed to compare frequencies. Significance was considered if *P* value was less than 0.05.

Ethical considerations: The study was authorized by the ethical committee of the Faculty of Medicine, Benha University, Egypt. The study procedure complied with the Institutional Animal Care and Use Committee's guidelines.

RESULTS

Serological results: Decreased levels of liver enzymes (SGPT and SGOT) were recorded in the three vaccinated groups with the significant highest reduction in the combined group (SLAP and SEA) (P<0.001) (Table 1).

Table (2) presents results of TNF-α and IL-10 levels, all vaccinated groups showed decreased TNF-α level with increased IL-10 level. The combination of SLAP and SEA gave the best result (the highest reduction in TNF-α level and highest increase in IL-10 with significant difference (P<0.001). Additionally, IgG antibody increased significantly in all vaccinated groups (P<0.001) with the highest level achieved in the combined SLAP/SEA group (2.20) (P<0.001).

Immunohistochemical results: Normal control mice (G1) showing no granulomas, mild sinusoidal expression of TNF-α and focal hepatocytic positivity (Fig.1 A and B). Compared to sections of liver tissues of normal non infected mice, similar sections from infected control mice (G2) showed increased

Table 1. Levels of liver enzymes in different studied groups.

Liver enzymes	Positive control	SEA	SLAP	SEA + SLAP
SGPT				
Mean ± SD	140.0±6.32	120.4±6.95	123.4 ± 30.09	95.4 ± 4.72
***P* value**		**0.002***	0.262	**<0.001***
SGOT				
Mean ± SD	139.6 ± 14.12	109.8 ± 14.03	101.2 ± 5.12	88.0 ± 4.06
***P* value**		**0.01***	**<0.001***	**<0.001***

***:** Significant: (P<0.05).

Table 2. TNF-α, IL10, and IgG antibody serum levels in the various study groups.

	Positive control	SEA	SLAP	SEA + SLAP
TNF-α				
Mean ± SD	846.6 ± 60.12	575.4 ± 21.0	575.4 ± 8.32	367.4 ± 25.52
***P* value**		**<0.001***	**<0.001***	**<0.001***
IL-10				
Mean ± SD	131.8 ± 2.39	133.8 ± 8.17	157.8 ± 3.56	302.4 ± 47.5
***P* value**		0.613	**<0.001***	**<0.001***
IgG				
Mean ± SD	0.558 ± 0.037	0.801 ± 0.007	0.879 ± 0.036	2.20 ± 0.344
***P* value**		**<0.001***	**<0.001***	**<0.001***

***:** Significant: ($P<0.05$).

expression of TNF-α in mononuclear inflammatory cells within the portal tract containing worms and within eggs granulomas (Fig. 1C and D, respectively). The SEA vaccinated groups showed decreased (Fig. 1E) and mild (Fig. 1F) expression of TNF-α in mononuclear inflammatory cells at the periphery of the eggs' granulomas. The SLAP vaccinated groups showed milder TNF-α positive expression in mononuclear inflammatory cells at the periphery of the fibro cellular eggs' granulomas (Fig. 1G) and to a lesser extent within the portal tracts (Fig. 1H). Meanwhile, vaccinated group with the combination SEA/SLAP cocktail (Fig. 1I, and J) showed the best results represented by infrequent positive expression of TNF-α in mononuclear inflammatory cells at the periphery of the fibro cellular egg granulomas besides, mild positive expression in portal tract.

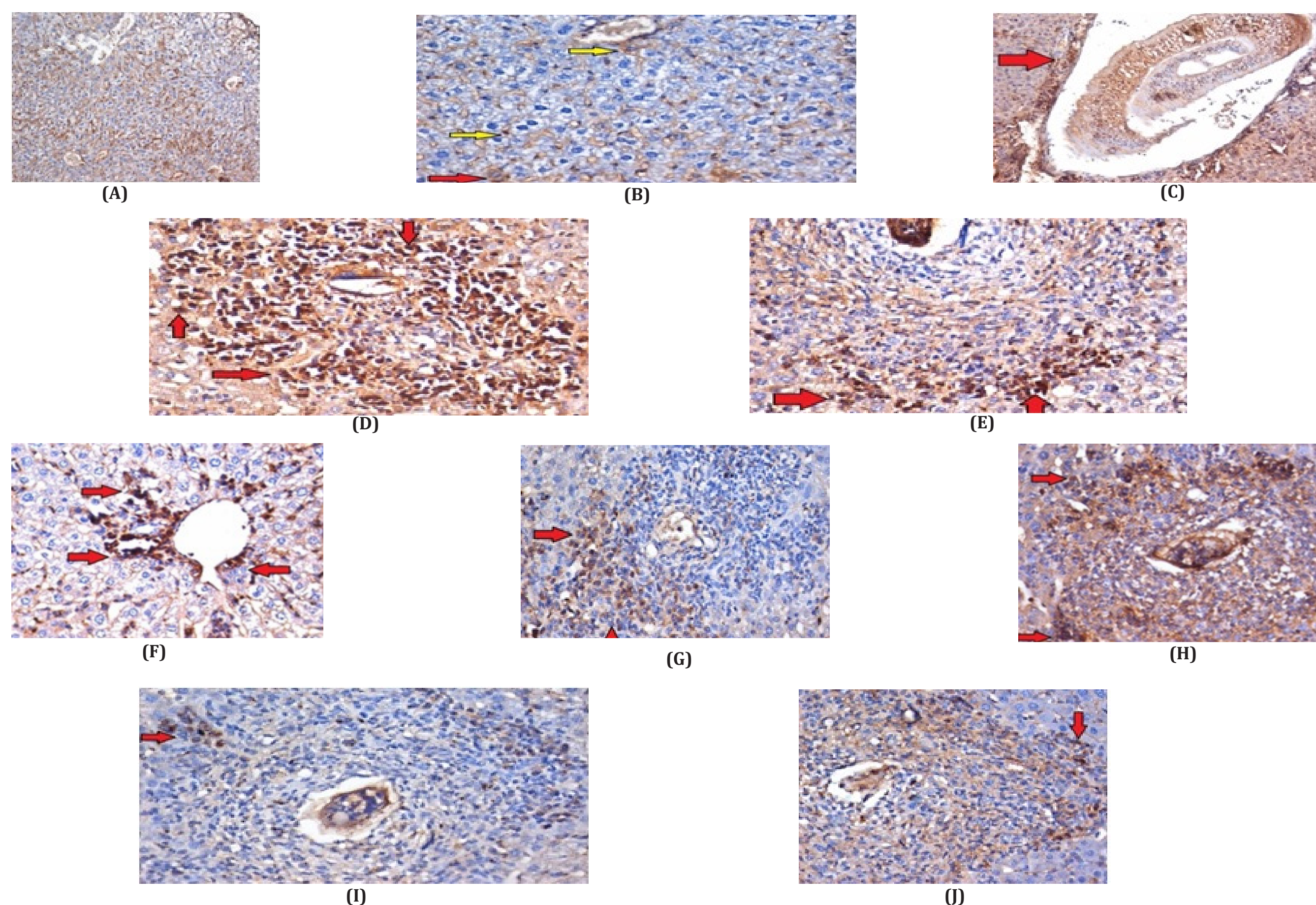

Fig. 1. Immunohistochemmical results of liver sections. **(A, B):** Negative control mice (G1) showing no granulomas, mild sinusoidal expression of TNF-α (yellow arrow) and focal hepatocytic positivity (red arrow) (IHC for TNF-α, DAB) X400. **(C, D):** Infected control mice (G2) showing increased expression of TNF-A in mononuclear inflammatory cells within the portal tract containing a worm **(C)** and within an egg granuloma **(D)** (IHC for TNF-α, DAB) X400. **(E, F):** Infected SEA immunized mice (G3) showing positive expression of TNF-α in mononuclear inflammatory cells at the periphery of the eggs granulomas **(E)** and to a lesser extent in portal tracts **(F)** (IHC for TNF-α , DAB) X400. **(G, H):** Infected SLAP immunized mice (G4) showing milder positive expression of TNF-α in mononuclear inflammatory cells at the periphery of the fibrocellular eggs granulomas **(G)** and to a lesser extent within the portal tracts **(H)** (IHC for TNF-α, DAB) X400. **(I, J):** Infected SEA/SLAP immunized mice (G5) showing infrequent positive expression of TNF-α in mononuclear inflammatory cells at the periphery of the fibrocellular eggs granulomas **(I)** and mild positive expression in portal tract **(J)** (IHC for TNF-α, DAB) X400. DAB: 3,3'diaminobenzidine tetrahydrochloride substrate chromogen solution.

DISCUSSION

Several experimental studies were conducted to identify vaccine candidate and develop a human vaccine. Unfortunately, due to the immunological evasive nature of adult schistosomes and schistosome-induced host immune-modulation, the creation of a new schistosome vaccine confronts significant scientific obstacles[10]. The creation of a novel combination vaccination is essential to boost protection since the outcome of protection evoked by a single antigen was insufficient[17]. In this research, we attempted to assess the prophylactic impact of SEA combined with SLAP on murine model of schistosomiasis as a new vaccine trial.

In our study, the liver enzymes were significantly decreased in different vaccinated groups with superiority for SLAP and SEA group. It is possible that the reactive oxygen species formation in the liver and antioxidant defenses changes that disturb hepatocyte function are the causes of the elevated liver enzymes in the infected control group compared to the normal group[31]. Administration of the combined antigens resulted in almost normal levels of enzymes that can be attributed to the development of the oxidant/anti-oxidant balance, indicating an improvement in liver function and providing further support to the vaccine's mechanism of action[32]. This agreed with Abdel-Hamed *et al*.[33] who reported that *B. alexandrina* antigen preparation (BAAP) combined with SLAP and BCG antigens for experimental vaccination produced the best decreased levels of liver enzymes.

In contrast to a Th2-type response, which offers resistance to helminth infections, a Th1-type immune response is critical for inducing immunological protection. Therefore, to avoid severe disease, a balance between Th1 and Th2 responses may be required[34]. The combination group was found to be the most fortunate vaccine in TNF-α level reduction. Our results were consistent with the findings of Hamed *et al.*[33], who demonstrated that all vaccination groups showed decrease in TNF-α level, but the combination of (BAAP + SLAP) resulted in the greatest decrease. El-Gawish *et al*.[35] showed that serum TNF-α in the infected group was significantly decreased reaching 33.05% as compared with control. Immunization with irradiated antigen also induced significant decrease in serum TNF-α, whereas, the lowest decrease was recorded in mice immunized with non-irradiated antigen. Additionally, Haseeb *et al*.[36] observed that inflammatory cytokines raised following egg excretion helps to explain their role in schistosomiasis complications, by increasing the formation of reactive oxygen species, which in turn causes tissue damage and fibrosis[36].

In the present study, SLAP+SEA group had the superiority regarding increased IL-10 level. This coincides with Hamed *et al*.[33] who stated that IL-10 was increased in all vaccinated groups, but the highest significant difference among all groups was obtained in infected group vaccinated with combined antigens (BAAP + SLAP) and supported by adjuvant. This was also endorsed by El-Gawish *et al*.[35] and Pearson *et al*.[37]. By inhibiting the emergence of Th1 & Th2-mediated diseases, Hoffmann *et al*.[38] demonstrated that IL-10 plays a critical regulatory function in immune responses during infection. The main element in preventing a rise in disease morbidity is this cytokine[39]. Additionally, type 2-associated cytokines such as IL-4, IL-13, and IL-10 are implicated in development of granulomas and fibrogenesis around tissue-deposited eggs and block classical macrophage activation[40].

In schistosomiasis, the predominant immunoglobulin elicited by an immunological response is IgG[41]. The IgG level was found to be much higher in the SLAP and SEA group compared to the infected control group (P<0.001). In a previous report, Mossallam *et al*.[42] agreed with our result, as in their study the combination of alum and *Schistosoma*'s extracellular vesicles (EVs) led to considerably greater antibody levels at all various research time points. In comparison to subgroups of infected controls, IgG levels were considerably raised by immunization with or without adjuvant. Additionally, Perera *et al*.[43] recently confirmed that SmCB expressing human adenovirus serotype 5 (AdSmCB) administration resulted in noticeably greater humoral and cell-mediated immune responses, which included IgG2c, polyfunctional $CD4^+$ T cells and Th1 effectors. An important degree of protection against *S. mansoni* infection was achieved by this combined Th1/Th2 immune response.

In our immunohistochemistry study, the combination group gave the best result concerning the decreased expression of TNF-α that was obvious in both egg granulomas and portal tract. This agreed with Hamed *et al*.[33] who reported that the group vaccinated with combined antigens and supported by adjuvant showed low TNF-α cytoplasmic expression. In conclusion, immunization with combined SLAP and SEA antigens vaccine have a protective potential to schistosomiasis better than administration of each antigen alone.

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