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IMMUNOGENICITY ASSESSMENT FOR LUNG-STAGE AND *BIOMPHALARIA ALEXANDRINA* COCKTAIL VACCINE IN *SCHISTOSOMA MANSONI* INFECTED MICE

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Abstract

The availability of a new vaccine is usually needed as an additional component to chemotherapy for control of schistosomiasis. Different strategies of different types of vaccines were assessed to decrease morbidity but did not give the best protection. The study assessed the efficacy of BAAP, SLAP and their combined preparations together with BCG adjuvant as an effective anti-schistosomal vaccine. Methodology: Six groups of Swiss albino mice were used; (G1) as a control, (G2) infected non immunized; (G3) infected and supported by Adj.; (G4) infected; vaccinated with BAAP and supported by Adj.; (G5) infected, vaccinated with SLAP and supported by Adj. and the target group (G6) infected, vaccinated with combined antigens (BAAP + SLAP) and supported by Adjuvant. Mice were sacrificed 8 weeks post infection for assessment the effect of our vaccine through parasitological, histopathological, serological and immunohistochemical study. The vaccination of mice with BAAP, SLAP and Adjuvant followed by challenge *S. mansoni* infection resulted in highest reduction percentages (92% & 86%) for mean numbers of adult burdens and fecal egg counts respectively,(82.4%, 81%) for granuloma number and diameter respectively compared with other groups. The improvement % of all measured enzymes was higher in G6 than other groups.IL10 was significantly increased in G6 than other groups; also, TNF was significantly decreased in G6 than other groups.

Keywords: Egypt, Sharkia Governorate, *Schistosoma mansoni*, Lung schistosomulum; *Biomphalaria alexandrina*, Vaccine, Immunohistochemistry, TNF-α, IL-10.

Introduction

Current estimation of human schistosomiasis suggested that 252 million people are infected with schistosome, with approximately two-thirds of the cases caused by *S. haematobium* and the rest of cases caused by *S. mansoni, S. japonicum* and *S. mekongi* (Hotez *et al,* 2014). With respect to the low mortality rate, it causes a chronic illness that can damage internal organs, and impair children growth and cognitive development; it may also have an impact on the etiology and transmission of HIV/AIDS, TB and malaria (Robin, 2015). Despite efforts to control this disease, based on treatment of infected people with praziquantel (PZQ) and elimination of snails, the incidence level has shown no significant decrease with continuous spread to new geographic areas particularly in the sub-Saharan Africa (Mahfouz *et al,* 2011).

Schistosomiasis is considered the strong immunological disease, which depend mainly on CD4+ T cells and accumulation of inflammatory cells at the egg deposition site during granuloma formation and also, antigen preparation from adult worms (SWAP) induce different cytokine responses (Dejani *et al,* 2014). The immunologic response of *S. mansoni* infection is controlled by two phases: the preliminary Th1 (IFN-γ) response which then switches to Th2 response (IL-5, IL-10 & IL-13) (Pearce *et al,* 1991; Wynn *et al,* 1995). Pro inflammatory cytokines (TNF-α & IL-6) were observed within the inflamed tissue (Street *et al,* 1999; Jenkins *et al,* 2005). The immune response during the acute stage of *S. mansoni* was controlled by a strong Th1 response with increased production of TNF-α and IFN-γ. In chronic stage of the disease Th2 response predominates with elevated levels of inter-

leukin IL-4, IL-5, & IL-10 and depressed levels of IFN-γ. The modulation was directed by *Schistosoma* eggs antigen driven IL-10 production (Alebie, 2014).

The possible role of TNF could be through recruitment of inflammatory cells, regulation of fibroblast growth, collagen synthesis and granulocyte activation (Sato *et al*, 1991). TNF appears to mediate protection from excessive hepatocellular damage and cachexia, but is not the cause of hepatocyte apoptosis (Davies *et al,* 2005). 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 *Schistosoma* infection (Wilson *et al,* 2011), also being a major cause of the spontaneous modulation of granuloma formation that can be observed in chronic schistosomiasis (Bourke *et al,* 2013).

Although the anti-schistosomal drugs are specific, there was no protection against reinvasion or even the formation of granuloma. Chemotherapeutic agents are ineffective in transmission control. Thus, development of vaccine is considered as an important issue for the future eradication of this disease (Rezende *et al,* 2011).

Vaccine strategies against schistosomes can be classified to three types: (1) prophylactic vaccine aimed to prevent or reduce infection and transmission. (2) Vaccine aimed to reduce reinfection or transmission by interrupting female worm survival or egg production. (3) A therapeutic vaccine to reduce disease but not affecting infection or transmission (Mo *et al,* 2014).

The ability of schistosome parasites for evasion the immunity of the host is considered one of the reasons for the slow progress in developing an effective schistosome vaccine. This may be due to the complex structure of the parasite and its ability to diverse its genetics and to variate its antigens during the multiple stages of life cycle. So, to protect host from schistosomiasis, a strong immunity is required involving both humoral and cellular responses that directed toward different stages of the parasites life cycle are essential (Mata *et al,* 2013).

In 1990, special program for Research and Training in Tropical Diseases (TDR/WHO), under coordination of UNDP/World Bank/ WHO evaluated six vaccine antigens from *S. mansoni*; Glutathione-*S*-transferase 28 (Sm 28-GST), paramyosin, Ir-V5, Triose phosphate isomerase (TPI), Sm 23 and Sm 14, although they yielded protective responses, but did not reach to the standard goal of the 40% protection (Wright *et al,* 1991; Bergquist *et al,* 2005). So, the development of an effective vaccine against human schistosomiasis remains a highly desirable goal.

Along the past two decades, several investigators have demonstrated the antigenic compatibility between *S. mansoni* and *Biomphalaria* (Weston *et al,* 1994). This finding attracted many scientists to think about antigen preparation from its intermediate host; that include protein, nucleoprotein, lipid and carbohydrate (Tolba et *al,* 1995). Hamed *et al.* (2010) found that nucleoproteins extracted from susceptible *B. alexandrina* were more effective in protection from schistosoma by significant reduction in worm burden and ova count. Mountford and Harrop (2003) identified proteins from *S. mansoni* lung-stage schistosomulum (SLAP) can stimulate protective Th1 cell-mediated immune responses. This purified SLAP induces marked decreased in number of worm burden, egg load, granuloma diameter and its collagen contents in association with an increase in percentage of degenerated ova in addition to improvement of pathological changes in pulmonary and hepatic tissue (El-Ahawany *et al,* 2006).

This study was carried out to evaluate the effect of combining shistosomula lung antigen preparations (SLAP) of *S. mansoni* and *B. alexandrina* antigen preparations (BAAP)

as an anti-schistosomal vaccine in murine models.

Materials and Methods

Type of study: Non randomized control-trial. This study was performed from November 2013 to May 2014.

Experimental design: Sixty Swiss albino male mice (6 to 8 weeks old) and *S. mansoni* cercariae of Egyptian strain which obtained from the Schistosome Biological Supply Program, Theodor Bilharz Research Institute (SBSP, TBRI, Giza, Egypt) were used in the present study. Mice were divided in ten groups. G1 (G1) as normal control, (G2) infected control, while (G3) infected and supported by Adj., (G4) infected and vaccinated with BAAP supported by Adj., (G5) infected and vaccinated with SLAP supported by Adj., (G6) infected and vaccinated with combined antigens (BAAP + SLAP) supported by Adj. Mice were supplied by standard diet and free accessibility to water and heat in standard cages all over the study.

Ethical aspects: All procedures related to animal experimentation in the present study met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences and approved by ethics committee of the Faculty of Medicine, Zagazig University.

Antigenic preparation from *S. mansoni* lung schistosomulum worms: Collection of *S. mansoni* eggs was done (Dresden and Payne, 1981). Each *B. alexandrina* snail was infected singly with 8-10 miracidia then left for 28 days at 25-27°C for complete maturation of *S. mansoni* cercariae which were collected (Liang *et al,* 1987) and their numbers were calculated (Webbe and James, 1971). Thirty Swiss albino mice were infected each with about 100 cercariae subcutaneously guided by Liang *et al.* (1987). Schistosomula were recovered from the lungs by a slight modification of the perfusion technique reported by (Imohiosen *et al,* 1978). Antigenic preparation of schistosomulum was done (Call *et al*, 1995) and determination of protein content (Smith *et al,* 1985).

Antigenic preparation from *S. mansoni* infected *Biomphalaria alexandrina* snails: *Schistosoma* Biological Supply Center (SBSC), Theodor Bilharz Research Institute, Cairo, Egypt was the source of laboratory bred infected *B. alexandrina* snails which infected in the laboratory (Becker and Lamprecht, 1977). Preparation of antigen was done (Nabih, 1981).

Adjuvants: Bacille Calmette-Guérin (BCG) was received from the Veterinary Serum and Vaccine Institute, Bacteria Diagnostic Research Department, Abbassia, emulsified in the PBS at 2:1 ratio (v/v).

Antigen administration protocol and assessment: Each mouse was sensitized with a single subcutaneous injection of 50µg protein from the selected antigen (Maghraby *et al,* 2007). After 15 days, a second inoculation with the same antigen concentration was performed; thus, each mouse received a total dose of 100µg protein. The antigen was combined with BCG100ul intradermally each time for each mouse (Boulanger *et al,* 1999). Two weeks after the last dose of vaccine, mice were infected by80±10 the Egyptian strain of *S. mansoni* cercariae using tail immersion method. Eight weeks post infection, animals were sacrificed by cervical dislocation. The parasitological, histopathological, serological and immunohistohemical studies were performed.

Worm count: Worms were recovered by perfusion of liver, intestine and portomesenteric system then counted (Duvall and DeWitt, 1967). The percentages of worm reduction were calculated according to this equation [P (%R)=C-V/C×100] where P was the percentage of protection, C is the mean number of parasites recovered from infected mice and V was the mean number of parasite recovered from the vaccinated mice (Tendler *et al,* 1996).

Egg counts: Egg number per gram stools was calculated using Kato-Katz thick smear

method as modified by Martin and Beaver (1968).

Histopathological study: Formalin-fixed paraffin embedded liver tissues were cut into sections 4um in thickness and stained by haematoxylin and eosin (H&E) for histopathological study (Von Lichtenberg, 1962). The number of granulomas per ten low power fields (/10 LPF) was counted. The mean number of granulomas per 10 LPF for each sample was recorded; the granuloma diameter (in µm) was calculated by the use of ocular micrometer lens fitted on a light microscope. The mean diameter of granulomas was measured in two perpendicular diameters to detect the granuloma size.

Serological study: Blood samples were collected from the corner of the mouse´s eyes (Hoff and Rlagt, 2000), and then sera were separated and divided into two part, one for liver enzymes measurement and the other stored at -20ºC until the determination of serum TNF-α and IL10 levels (R&D systems, Minneapolis, Quantikine M,MN, USA) by ELISA according to manufacturer's instructions using polyclonal antibody and monoclonal antibody specific for TNF-a, IL10 and measured at 450nm optical density (OD). Concentrations were determined by available standard curves.

Immunohistohemical study: Immunohistochemical staining was carried out using the EnVision (USA) method. Tissue sections (3-5µm) were deparaffinized in xylene and rehydrated in graded alcohol. Slides were incubated for 10 minutes in 0.3 % hydrogen peroxide to block endogenous peroxidase activity. Dakotargetretrieval solution (pH 6.0) was used for 20 minutes. Antibody binding was detected by Dako's HRP Envision kit (Dako, Cytomation, Denmark). Primary antibodies are CD4 (ready monoclonal mouse, Clone 4B12, Dako, USA); CD8 (ready to use, monoclonal mouse, Clone C8/144B, Dako, USA); Anti-TNF alpha antibody (ab9739, abcam, Cambridge, UK); IL10 Positive and negative controls were stained at the same setting simultaneously human tonsils, TNF alpha and IL10.

Statistical analysis: Data were introduced to SPSS program version 16.0 and presented as M±SD. ANOVA test was used for significant differences between groups. P=0.05 was considered significant.

Results

Table 1: Parasitological parameters detected eight weeks post-infection in groups:

Parameter	egg count/g stool	Reduction%	Worm burden	Reduction%
G1	0	--	0	---
G2	200 ±42.16 [a]	--	21.4± 5.37[a]	---
G3	160 ±37.71 [b]	20%	20.6 ±3.50 [a]	24%
G4	137 ±32.68 [b]	31.5%	15.5 ±4.03 [b]	26%
G5	97 ±14.94 [c]	51.5%	11.4 ±2.55 [c]	84%
G6	28 ±19.89 [d]	86%	1.6 ±1.07 [d]	92%
K	39.21		37.04	
P	<0.001**	----	<0.001**	----

Groups with different letters= significantly different

Table 2: Mean granuloma number, diameter and reduction% at week 8 post-infection in control and tested groups:

Parameter	Granuloma number	Reduction%	granuloma diameter	Reduction%
G1	0	---	0	---
G2	9.7 ± 4.69 [a]	---	365.19 ± 58.76 [a]	---
G3	8.5 ± 4.09 [a,b]	12.3%	314.20 ± 17.37 [b]	13%
G4	7.1 ± 2.73 [a,b]	26.8%	276.47 ±35.82[c]	24%
G5	5.7 ± 1.83 [b]	41.2%	184.68 ±49.72[d]	49%
G6	1.7 ± 0.82 [c]	82.4%	67.46± 5.85 [e]	81%
Test	K=26.95		F=91.67	
P	<0.001**	-------	<0.001**	------

Table 3: Levels of liver enzymes in different groups:

Parameters	G1	G2	G3	G4	G5	G6	F	P
Succinate dehydro-genase	a 0.76±0.17	b 0.34±0.03 (-57.5)	b,c 0.38±0.03 [6.5]	c 0.43±0.04 [13.1]	d 0.56±0.03 [28.9]	a 0.71±0.08 [48.6]	41.98	<0.001 **
Lactate dehydro-genase	a 111.5±22.98	b 72.72±10.08 (-34.7)	b,c 75.26±8.57 [2.2]	b,c 80.8±6.28 [7.2]	c 85.79±7.33 [11.7]	a 109.08±16.12 [32.6]	16.36	<0.001 **
Glucose-6-phospha-tase	a 58.48±3.97	b 90.73±3.68 (+55)	b 88.341±2.92 [4]	c 80.55±3.35 [17.4]	d 71.77±3.86 [32.4]	a 57.23±4.98 [57]	142.5	<0.001 **
Acid phos-phatase	a 14.53±2.44	b 25.70±3.04 (+76.8)	b,c 24±2.74 [11]	c,d 22.16±2.76 [24.3]	d 20.72±3.93 [42.4]	a 16.24±3.71 [65.1]	19.3	<0.001 **
5'-nucleotidas	a 184.1±35.07	b 295.81±10.68 (+60.6)	b,c 289.93±7.29 [3]	c 278±7.71 [9]	d 259.01±15.11 [19.9]	a 190.16±8.55 [57.7]	85.17	<0.001 **

Groups with different letters are significantly different

Enzyme units: µmol/min/mg of protein. Unshared superscript letters between all groups are significant values at $p \leq 0.0001$. Numbers between round brackets are percentage changes over control group. Values between square brackets improved% (i.e. mean vaccinated-mean infected/ mean control x 100).

Table 4: Serum levels of TNFα and IL10, 8 weeks post-infection in tested groups:

Groups Parameter	G1	G2	G3	G4	G5	G6	F	P
TNF-α (pg/ml). M± SD	a 58.12±6.14	b 503.06±57.81	c 448.8±45.46	d 400.48±27.66	e 319.43±49.23	f 104.02±32.23	211	<0.001**
IL-10 (pg/ml). M ± SD	a 43.22±7.82	b 73.61±18.73	c 107.59±13.71	d 213.20±49.31	e 414.35±43.4	f 667.52±56.96	436	<0.001**

Groups with different letters are significantly different

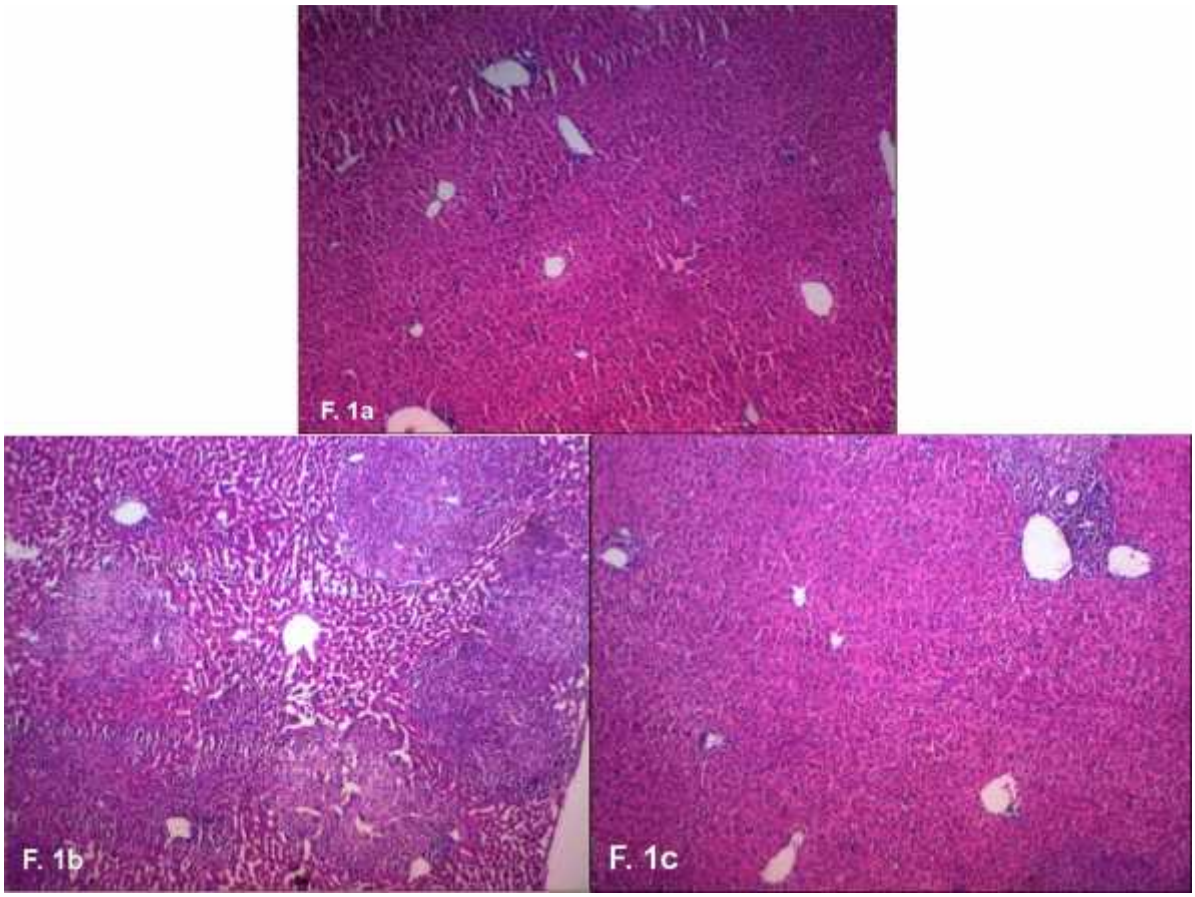

Fig.1: Haematoxylin and eosin staining of mice livers (x200) a-G1 showed normal liver tissue, b-G5 showed large number of granuloma with extensive reaction, and c- G6 showed smallest number of granuloma and the least reaction.

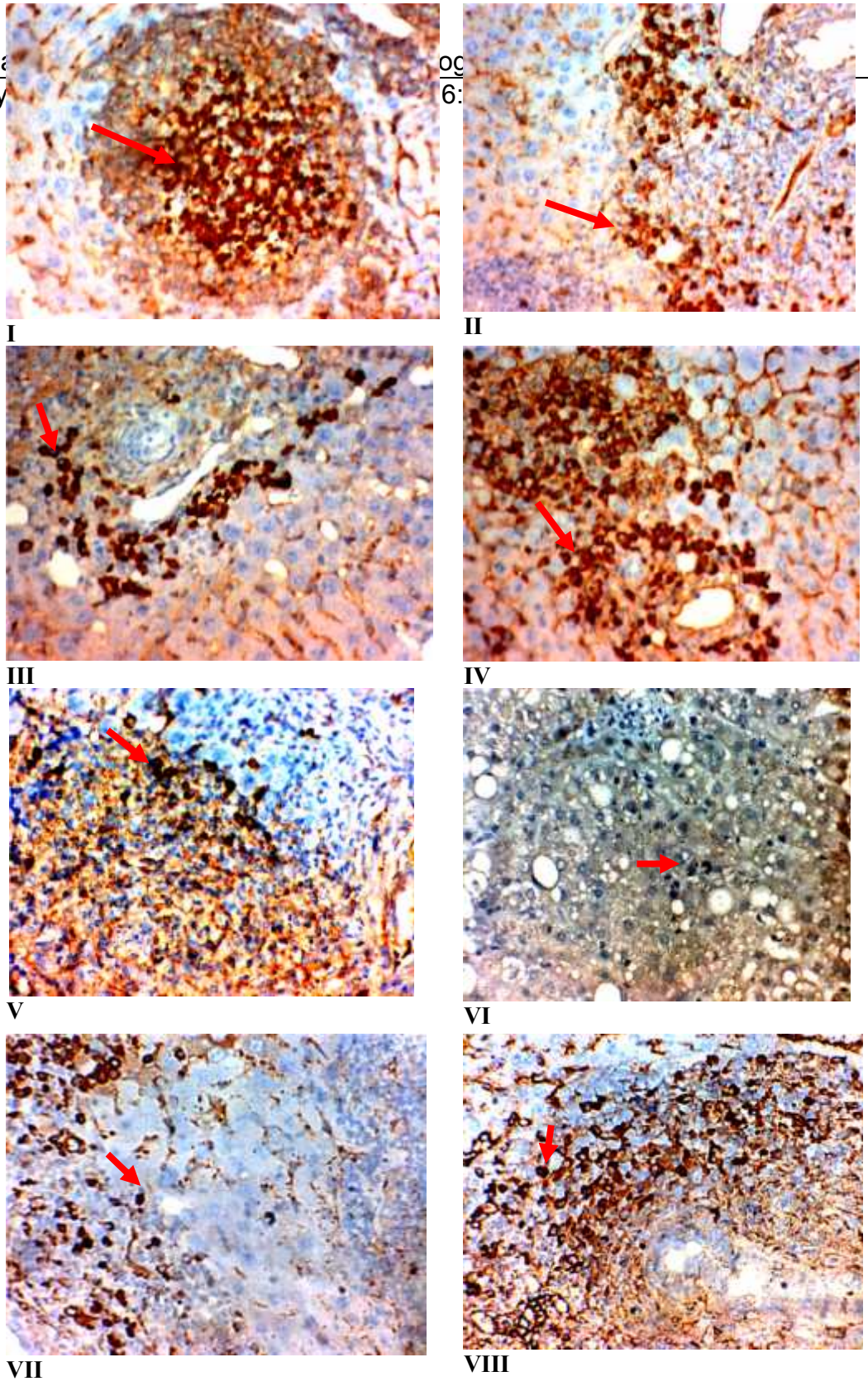

Fig. 2: Immunohistochemical staining of mice livers (immunoperoxidase×400) (I) G3 showed an increased number of CD4 (membranous and cytoplasmic stain), (II) G6 showed decreased number of CD4, (III)G3 showed decreased number of CD8 (membranous and cytoplasmic stain), (IV) G6 showed increased number of CD8, (V) G3 showed high TNF alpha cytoplasmic expression,(VI) G6 low TNF alpha cytoplasmic expression, (VII)G3 showed low IL10, (VIII) G6 showed high IL10.

Discussion

Generally, in spite of the presence of an anti-*Schistosoma* medication and the control programs do the disease is increasing in in-

cidence and prevalence into new countries (Sulbara *et al,* 2013). Basyoni and Abd El-Wahab (2013) reported that *B. alexandrina* antigen was good one among other antigens that can replace adult *S. mansoni* crude antigen in diagnosis and thus for vaccination.

As the outcome of protection elicited by a single antigen was insufficient, development of new combination vaccine is mandatory to increase protection (Khalifa *et al,* 2011).

The BCG as an adjuvant is known to promote infection resistance associated with the stimulation of schistosome specific T cell dependent cell-mediated immune responsiveness, and activate macrophages for larval schistosome killing (Pearce *et al,* 1988).

In the current study, parasitological assessment revealed a reduction in the fecal egg count (20%, 31.5%, 51.5%, 86%) respectively in all vaccinated groups. The mean number of eggs per gram stool decreased in immunized groups as compared to non-immunized infected control group. Teixeira De Melo *et al.* (2010) in Brazil who reported that mice immunization with schistosomula tegument (Sm-teg) induced significant reduction in fecal egg counts (59-60%) and agreed with Mountford amd Harrop (1998) who concluded that the delivery of two doses of SLAP and IL-12 induced significant levels (37.9–53.4%) of protective immunity to challenge infection, whereas Mountford *et al.* (1996) stated that vaccination with SLAP alone failed to induce any protection. Reduction in worm burden is the gold standard of anti-*Schistosoma* vaccine development (Capron *et al,* 2002).

The present study results showed variable degrees of reduction in worm burden number and in egg count per gram stool. The percentage reduction of worm burden (G3, G4, G5, G6) gave (24%, 26%, 84%, 92%) respectively. Also, combination of SLAP, BAAP and BCG in (G6) recorded the highest reduction in worm burden. These data agreed with Teixeira De Melo *et al.* (2010) who reported that mice immunization with schistosomula tegument (*Sm*-teg) induced significant reduction in worm burden (43-48%); the variation between their data and ours may be as a result of using different antigen preparations. Also, Hamed *et al.* (2010) reported 70.96% reduction in worm burden by using the nucleoprotein of susceptible snails as antigen. El Ridi and Tallima (2013) reported that mice immunized with glyceraldehyde 3-phosphate dehydrogenase (SG3PDH), peroxiredoxin (TPX), and other larval excretory–secretory products (ESP) caused percentage reduction of 62-78% in challenge worm burden with highly significant difference. However, El-Ahwany *et al.* (2006) reported a highly significant reduction (52.4%) in the mean number of adults in group infected and immunized with purified schistosomulae antigen compared to the infected controls; their lower results than ours because we used 18 day old schistosomulum but they used 14 day old schistosomulum and also a cocktail of different antigen preparation. These data agreed with Melo *et al.* (2014) who found that mice immunization with *S. mansoni* schistosomula tegument (Sm-teg) formulated with Freunds adjuvant induced partial protection against infection associated with an increased antibody production that were able to bind to surface of recently transformed schistoso- mula. Naturally or vaccine-acquired immunity could significantly decrease human pathology and disease transmission (Siddiqui *et al,* 2011).

In the present study the cocktail vaccine showed the highest reduction in granuloma count (12.3%-82.4%) and granuloma diameter (13%-81%). This agreed with Chitsulo *et al.* (2004) who reported that the vaccine that can induce partial reduction in worm burdens and capable to reduce the pathology and limited transmission. Also, El-Ahwany *et al.* (2006) stated that experimentally induced liver granulomas were significantly reduced with immunization of 14 days old schistosomulae antigenic preparation and attributed that to reduction of CD4+ cells

and increase in CD8+ in the immunized groups. Pinho *et al. (*2010) reported that mice immunized by tegument protein of *S. mansoni* (rSmIg) had a significant reduction of liver granuloma size and thus reduced pathology induced by infection. Also, Hamed *et al.* (2010) used *Schistosoma* tegmental antigens as a vaccine and gained reduction of granuloma count and diameter (56.3%, 57.9%) respectively.

In the present work, group (G6) vaccinated by combined antigen (SLAP+BAAP+BCG) gave the best result (32.6%, 48.6%, 57%, 57.7%, 65.1%) in the lactate dehydrogenase, succinate dehydrogenase, glucose-6-phosphatase, 5'-nucleotidas and acid phosphatase enzymes respectively.

In the present study, the levels of lactate dehydrogenase and succinate dehydrogenase enzymes (G2) were significantly decreased in infected mice (34.7% & 57.5%) respectively. But, both glucose-6- phosphatase, 5`-nucleotidase and acid phosphatase enzymes were significantly increased (55%, 60.6%, 76.8%) respectively. These results agreed with Hamed and Hetta (2005) who reported that variation in enzyme activities after *S. mansoni* infection attributing to reduction in levels of succinate and lactate dehydrogenases to *Schistosoma* toxins that affected both mitochondrial and plasma membranes that led to enzyme leakage. The mitochondrial changes could be due to the limited amount of oxygen present as a result of inflammation; hence mitochondrial oxidation, Krebs cycle intermediates, and enzyme activities are repressed (Wang *et al,* 2006). In the current study the increased level in Glucose-6-phosphatase activity agreed with El-Banhawey *et al.* (2007) and the explanation may be due to the effect of *Schistosoma* toxins on the endoplasmic reticulum by the located enzyme, or due to the elevation of cytosolic calcium that convert the inactive form of the enzyme phosphorylase-b to the active form phosphorylase-a, so degrading glycogen into glucose. Also, Bashtar *et al.* (2006) attributed the increased level of acid phosphatase in tissue catabolism to the effect of metabolic worm products on lysosomal enzymes. The elevation of the liver enzymes in infected control group as compared to normal group may be due to the production of reactive oxygen species in the liver and the alteration of antioxidant defenses leading to the disruption of hepatocyte (*Yang et al,* 1986). So, administration of the combined antigens in the present research lead to decreased levels of enzymes to almost normal that can be endorsed to the elaboration of the oxidant/antioxidant balance indicating an improvement in liver function that provide further support to the mechanism of action of the vaccine (Abdel-Hafeez *et al,* 2012).

The most effective therapy for schistosomiasis hepatic pathology was to remove the causative agent (Fang *et al.,* 2010), so in this study used SLAP and BAAP vaccines trying to eliminate the infection from the beginning. Caldas *et al.* (2008) and Aly *et al.* (2010) reported that in the acute phase there was a mixed expression of Th1 & Th2 cytokines with predominance of Th1 during the early stage that cytokines modulate granuloma size and the fibrosis amount played a main role in the pathology of schistosomiasis.

In the current study, there was a reduction in TNF-α level in all vaccinated groups, but combination of SLAP, BAAP & BCG in (G6) gave the highest reduction with highly significant difference between all groups. Also, Haseeb *et al.* (2001) reported that inflammatory cytokine usually increased after the excretion of egg explaining its effect in complications of schistosomiasis, as it able to induce tissue injury and fibrosis through stimulating reactive oxygen species production, but Joseph and Boros (1993) reported that TNF-α has a fundamental role in the modulation of granulomatous reaction induced by eggs, and neutralization of TNF-α decreased granuloma size during the acute stage of infection. So, these cytokines play

distinct roles in granuloma formation and hepatic fibrosis depending on the articular cytokine milieu it expressed (Hoffmann *et al,* 1998).

In the present study, IL-10 was increased in all vaccinated groups, but the highest significant difference among all groups was obtained in (G6).This agreed with Hoffmann *et al.* (2002) and Pearce and MacDonald (2002) who reported that IL-10 plays a crucial regulating role in immune responses during infection, by preventing the development of Th1 & Th2-mediated pathologies. This cytokine is the key factor in avoiding an increase in disease morbidity (Caldas *et al,* 2008; Aly *et al,* 2010). Also, type 2-associated cytokines such as IL-4, IL-13 & IL-10 inhibit classical macrophage activation and implicated in granuloma formation and fibrogenesis around tissue-deposited eggs (Morais *et al,* 2002; Burke *et al,* 2009).

S. mansoni tegument interaction with the immune system plays a key role in the establishment or elimination of the disease; immunohistochemical staining of mice livers in the present study showed an increased number of CD4 in G3 while G6 decreased in number, and G3 decreased number of CD8 while G6 increased in number. Also, G3 showed high cytoplasmic expression of the TNF-α but G6 showed low cytoplasmic expression, the same was gained in IL10 where G3 showed low level and G6 showed high level. El-Ahwany *et al.* (2006) reported that experimentally induced liver granulomas were significantly reduced with immunization of 14 days old schistosomulae antigenic preparation compared to infected control and attributed that to reduction of CD4+ cells and increase of the CD8+ in immunized groups with purified schistosomulae antigen. Also*, Schistosoma* vaccine could cause proliferation of splenocytes, induce immune protection to its host by up regulating CD4 + & CD8 + T cells and inhibited the apoptosis of mice splenocytes (Xiang *et al,* 2013). Araujo *et al.* (2012) demonstrated that a significant antibody production, increased percentage of CD4 + IFNγ and CD4 + IL-10 cells in spleen and increased production of IFN-γ & IL-10 by spleen cells in the immune mice. They added that adjuvant did not only promote immune responses but also considered as molecule that slowly release the antigen to the half-life of the antigen and improved its uptake by phagocytes.

The reduction in granuloma size could be explained by the increased level of CD4T-cells indicating an improvement of the immunity of mice. The Th1 immune response induced by vaccination, results in the production of T-regulatory cells creating interleukin-10 (IL-10), that help in the prevention of an exacerbated granulomatous reaction (Wynn and Cheever, 1995; Jarnicki *et al,* 2008). Wilson *et al.* (2007) suggested that IL-10 plays a regulatory role in preventing the expansion of rigorous pathology. Immunologically *S. mansoni* radiation attenuated (RA) cercariae vaccine showed that the migration of schistosomulae to terminate in the lungs of the protected animals formatting the inflammatory foci of monocytes and the CD4+ T-cells with Th1 characteristics (Hewitson *et al,* 2005; Wilson and Coulson, 2009).

Badr *et al.* (2015) immunized mice and rats with purified recombinant lung-stage ESP vaccine antigens to compare between the immune response during critical lung schistosomiasis larval stage in a susceptible host mouse as and less-susceptible rat for schistosomiasis infection. They screened Th1, Th2 and Th17 responses and levels of serum antibodies. They found a detectable amount of IL-17 and IL-4, but no IFNγ in splenic cell of rats. They predict that schistosomes invading rats are met with type 2 cytokines upon development and migration, resulting in their attrition. El Azzouni *et al.* (2016) used cercarial transformation fluid (CTF) and crude cercarial antigen (CCA) with alum as an adjuvant to stimulate Th2 immune responses and macrophages for

producing prostaglandins (PGs), having an active role in the regulation of immune responses. Ricciardi *et al.* (2016) tried a vaccine formulation formed of recombinant *S. mansoni* (Sm) cathepsin B and reported that this formulation can produce antigen-specific antibodies together with stimulation of Th1 cytokines (IFN-γ, TNF-α & IL-12).

Conclusion

The outcome data showed that the combination of SLAP, BAAP and BCG increased the protective immunity and reduced the immunopathological changes. This new cocktail represents a promising approach towards the future development of vaccine strategy

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