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Induction of Protective Cellular and Humoral Responses against Fasciolosis in Rabbits using Immunoaffinity Fraction of *Fasciola gigantica* Excretory Secretory Products

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Abstract

Fasciolosis due to *Fasciola gigantica* or *F. hepatica* causes significant production losses in animals, as well as being a zoonotic disease of global importance. In an attempt to develop vaccine against fasciolosis in rabbits, an immunoaffinity fraction of *F. gigantica* excretory secretory products was isolated. The fraction possesses 87.67% of the initial antigenic activities with 2051.5 fold increase in specific activity compared to crude extract. It consists of two bands of molecular weight 27 kDa and 23.5 kDa as revealed by SDS-PAGE. The detailed structural analyses of the pure fraction showed O-glycan [Ser-Arg-Ser-Arg-Ser-GlucNAc] using mass spectrometry. Vaccination of rabbits twice with the fraction resulted in 85% reduction in worm burden. It is also resulted in high antibody IgG levels as proved by ELISA. The highest IgG response was observed in vaccinated rabbits at two weeks post infection and remained stable to the end of the experiment. A significant expression of IL-4 and INF-γ was observed in vaccinated rabbits starting one week until thirteen weeks post infection. The level of IL-4 was significantly higher than the level of INF- γ throughout the experiment as measured by ELISA. Collectively, the current results suggest promising immunoprophylactic potentials of the immunoaffinity fraction of *Fasciola gigantica* excretory secretory products against fasciolosis in rabbits through induction of both cellular and humoral responses.

Keywords: *F. gigantica*; Excretory secretory products; Vaccine; IgG; IL-4; INF-γ; Rabbits

Introduction

Fasciolosis is a worldwide zoonotic disease caused by trematode parasite of the genus *Fasciola*. WHO estimates that at least 2.4 million people are infected in more than 70 countries worldwide, with several million people at risk. No continent is free from fasciolosis, and it is likely that where animal cases are reported, human cases also exist [1]. Recently, *Fasciola* spp. was added to the WHO list of neglected tropical diseases after decades of neglect [2].

In Egypt, fasciolosis becomes hyperendemic and problematic where animal reservoir and snail vector are available [3]. The emerging situation of both human and livestock fasciolosis in Egypt has increased significantly due to both *F. gigantica* and *F. hepatica* [4-6]. Nearly 800,000 Egyptians are suffering from fasciolosis and about 24 millions are at risk [7,8].

The liver fluke secretes molecules, known as excretory-secretory products (ESPs) that modulate or suppress host immune responses [9,10]. During early chronic infections, there is a predominance of a Th2 response, which decreases in advanced chronic infections characterized by a persistent immune suppression [11].

$CD4^+$ T cells can be separated into 2 major subsets, Th1 and Th2, on the basis of their cytokine secretion patterns and function. Th1 cells produce many cytokines, including IFN-γ and TNF-α, and promote the activation of macrophages which lead to the production of opsonizing antibodies. Also, Th1 cells promote mediation of a delayed-type hypersensitivity reaction and inflammatory responses. Th2 cells produce many other cytokines, including IL-4, IL-6, and IL-10, and promote immediate type hypersensitivity reactions, involving IgE, eosinophils, and mast cells [10]. Generally, helminth infections are manifested by suppression of Th1 function and induction of T cells, which express cytokines characteristic of the Th2 subset [12].

To control fasciolosis, a significant data suggests that a number of *Fasciola* molecules, including cathepsins L, glutathione S-transferase, leucine aminopeptidase and fatty acid binding proteins have the potency of inducing a protective response against the infection in laboratory animals [13] and large animal models [14].

Vaccination studies with purified native or recombinant *Fasciola* antigens suggest that this approach, which diminished morbidity and mortality and reduced transmission, is a realistic goal [15].However, despite long-standing research, a vaccine against this parasite has not yet been developed to the level of commercialization [16]. This can be largely attributed to a fact that immune responses to vaccines are influenced by the route of administration, type of antigen and adjuvant [17,18].

The objective of the current study is to identify *Fasciola gigantica* ESPs fraction with potential for use as vaccine, and to evaluate the protective humoral and cellular immune responses in *F. gigantica*-infected rabbits. In our laboratory, rabbit is often used as a model for

experimental fasciolosis to evaluate the effectiveness of *Fasciola* vaccine candidates [19-21].

Materials and Methods

Animals

Thirty native rabbits, 1.25-1.5 Kg each, were used in the current study. They were proved to be free from any parasitic infections by examining them parasitologically using fecal egg analysis and serologically with indirect ELISA using antigens of most common parasites that infect rabbits (*Eimeria steadi, Fasciola gigantica* and *Trichostrongylus colubriformis*). Animals were housed, fed and kept under conventional germ-free conditions in the animal house of the Veterinary Division, National Research Centre. All procedures related to animal experimentation met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences.

Parasites

Metacercariae of *F. gigantica* were purchased from the Schistosome Biology Supply Center (SBSC) of Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Adult *F. gigantica* worms were collected from the biliary tracts and gall bladders of condemned bovine livers from local slaughter-house.

Preparation of ESPs

The live intact *F. gigantica* worms were washed several times with cold 0.01 M PBS (pH 7.4) to eliminate any traces of bile, blood, and contaminated microorganisms. They were then incubated for 16 hr at 37°C in RPMI 1640 medium (pH 7.4) containing 100 IU of penicillin and 100 mg streptomycin/ml of medium. Following incubation, the medium was removed and centrifuged at 12,074 xg for 30 min at 4°C [22]. The supernatant containing ESPs was collected, and the protein content was measured using the method of Lowry et al. [23]. It was then stored at -20°C until use.

Preparation of Rabbit Hyperimmune Serum

100 µg of ESPs was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into each of 5 rabbits. A booster dose of 100 µg ESPs in Freund's incomplete adjuvant was injected two weeks later. Second and third booster doses were given on days 21 and 28, respectively [24]. Blood samples were collected 4 days post last injection from rabbit's ear vein. Prepared antiserum was stored at -20°C until use.

Purification of *F. gigantica* ESPs

The prepared rabbit hyperimmune serum was dialyzed against 100 mM $NaHCO_3$ buffer pH 8.3 containing 500 mM Nacl and 0.02% (w/v) NaN_3 and coupled to the cyanogen bromide (CNBr)-activated Sepharose-4B at the ratio of 2 mg/ml-swollen beads by strictly following the manufacturer instructions. Crude ES (FgESPs) was applied to the column. After washing the column with 0.15 M PBS pH 7.3 several times, the bound material was eluted with 50 mM glycine-500 mM Nacl-0.02 % (w/v) NaN_3 PH 2.3. The eluted fraction was immediately brought to PH 8.0 with solid $NaHCO_3$ and then dialyzed against 0.03 M PBS-0.02% (w/v) NaN_3 PH 8.0. The isolated ES fraction was assayed for protein content by the method of Lowry et al. [22] and its immunogenic activity was investigated compared to unbound fraction and crude extract by ELISA.

SDS-PAGE

Both crude and isolated fraction of ESPs were separately mixed with reducing sample buffer containing 5% 2-mercaptoethanol and electrophoresed on SDS-PAGE 10% slab gel (60 µg protein/well)according to procedures of Laemmli [25]. After separation slab gel was stained with silver stain according to Wray et al. [26]. High and low molecular weight standards (Sigma) were electrophoresed on the same gel to calculate the relative molecular weights of the examined antigens. Gel was photographed wet using Kodak Tri-X-pan films.

Chemical elucidation

The analytical sample was homogenous by Thin-Layer Chromatography (TLC), which was performed on EM silica gel 60 F sheet (0.2 mm) with $CHCl_3/CH_3OH$ (9:1), (v/v); as the developing elute livers nt, rate of flow (R_f)=0.2. The spot was detected with U.V model UVGL-58 as a violet spot. EI Mass spectra recorded on a Varian MAT 311A spectrometer.

Vaccination protocol

Rabbits were divided into 3 groups. The first group (five rabbits) was a normal control group. The second group was the infected control group (ten rabbits). The third group was the immunized-challenged group (ten rabbits). Rabbits were immunized intramuscularly twice at 2 weeks interval, with 40 µg of purified fraction diluted in 250 µl 0.01M PBS and 250 µl of complete and incomplete Freund adjuvants (CFA) separately. Two weeks after the last booster dose of immunization, rabbits were infected orally with 30 *F. gigantica* metacercariae [27]. Rabbits were bled at 2 weeks intervals after infection until the end of the experiment for the collection of sera. All rabbits were necropsied at 13 weeks post challenge for the determination of worm burdens. Whole blood was collected from each rabbit and centrifuged at 214 xg at 4°C for 10 min, and the obtained serum samples were stored at -20°C until analysis.

Assessment of the protective value of the vaccine candidate

Fluke count: Worms were recovered from the gall bladder and livers of both vaccinated and non-vaccinated infected rabbits. The mean total number of flukes/group was calculated. Worm burden reduction in immunized animals was calculated by the formula (C-E)/C-100, where C is the average number of flukes in the control group and E is the average number of flukes in the vaccinated group of animals.

IgG response: ELISA was adopted to evaluate the success of the purification process by determination of the antigenic activities of the eluted fraction compared with unbound one and crude extract. The assay was also adopted to evaluate the protective IgG level due to the fraction in rabbit serum samples collected at different intervals of infection based on the method of Engvall and Perlmann [28]. Plates were coated, separately, with 5 µg /ml isolated fraction, unbound fraction or crude ES in carbonate buffer. Positive and negative rabbit serum samples diluted at 1:100 was added to the coated plates separately. Anti-rabbit IgG horse radish peroxidase labeled-conjugate (1:1000) and ortho-phenylenediamine (OPD) 1 mg/ml substrate buffer

(Sigma) were used. The optimum antigen concentration, antibody and conjugate dilutions were determined by checkerboard titration. Plates were read spectrophotometrically at 450 nm and the cut off values of Optical Densities (OD) were calculated according to Hillyer et al. [29].

Assessment of cytokines level: Serum levels of IL-4 and IFN-γ were measured with double-antibody sandwich ELISA kit (Glory Science Co., Ltd. Del,Rio,Tx 78840,USA). The concentration was calculated from the standard curve that was performed in the same assay.

Statistical analysis

Data are expressed as mean ± SD. Comparison between the mean values of different parameters in the studied groups was performed using 1-way ANOVA test.

Results

Purified vaccine candidate

As summarized in Table 1, 87.67% of the initial antigenic activities in *F. gigantica* ESPs were recovered in the bound and eluted fraction which represents only 0.65% of total protein in crude ES applied to the column giving 2051.5 fold increases in the specific activities compared to crude ESPs.

Electrophoretic profile of the candidate

Under reducing conditions in SDS-PAGE 10% slab gel, the vaccine candidate was resolved in only two bands of molecular weight 27 kDa and 23.5 kDa compared to multiple bands in the crude extract ranged from 205 kDa to 14.2 kDa.

Chemical structure of the candidate

The Thin Layer Chromatography (TLC) gave one spot in MeOH/$CHCl_3$ (80:20 v/v) as an eluent. By immersing the TLC sheet in 5% H_2SO_4/MeOH solution and heating it gave burning red color for GlucNAc sugar moiety. The mass spectrum of the monomer m/z=1,000 [M+3H]. Ser-Arg-Ser-Arg-Ser-GlucNAc. The fragmentation or peak at m/z=106 is an indication for the Serine (Ser) residue [M+H] (Figure 1). The fragmentation or peak at m/z=170 is an indication for the Arginine (Arg) residue [M-4H]. The fragmentation or peak at m/z=223 is an indication for the GlucNAc residue [M+2H]. The fragmentation or peak at m/z=792 is an indication for the [monomer-OglucNAc]. EI-Mass: (m/z%) =1000 (M+3H, 50) (Figures 2 and 3).

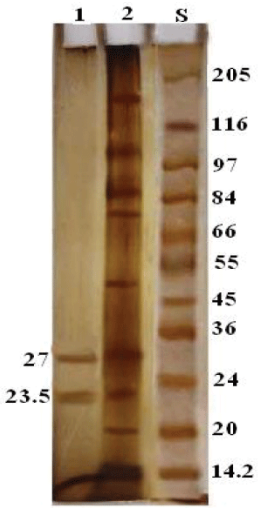

Figure 1: Electrophoretic profile of the vaccine candidate (Lane 1), ESPs (Lane 2) and Molecular weight standards (lane S).

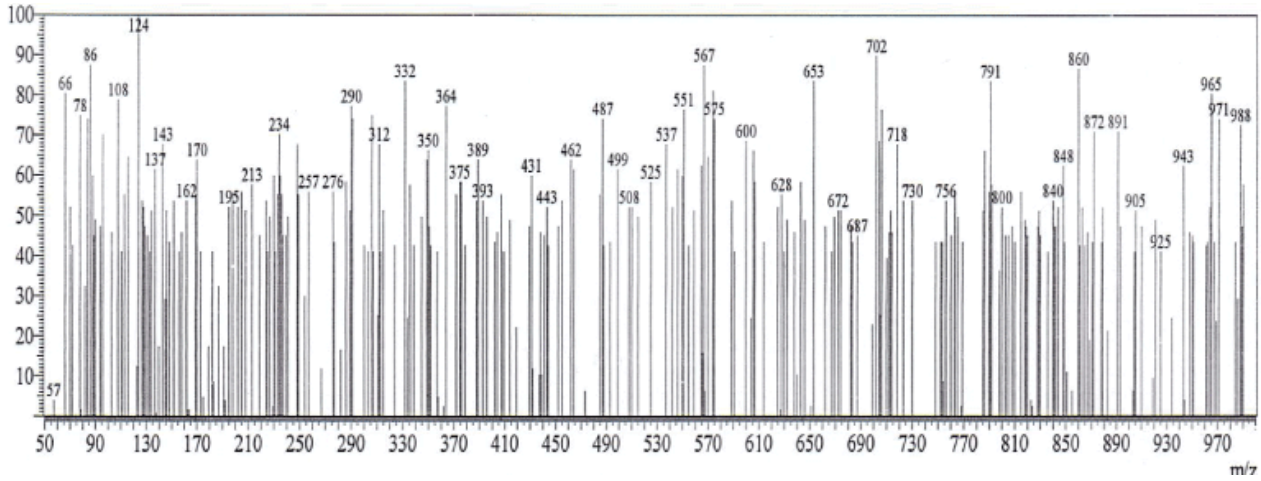

Figure 2: Mass spectrum of *F. gigantica* isolated fraction

Fraction	Total proteina (µg × 104)	Activity unitb (Au × 106)	Specific activityc (Au/µg × 102)	Purification fold	Yield (%)
Crude ES	29.2	7.3	0.25	1.00	100.00
Unbound to column	16.3	0.5	0.031	0.22	6.85
Bound and eluted fraction	0.19	6.4	33.53	2051.5	87.67

Table 1: Quantitative summary of *F. gigantica* ESPs purification. [a]Protein was measured as described by Lowry et al. [22]. [b]A unit of activity defined as the amount of protein required to give one well of agglutination. [c]Specific activity which is the number of activity per µg of protein and is related to the starting crude ESPs.

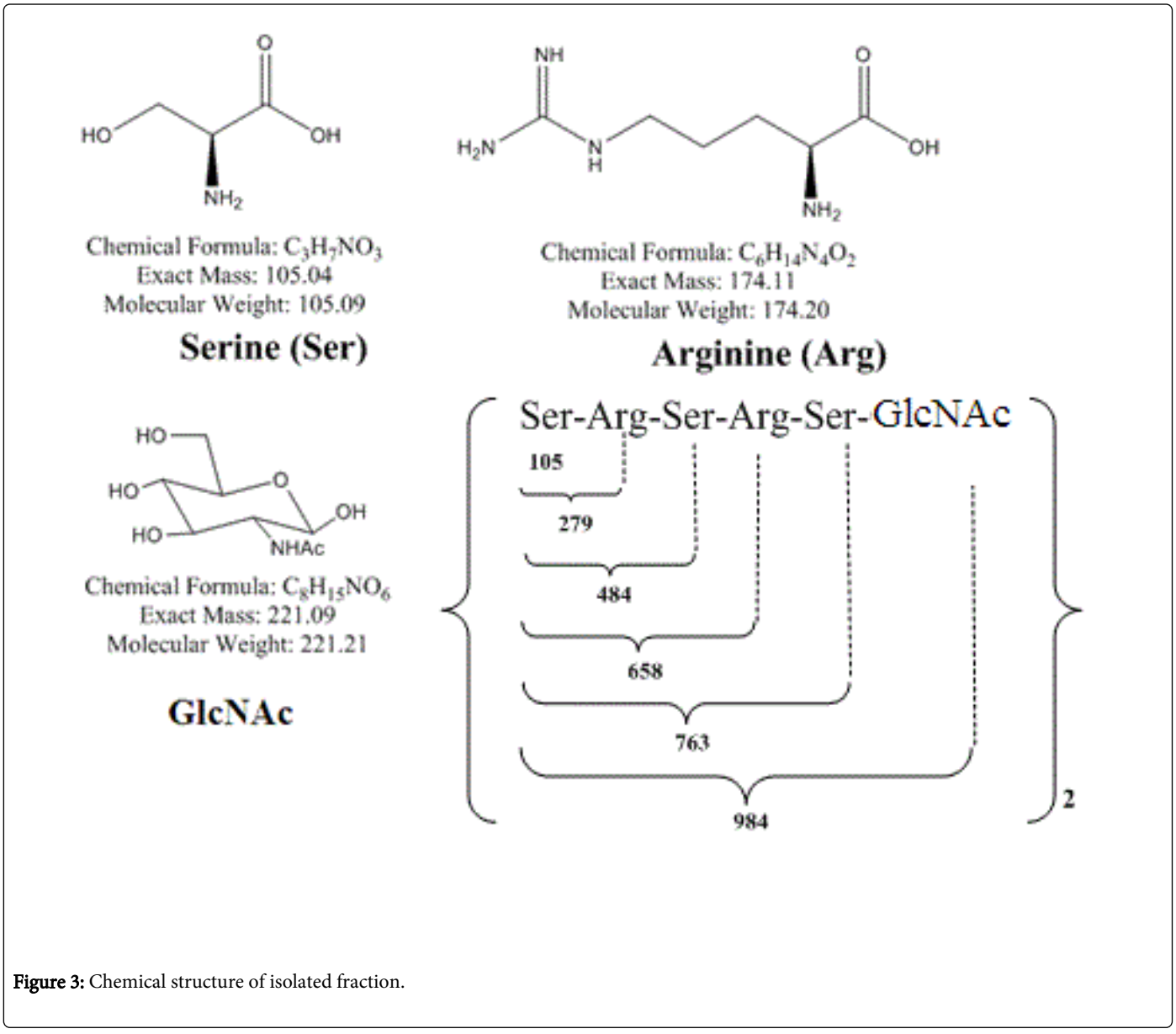

Figure 3: Chemical structure of isolated fraction.

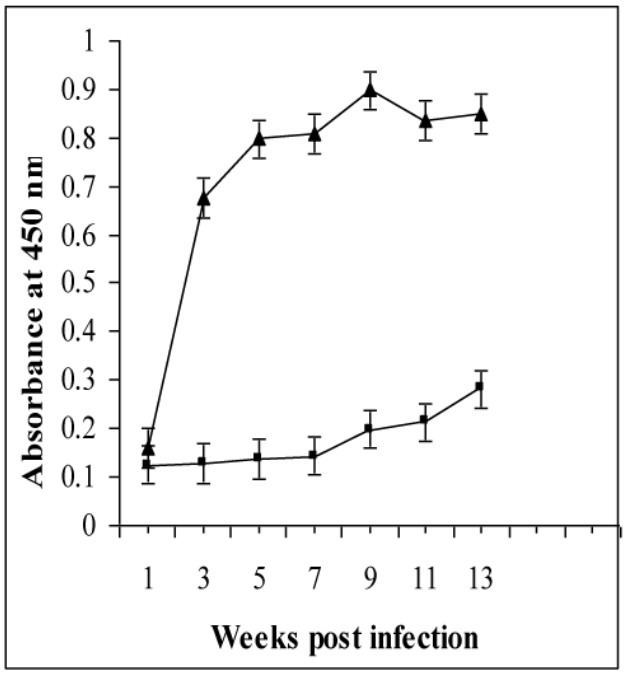

Figure 4: Levels of IgG response measured by ELISA against vaccine candidate in vaccinated challenged rabbit serum samples (▲) and non-vaccinated normal rabbit samples (■). Bars in the figure represent SD.

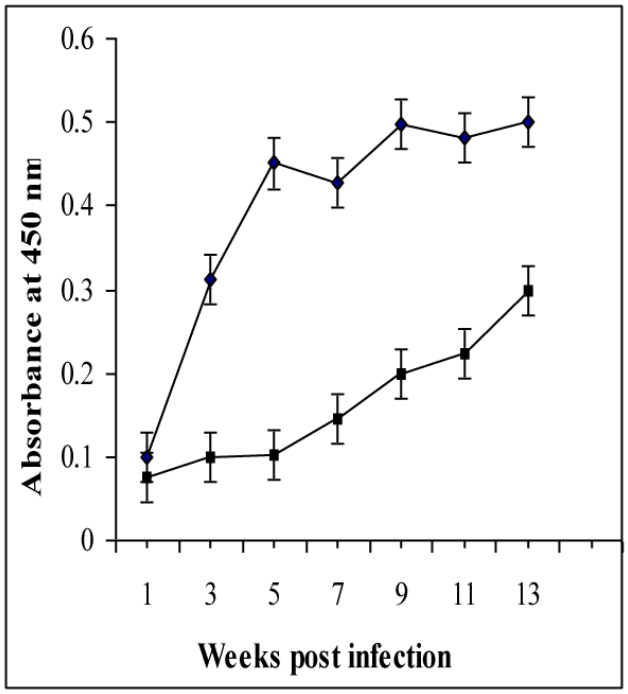

Figure 5: levels of IgG response measured by ELISA against crude ESPs in vaccinated challenged rabbit serum samples (◆) and in non-vaccinated normal rabbit samples (▮). Bars in the figure represent SD.

Protective Response of the Vaccine Candidate in Rabbits

Parasitological response: Mean number of recovered worms in vaccinated infected rabbits was reduced to only 3 ± 1.04 worms compared to 20 ± 1.019 worms in infected non vaccinated animals recording 85% reduction in worm burden in vaccinated rabbits.

Humoral response: The level of developed IgG response due to bound and eluted fraction increased to record OD values 0.885 ± 0.0013 two weeks post first dose of vaccination and reached to OD value 0.969 ± 0.0014 four weeks post vaccination and before challenge. This IgG profile is higher than that due to ESPs which recorded OD value 0.568 ± 0.0012 after two weeks of vaccination and increased to 0.586 ± 0.0013 after four weeks. After challenge the IgG in the vaccinated rabbits with the fraction was significantly higher than that in vaccinated rabbits with crude ESPs at all intervals of the experiment starting one week post challenge (0.15 9 ± 0.0012) and reached its maximum level (0.898 ± 0.0013) nine weeks post challenge (Figures 4 and 5).

Cellular response: Levels of IFN-γ were significantly higher in vaccinated infected rabbits than in non-vaccinated infected control throughout the experiment recording the highest value (189.94 ± 0.268) one week post infection (Figure 6). Levels of IL-4 were also higher in vaccinated infected rabbits than non-vaccinated infected control except 13 weeks post infection recording the highest value (342.04 ± 0.261) 11 weeks post infection (Figure 7). Significant higher level of IL-4 than IFN-γ was observed throughout the experiment (Figures 6 and 7).

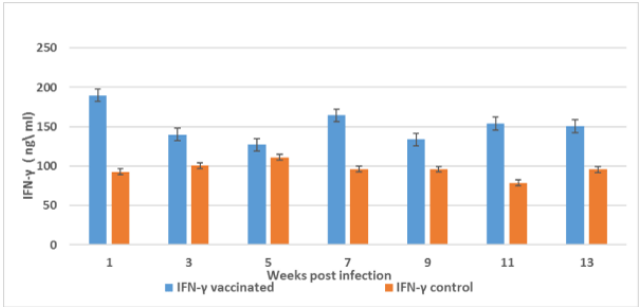

Figure 6: Levels of IFN-γ in vaccinated challenged rabbits and non-vaccinated infected ones as measured by ELISA. Bars in the figure represent SD.

Discussion

Based on the importance of *Fasciola* ESPs in eliciting humoral and cellular host immune responses [9,30-32], the main facet of the current study is to evaluate the protective responses to some of these products against rabbit fasciolosis. In the present research, a fraction with most antigenic activities of ESPs was isolated by immunoaffinity column chromatography with 2051.5 purification folds than whole products. The success of the purification process was further supported by the low antigenic activity in the unbound fraction which means that most antigenic activity was associated with the pure bound fraction.

SDS-PAGE showed that the purified fraction consists of two bands of molecular weight 27 and 23.5 kDa. 27 kDa was previously isolated from *Fasciola* ESPs [33,34]. It was previously supposed that 27 kDa appeared to be released from cells lining the gut [15,35] and the 28 and 26-27 kDa molecules were shown to essentially consist of cathepsin CP [36,37].

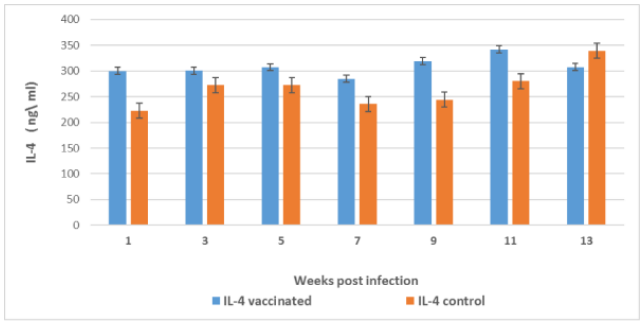

Figure 7: Levels of IL-4 in vaccinated challenged rabbits and non-vaccinated infected ones as measured by ELISA. Bars in the figure represent SD

The detail chemical structure of the current ES fraction is O-linked GLcNAc Ser-Arg-Ser-Arg-Ser-GlcNAc. Previous research on glycans in *F. gigantica* adult worms proved the existence of O-GlcNAc in a fraction of *F. gigantica* that can be used successfully in the diagnosis of acute and chronic rabbit fasciolosis [38]. O-GlcNAc is a widespread dynamic carbohydrate modification of cytosolic and nuclear proteins with features analogous to phosphorylation. O-GlcNAc acts critically in many cellular processes, including signal transduction, protein degradation, and regulation of gene expression. However, the study of its specific regulatory functions has been limited by difficulties in mapping sites of O-GlcNAc modification [39].The existence of O-GlcNAc was previously documented in parasites [40] *Plasmodium falciparum* expresses its own O-GlcNAc transferase during intraerythrocytic development [41]. So, O-GlcNAc glcosylation of enzymes is recorded which supports glycosylation of *Fasciola* ES fraction in the current study which probably includes Cathepsin based on the existence of 27 kDa.

Vaccination of rabbits with the purified ES fraction emulsified in friend's adjuvant resulted in 85% reduction in worm burden. Selection of rabbits in the current study was based on its success as laboratory animal model in different researches as assessment of drug against fasciolosis [42] and in vaccination studies against fasciolosis [27].In addition, rabbits are cheaper than goats and sheep, easy to handle, raise and infect with fasciolosis.

In previous vaccination trials in rabbits with *F. hepatica* saposin emulsified in TiterMax adjuvant resulted in 81.2% reduction in worm burden after challenge with *F. hepatica metacercariae* [43]. In later study, FhSAP2 emulsified in Freund's adjuvant induced an 83.3% reduction in liver fluke count in rabbits [13].Vaccination of sheep with *F. hepatica* Cysteine proteinases emulsified in Freund's adjuvant resulted in 56.9% reduction in worm count [34]. The authors in the previous trials attributed worm reduction due to vaccination with ES components to high level of IgG. This explanation could be assumed in the current study particularly high level of IgG was recorded. In the present research, the level of developed IgG response due to vaccine candidate in vaccinated challenged rabbits was higher than that in control non vaccinated rabbits and also higher than IgG response to crude extract at different intervals of infection. This is consistent with observations made by other authors who, used immunogens of *Fasciola* ESPs and found associations between protection and the levels of antibodies [44-46].

In our study, the serum levels of pro-inflammatory cytokine, IFN-γ showed lower level than the anti-inflammatory cytokine level, IL-4. So, the T-cell response due to vaccine candidate is mainly Th2 although, Th1 also exists.

Adoption of anther vaccine candidate, FhSAP2, against rabbit fasciolosis induced protection associated with high levels of IFN-γ, TNFα, IL-2 and IL-10, which suggests a mixed Th1/Th2 mechanism with dominance of Th1-cytokines [13]. Involvement of both Th1/Th2 responses in protection against fasciolosis was previously reported using a cathepsin L-based vaccine [14].

Although the mechanism of protection induced by the candidate is unknown, it is speculated that it may be related to the antibodies produced after vaccination, which may activate cellular mechanisms that directly affect the parasite. Moreover, the current vaccine candidate was emulsified with FCA, so it is likely that the protection was achieved through an effective stimulation of TLR2, which is important for the production of inflammatory cytokines and interferon stimulated genes.

In conclusion, *F. gigantica* releases a glcosylated protein that could be successfully utilized in eliciting protective cellular and humoral responses against rabbit fasciolosis resulted in significant reduction in worm burden. The candidate deserves further investigations to evaluate different vaccination protocols, other adjuvants and protective effect in other hosts.

References

1. WHO (2011) Foodborn trematode infection, Fascioliasis.
2. WHO (2010) First WHO report on neglected tropical diseases 2010: working to overcome the global impact of neglected tropical diseases. Geneva 172.
3. Rashed AA, Khalil HH, Morsy AT (2010) Zoonotic ectopic fascioliasis: review and discussion. J Egypt Soc Parasitol 40: 591-608.
4. Mas-Coma S (2005) Epidemiology of fascioliasis in human endemic areas. J Helminthol 79: 207-216.
5. Mas-Coma S (2007) Lymnaea cousinni (Gastropoda: Lymnaeidae) as transmitter of fascioliasis. Mem Inst Oswaldo Cruz 102: 241-242.
6. Mas-Coma S, Valero MA, Bargues MD (2009) Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Vet Parasitol 163: 264-280.
7. WHO (1995) Control of food born trematode infections, Technical sheet.
8. Haseeb AN, el-Shazly AM, Arafa MA, Morsy AT (2002) A review on fascioliasis in Egypt. J Egypt Soc Parasitol 32: 317-354.
9. Brown W, Davis W, Dobbelaere D, Rice-Ficht A (1994) CD4+ T-cell clones obtained from cattle chronically infected with Fasciola hepatica and specific for adult worm antigen express both unre-stricted and Th2 cytokine profiles. Infect Immun 62: 818-827.
10. Molina EC (2005) Serum interferon-gamma and interleukins-6 and -8 during infection with Fasciola gigantica in cattle and buffaloes. J Vet Sci 6: 135-139.
11. Borish LC, Steinke JW (2003) Cytokines and chemokines. J Allergy Clin Immunol 111: S460-475.
12. Clery D, Torgerson P, Mulcahy G (1996) Immune responses of chronically infected adult cattle to Fasciola hepatica. Vet Parasitol 62: 71-82.

13. Espino AM, Rivera F (2010) Quantitation of cytokine mRNA by real-time RT-PCR during a vaccination trial in a rabbit model of fascioliasis. Vet Parasitol 169: 82-92.
14. Hoyle D, Dalton J, Chase-Topping M, Taylor D (2003) Pre-exposure of cattle to drug-abbreviated Fasciola hepatica infections: the effect upon subsequent challenge infection and the early immune response. Vet Parasitol 111: 65-82.
15. Hillyer GV (2005) Fasciola antigens as vaccines against fascioliasis and schistosomiasis. J Helminthol 79: 241-247.
16. Małgorzata K, Depa-Martynów M, Butowska W, Filipiak K, Pawelczyk L, et al. (2007) Human spermatozoa ultrastructure assessment in the infertility treatment by assisted reproduction technique. Arch Androl 53: 297-302.
17. Walker RI (1994) New strategies for using mucosal vaccination to achieve more effective immunization. Vaccine 12: 387-400.
18. Wedrychowicz H, Lamparska M, Kesik M, Kotomski G, Mieszczanek J, et al. (2003) The immune response of rats to vaccination with the cDNA or protein forms of the cysteine proteinase of Fasciola hepatica. Vet Immunol Immunopathol 94: 83-93.
19. Abdel-Rahman EH, Abdel-Megeed KN (2004) Fasciola gigantica: immunization of rabbits with proteins isolated from coproantigen. J Egypt Soc Parasitol 34: 631-642.
20. Abel Rahman EH, Abdel Megeed KN (2005) Cross-protection induced by cross-reactive antigen against Fasciola gigantica and Trichinella spiralis infections. J Egypt Soc Parasitol 35: 281-294.
21. Abdel-Rahman E, Bashtar A, Hassanain M, Hassanain N, Toaleb N (2014a) Evaluation of a vaccine candidate isolated from Fasciola gigantica excretory-secretory products in rabbits. Global Veterinaria 13 : 720-727.
22. Dalton JP, Heffernan M (1989) Thiol proteases released in vitro by Fasciola hepatica. Mol Biochem Parasitol 35: 161-166.
23. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
24. Fagbemi BO, Obarisiagbon IO, Mbuh JV (1995) Detection of circulating antigen in sera of Fasciola gigantica-infected cattle with antibodies reactive with a Fasciola-specific 88-kDa antigen. Vet Parasitol 58: 235-246.
25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
26. Wray W, Boulikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem 118: 197-203.
27. Muro A, Ramajo V, López J, Simón F, Hillyer GV (1997) Fasciola hepatica: vaccination of rabbits with native and recombinant antigens related to fatty acid binding proteins. Vet Parasitol 69: 219-229.
28. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871-874.
29. Hillyer G, Soler De Galanes M, Rodriguez- Perez J, Bjorland J, De Lagrava M, et al. (1992) Use of the falcon assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immunoelectrotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian Altiplano. Am J Trop Med Hyg 46: 603-609.
30. Oldham G (1985) Immune responses in rats and cattle to primary infections with Fasciola hepatica. Res Vet Sci 39: 357-363.
31. Phiri IK, Phiri AM, Harrison LJ (2006) Serum antibody isotype responses of Fasciola-infected sheep and cattle to excretory and secretory products of Fasciola species. Vet Parasitol 141: 234-242.
32. Ghazy A, Abdel-Rahman E, Abd El-Aziz M (2007) Evaluation of ES-F5 serum and milk ELISAs for diagnosis of Fasciola gigantica infection in lactating buffaloes. OJVR 11: 1-11.
33. Sobhon P, Apinhasmit W (1996) Opisthorchis viverrini: the effects of colchicine and cytochalasin B on the adult tegument. Southeast Asian J Trop Med Public Health 27: 312-318.
34. EL-Ahwany E, Rabia I, Nagy F, Zoheiry M, Diab T, et al. (2012) Protective Role of Purified Cysteine Proteinases against Fasciola gigantica Infection in Experimental Animals. Korean J Parasitol 50: 45-51.
35. Anuracpreeda P, Wanichanon C, Chaithirayanon K, Preyavichyapugdee N, Sobhon P (2006) Distribution of 28.5 kDa antigen in the tegument of adult Fasciola gigantica. Acta Trop 100: 31-40.
36. Dixit AK, Yadav SC, Sharma RL (2002) 28 kDa Fasciola gigantica cysteine proteinase in the diagnosis of prepatent ovine fasciolosis. Vet Parasitol 109: 233-247.
37. Dixit AK, Yadav SC, Sharma RL (2004) Experimental bubalian fasciolosis: kinetics of antibody response using 28 kDa Fasciola gigantica cysteine proteinase as antigen. Trop Anim Health Prod 36: 49-54.
38. Abdel-Rahman E, Mohamed A, Abdel-Rahman A, El Shanawany E (2014) The role of Ser-(Arg-Ser-Arg-Ser-GlucNAc) 19-GlucNAc Fasciola gigantica glycoprotein in the diagnosis of prepatent fasciolosis in rabbits. J Parasit Dis.
39. Vosseller K, Trinidad J, Chalkley R, Specht C, Thalhammer A, et al. (2006) O-Linked N-Acetylglucosamine proteomics of postsynaptic density preparations using lectin weak affinity chromatography and mass spectrometry. Mol Cell Proteomics 5: 923-934.
40. Silmon de Monerri N, Nardelli S, Calloway M, Nieves E, Angeletti R (2014) O-GlcNAc occurs in the protozoan parasite Toxoplasma gondii and is part of the histone code (790.4). The FASEB J 28: 790.
41. Dieckmann-schuppert' A, Bause' E, schwarz R (1993) Studies on 0-glycans of Plasmodium-falciparum-infected human erythrocytes Evidence for 0-GlcNAc and 0-GlcNAc-transferase in malaria parasites. J Biochem 216:779-788.
42. el-Bahy MM (1997) Comparative studies on the effect of bithionol, praziquantel and triclabendazole in rabbit's fascioliasis. J Egypt Soc Parasitol 27: 615-616.
43. Espino AM, Hillyer GV (2004) A novel Fasciola hepatica saposinlike recombinant protein with immunoprophylactic potential. J Parasitol 90: 876-879.
44. Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, et al. (2008) Fasciola hepatica leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol Biochem Parasitol 158: 52-64.
45. Zafra R, Buffoni L, Martinez-Moreno A, Perez-Ecija A, Martinez-Moreno F, (2008) A study of the liver of goats immunized with a synthetic peptide of the Sm14 antigen and challenged with Fasciola hepatica. J Comp Pathol 139: 169-176.
46. Reszka N, Cornelissen J, Harmsen M, Bienkowska-Szewczyk K, deBree J (2005) Fasciola hepatica procathepsin L3 protein expressed by a baculovirus recombinant can partly protect rats against fasciolosis. Vaccine 23: 2987-2993.