Journal of the Egyptian Society of Parasitology, Vol.50, No.2, August 2020
J. Egypt. Soc. Parasitol. (JESP), 50(2), 2020: 281 - 292

A STUDY OF IMMUNOTHERAPEUTIC EFFICACY OF *TRICHINELLA SPIRALIS* EXCRETORY-SECRETORY PROTEINS IN MURINE TRICHINELLOSIS

By
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Abstract

Trichinellosis is a serious zoonotic parasitic and globally endemic disease. Benzimidazole derivatives are apparently unable to kill encapsulated larvae and its effectiveness depends on solubility, dosage of therapy, host biotransformation, selectivity patterns as well as onset of treatment after infection. Excretory-secretory (ES) proteins released by *Trichinella* induce a strong immune response when tested as a vaccine. The current study investigated both therapeutic and protective effects of adult worm excretory secretory protein (AW/ES), or larval excretory secretory protein (LES) on *T. spiralis* infected mice, conducted by parasitological, histopathological, immunohistochemical, serological and molecular investigations. Larval excretory secretory protein achieved more significant therapeutic and protective effects than adult worm excretory secretory protein in experimentally infected mice, causing reduction in larval counts and decrease in pathological changes of both muscular and intestinal tissues. It increased serum IgG OD values, decreased Foxp3 expressions and increased the mean cycle thres- hold values of muscular tissues but it had lesser effects on adult worm counts than albendazole.

Key words: Mice, *Trichinella spiralis,* Immunotherapeutic efficacy, Excretory-secretory proteins

Introduction

Trichinellosis is a parasitic zoonosis caused by *Trichinella spiralis,* a serious endemic disease worldwide (CDC, 2017). Human infection commonly occurred by eating raw or undercooked pork containing *Trichinella* larvae. Pork and its products were associated with human trichinellosis outbreaks (Wang *et al*, 2017). The global prevalence of trichinellosis is difficult to be evaluated, but about 11 million people may be infected (Lee *et al*, 2016). Trichinellosis was reported in at least 55 countries (Pozio, 2007), where the annual number of clinical cases was estimated to be 10000 with 0.2% death rate (Got-tstein *et al*, 2009). Life cycle alternates bet-ween enteric and muscle phases (Zhan *et al*, 2013).

Trichnella antigens were divided into the surface, excretory secretory (ES) and residual somatic antigens. Excretory secretory antigens are glycoproteins in nature excreted/ secreted from larvae or adults of *Trichinella.* The major sources of ES products include not only the molecules released from specialized excretory or secretory organs but also the material shed from the cuticular and tegumental surfaces (Zhang *et al*, 2018).

Immunization with ES protein elicits a robust immune response and high protection against trichinellosis. Some studies showed that pig's inoculation with *T. spiralis* larval ES antigens could significantly reduce the adult worm burden. *T. spiralis* ES proteins offer promising targets for vaccines development (Goyal *et al*, 1997). The protective effect was via active lymphocyte proliferative responses & cytokine production. The Th2 type interleukin (IL)-10, IL-4 & IL-5 were the predominant cytokines (Robinson *et al*, 1995a; Quan *et al*, 2004; Yang *et al*, 2018). Administration of efficacious antihelminthic drugs at the stage of intestinal invasion was very important to have a better outcome. Antihelminthic drugs such as mebendazole and albendazole are commonly used in treatment but none was fully effective against the encysted *T. spiralis* or the newborn larvae. So, there was a great need to develop new safe antihelminthic to treat trichinellosis (Saad *et al*, 2016).

Generally, immunotherapy is the therapeu-

tic approach that targets or manipulates the immune system. It controls the host's adaptive and innate immune response to provide a long-lived elimination of diseased cells. It is categorized broadly into passive (adoptive & antibody-based) and active (vaccine therapy & allergen-specific) approaches (Papaioannou *et al*, 2016). Active immunotherapy induces the patient's immune response resulting in development of specific immune effectors (antibodies and T cells) which control the disease (Naran *et al*, 2018).

The present study aimed to evaluate the therapeutic and protective effects of *Trichnella* excretory/secretory (ES) proteins on enteral and muscle phase in *Trichinella spiralis* infected mice.

Materials and Methods

Preparation and isolation of ES antigens: *Trichinella spiralis* strain was obtained from laboratory bred infected albino mice in Parasitology Department, Faculty of Medicine, Tanta University. Infected muscles were cut excised (2cm), and digested with pepsin solution (Pepsin 1g/HCl 1ml/distilled water to 100ml) for 12 hours. Freshly *T. spiralis* larvae were isolated by the stereomicroscope. Twenty mice were infected for ES antigens preparation, each with about 300 *T. spiralis* infective larvae **(**Korenaga *et al*, 2001).

Ten mice were sacrificed on 8th day post infection (dpi), adult worms (AW) were collected from the upper two-thirds (duodenum and jejunum) of small intestine. They were washed five times in PBS with 100U penicillin/ml and 100µg streptomycin/ml and then cultured at 37◦C in RPMI 1640 (medium containing 10% fetal bovine serum) and 5% CO_2 for 18h. After incubation, the supernatant contained AW/ES antigens were obtained by centrifugation at 4°C, 11,000g for 20 min (Wang *et al*, 2017).

At 35th day post infection (dpi), another ten mice were sacrificed; larvae of *T. spiralis* were isolated from mice muscle tissue by artificial digestion, and then washed. Clean larvae were incubated in a CO_2 incubator for 24h at 37°C in Petri-dish contained RPMI-1640 culture medium. Supernatants were collected by centrifugation, and dialyzed to get larval ES antigens (Wang *et al*, 2014).

Ethics Statement: All animals were treated according to Theodor Bilharz Research Institute ethics. They were kept under standard conditions in TBRI animal house maintained on a standard commercial pelleted diet in an air-conditioned room at 20-22°C. All experimental procedures were performed in accordance with the international ethical guidelines approval by TBRI Ethical Committee.

Animals and Study Design: A total of 140 Swiss male albino mice aged two months and weighed 25±0.2gm were used. They were divided into 7 groups of 20 mice each. GI: Non-infected, non-vaccinated and non-treated mice, GII: Infected non-vacciated and non-treated mice, each one received 300 *T. spiralis* infective larvae. GIII: Vaccinated with AW/ES antigen, 100µg in PBS injected subcutaneously 7 days before oral infection with 300 *T. spiralis* muscle larvae (Dea-Ayuela and Bolas-Fernández, 2000). GIV: Vaccinated with larval ES antigen 100µg in PBS injected subcutanously 7 days before oral infection with 300 *T. spiralis* muscle larvae. GV: Infected and treated with 25µg of AW/ES antigen injected intraperitoneally for 7 days starting from the 1st infection day. GVI: Infected and treated with 25µg of larval ES antigen injected intraperitoneally for 7 days starting from the1st infection day (Yang *et al*, 2014). Both GV & GVI were immunotherapy groups.

GVII: Infected and treated with albendazole (suspension 20mg/ml, Egyptian International Pharmaceutical Industries Co.) 50 mg/kg/day orally starting from the 1st infection day for three successive days (Bakir *et al*, 2017). Each group was further subdivided according to the time of scarification into 2 equal subgroups (10 mice each) at the 8th dpi named (SGs Ia, IIa, IIIa, IVa, Va, VIa, VIIa) to evaluate the enteral phase and at the 35th dpi named (SGs Ib, IIb, IIIb, IVb, Vb, VIb, VIIb) to evaluate the muscular phase.

Parasitological assessment was carried out

by isolation and counting of adult worms in the small intestine of all groups at 8th dpi by a microscope ×10 (Quan *et al*, 2008). Also, by collection and counting of larvae from all groups at 35th dpi, larvae were microscopically counted using a McMaster counting chamber (Saad *et al*, 2016).

Histopathological study: Mice of first subgroups were sacrificed at 8th dpi and specimens from small intestine were collected. Mice of second subgroups were sacrificed at 35th dpi and muscles specimens were collected, fixed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (Goyal *et al*, 2013).

Immunohistochemical study: Paraffin sections 4µ thickness from muscle tissue blocks of all groups were stained by immunohistochemical method for Forkhead box (Foxp-3+) T regulatory (T-regs**)** after Eissa *et al*, (2016). Immunohistochemical identification of foxP3+Treg cells was done using rat anti-foxp3 antibody, clone 150D/E4 (eBioscience, Vienna, Austria). Kit was ultravision detection system anti-polyvalent horseradish peroxidase/diaminobenzidine tetra-hydrochloride (HRP/DAB). Muscle tissue sections were deparaffinized, hydrated, subjected to microwave antigen retrieval in citrate buffer for 15min., blocked for endogenous peroxidase, and then they were exposed to primary antibody (antifoxp3) for 3hrs at a dilution of 1:50. Biotinylated goat anti-polyvalent was applied for an hr, and then streptavidin biotin was applied for 20 min. The chromogen used was DAB. A minimum of 3 high power fields (HPFs x400) were counted for each section and positive cells were expressed as mean number of positive cells /HPF. Positive Foxp3 staining was identified when cell membrane alone or together with cytoplasm showed brown staining, whereas, negative was considered without membrane staining.

The staining intensity (Fraser *et al*, 2003) was scored**:** mild=1+, moderate=2+, strong= 3+. The stained slides were evaluated quantitatively using the H-score. Briefly, staining intensity was given a number (1+, 2+ & 3+) for mild, moderate and strong staining respectively. Stained cells percent in each tissue was multiplied by staining intensity. A score of 0-300 was given for stained biomarker after the following equation H score = [1 × (% cells 1+) + 2× (% cells 2+) + 3 × (% cells 3+)].

ELISA for serum total specific IgG level (Arana *et al*, 2012): Before scarification of mice at 35th dpi, blood samples were taken for estimation of serum level of specific indirect ELISA IgG antibodies in sera of mice. Maxsorp 96-well microtitre plates (Nunc, Denmark) were coated with 125µg/ml of ES antigen in carbonate-bicarbonate buffer, pH 9.4 overnight at 4°C. Plates were blocked with 250µl/well of 2% bovine serum albumin diluted in PBS, pH 7.2, with 0.05% Tween-20 (PBS/BSA/T-20) for 2hr at room temperature. Sera were diluted 1:100 in PBS/T-20, loaded into plates (100µl/well), and incubated at room temperature for an hr. After washing, 100µl/well of alkaline phosphatase (AP)-conjugated anti-mouse IgG (Southern Biotech, USA, 1mg/ml each) diluted in PBS/T-20 (1:5000 for IgG) were added for 90min. Reaction developed by adding100µl/well of p-nitrophenyl phosphate substrate (Sigma-Aldrich, UK) and incubation until appearance of yellow color. Absorbance was read at 405 nm using an ELISA reader (Bio-Rad, UK) **(**El-Aswad *et al*, 2019).

Molecular study: By real-time polymerase chain reaction (PCR) for detection of *T. spiralis* larva DNA in muscular tissue (Cuttell *et al*, 2012).

Genomic DNA was extracted from muscular frozen tissue using JET™ Genomic DNA Purification Mini Kit (THERMO SCIENTIFIC, EU/Lithuania). Samples were digested with Proteinase K in lysis solution. The lysate was mixed with ethanol and loaded on the purification column where DNA binds to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the elution buffer. PCR for

T. spiralis gene was evaluated to a total volume of 25ul contained 10ul of genomic DNA, 2.5ul of 10x Taq polymerase buffer, 1.5ul 2mM MgCl2, 0.25ul Taq DNA polymerase (5units/µl) (Genecraft, Germany), 0.5ul of dNTPS (10mM) (Stratagene, USA), 1ul of each primer (20µM) (Midland, Texas) & 8.25ul of H_2O using the primers, Forward primer 5- CATGGTTAGGTGAGATATTG-CCTGC-3, Reverse primer 5-GGTCCTCC-TTCCAGAAGATCTACTTTG-3. Real-time PCR assay was performed on a Rotor-Gene 6000 (Corbett Research) real-time PCR cycler in the Central Lab, Faculty of Medicine, Menoufia University. Total reaction volume was 10µl contained 5µl 2×Sensi-Mix Plus SYBR® Green (Quantace Ltd.). PCR primers were optimized for use at 300nM & 100 ng of DNA was added to each reaction. Samples were always twice PCR replication. Cycling profile included an initial denaturation at 95°C/10min, then 40 cycles of 95°C/ 10 s, 62°C/15 s & 72 ◦C/20 s. Fluorescence was measured in green channel and data collected at the extension step. Cycle threshold (Ct) values were individually calculated by internal software using a manual threshold setting of 0.2 & 'dynamic tube' and 'ignore first 10 cycles' functions were activated. A melt-curve analysis was done after each run in order to match amplicons with positive control melt curve peaks and confirm specificity. Cycle threshold in muscular tissue was inversely proportional to the amount of larval DNA detected by real-time PCR.

Statistical analysis: Data were coded, tabulated and analyzed using SPSS (20), IBM Corp. Released 2011 (IBM SPSS Statistics for Windows, Version 20.0). Armonk, NY: IBM Corp. Data were of quantitative type, expressed in mean and standard deviation (SD). Mann Whitney's test was used for comparison of quantitative variables between two groups. Post Hoc Value was used to assess the difference in two means of two individual groups. Significance level was 95%, so, p-value >0.05 was not significant difference; p-value < 0.05 was considered a statistically significant difference.

Results

A significant reduction of *T. spiralis* adult worm count was recorded in all vaccinated and treated subgroups as compared to infected subgroup. There was a significant difference between both vaccinated SGs (IIIa & Iva respectively) (P6<0.05) and a significant difference between both groups treated with ES (Va & VIa respectively) (P13 < 0.05). There was a significant difference between albendazole treated SG VIIa & both treated SGs with AW/ES (Va) & larval ES antigens (VIa) (P14& P15<0.001) & vaccinated SGIIIa with AW/ES (P9 <0.001), but when comparing albendazole treated SG VIIa with vaccinated SG IVa with larval ES antigen, without significant difference (P12 >0.05). A significant difference was between vaccinated SGIVa with larval ES & treated SGVa with AW/ES (P10 <0.001), but without significant difference with treated with larval ES SGVIa (P11 >0.05). But, no significant difference was between vaccinated SGIIIa by AW/ES with treated SGs with AW/ES or larval ES (Va & VIa respectively) (P7>0.05& P8 >0.05).

Larval count, a significant reduction in *T. spiralis* larval percent was in all vaccinated (IIIb & IVb) and treated (Vb & VIb) as compared with infected (SGIIb). Also, a significant difference was between all vaccinated and treated mice, except difference between AW/ES treated SGVb & albendazole treated SGVIIb was non-significant (P14 >0.05).

Histopathological results**:** There was a significant decrease in the inflammation intensity in intestinal tissues in all vaccinated and treated groups as compared to infected control (SGIIa). Also, significant differences were detected between albendazole treated (SGVIIa) and both SGIIIa vaccinated with AW/ES antigen (P9<0.05) & SGVa treated with AW/ES antigen (P14<0.05).

Histopathological results: muscle tissues showed a decrease in intensity of larval deposition in all vaccinated and treated subgroups with significant differences between

them and infected non-vaccinated non-treated (SGIIb). There were significant differences between vaccinated with larval ES antigen (SGIVb) & treated with AW/ES antigen (SGVb) (P10 <0.05) and albendazole treated (SGVIIb) (P12<0.05). There were significant differences between larval ES antigen treated (SGVIb) and those treated with AW/ES antigen (SGVb) (P13< 0.05) or albendazole treated SGVIIb (P15< 0.05).

Immunohistochemical results: Highest % of FoxP3 expression and intensity (H-Score) was in infected non-treated (SGIIb) (136± 9.66). Differences among subgroups were significant except mice vaccinated with larval ES antigen (SGIVb) and larval ES treated (SGVIb) was non-significant (P11>0.05).

Serological results: Serum IgG OD values in vaccinated subgroups showed the highest one was in subgroup vaccinated with larval ES antigen SGIVb (0.69±0.094), followed by vaccinated with AW/ES antigen SGIIIb (0.5760±0.11). In treated subgroups, highest serum IgG OD value was in mice treated with larval ES antigen SGVIb (0.39±0.056) followed by mice treated with AW/ES antigen SGVb (0.29±0.06) then albendazole treated mice SGVIIb (0.15±0.045). There were significant differences between all subgroups except between the infected SGIIb and albendazole treated SGVIIb.

Real time PCR: Value of cycle threshold was inversely proportional to DNA concentration. There was significant reduction of *T. spiralis* larval DNA concentration in vaccinated SGIIIb & SGIVb. Cycle threshold highest value was in mice vaccinated with larval ES protein SGIVb (35.6±1.32), but mice vaccinated with adult ES protein SG IIIb the cycle threshold value was (29.95± 0.00). As to treatment, highest value was in mice treated with larval ES protein (SGVIb) (30.8±0.042). In mice treated with albendazole (SGVIIb), cycle threshold value was (23.57±0.013) but mice treated with adult ES antigen (SGVb) was 15.2±0.08, with significant reductions of *T. spiralis* larval DNA concentrations in all vaccinated and treated subgroups as compared to SGIIb. Details were given in tables (1, 2 &3) and figures

Table 1: Comparison of the mean count of *T. spiralis* adult worm at 8th dpi among all groups:

Groups (n=10 each)	Adult count			Reduction %	Mann Whitney test	P.value
	M± SD	Median	Range			
SGIIa (Infected group)	50.2±5.09	50	40-55	0%	IIa *vs* IIIa =3.814	P1<0.001**
					IIa *vs* IVa =3.822	P2<0.001**
SGIIIa (Vaccinated with AW/ES & infected)	8.2±3.4	9.5	0-12	83.7%	IIa *vs* Va =3.817	P3<0.001**
					IIa *vs* VIa =3.810	P4<0.001**
SGIVa (Vaccinated with L/ES & infected)	4.2±3.1	5	0-8	91.6%	IIa *vs* VIIa =3.857	P5<0.001**
					IIIa *vs* IVa =2.758	P6<0.05*
SGVa (Infected & treated with AW/ES)	10±2.35	10.5	5-12	80.1 %	IIIa *vs* Va =1.469	P7>0.05
					IIIa *vs* VIa = 1.657	P8 >0.05
SGVIa (Infected & treated with L/ES)	6.6±2.45	6	2-10	86.9%	IIIa *vs* VIIa = 3.340	P9 <0.001**
					IVa *vs* Va =3.307	P10 <0.001**
SGVIIa (Infected & treated with Albendazole)	1.7±2.35	0	0-5	96.6%	IVa *vs* VIa =1.818	P11 >0.05
					IVa *vs* VIIa =1.867	P12 >0.05
					Va *vs* VIa =2.608	P13<0.05*
					Va *vs* VIIa =3.747	P14<0.001**
					VIa *vs* VIIa =3.368	P15<0.001**

P1: comparison between SGIIa & IIIa, P2: between IIa & IVa, P3: between IIa & Va, P4: between IIa & VIa, P5: GIa & VIIa, P6: between IIIa & IVa, P7: between IIIa & Va, P8 between IIIa & VIa, P9: between IIIa & VIIa, P10: between IVa &Va, P11: between IVa & VIa, P12: between IVa & VIIa, P13: between Va & VIa, P14: between Va & VIIa, P15: between VIa & VIIa.

Table 2: Comparison of mean count of *Trichinella spiralis* larvae at 35th dpi among all groups:

Groups (n=10 each)	Larval count			Reduction %	Mann Whitney test	P. value
	Mean± SD	Median	Range			
SGIIb (Infected group)	101650±9945.6	100200	90000-120000	0	IIb vs b =3.785 IIb vs IVb =3.79 IIb vs Vb =3.787 IIb vs VIb =3.79 IIb vs VIIb =3.784 IIIb vs IVb =3.375 IIIb vs Vb= 2.245 IIIb vs VIb =3.192 IIIb vs VIIb =2.113 IVb vs Vb =3.576 IVb vs VIb =2.975 IVb vs VIIb =3.429 Vb vs VIb =3.094 Vb vs VIIb =1.634 VIb vs VIIb =3.091	P1<0.001** P2<0.001** P3<0.001** P4<0.001** P5<0.001** P6<0.001** P7<0.05* P8<0.001** P9<0.05* P10<0.001** P11<0.001** P12<0.001** P13<0.001** P14>0.05 P15<0.001**
SGIIIb (Vaccinated with AW/ES & infected)	32350±2505.6	31150	30000-7000	68.2%		
SGIVb (Vaccinated with L/ES & infected)	3972±1681.6	3650	1200-5000	96.1%		
SGVb (Infected & treated gr with AW/ES)	37130±1965.28	37100	35000-40000	63.5%		
SGVIb (Infected & treated with L/ES)	12230±6002.97	12600	5000-20000	87.96%		
SGVIIb (Infected & treated with Albendazole)	35760±1742.41	35100	33000-38200	64.8%		

P1: comparison between Subgroups IIb & IIIb, P2: between IIb & IVb, P3: between IIb & Vb, P4: between IIb & VIb, P5: IIb & VIIb, P6: between IIIb & IVb, P7: between IIIb & Vb, P8: between IIIb & VIb, P9: between IIIb & VIIb, P10: between IVb & Vb, P11: IVb & VIb, P12: between IVb & VIIb, P13: between Vb & VIb, P14: between Vb & VIIb, P15: between VIb & VIIb.

Table 3: Comparison of mean of IgG OD serum values among all groups at the 35th dpi:

Groups (n=10 each)	Serum IgG (OD at 450 nm)			MannWhitney Test	P. value
	Mean± SD	Median	Range		
SGIb (Normal control)	0.065	0.065	0.065	IIb *vs* IIIb =3.787 IIb *vs* IVb =3.804 IIb *vs* Vb =2.395 IIb *vs* VIb =3.732 IIb *vs* VIIb =1.469 IIIb *vs* IVb =2.369 IIIb *vs* Vb =3.767 IIIb *vs* VIb =3.326 IIIb *vs* VIIb=3.803 IVb *vs* Vb =3.822 IVb *vs* VIb =3.783 IVb *vs* VIIb =3.824 Vb *vs* VIb =2.867 Vb *vs* VIIb =3.419 VIb *vs* VIIb=3.802	P1<0.001** P2<0.001** P3 <0.05* P4<0.001** P5>0.05 P6<0.05* P7<0.001** P8<0.001** P9<0.001** P10<0.001** P11<0.001** P12<0.001** P13 <0.05* P14<0.001** P15<0.001**
SGIIb (Infected)	0.1950±0.074	0.2	0.1- 0.3		
SGIIIb (Vaccinated with AW/ES & infected)	0.5760±0.11	0.6	0.4- 0.73		
SGVb (Vaccinated with L/ES & infected)	0.69±0.094	0.7	0.5- 0.8		
SGVb (Infected and treated with AW/ES)	0.29±0.06	0.30	0.20-0.40		
SGVIb (Infected and treated with L/ES)	0.39±0.056	0.40	0.30-0.50		
SGVIIb (Infected and treated with Albendazole)	0.15±0.045	0.14	0.10-0.20		

P1: comparison between Subgroups IIb & IIIb, P2: between IIb & IVb, P3: between IIb & Vb, P4: between IIb & VIb, P5: IIb & VIIb, P6: between IIIb & IVb, P7: between IIIb & Vb, P8: between IIIb & VIb, P9: between IIIb & VIIb, P10: between IVb & Vb, P11: between IVb & VIb, P12: between & VIIb, P13: between Vb & VIb, P14: between Vb & VIIb, P15: between VIb & VIIb.

Discussion

Trichinellosis is a zoonotic disease associated with severe neurological, ocular and cardiovascular complications and may end fatally. Classical medical treatment included mebendazole or albendazole had a limited effect in treating the muscular phase of *T. spiralis* (Othman and Shoheib, 2016).

The present study evaluated the protective and therapeutic effects of *Trichnella* ES proteins on enteral and migratory phase of trichinellosis**.** The protective effect was previously evaluated by many authors, but the therapeutic effect was no used as a therapeutic agent. Crude ES antigens were tested as a vaccine either alone or with adjuvant by many authors (Robinson *et al*, 1995a; Dea-Ayuela and Bolas-Fernández, 2000; Quan *et al*, 2004). These authors reported that immunization might elicit an effective immune response, resulting in complete protection against infective larvae. Besides, crude ES antigens at variable molecular size had been investigated as immunogens against *T. spiralis* infection and showed effective immune actio in reduction of worm burden (Robinson *et al*, 1995b; Lightowlers and Rickard, 1998; Nagano *et al*, 2008; Bi *et al*, 2015).

In the current study, there was significant reduction of the mean count of adult worms in all vaccinated and treated groups in comparison with the infected group. The better

outcome was in albendazole treated group followed by the group vaccinated with larval ES. Also, significant *T. spiralis* larval reduction percent was observed in all vaccinated and treated groups in comparison with the infected group. The better outcome was in the group vaccinated with larval ES. The results agreed with Cvetkovic *et al*. (2016) who reported significant effects of several components of excretory-secretory antigens on dendritic cells in vitro and Lee *et al.* (2016) who tested protective effect of virus-like particles containing the 53 KDa excretory/secretory proteins of *T. spiralis* and the influenza matrix protein 1 as a core protein, and reported that (ES) proteins played a critical role in modulating host immune system, and elicited a strong immune response and high protection against *T. spiralis* infection.
Also, the present results agreed with Quan *et al*. (2004) who found that immunization of rats with ES Ag of *T. spiralis* Korean isolate without any adjuvant elicits effective protective immunity against challenge infection. The protective effects involved both adults and larvae and *T. spiralis* ES proteins offer promising targets for vaccines development.
The therapeutic effects of ES products of *T. spiralis* were tested in the amelioration of autoimmune, allergic and malignant diseases in vitro and in animal models (Liao *et al*, 2018). Also, ES products used for treatment of colitis (Smith *et al*, 2007; Yang *et al*, 2014), experimental autoimmune encephalomyelitis (Vukman *et al*, 2016), type 1 diabetes (Liu *et al*, 2009) and rheumatoid arthritis (Pineda *et al*, 2012). The results also agreed with Roatt *et al*. (2017**)** who evaluated the therapeutic vaccine of antigens of *Leishmania braziliensis* associated with monophosphoryl lipid A adjuvant for treatment of visceral leishmaniasis, and found that immunotherapy was a significant approach to be used as potential treatment strategy.

In histopathological results of intestinal tissue, there were decrease in the intensity of inflammation in intestinal tissues and larval deposition in muscular tissues of all vaccinated group and treated groups compared to infected non-vaccinated non-treated mice. This agreed with Eissa *et al*. (2003) who used autoclaved *T. spiralis* larval vaccine in combined with BCG as an adjuvant and tested histopathological changes. They found sharp reduction in number of muscle encysted larvae, and were hardly seen as hyalinized cyst surrounded by precystic fibrosis & mild chronic inflammatory infiltrate, with different inflammatory infiltration surrounding muscle. Also**,** Yang *et al*. (2014) found that AW/ES treatment decreased the damage score for induced mouse colitis. AW/ES treatment reduced epithelial destruction, edema, and infiltration of inflammatory cells on the colon histological sections.

In the current study, the highest intensity of FoxP3 expression was in infected non-treated mice. Kang *et al*. (2012) and Cho *et al*. (2012) reported enhanced production of IL-10, transforming growth factor beta (TGF-β) and proliferation of T reg cells in *T. spiralis* infected mice. The present data agreed with Ahn *et al*. (2016) who studied the T reg cells distribution in mice intestine and muscle tissues during *T. spiralis* life cycle. They found the number of activated T reg cells did not change in muscle tissue for 0-1 week after infection, and then rapidly increased around the parasite to 4th week of infection when nurse cells were fully developed. Also, The present results agreed with Guo *et al*. (2016) where BALB/c mice were immunized with recombinant *T. spiralis* paramyosin & CD4+ CD25+Foxp3+ T cells were sorted in immunized mice splenocytes and immunization did not increase in CD4+CD25+Foxp3+ T cells. However, Radovic *et al*. (2015) found that the percentage of CD4+CD25+Foxp3+ T cells was higher in the larval ES immunized group than in control one. The present results disagreed with Xu *et al*. (2018) who studied the protective and therapeutic role of 2 recombinant *T. spiralis* serine protease inhibitors on induced experimental colitis in mice model, and found percentage of CD4+ CD25+Foxp3+T reg cells in CD4+ T lymph-

ocytes in spleen and mesenteric lymph nodes were higher than in control ones. This difference may be due to different in studied tissues or different protein used.

In the present study, the highest IgG OD value was in mice vaccinated with larval ES antigen (0.69±0.094), followed by mice vaccinated with AW/ES antigen (0.5760±0.11). In treated groups, t highest serum IgG OD value was in mice treated with larval ES antigen (GVI) (0.39±0.056) followed by mice treated with AW/ES antigen (GV) (0.29±-0.06) then albendazole treated mice (GVII) (0.15±0.045), with significant differences between all groups except between the infected (GII) and albendazole treated (GVII) was non-significant (P5>0.05).

The present results agreed with Bi *et al*. (2015) who reported that mice immunized with one 20-kDa protein secreted by *T. spiralis* muscle larvae and adults ES induced protection associated with a high IgG antibody level, as well as increased total IgG, IgG1 & IgG2a subtypes. Humoral response contributed greatly to resistance against trichinellosis by entrapping and expulsing infective larvae, reducing adults' fecundity & eliminating newborn larvae. Also, intramuscular immunization of mice with virus-like particles (VLPs) contained 53 kDa ES antigen of *T. spiralis* plus cholera toxin (CT) adjuvant induced specific IgG, IgG1 & IgG2a antibody responses and significantly reduce worm burden (Lee *et al*, 2016).

The present results agreed with Kołodziej-Sobocińska *et al*. (2006) and Dvorožňáková *et al*. (2010) who found that IgG was the most abundant immunoglobulin in mice and humans. During trichinellosis, IgG- antibodies were involved in inflammatory response to infection, showing an increase during the muscular phase (Pinelli *et al*, 2007).

As to real time PCR results of this study, there was a decrease in the amount of larval DNA in muscular tissues of all vaccinated and treated groups detected by real-Time PCR. The better outcome was reported in mice vaccinated with larval ES protein.

The present results agreed with Quintana *et al*. (2016) who used specific primers for detection of *T. spiralis* DNA in muscle by real-time PCR. It has been suggested that specific PCR for *T. spiralis* may be useful for detection of infection at early stages in humans and food animals. The present results agreed with Cuttell *et al*. (2012) who used the same primers to measure cycle threshold, and selected to target a conserved region of gene allowing putative amplification of a 195bp fragment of *Trichinella*. Real-time PCR allowed not only qualitative detection and/or quantitative measurement of parasite DNA, but also the potential to simultaneously differentiate isolates to the species or genotype level. DNA extraction method is sensitive and specific to detect *Trichinella* larvae in muscle tissue. This agreed with Tantrawatpan *et al*. (2013) who used a developed probes-based real-time FRET PCR combined with a melting curve analysis to detect *T. spiralis* DNA sequence for mitochondrial small-subunit ribosomal RNA (rRNA) directly in muscle tissue from *T. spiralis* experimentally infected mice by specific prim ers, which proved be used for differentiation of *T. spiralis*, *T. pseudospiralis*, and *T. papuae* by the different Tm values.

Conclusion

Larval excretory secretory protein has both therapeutic and protective effects on experimental trichinellosis through causing reduction in adult, larval counts and decrease in pathological changes of both muscular and intestinal tissues. Besides, it increased serum IgG OD values, decreased Foxp3 expressions and increased the mean cycle threshold values of muscular tissues.

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Explanation of figures

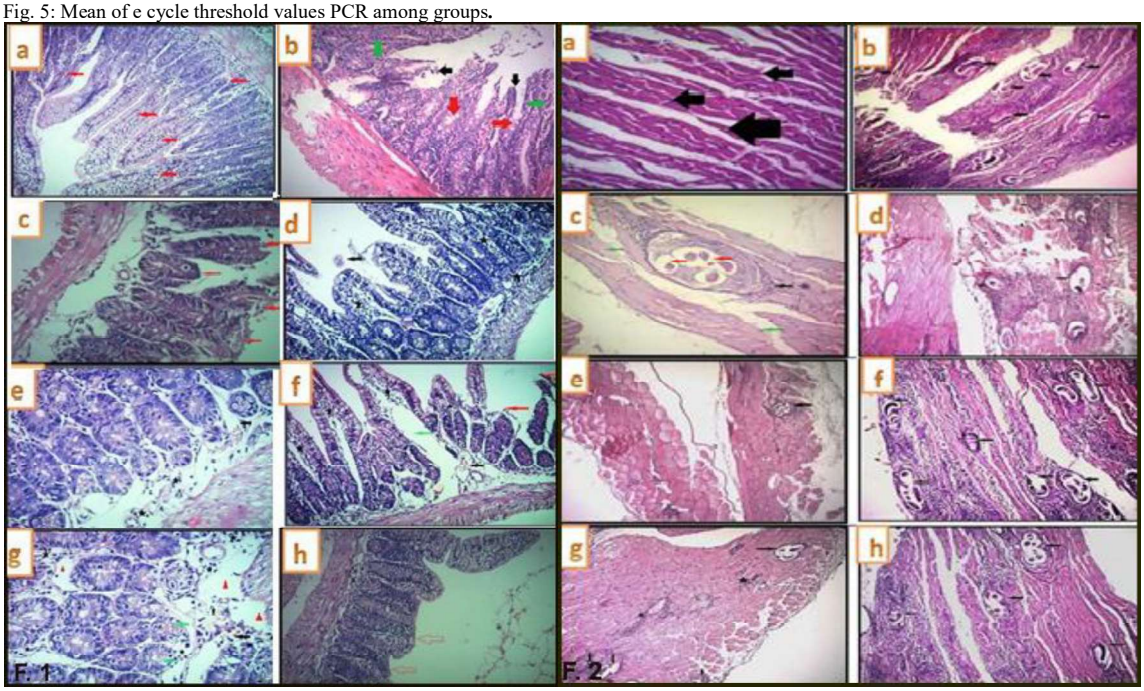
Fig. 1: Histopathology of intestinal tissues. a- Transverse section in intestinal tissue normal mice (GIa), showed normal leaf like villi and crypts (red arrow) (H&E) (x100), b- TS in intestinal tissue of infected mice (GIIa), showed severe atrophy and degeneration of crypts (black arrow), severe inflammatory infiltrate (green arrow) and goblet cell hyperplasia (red arrow) (H&E) (x100), c- TS in intestinal tissue of infected mice (GIIa) showed severe atrophy and degeneration of villi and crypts (red arrow) (H&E) (x200), d- TS in intestinal tissue of AW/ES vaccinated (GIIIa) showed moderate atrophy and degeneration of some villi (black arrow), moderate inflammatory infiltrate of villi (star) (H&E) (x400), e- TS in intestinal tissue of LES vaccinated (GIVa) showed mild to moderate inflammatory infiltrate (star) and moderate congestion (black arrow) (H&E) (x400), f- TS in intestinal tissue of AW/ES treated (GVa) showed moderate to severe atrophy and degeneration of some villi (red arrow), inflammatory infiltrate in corium of villi (star), oedema (green arrow) and congestion (black arrow) (H&E) (x400), g- TS in intestinal tissue of LES treated (GVIa) showing moderate degeneration of crypts (green arrow), inflammatory infiltrate (star), oedema (red arrow) and congestion of blood vessels (black arrow) (H&E) (x400), and h-TS in intestinal tissue of albendazole treated group (GVIIa) showing mild atrophy and degeneration of some villi (red arrow) and mild inflammatory (H&E) (x400).

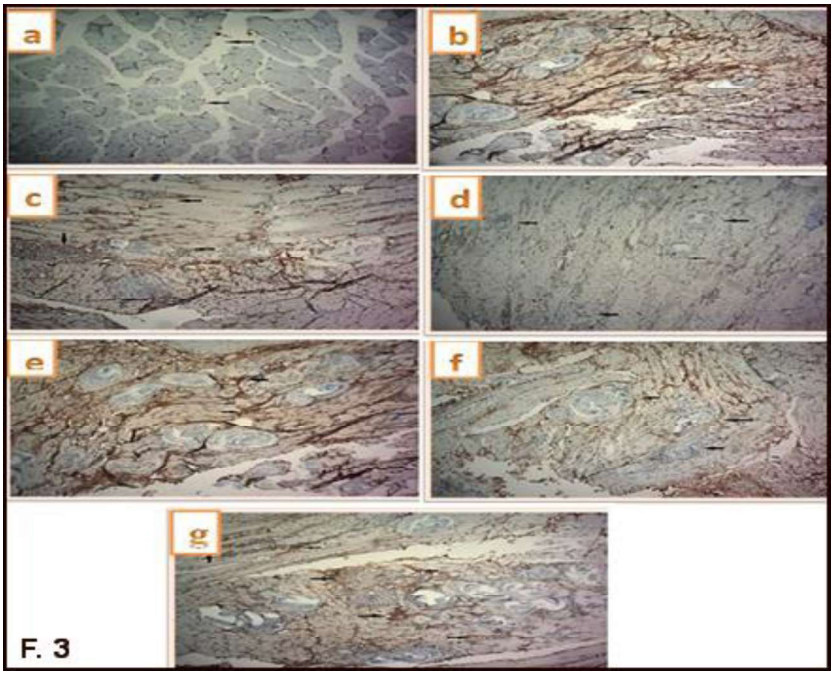
Fig.2: Histopathology of muscle tissues: a- TS in skeletal muscle tissue of normal (GIb), showed normal appearance of muscle fibers, average distribution of peripherally situated and flattened nuclei (black arrows) (H& E) (x100), b- TS in skeletal muscle tissue of infected g (GIIb), showed heavy larval deposition (+3) surrounded by dense inflammatory reaction (+3) (black arrows) (H& E) (x100), c- TS in skeletal muscle tissue of infected (GIIb), showed larval deposition (red arrows) surrounded by dense inflammatory reaction (black arrows) (+3) with degeneration of fibers (green arrow) (H& E) (x200), d- TS in skeletal muscle tissue of AW/ES vaccinated (GIIIb), showed moderate larval deposition (+2) surrounded by moderate inflammatory reaction (black arrows) (H& E) (x100), e- TS in skeletal muscle tissue of LES vaccinated (GIVb), showed mild larval deposition (+1) surrounded by mild inflammatory reaction (black arrows) (H& E) (x100), f- TS in skeletal muscle tissue of AW/ES treated (GVb), showed moderate larval deposition (+2) surrounded by intense inflammatory reaction (black arrows) (H& E) (x100), g- TS in skeletal muscle tissue of LES treated (GVIb), showed mild larval deposition (+1) (black arrows) surrounded by moderate inflammatory reaction (star) (H& E) (x100), and h-TS in skeletal muscle tissue of albendazole treated GVIIb), showed moderate larval deposition (+2) surrounded by moderate inflammatory reaction (black arrows) (H& E) (x100).

Fig.3. Comparison regarding FoxP3 expression: a- TS in skeletal muscle tissue stained with immunohistochemical stain of normal (GIb) showed negative FoxP3 stain expression (black arrows) (x100), b- TS in skeletal muscle tissue of infected (GIIb) showed strong FoxP3 (+3) expression (black arrows) (x100), c- TS in skeletal muscle tissue of AW/ES vaccinated (GIIIb) showed mild to moderate FoxP3 expression (black arrows) (x100), d- TS in skeletal muscle tissue of LES vaccinated (GIVb) showed very mild FoxP3 (+1) expression (black arrows) (x100), e- TS in skeletal muscle tissue of AW/ES treated (GVb) showed moderate FoxP3 (+2) expression (black arrow) (x100), f- TS in skeletal muscle tissue of LES treated (GVIb) showed mild FoxP3 (+1) expression (black arrows) (x100), and g-TS in skeletal muscle tissue stained with immunohistochemical stain of albendazole treated (GVIIb) showed moderate FoxP3 (+2) expression (black arrows) (x100).

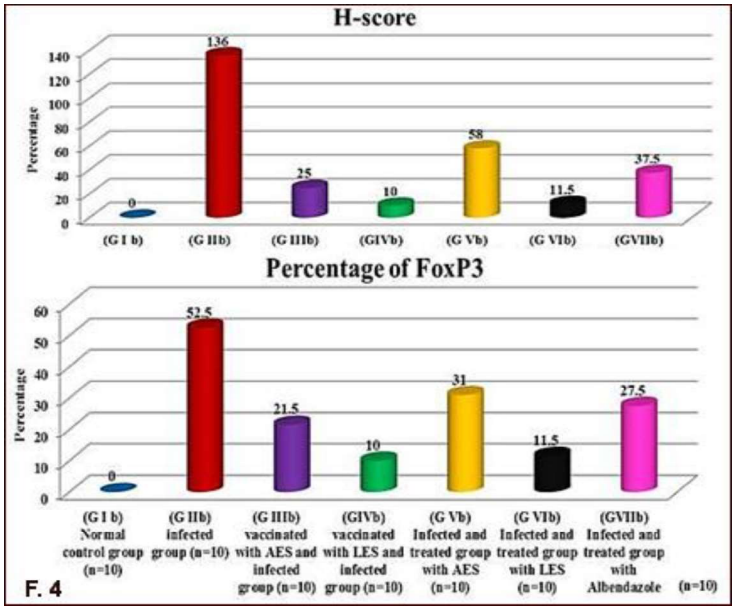
Fig 4: H score and percentage of FoxP3 expression in muscular tissue among groups

Fig. 5: Mean of e cycle threshold values PCR among groups**.**

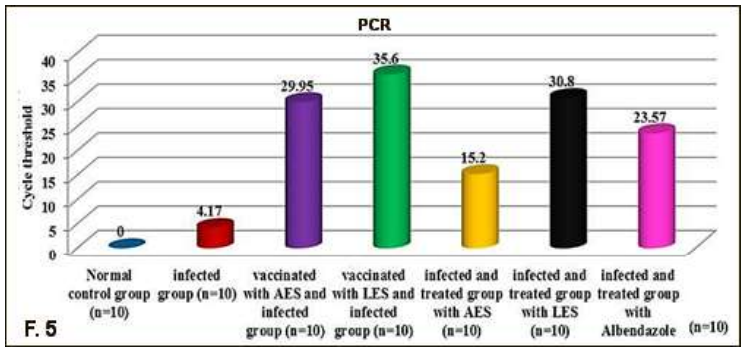



a
b
c
d
e
f
g

F. 3


H-score
Percentage
140
120
100
80
60
40
20
0
136
0
25
10
58
11.5
37.5
(G I b)
(G IIb)
(G IIIb)
(GIVb)
(G Vb)
(G VIb)
(GVIIb)
Percentage of FoxP3
Percentage
60
50
40
30
20
10
0
52.5
0
21.5
10
31
11.5
27.5
(G I b) Normal control group (n=10)
(G IIb) infected group (n=10)
(G IIIb) vaccinated with AES and infected group (n=10)
(GIVb) vaccinated with LES and infected group (n=10)
(G Vb) Infected and treated group with AES (n=10)
(G VIb) Infected and treated group with LES (n=10)
(GVIIb) Infected and treated group with Albendazole (n=10)

F. 4


PCR
Cycle threshold
40
35
30
25
20
15
10
5
0
0
4.17
29.95
35.6
15.2
30.8
23.57
Normal control group (n=10)
infected group (n=10)
vaccinated with AES and infected group (n=10)
vaccinated with LES and infected group (n=10)
infected and treated group with AES (n=10)
infected and treated group with LES (n=10)
infected and treated group with Albendazole (n=10)

F. 5