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THERAPEUTIC EFFICACY OF CHITOSAN NANOPARTICLES AND ALBENDAZOLE IN INTESTINAL MURINE TRICHINELLOSIS

By

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Abstract

Most of the drugs used for the treatment of trichinellosis showed a limited bioavailability and a high degree of resistance. Therefore, there is an urgent need to develop new agents to improve the bioavailability of these drugs. This work assessed the use Chitosan (CH) nanoparticles singly or combined with albendazole (ABZ) for the treatment of experimental trichinellosis. Fifty male albino mice were used. They were divided into six experimental groups. Chitosan nanoparticles were used orally at a dose of 100mg/kg/day starting from 3rd dpi for three successive days for the treatment of the intestinal phase of infection either alone or loaded with full dose or half dose of ABZ. Results showed significant reduction in all treated groups with the highest reduction rate of adult 99.1%, improvement of the intestinal histopathological changes and a significant decrease in iNOS expression of the intestinal tissue were observed in groups treated by CH loaded with full dose of ABZ compared to control group

Key words: *Trichinella spiralis*, Albendazole, Chitosan Nanoparticles, Nitric oxide, SEM.

Introduction

Trichinellosis is a foodborne zoonotic parasite caused by *Trichinella spiralis*, which has a wide range of hosts including man (Ashour and Elbakary, 2011). It is widely distributed all over the world in most climates except for deserts with a burden of approximately 10,000 people per year and 0.2% mortality rate (Garcia *et al*, 2014). Humans acquire the infection by ingesting raw or insufficiently cooked meat of pigs or other animals containing *Trichinella* larvae. Following the ingestion of infected meat, *Trichinella* larvae are released from their capsules to invade the epithelial cells of the upper part of the small intestine and mature into adult worms. In 2–3 weeks, the fertilized females produce 1500 newborn larvae that migrate via the blood and lymphatic systems to invade and encapsulate in the skeletal muscles (Saad *et al*, 2016). Within the skeletal muscle, the *Trichinella* larvae induced the myocyte to transform into a new cell type called nurse cell, which maintains the life of the larvae for months to years (Despommier, 2009).

The life cycle of *T. spiralis* consists of two phases; enteral and muscular (Abou Rayia *et al*, 2017). The enteral phase is manifested by abdominal symptoms and gastroenteritis with diarrhea and abdominal pain (Wilson *et al*, 2015). It is usually diagnosed as acute food poisoning (Yu and Qi, 2015), while, the muscular phase is manifested by periorbital edema, myalgia and muscle weakness (Gottstein *et al*, 2009). Low-intensity of infection can remain asymptomatic but parasite burdens greater than a few hundred larvae can initially cause gastroenteritis with diarrhea and abdominal pain approximately two days post infection (p.i.) (Gottstein *et al*, 2009).The administration of efficacious anthelmintic drugs at the stage of intestinal invasion is remarkably important to obtain a better outcome. Though anthelminthics such as mebendazole and ABZ are commonly used to treat it but none of these drugs are fully effective against the encysted or newborn larvae of *T. spiralis* have a high degree of resistance and a weak activity against encapsulated larvae (Garcia *et al*, 2014). Also, it was poorly water soluble and highly lipophilic drug and consequently, it can exhibit unfavorable bioavailability after oral administration, leading to variable degree of oral absorption (Piccirilli *et al*, 2014).

Nanoparticles have been used widely in previous studies as vehicles to deliver drugs or vaccines, to improve their therapeutic efficacy (Jiang *et al*, 2013; Gaafar *et al*, 2014). Chitosan (CH) is a natural polysaccharide produced by deacetylation of chitin (Abdel-Latif *et al*, 2017). The chitosan has anti-inflammatory and antimicrobial activities due to its role in stimulating cellular, humoral immune response, strong inducer for mixed Th1/2 responses and for type I interferon (Neimert-Andersson *et al*, 2011; Xia *et al*, 2015). It has several properties, a) non-toxic, b) with various molecular weights, c) ability to form complexes with DNA as it effectively protects DNA from degradation, d) ability to enhance the penetration of large molecules across the mucosal surface, e) antibacterial and f) capability to be taken up by the payer's patches (Ali and Ahmed, 2018). Ghaly *et al*. (2019) found that crustacean derive chitosan have anticancer activity by induced cell toxicity that was cell type and concentrations dependent. Also, cell cycle, cell arrest profile was accompanied with the up and down regulation of the pro-apoptotic and the anti-apoptotic gene

The aim of the present study was to assess the use of chitosan nanoparticles alone or loaded with albendazole full and half dose in the treatment of mice experimentally infected with *T. spiralis* during the intestinal phase of the parasite cycle.

Materials and Methods

A total of 50 laboratory-bred free parasites, Swiss Albino mice weighing 18–20 g each, were used. Experimental animals were obtained from the animal house of Theodore Bilharz Research Institute, Giza, Egypt. Mice were fed on a standard pellet diet and water. The isolate of *T. spiralis* used was originally obtained from infected pork from Cairo abattoir and maintained in the laboratory of Medical Parasitology Department, Tanta Faculty of Medicine by consecutive passages in rats and mice. *Trichinella* isolate used was genotyped as *T. spiralis* by the European Union Reference Laboratory for Parasites, Superior Institute of Health, Rome, Italy. They maintained in accordance with the institutional and national guide lines and were kept in the animal house exposed to 12 hours light / 12 hours dark and fed on standard diet and tap water. Stool examination was done prior to study to be sure that mice were free from any intestinal parasitic infection. Mice were orally infected with 200 *T. spiralis* larvae/mouse (Dunn and Wright, 1985) and maintained in accordance with the institutional and national guidelines.

Drug administration: Albendazole drug was purchased as Alzental (Epico) from the pharmacy. One tablet (100mg) was dissolved in 50ml distilled water and given orally in a dose of 50mg/kg/day (Attia *et al*, 2015). Chitosan nanoparticles: degree of deacetylation 93%. From Sigma-Aldrich, USA, was used as a solution, given orally in a dose of 100 mg/kg/day. Chitosan nanoparticles loaded with either full dose or half dose of ABZ, was administered orally in a dose of 100 mg/kg/day (Akhtar *et al*., 2012).

Experimental design and sampling: The experimental animals were divided into six groups: GI (control negative) included 5 mice, GII (control positive) included 5 mice, GIII (infected treated by ABZ) contained 10 mice, GIV (infected treated by CH nanoparticles) contained 10 mice, GV (infected treated by CH nanoparticles loaded with full dose of ABZ) contained 10 mice and GVI (infected treated by CH nanoparticles loaded with half dose of ABZ) contained 10 mice. The drugs were given three days post infection in all groups for three consecutive days and mice were sacrificed on 6^{th} day post infection to evaluate the drugs action on the intestinal phase. Small intestines were dissected out, opened longitudinally, washed and one piece of the middle third of the ten mice were stored in 10 % formalin for histopathological & immunohistochemical studied. *T. spiralis* adults were counted, isolated and preserved in glutraldehyde solution for SEM study.

T. spiralis adult count in the intestine: The

washed intestine was cut into small pieces 1 cm each and incubated at 37°C in a 250ml beaker containing 100ml of Hanks' balanced salt solution for 2hrs to allow the worms to migrate out of the tissue and collect in the container. The solution was pipetted and the intestine was washed several times with saline. All fluid was collected in tubes and centrifuged at 1500rpm for 5min. To count worms, the supernatant was decanted and the sediment reconstituted in 3-5 drops of saline and then examined drop by drop at a magnification of ×10 (Issa *et al*, 1998).

Efficacy of treatment (%) = 100 x mean No. recovered in controls minus mean number recovered in treated mouse/ mean No. recovered in controls (Ashour *et al*, 2016).

Histopathological examination: For histopathological examination, intestinal specimens (1 cm from the small intestine at the junction of the proximal 1/3 and distal 2/3) were taken from mice sacrificed on the 7^{th} dpi. These specimens were fixed in 10% formalin, dehydrated, cleared and then embedded in paraffin blocks. Formalin-fixed, paraffin-embedded sections (5µm thickness) were prepared and stained with hematoxylin and eosin (Nassef *et al*, 2010).

Immunohistochemical staining of iNOS: The iNOS protein was analyzed by immunehistochemical staining using avidin biotin immunoperoxidase complex technique (Ultravision Plus Detection System antipolyvalent HRP/DAB, ready to use; Thermo Scientific Corporation, USA). Immunohistochemistry was performed according to the manufacturing protocol. Tissue sections (4-µm thick) of the previously formalin-fixed, paraffin-embedded specimens were cut and mounted. The sections were de-paraffinised and rehydrated in graded alcohol and endogenous peroxidase was blocked by using 3% hydrogen peroxide in methanol for 5min. For antigen retrieval, slides were immersed in a citrate buffer and put in the microwave for 8min. Samples were then incubated for 1 hr at room temperature with iNOS (rabbit polyclonal antibody; Thermo Scientific Corporation) at a dilution of 1:100. After secondary antibody application, slides were developed using 3-3′-diaminobenzidine chromogen and counterstained with hematoxylin. Negative control slides were made by omitting primary antibody. Sections from lung were stained as a positive control (Attia *et al*, 2015).

Evaluation of iNOS expression: iNOS immune stains were assessed microscopically in 10 HPF (x400) in each section. Positive cells for iNOS showed brownish cytoplasmic staining semi-quantitatively scored based on the percentage of positive cells as follows: 0 (0- 9%), +1 (10-49% positive cells), +2 (50-89% positive cells), +3 (>90% positive cells). Staining intensity was graded as follows: 0 (no staining), +1: weak, +2: moderate and +3: strong. The final scores were calculated by multiplying the quantity score with intensity score, divided by 3, with a range from 0 to 9. A score of 7-9 was considered a strong immunoreactivity (+3); 4-6: moderate (+2); 1-3: weak (+1); and 0: negative (Ravn *et al*, 1993; Lee *et al*, 2008).

SEM: Worms were directly pipetted into a fresh fixation solution of 2.5% glutaraldehyde (w/v) in 0.1M sodium cacodylate at pH 7.2 and left overnight at 3 °C. Worms were washed in 0.1M sodiumcacodylate buffer at pH 7.2 for 5min, post-fixed in a 2% (w/v) osmiumtetroxide in sodium cacodylate buffer for 1hr. The specimens were dehydrated in an alcohol series, dried using liquid carbon dioxide and mounted on stub. After sputter coating with gold, they were examined in Jeol SEM (Mitaka, Japan) after Bughdadi (2010).

Statistical analysis: Data collected were tabulated and analyzed by SPSS version 22.0 on IBM compatible computer. Quantitative values of the measured parameters were expressed as mean ± standard deviation (SD). Mann-Whitney test and chi-square (χ^2) test were used. Difference was significant when $P < 0.05$, highly significant when $p < 0.01$ and not significant when $p > 0.05$.

Results

The lowest mean adult count was in GV received chitosan nanoparticles loaded with albendazole (1.8±1.03) and showed the most effective eradication of *T. spiralis* worms, with drug efficacy of 99.1% (Tab. 1).

Concerning the histopathological changes in the intestinal phase of the infection, examination of the infected control (GII) revealed severe inflammation, dense inflammatory infiltrate in lamina propria, edema, destruction of intestinal villi and ulceration. Marked improvement of inflammation was observed in group treated with CH nanoparticles loaded with full dose of ABZ (GV). Most of the infected mice that were treated with ABZ (GIII), showed moderate degree of inflammation, while CH treated group (GIV) showed severe inflammation.

The immunohistochemical expression of the iNOS in the intestinal sections of the infected control group revealed strong cytoplasmic staining (+3) in the inflammatory cellular infiltrate within the villous core. The lowest intensity of iNOS expression (80 %) was noticed in group treated with CH nanoparticles loaded with full dose of ABZ (GV) with weak expression (+1) and mild cytoplasmic staining in the inflammatory cellular infiltrate within the villous core and around the crypts. Most of the infected mice that were treated with ABZ (GIII), showed moderate degree of inflammation, while CH treated group (GIV) showed strong iNOS expression (+3). No significant difference was observed between groups received CH loaded with full dose of ABZ or CH loaded with half dose of ABZ either as regards histopathological or immunohistochemical changes in the intestine.

SEM examination of adult worms in infected untreated group showed normal morphology with preserved cuticle, characteristic transverse creases, ridges and annulations with normal appearance of the hypodermal gland openings (GII). While marked destruction of the adult worm's cuticle in all treated groups with more destruction in the subgroup treated by CH loaded with full dose of ALB (GVI). Cuticle showed areas with marked swellings, complete disappearance of the internal content, loss of the normal creases, ridges and annulations of the cuticle. Sloughing of some areas of the cuticle was observed in the group treated by chitosan nanoparticles (GIV).

The details were given in tables (1, 2 & 3) and figure (a, b, c, d, e, f, g & h).

Table 1: Comparison between control (n=5) and treated (n=10) regarding adult count (N= 10)

Groups	Adult count		Reduction %	Mann Whitney test	P value of mean
	M±SD	Range			
GI (Control -ve)	No	No	No	II vs. III =3.15 II vs. IV =3.09 II vs. V =3.16 II vs. VI =3.26 III vs. IV =3.79 III vs. V =3.84 III vs. VI =3.89 IV vs. V =3.82 IV vs. VI =3.87 V vs. VI =1.84	P1=0.002* P2=0.002* P3=0.002* P4=0.001** P5<0.001** P6<0.001** P7<0.001** P8<0.001** P9<0.001** P10=0.07
GII (Control +ve)	202.0±17.9	170- 210	0%		
GIII (TTT ABZ)	19.8±5.6	13-25	90.2 %		
Group IV(TTT CH)	73.9±9.7	62- 89	63.3%		
GV (TTT CH + full ABZ)	1.8±1.03	1 – 4	99.1%		
GVI (TTT CH +half ABZ)	2.5±0.97	2 – 5	98.8 %		

Table 2: Histopathological examination of intestinal tissues of control (n=5) and treated (n=10)

groups	Inflammation Degree	Inflammatory Intensity		χ^2 test	P value
GI (Control –ve)		No.	%	I versus II =10.0	P1 =0.007*
	0	5	100	I versus III =15.0	P2=0.002*
GII (Control +ve)	+2	1	20.0	I versus IV =15.0	P3=0.001**
	+3	4	80.0	I versus V =8.57	P4=0.01*
GIII (infected TTT ABZ) (n=10)	+ 1	1	10.0	I versus VI =15.0	P5=0.002*
	+ 2	8	80.0	II versus III =7.40	P6=0.02*
	+ 3	1	10.0	II versus IV =0.17	P7=0.68
GIV (infected TTT CH) (n=10)	+ 2	3	30.0	II versus V =12.75	P8=0.005*
	+ 3	7	70.0	II versus VI =8.40	P9=0.01*
GV (infected TTT CH + full ABZ)	0	2	20.0	III versus IV =7.77	P10=0.02*
	+ 1	7	70.0	III versus V =12.94	P11=0.005*
	+ 2	1	10.0	III versus VI =8.10	P12=0.01*
GVI (infected TTT CH + half ABZ)	+ 1	7	70.0	IV versus V =17.0	P13=0.001**
	+ 2	2	20.0	IV versus VI =11.70	P14=0.003*
	+ 3	1	10.0	V versus VI =3.33	P15=0.34

Table 3: iNOS expressions in intestinal tissues of control (n=5) and treated (n=10)

Groups	iNOS intensity expression	iNOS% expression		χ^2 test	P value
GI (control -ve) (n=5)		No.	%	I versus II =10.0	P1 =0.007*
	0	5	100	I versus III =15.0	P2=0.002*
GII (control +ve) (n=5)	+ 2	1	20.0	I versus IV =15.0	P3=0.001**
	+ 3	4	80.0	I versus V =8.57	P4=0.003*
GIII (infected TTT ABZ) (n=10)	+ 1	1	10.0	I versus VI =15.0	P5=0.001**
	+ 2	8	80.0	II versus III =7.40	P6=0.02*
	+ 3	1	10.0	II versus IV =0.00	P7=1.00
GIV (infected TTT CH) (n=10)	+ 2	2	20.0	II versus V =15.0	P8=0.002*
	+ 3	8	80.0	II versus VI =11.63	P9=0.003*
GV (infected TTT CH + full ABZ) (n=10)	0	2	20.0	III versus IV =10.04	P10=0.007*
	+ 1	8	80.0	III versus V =16.44	P11=0.001**
GVI (infected TTT CH + half ABZ) (n=10)	+ 1	7	70.0	III versus VI =7.77	P12=0.02*
	+ 2	3	30.0	IV versus V =20.0	P13<0.001**
				IV versus VI =15.20	P14=0.001**
				V versus VI =5.07	P15=0.08

Discussion

Albendazole is extensively used against intestinal parasites due to its extended spectrum activity and low cost (Priotti *et al*, 2017). But, its effectiveness influenced by several key factors, such as oral bioavailability, which mainly depends on the solubility, dosage of therapy and the host biotransformation as well as time of onset of treatment after infection (Virkel *et al*, 2002; Solana *et al*, 2009). Therefore, this work aimed to assess the use of chitosan nanoparticles alone or loaded with albendazole full and half doses in the treatment of mice experimentally infected with trichinellosis during the intestinal of the parasite cycle.

The results agreed with Priotti *et al*. (2017) who indicated that the microcrystals made with chitosan appear to be the best options to optimize oral absorption of the active pharmaceutical ingredient. Also, Garcia *et al*. (2013) reported that microencapsulated formulations (based on chitosan particles with different concentrations), designed to improve albendazole dissolution rate in treating *T. spiralis* infected mice during the intestinal phase of the parasite cycle. The high therapeutic response observed was due to a better bioavailability of albendazole and improvement of the intestinal bioavailability of the drug in these formulations and improving the drug dissolution rate and consequently, its absorption by intestinal *T. spiralis* worms. It is well-known that the smaller the particle size, the larger the surface area and the faster the dissolution rate of drug particles (Priotti *et al*, 2017).

Chitosan results agreed with Hoseini *et al.* (2016) who reported that treatment with chitin and chitosan MPs induced a protective response in *L. major*-infected BALB/c mice, but lower than that obtained by Abdel-Latif

et al. (2017), who reported reduction in both adult and egg count in mice infected with *H. nana* after treatment by chitosan particles (95% for adult and 77% for eggs).

The present results disagreed with Gaafar *et al*. (2014), who did not report significant reduction in the parasite count in the infected group treated with chitosan nanoparticles compared to controls in the treatment of murine toxoplasmosis.

Histopathological changes correlated with previous studies (Shalaby *et al*, 2010; Abou Rayia *et al*, 2017). Control of inflammation reduced systemic cytokine release from activated immune cells that relieved systemic manifestations like fever, tissue edema and vasculitis (Kociecka, 2000). Besides, Abdel-Latif *et al*. (2017) was against the present chitosan results. Where chitosan induced improvement in intestinal morphometric measurements and reversed alterations to nearly normal length and width as well as crypts in *Hymenolepis nana* infected mice.

Nitric oxide (NO) production during the intestinal phase of *Trichinella* showed a preventive effect against the parasite establishment (Kolodziej-Sobocinska *et al*, 2006). Also, NO was associated with the pathogenesis of enteropathy and smooth muscle hypermotility during *T. spiralis* infection, and inhibition of inducible nitric oxide synthase (iNOS) abolished intestinal hypermotility (Torrents *et al*, 2003;Othman *et al*, 2016).

The present results agreed iNOS expression in mice infected with *T. spiralis* (Zeromski *et al*, 2005; Yu *et al*, 2013; Attia *et al*. 2015). Also, many studies reported the stimulatory effect of ALB on enzymes involved in oxygen and nitrogen free radical-based host defense (iNOS) in trichinellosis (Derda *et al*, 2003, Boczon *et al*, 2004; Zeromski *et al*, 2005), which explained iNOS expression in ALB treated groups. Also, the effects of chitosan on iNOS expression agreed with Peluso *et al*. (1994) and Li *et al*. agreed with Peluso *et al*. (1994) and Li *et al*. (2009); Porporatto *et al*. (2003) and Yu *et al*. (2004) who reported that the increased activity of iNOS and induced synthesis of NO in macrophages was influenced by chitosan. But, Abdel-Latif *et al*. (2017) reported that, the expression level of iNOS was decreased in the intestinal tissue by chitosan particles in mice experimentally infected with *H. nana*.

During the intestinal phase of *T. spiralis*, NO contributed to the intestinal pathology and prevented effect against the infection establishment (Boczon *et al*, 2004; Kolodziej-Sobocinska *et al*, 2006). Inhibition of the iNOS abolished intestinal hypermotility (Othman *et al*, 2016).

SEM results agreed with Abou Rayia *et al*. (2016) who found significant degeneration and destruction in adult teguments in mice infected with *T. spiralis* and treated with the artemisinin and mebendazole. Hammouda *et al*. (1992) found that changes in organisms shape were secondary to changes due to interference of drugs with DNA synthesis of parasite or interference with folic acid cycle. Also, Gaafar *et al*. (2014) reported disorganized conoid in SEM in the treatment of murine toxoplasmosis with chitosan and silver nanoparticles.

Conclusion

Chitosan nanoparticles loaded with ABZ proved a promising drug against adults of *T. spiralis* in experimentally infected mice.

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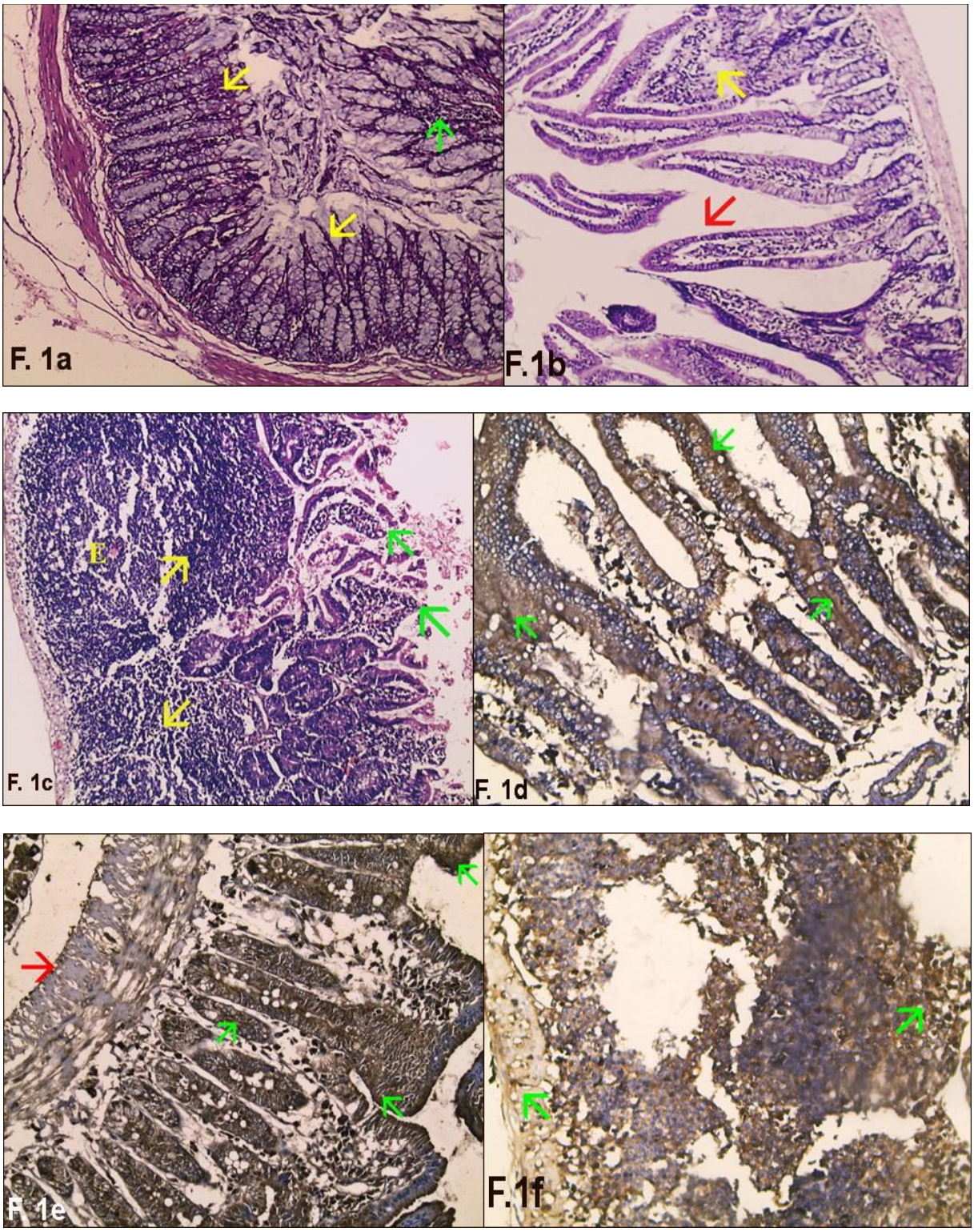
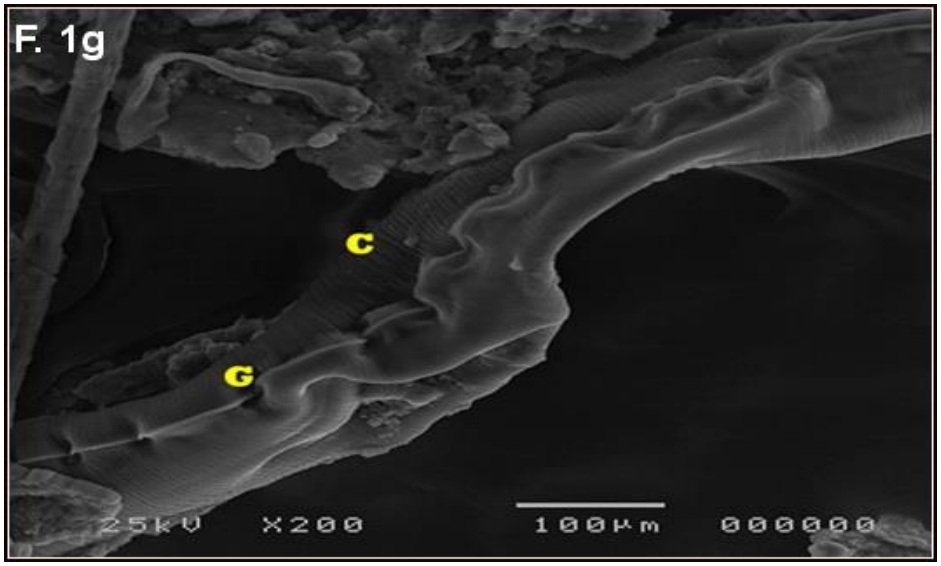
Explanation of figures

Fig. 1a: TS in intestine of GV (TTT by CH + full ABZ) showed very mild inflammation with scarce inflammatory infiltrate in lamina propria (green arrow) mainly lymphocytes with intact mucosa, intestinal villi and crypts (yellow arrows) and muscularis mucosa in addition to mild edema (H&E, x100).
Fig. 1b: TS in intestine of GIII (TTT by ABZ) showed mild to moderate inflammation, dense inflammatory infiltrate in lamina propria mainly lymphocytes and mast cells, plasma cells, edema (yellow arrow) and intact cylindrical intestinal villi (red arrow) (H&E, x100).
Fig. 1c: TS in intestine of GIV (TTT by CH) showed severe inflammation with dense inflammatory infiltrate in lamina propria (green arrows) mainly lymphocytes, plasma cells and eosinophil's (E) in addition to moderate edema and ulceration (yellow arrows) (H&E) (x100).
Figu.1d: TS in intestine of GV (TTT by CH + full ABZ) showed diffuse mild iNOS expression in lining epithelial cells, cytoplasm, inflammatory cells in lamina propria, and in muscularis mucosa (green arrows) (x200).
Fig. 1e: TS in intestine of GIII (TTT by ABZ) showed diffuse moderate iNOS expression in lining epithelial cells, cytoplasm, inflammatory cells in lamina propria (green arrows) and negative expression in muscularis mucosa (red arrow) (x200).
Fig. 1f: TS in intestine of GIV (TTT by CH) showed diffuse moderate iNOS expression in lining epithelial cells, strong expression in cytoplasm, inflammatory cells in lamina propria and in muscularis mucosa (green arrows) (x200).

Fig. 1g: SEM of *T. spiralis* adult of an infected control mouse showing primary folds with large spacing. Transverse creases (C) and hypodermal glands opening (G).
Fig. 1h: SEM of *T. spiralis* adult of an infected treated with CH+ full ABZ showed sloughing of wall with complete large opening (O) due to rupture of cuticle with complete disappearance of internal content and multiple fissures with loss of normal annulations of cuticle (green arrows).
Fig. 1i: SEM of *T. spiralis* adult of an infected treated with CH showed sloughing and erosion of some areas of cuticle and large blebs (yellow arrow) with fissure and loss of normal annulations of cuticle (green arrow).

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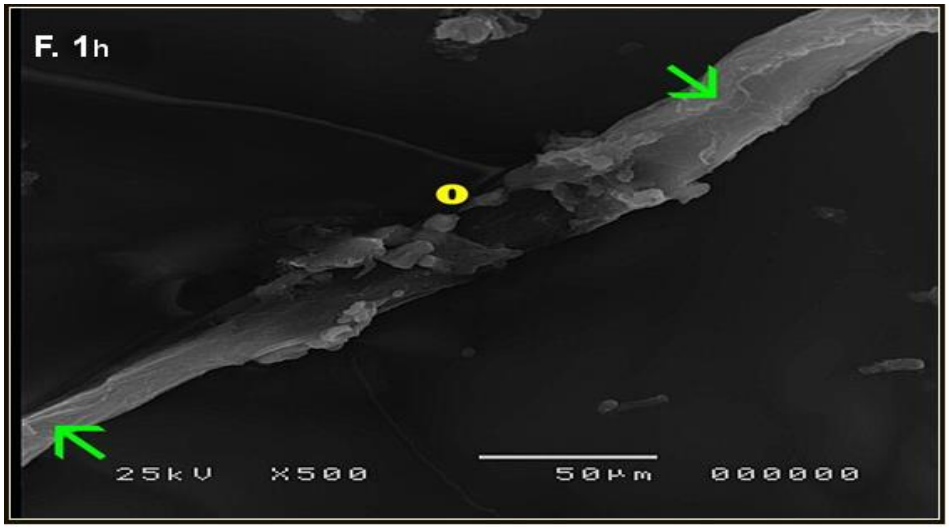
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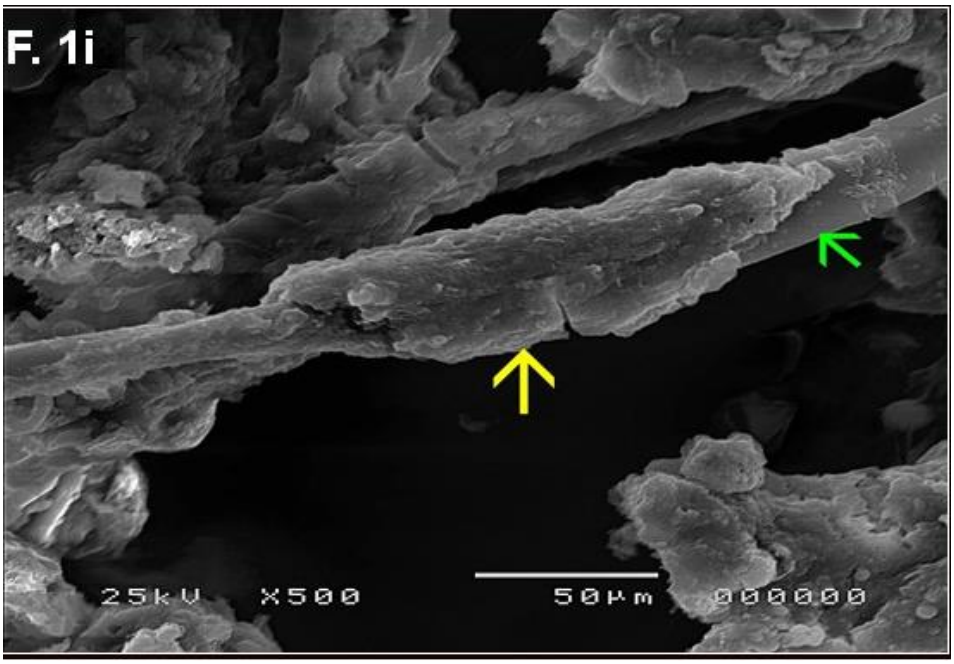
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