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The Effect of Aminoguanidine Versus Albendazole on Expression of TGF-β1 in Inflammatory Cells and Collagen Accumulation in the Small Intestine of Mice Infected with *Toxocara canis* Eggs

Salwa Fouad Oshiba and Hayam Abdel Samie Aiad

Parasitology Department, Faculty of Medicine, Menoufia University, Egypt
Pathology Department, Faculty of Medicine, Menoufia University, Egypt

Abstract: Toxocariasis is considered an important worldwide zoonosis. In humans, the principal route of transmission is by ingestion of embryonated *Toxocara canis eggs*. The aim of the present study was to determine the effect of aminoguanidine versus albendazole on expression of TGF-β1 in inflammatory cells and collagen accumulation in the small intestine of mice infected with *Toxocara canis* eggs. The present study was conducted on four groups of albino mice: GI (None infected) which was subclassified into GIa, GIb and GIc, GII (infected with *Toxocara canis* eggs), GIII (infected & treated with albendazole) and GIV (infected & treated with aminoguanidine). Mice from each group were scarificed at the 2nd, 10th dpi (days postinfection) and 12th wpi (weeks postinfection). The small intestine of mice from each group was obtained; part of it was digested for detection of *Toxocara canis* larvae and another part was subjected to histopathlogical study and then stained with both haematoxylin and eosin and masson's trichrome stains. Also, immunohistochemical staining was performed for intensity of expression of TGF-β1 in the tissue of the small intestine. Histopathological changes were more severe at the 2nd dpi than 10th dpi and 12th wpi in infected group (GII) and in infected and albendazole treated group (GIII) than in infected and aminoguanidine treated group (GIV). Collagen fiber accumulation was detected more in GIII (infected and albendazole treated group) than GII (infected non treated group) and GIV (infected and aminoguanidine treated group). Enhanced expression of transforming growth factor-β1 by immunohistochemical study was observed in GIII which treated with albendazole while decreasing in expression was noticed in GIV which treated with aminoguanidine. It was concluded that aminoguanidine is better than albendazole for treatment of toxocariasis as it decreases collagen fiber accumulation through decrease in expression of TGF-β1 while albendazole increases collagen fiber accumulation due to enhanced expression of TGF-β1 in inflammatory cells of the small intestine of mice infected with *Toxocara canis* eggs.

Key words: *Toxocara* · TGF-β1 · Albendazole · Aminoguanidine · Fibrosis

INTRODUCTION

Toxocariasis is considered an important worldwide zoonosis [1]. Dogs and cats infected with *Toxocara* shed parasite eggs that contaminate the soil [2, 3]. Ingestion of embryonated *Toxocara* eggs by humans leads to hatching of larvae, penetrating the wall of the small intestine and dissemination in blood stream towards eyes, lungs, liver, muscles and central nervous system [1, 4]. Most infections with *Toxocara* in humans are asymptomatic, but a severe disease may occur in some individuals [1, 5, 6]. Transforming growth factor-beta (TGF-β) is a multifunctional peptide that controls proliferation, differentiation and other functions in many cell types [7]. It is a type of cytokine which plays a role in immunity, cancer, bronchial asthma, lung fibrosis, heart disease, diabetes, hereditary hemorrhagic telangiectasia, Marfan syndrome, Vascular Ehlers-Danlos syndrome, Loeys–Dietz syndrome, Parkinson's disease, chronic kidney disease, multiple sclerosis and AIDS [8]. TGF-β is secreted by many cell types, including macrophages, in a latent form in which it is complexed with two other polypeptides, latent TGF-beta binding protein (LTBP) and latency-associated peptide (LAP). Serum proteinases

Corresponding Author: Salwa Fouad Oshiba, Parasitology Department, Faculty of Medicine, Menoufia University, Egypt. E-mail: sal_131977@yahoo.com.

such as plasmin catalyze the release of active TGF-β from the complex. This often occurs on the surface of macrophages where the latent TGF-β complex is bound to CD36 via its ligand, thrombospondin-1 (TSP-1). Inflammatory stimuli that activate macrophages enhance the release of active TGF-β by promoting the activation of plasmin. Macrophages can also endocytose IgG-bound latent TGF-β complexes that are secreted by plasma cells and then release active TGF-β into the extracellular fluid [9, 10].

TGF-β exists in at least three isoforms called TGF-β1, TGF-β2 and TGF-β3. Until the three isoforms were discovered, TGF-β referred to TGF-β1, as it was the first member of this family to be discovered [11]. TGF-β4 precursor was discovered as a gene upregulated during pre-menstrual phase in the endometrial stroma and called EBAF (Endometrial bleeding associated factor) [12]. TGF-β-1 is a peptide of 112 amino acid residues derived by proteolytic cleavage from the c-terminal of a precursor TGF-β [13]. Most tissues have high expression of the genes encoding TGF-β. In contrast, other anti-inflammatory cytokines such as IL-10 show minimal expression in unstimulated tissues and seem to require triggering by commensal or pathogenic flora [14]. Nitrosative stress influences the regulation of TGF-β. Nitric oxide (NO) production may augment TGF-β1 activity by modification of a naturally occurring neutralizing peptide [15]. The aim of the present study was to compare the effect of albendazole and aminoguanidine on collagen fiber accumulation through their effect on TGF-β1 in small intestine of mice infected with *Toxocara canis* eggs.

MATERIALS AND METHODS

Egg Culture: Method of *Toxocara canis* egg culture and inoculation protocol was previously described by Fan *et al*. [16]. Briefly, adult worms were obtained from the small intestine of necropsied stray dogs. The anterior abdominal wall of each adult worm was opened and both uteri were removed then, cut into small fragments from which eggs were teased by gentle pressure using a dissecting needle and stirred into in 1% of sodium hypochlorite solution for 5 minutes. Then, a clear suspension of the eggs was obtained by straining the fluid through two layers of gauze to remove tissue debris then centrifuged for 5 min at 2000×*g*. The resulting pellet was washed twice with distilled water, once with 2% formalin and then resuspended in 2% formalin in petri-dishes in thin layers at 28-30°C for 4-8 weeks to induce embryonation with gentle weekly agitation. Embryonated eggs were kept at +4°C until used. The eggs were washed with water to remove the formalin before use. Viability of the *T. canis* embryonated eggs was assessed by the light stimulation method before use [17].

Experimental Animals: Three months old male Balb/c albino mice weighing 25-30 g were used in this study. They were kept in the animal house at the Faculty of Medicine Menoufia University; exposed to 12 hours light / 12 hours dark and fed *ad libitum* on standard diet and tap water. Each mouse was inoculated orally with 1000 embryonated *Toxocara canis* eggs counted by hemocytometer [16].

Experimental Design: This study was conducted on 126 Balb/c albino mice. They were classified into 4 main groups I, II and III and IV. Group I was formed of 36 albino mice which were then subclassified into 3 subgroups (GIa, GIb and GIc), each was formed of 12 mice: subgroups Ia (Non infected & non treated mice), Ib (Non infected & treated with albendazole) and Ic (Non infected & treated with aminoguanidine). Each of groups II, III and IV was formed of 30 mice; group II (infected with *Toxocara canis* eggs), Group III (infected with *Toxocara canis* eggs and treated with albendazole) and group IV (infected with *Toxocara canis* eggs and treated with aminoguanidine). Four mice from each subgroup of group 1 (GIa, GIb and GIc) and 10 mice from each group of groups II, III and IV were scarificed at 2, 10 dpi and 12 wpi.

Drug Regimens: Albendazole in the form of Alzental (Epico Pharm Co., 10th Ramadan city, Egypt) as a white suspension of 100 mg/5 ml was given orally at a dose of 100 mg/kg once daily for 5 consecutive days, diluted in 0.1 ml distilled water from the first day of infection [18].

Aminoguanidine in the form of aminoguanidine bicarbonate as a white powder (Sigma Chemicals, Cairo, Egypt) was dissolved in distilled water and given intraperitoneally at a dose of 50 mg/kg on 1, 3, 5, 8, 10, 12, 14, 16, 18, 22, 27, 32, 37, 40 and 44 dpi [19].

Parasitological Study for *T. canis* Larval Recovery: One fourth of the tissue of the small intestine from each mouse was sliced and digested in 50 ml pepsin-HCl solution (2.5 g pepsin, 3.5 ml HCl and 500 ml water) and was incubated at 37°C for 24 h [20]. The digests were filtered through sieves and the sedimental liquids were then centrifuged for 2 min at 1500×g rpm. The sediments were then collected and transferred to petri-dishes and larvae were counted at × 40 magnifications under an inverted microscope (Olympus, Tokyo, Japan) [21, 22].

Table 1: Description of histologic degree of inflammation and fibrosis [25]:

Degree	Inflammation	Fibrosis
None	None	None
Mild	Focal mural or subserosal inflammation	Mild increase in collagen, focal distribution of submucosal fibrosis
Moderate	Patchy mural or subserosal inflammation	Patchy distribution, thicker layer, no expansion of lamina propria
Marked	Diffuse mural inflammation	Thick layer, slight expansion of lamina propria
Severe	Diffuse mural and subserosal inflammation	Expansion of lamina propria involving muscularis propria

Table 2: Description of TGF-β1 staining score [27]

Score of expression of TGF-β1	Extensiveness (frequency)
0 (negative)	Positive cells accounted for less than 50%.
+1 (weakly positive)	Positive cells accounted for 55-60%
+2 (moderately positive)	positive cells were 61-80%
+3 (diffusely positive)	Positive cells accounted for more than 80%

Histopathological Study

Haematoxylin and Eosin Staining Procedure: Part of the tissue of the small intestine from each mouse was fixed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin stain [23].

Masson's Trichrome Staining Procedure: This type of staining is a recommended method for connective tissue staining. Trichrome stains have historically been used to distinguish collagen from muscle tissue. The term "trichrome" refers to a mix of three stains. These dyes often stain nucleus, collagen and cytoplasmic structures in mordants such as phosphotungstic or pshosphomolybdic acid. The sections were deparaffinized and rehydrated. Biebrich scarlet acid fuchsin was first used to stain all tissue elements to red color in the section because of small size. The red stain developed was selectively removed from unwanted areas by differentiating with phosphomolybdic acid. Light green was then progressively applied to the section which is a large molecule dye. Celestin blue was used to stain the nuclei as it is more resistant to removal by the acid solutions used in the method. By examination under microscope; cytoplasm, keratin, muscle fibers and acidophil granules were stained red; erythrocytes were stained yellow; collagen, mucus, basophil granules of hypophysis were stained green and nuclei were stained black [24].

Immunohistochemical Localization of TGF-β1 in the Small Intestine: Each paraffin embedded tissue section was deparaffinized with subsequent immersion in xylene and rehydration in solutions of decreasing ethanol (100, 95 & 80%). Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide (H2O2) (Sigma) at room temperature for 10-15 min. The slides were washed twice with phosphate buffered saline (PBS) solution 5 min each. Subsequently, the tissues were submitted to heat induced antigen retrieval protocols. The slides were submerged in a 10 mM sodium citrate buffer at pH 6.0 and incubated in an 830-W microwave oven (Sunpentown, Chiba, Japan) for at least 15 min. The slides were left to cool then they were incubated in PBS solution twice with 5 min each. The ready to use primary antibody (Thermo Sientific) (100 µl for each tissue section) was applied and the slides were then incubated in a humidified chamber for 30 minutes. Then, the slides were washed twice with PBS solution 5 min each. A set of immunohistochemical detection kit was employed to detect the TGF-β1 by incubating each tissue section with 100 µl of the ready to use goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Thermo Scientific, Cat. N: 36000) in a humidified chamber for 10 minutes. Then, the slides were washed twice with PBS solution 5 min each. Thereafter, the sections with oxidase was detected with the chromogen 3, 3-diaminobenzidine (DAB) and incubated in a humidified chamber for 10 minutes which resulted in a brown color. Then the slides were washed with tap water. Sections were counter-stained with Harris hematoxylin (Vector Laboratories, Burlingame, CA, USA), dehydrated and mounted with mounting medium (Neomarkers). Additionally, to ascertain the specificity of the tissue staining, negative and positive control sections were treated as above to evaluate results [26].

RESULTS

Results of Larval Recovery: Regarding the mean number of larvae obtained after digestion of tissues of the small intestine, no larvae were detected in anyone of the three subgroups of group I in all days; the highest number of larvae was detected at 2 dpi in group II followed by groups III and IV while no larvae were detected at 10 dpi

Table 3: Comparison between the mean numbers of *Toxocara canis* larvae in the tissues of the small intestine at different days in all mice groups

Day of scarification	Group I (n=36) Mean ± SD	Group II (n=30) Mean ± SD	Group III (n=30) Mean ± SD	Group IV (n=30) Mean ± SD	*f*-test	*P*-value
2 dpi	ND	17.7±1.49	16.8±1.68	10.1±2.02	56.36	P1<0.05*, P2 <0.001**, P3 <0.001**
10 dpi	ND	ND	ND	ND		
12 wpi	ND	ND	ND	ND		

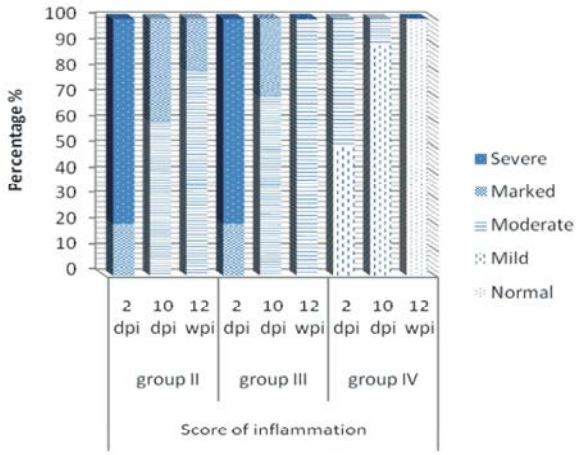
P<0.05* … significant & P <0.001** … highly significant

P1 between group II and III, P2 between group II and IV, P3 between group III and IV

and 12 wpi in all groups with a highly significant difference (p<0.001**). Little decrease in the larval count was detected in the infected and albendazole treated group (GIII) compared to the infected group only (GII) (p1<0.05*). While in infected and aminoguanidine treated group (GIV), the mean number of larvae was significantly lower than that of GII and GIII (p2 & p3 <0.001**) respectively. So, the present study showed that *Toxocara canis* larvae disappeared from the intestine at 10 dpi and onwords and also, the drug, aminoguanidine could decrease *Toxocara* larval count in early days after infection.

Histopathological Results

Hx & E Staining: Histopathological results of the present study showed that in group I, no histopathological changes were detected in any of the three subgroups GIa, GIb or GIc. In group II (infected and non treated mice), the degree of inflammation was mostly severe in 80% and marked in 20% of mice at the 2nd dpi then improvement occurred at the 10th dpi as the degree of inflammation becomes moderate in 60% and marked in 40% of mice then more slight improvement occurred at the 12th wpi where 80% had moderate and 20% had marked degree. In group III (infected and treated with albendazole), the degree of inflammation was mostly severe in 80% and marked in 20% at the 2nd dpi then improvement occurred at the 10th dpi as the degree of inflammation becomes moderate in 70% and marked in 30% of mice then at the 12th wpi all the mice (100%) showed moderate degree of inflammation with a highly statistically significant difference (p<0.001)**. While in group IV (infected and treated with aminoguanidine), eminant improvement was noticed in the degree of inflammation started from the 2nd dpi where 50% of the tissues had moderate degree and the other 50% had mild degree of inflammation. At the 10th dpi, most of the cases (90%) had mild and 10% had moderate degree of inflammation (p<0.05)*. At the 12th wpi, all the mice (100%) were normal with a highly statistically significant difference (p<0.001)**. So, we noticed that there was no obvious difference regarding the degree of inflammation in the small intestine comparing group II (Infected non treated mice) to group III (infected and treated with albendazole). While, it was noticed that in group IV (infected and treated with aminoguanidine), there was marked improvement of inflammation began from the 2nd dpi and the 10th dpi then complete resolution of inflammation occurred at the 12th wpi compared to group II (infected non treated) and group III (infected and treated with albendazole) (Graph 1).



Graph 1: Comparison between the three studied groups (GII, GIII and GIV) regarding the degree of inflammation in the small intestine at different days postinfection

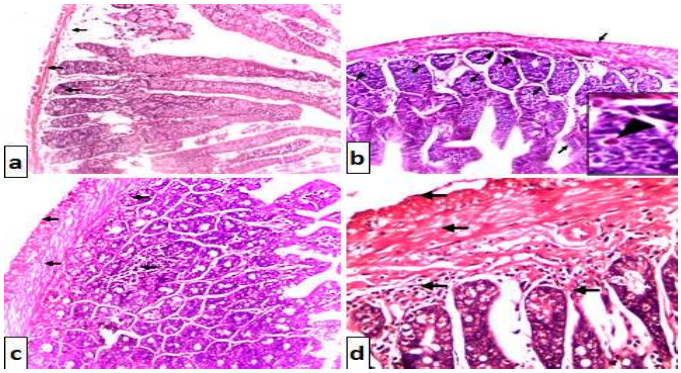


Fig. 1: a) Normal histology of the small intestine of GI with no infiltration of the mucosa and normal thickness of muscularis mucosa (Hx & E stain) x (200). b) Tissue of the small intestine of infected non treated mice (GII) and scarificed 2 dpi showing: severe degree of histopathological changes with diffuse mural and subserosal inflammation, polymorphic infiltration, some destruction of intestinal villi, deposited larva in the submucosa (head of the arrows) but normal thickness of muscularis mucosa (Hx. & E. X200). Lower inset: (arrowhead) *Toxocara canis* larva at a higher magnification. c) Tissue of the small intestine of infected non treated mice (GII) and scarificed 10 dpi showing: moderate degree of histopathological changes with patchy mural and subserosal inflammation and moderate thickening of muscularis mucosa (Hx. & E. X200). d) Tissue of the small intestine of infected non treated mice (GII) and scarificed 12 wpi showing: moderate degree of histopathological changes with patchy mural and subserosal inflammation and mild thickening of muscularis mucosa (Hx. & E. X200)

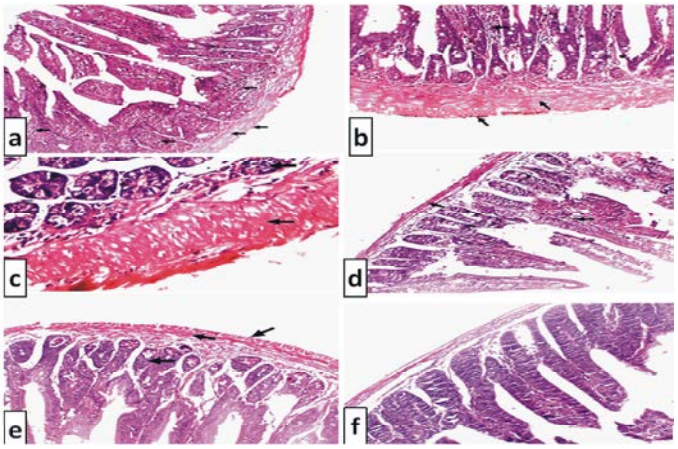
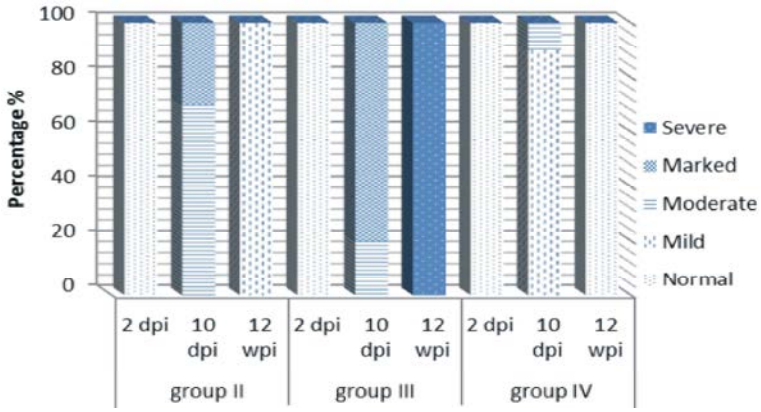


Fig. 2: a) Tissue of the small intestine of mice infected and treated with albendazole (GIII) and scarificed 2 dpi showing: severe degree of histopathological changes with diffuse mural and subserosal inflammation, polymorphic infiltration and normal thickening of muscularis mucosa (Hx. & E. X200). b) Tissue of the small intestine of infected mice and treated with albendazole (GIII) and scarificed 10 dpi showing: moderated degree of histopathological changes with patchy mural and subserosal inflammation with marked thickening of muscularis mucosa (Hx. & E. X200). c) Tissue of the small intestine of infected mice and treated with albendazole (GIII) and scarificed 12 wpi showing: moderated degree of histopathological changes with patchy mural inflammation with severe thickening of muscularis mucosa (Hx. & E. X200). d) Tissue of the small intestine of infected mice and treated with aminoguanidine (GIV) and scarificed 2 dpi showing: mild degree of histopathological changes with focal mural inflammation with no thickening of muscularis mucosa (Hx. & E. X200). e) Tissue of the small intestine of infected mice and treated with aminoguanidine (GIV) and scarificed 10 dpi showing: mild degree of histopathological changes with focal mural and subserosal inflammation with mild thickening of muscularis mucosa (Hx. & E. X200). f) Tissue of the small intestine of infected mice and treated with aminoguanidine (GIV) and scarificed 12 wpi showing: no histopathological changes with no thickening of muscularis mucosa (Hx. & E. X200)



Graph 2: Comparison between the three studied groups (GII, GIII and GIV) regarding the degree of fibrosis in the tissues of the small intestine at different days postinfection

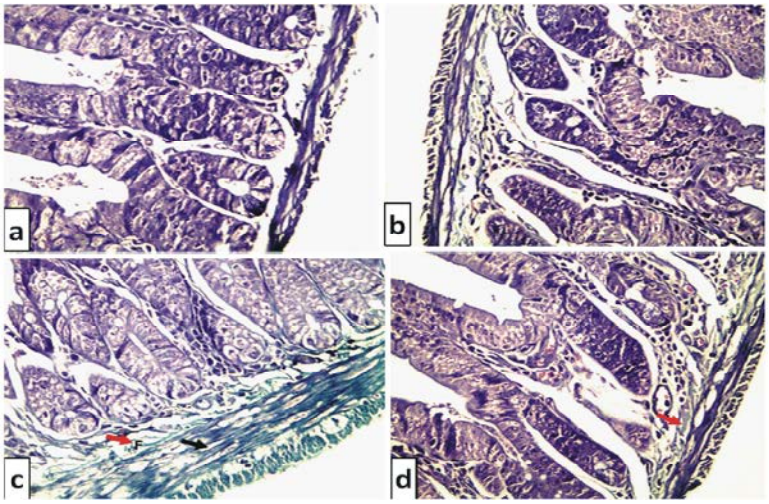


Fig. 3: a) Tissue of the small intestine of non infected & non treated mice (GI) showing: no collagen fiber accumulation (Masson's trichrome X200). b) Tissue of the small intestine of infected mice (GII) and scarificed at 2 dpi showing: no collagen fiber accumulation (Masson's trichrome X200). c) Tissue of the small intestine of infected mice (GII) and scarificed at 10 dpi showing: moderate degree of collagen accumulation with patchy distribution (red arrow), thick muscularis mucosa (black arrow), no expansion of lamina propria (Masson's trichrome X200); F: fibrosis. d) Tissue of the small intestine of infected mice (GII) and scarificed at 12 wpi showing: mild degree of collagen accumulation, focal distribution in the submucosa (red arrow) (Masson's trichrome X200)

Masson's Trichrome Staining: Concerning masson's trichrome staining for detection of collagen fiber accumulation, the present study showed that in group I, no collagen fibers were detected in all subgroups at different days of scarification. In group II (infected non treated mice), no collagen fibers were detected at the 2nd dpi while at the 10th dpi, most of the cases (70%) had moderate and 30% had marked degree of collagen accumulation. At the 12th wpi, there was decrease in degree of collagen accumulation where all mice (100%) had mild degree ($p<0.001$)**. In group III (infected and treated with albendazole), no collagen fibers were detected at the 2nd dpi in all mice (100%) then at the 10th dpi, most of the cases (80%) had marked and 20% had moderate degree of collagen accumulation. Unexpectedly at the 12th wpi, all mice (100%) showed severe degree of collagen fiber accumulation insipite of albendazole treatment with a highly statistically significant difference ($p<0.001$)**. Inversely, in group IV (infected and treated with aminoguanidine), all mice (100%) were normal with no collagen accumulation at the 2nd dpi. At the 10th dpi, marked improvement was noticed where most of the mice

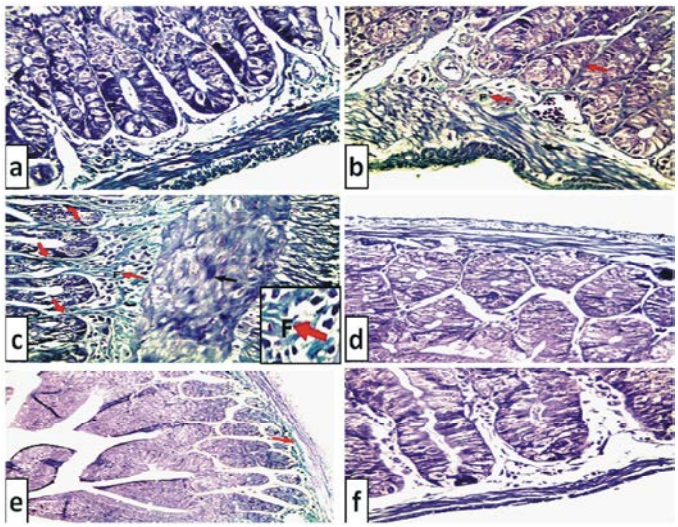


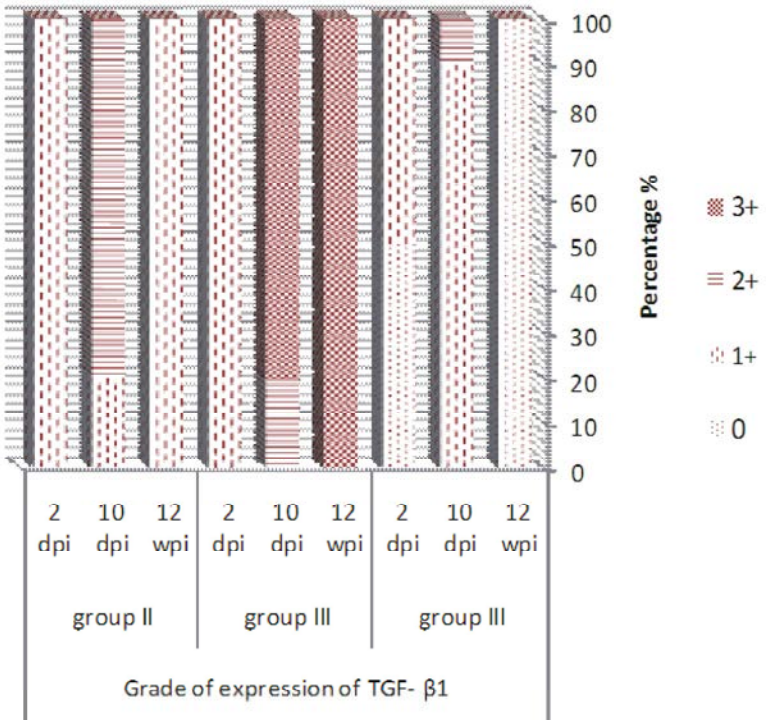
Fig. 4: a) Tissue of the small intestine of mice infected and treated with albendazole (GIII) and scarificed at 2 dpi showing: no collagen fibers (Masson's trichrome X200). b) Tissue of the small intestine of mice infected and treated with albendazole (GIII) and scarificed at 10 dpi showing: marked degree of fibrosis, slight expansion of lamina propria (Red arrows) with thick layer of muscularis mucosa (Black arrow) (Masson's trichrome X200). c) Tissue of the small intestine of mice infected and treated with albendazole (GIII) and scarificed at 12 wpi showing: severe degree of fibrosis with expansion of lamina propria (Red arrows) and severe thickening of muscularis propria (Black arrow); F: fibrosis (Masson's trichrome X200). Lower inset: (red arrow) collagen fiber deposition at a higher magnification. d) Tissue of the small intestine of mice infected and treated with aminoguanidine (GIV) and scarificed at 2 dpi showing: no collagen fiber accumulation (Masson's trichrome X200). e) Tissue of the small intestine of mice infected and treated with aminoguanidine (GIV) and scarificed at 10 dpi showing: mild increase in collagen fiber accumulation, focal distribution in the submucosa (Red arrow) (Masson's trichrome X200). f) Tissue of the small intestine of mice infected and treated with aminoguanidine (GIV) and scarificed at 12 wpi showing: no collagen fiber accumulation (Masson's trichrome X200).

(90%) showed mild degree of collagen accumulation and 10% showed moderate degree with a significant difference ($p<0.05$)*. At the 12th wpi, complete recovery of the all mice occurred where 100% of mice were normal with no collagen accumulation with a highly statistically significant difference ($p<0.001$) ** (Graph 2 and Fig. 3 & 4).

Immunohistochemical Expression of TGF-β1: Immunohistochemical results of the present study showed that, in group II (infected non treated mice), the grade of expression of TGF-β1 in inflammatory cells was 1+ (mildly positive) in 100% of the mice at the 2nd dpi. While at the 10th dpi, most of the mice (80%) showed moderate grade of expression (2+) and 20% showed mild grade (1+). At the 12th wpi, all mice (100%) showed mild grade of expression ($p<0.001$)**. In group III (infected and treated with albendazole), the grade of expression of TGF-β1 was 1+ (mildly positive) in 100% of the mice at the 2nd dpi. While at the 10th dpi, there was an increase in grade of TGF-β1 expression in inflammatory cells where most of the mice (80%) were diffusely positive (3+) and 20% were moderately positive (2+). At the 12th wpi, there was a more increase in the grade of TGF-β1 expression where all mice (100%) were diffusely positive (3+) with a highly statistically significant difference ($p<0.001$)**. In group IV (infected and treated with aminoguanidine), it was noticed that there was a decrease in the grade of expression of TGF-β1 starting from the 2nd dpi where 50% showed negative staining (0) and the other 50% showed mildly positive staining (1+). At the 10th dpi, there was also a marked decrease in the grade of expression where most of the mice (90%) showed mild grade (1+) and 10% showed moderate grade (2+) ($p<0.05$)* compared to group II and group III. While at the 12th wpi, it was noticed that there was negative scaining (0) of TGF-β1in all the mice (100%) with a highly statistically significant difference ($p<0.001$)**. This means that the drug aminoguanidine inhibited expression of TGF-β1 in inflammatory cells of the small intestine while albendazole stimulated this expression (Table 4, Graph 3 and Fig. 5 & 6).

Table 4: The grade of expression of TGF-β1 in the small intestine of mice infected with *Toxocara canis* embryonated eggs (Group II), infected with *Toxocara canis* embryonated eggs and treated with albendazole (Group III), infected with *Toxocara canis* embryonated eggs and treated with aminoguanidine (Group IV) and scarificed at 2, 10 dpi and 12 wpi

	Grade of expression of TGF- β1in group II									
	0		1+		2+		3+			
Day of scarification (PI)	N	%	N	%	N	%	N	%	Z test	P value
2dpi	0		10	100%	0		0		4.2	<0.001**
10 dpi	0		2	20%	8	80%	0		2.24	0.025
12 wpi	0		10	100%	0		0		4.2	<0.001**
	Grade of expression of TGF- β1 in group III									
	0		1+		2+		3+			
Day of scarification (PI)	N	%	N	%	N	%	N	%	Z test	P value
2dpi	0		10	100%	0		0		4.2	<0.001**
10 dpi	0		0		2	20%	8	80%	2.24	0.025
12 wpi	0		0		0		10	100%	4.2	<0.001**
	Grade of expression of TGF- β1 in group IV									
	0		1+		2+		3+			
Day of scarification (PI)	N	%	N	%	N	%	N	%	Z test	P value
2dpi	5	50%	5	50%	0		0		0.45	0.654
10 dpi	0		9	90%	1	10%	0		3.13	0.001**
12 wpi	10	100%	0		0		0		4.2	<0.001**



Graph 3: Comparison between the three studied groups (GII, GIII and GIV) regarding the grade of expression of TGF-β1 in the tissues of the small intestine at different days postinfection

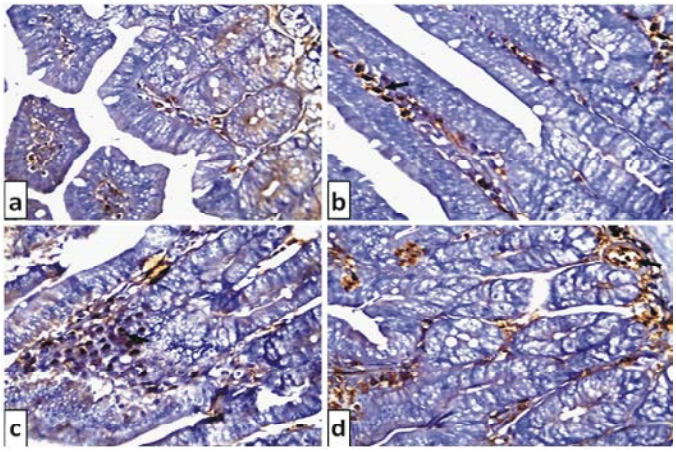


Fig. 5: a) Tissue of non infected mice (GI) showing: negative staining of TGF-β1 (TGF-β1 immunohistochemistry X200). b) Tissue of infected mice with *Toxocara canis* eggs (GII) showing: mildly positive staining (1+) (TGF-β1 immunohistochemistry X200). c) Tissue of infected mice with *Toxocara canis* eggs (GII) at 10 dpi showing: moderately positive staining (2+) (TGF-β1 immunohistochemistry X200). d) Tissue of infected mice with *Toxocara canis* eggs (GII) at 12 wpi showing: mildly positive staining (1+) (TGF-β1 immunohistochemistry X200)

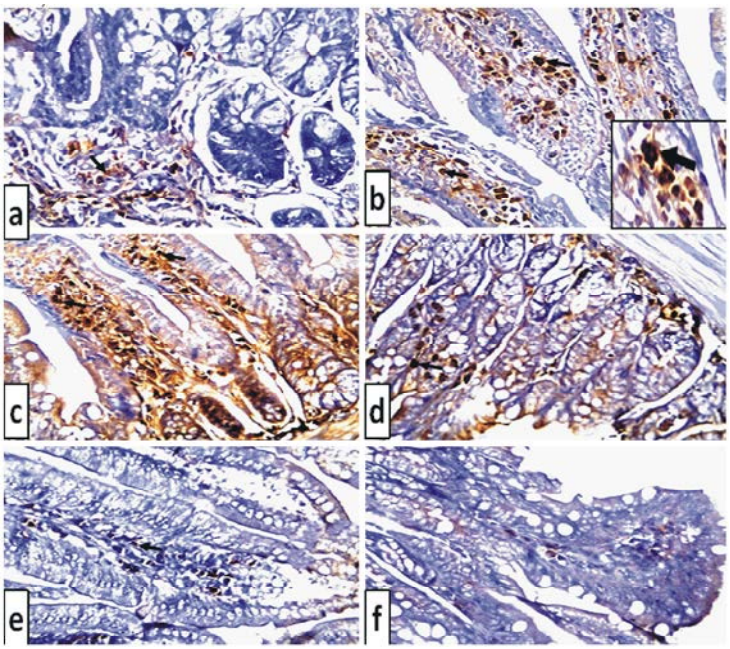
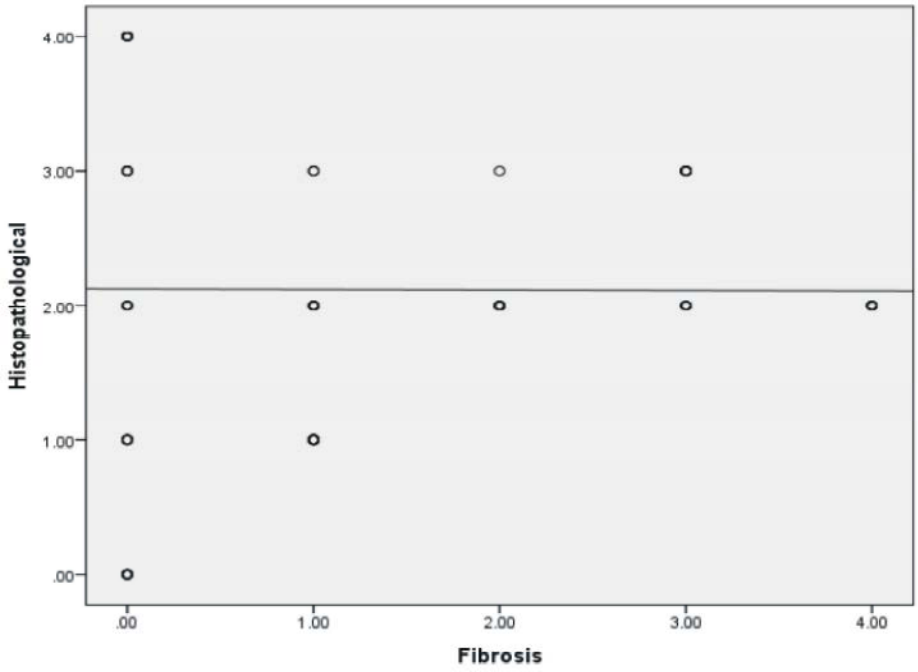


Fig. 6: a) Tissue of infected mice with *Toxocara canis* eggs & treated with albendazole (GIII) at 2 dpi showing: mildly positive staining (1+) (TGF-β1 immunohistochemistry X200). b) Tissue of infected mice with *Toxocara canis* eggs & treated with albendazole (GIII) at 10 dpi showing: diffusely positive staining (3+) (TGF-β1 immunohistochemistry X200). Lower inset: (black arrow) diffusely positive staining (3+) (TGF-β1) at a higher magnification. c) Tissue of infected mice with *Toxocara canis* eggs & treated with albendazole (GIII) at 12 wpi showing: diffusely positive staining (3+) (TGF-β1 immunohistochemistry X200). d) Tissue of infected mice with *Toxocara canis* eggs & treated with aminoguanidine (GIV) at 2 dpi showing: mildly positive staining (1+) (TGF-β1 immunohistochemistry X200). e) Tissue of infected mice with *Toxocara canis* eggs & treated with aminoguanidine (GIV) at 10 dpi showing: mildly positive staining (1+) (TGF-β1 immunohistochemistry X200). f) Tissue of infected mice with *Toxocara canis* eggs & treated with aminoguanidine (GIV) at 12 wpi showing: negative staining (0) (TGF-β1 immunohistochemistry X200)

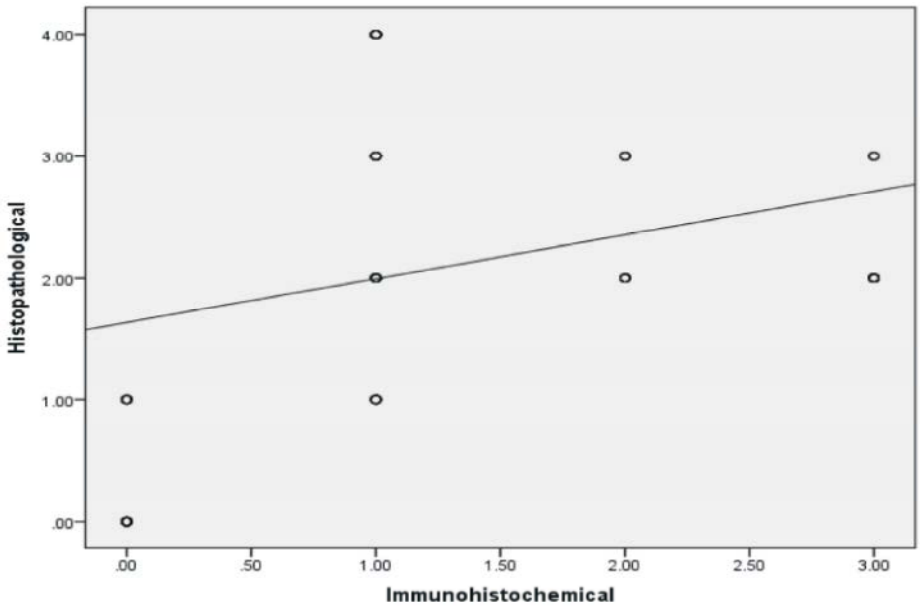
Table 5: Correlations between histopathological changes, degree of fibrosis and grade of TGF-β1 expression in tissue of the small intestine of mice at different days in different groups

P- value	R	Parameters	
0.753	0.034	degree of fibrosis	Histopathological changes
<0.001**	0.361	immunohistochemical staining	Histopathological changes
<0.001**	0.859	immunohistochemical Staining	degree of fibrosis

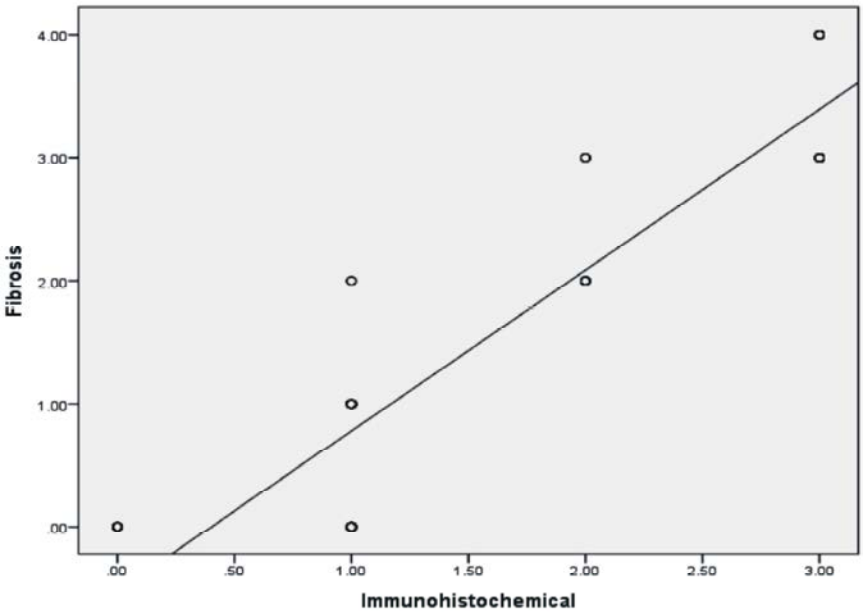
r = Pearson's correlation coefficient



Graph 4: No significant correlation between histopathological changes and degree of fibrosis in the tissue of the small intestine of different groups at different days postinfection



Graph 5: Positive Pearson's correlation between histopathological changes and the grade of TGF-β1 expression in the tissue of the small intestine of different groups at different days postinfection



Graph 6: Positive Pearson's correlation between degree of fibrosis and the grade of TGF-β1 expression in the tissue of the small intestine of different groups at different days postinfection

DISCUSSION

Human toxocariasis is a parasitic zoonosis with a worldwide distribution but is underdiagnosed with an underestimated impact on human health [28].

Parasitological results of the present study showed that the highest number of larvae was detected at 2 dpi in groups II, III and IV while no larvae were detected at 10 dpi or 12 wpi in all groups with a highly significant difference (Table 3). It was noticed that the drug, aminoguanidine markedly decreased larval count in the tissue of the small intestine in GIV while very little decrease appeared in albendazole treated group (GIII) (Table 3). These results coincide with Carlos *et al*. [29] who concluded that larval count decreased in the tissues of the small intestine of rats after the first 24 hours from infection with 1000 *Toxocara canis* eggs and onwords. The study of Taira *et al*. [30] showed that 56.3% of larvae recovered were detected in the intestines at 1 dpi of rats experimentally inoculated with 1000 eggs. Also, the studies of Taira *et al*. [31] and Flecher *et al*. [32] showed similar results.

Albendazole acts by causing degenerative alterations in the tegument and intestinal cells of the worm thus inhibiting its polymerization into microtubules. The loss of these cytoplasmic microtubules leads to impairment of glucose uptake and depleting glycogen stores by the larval stages of the susceptible parasites. This decreases energy required for survival causing immobilization and death of the parasite [33]. In addition, free albendazole was poorly absorbed from the small intestine so that, its effect appeared late after several days of treatment or after combination with other particles as chitosan [34]. This explains that in the current study, albendazole treatment had a very little effect on decreasing larval count in the tissues of the small intestine of GIII at 2 dpi (Table 3). On the other hand, aminoguanidine treatment in GIV was more effective in decreasing larval count from 2 dpi (Table 3) which could be explained by its vasoconstrictor effect on blood vessels as recorded by Mukohda *et al*. [35] and Gericke *et al*. [36] thus decreasing the number of larvae reaching the organs. Another explanation is that nitric oxide (NO) secretion in some parasitic infections impairs host lymphocytic proliferation causing immunosuppression that may help survival of the parasite so that inhibition of NO secretion by aminoguanidine as an inhibitor of inducible nitric oxide synthase (iNOS) enzyme may restore proliferative capacity of lymphocytes that may interfere with survival of the parasite [37].

Histopathological results of the present study showed that in group II (infected non treated mice), the degree of inflammation was mostly severe at 2 dpi then it improved to moderate and marked at 10 dpi then it became mostly moderate at 12 wpi with polymorphic infiltration and thickening of the muscularis mucosa. These results

coincide with the study of Ali [38] who showed that the tissue of small intestine of rats infected with 1000 ml of *T. cati eggs* after two months of infection showed molting of epithelial cells lining the villi, expansion of villi and polymorphocytic infiltration. Also, these results corresponding to the study of Fan *et al*. [21] who detected that inflammatory changes due to infection of mice with 250 *Toxocara canis* eggs were more severe from 1-2 dpi then improved onwords.

In group III (infected and treated with albendazole), improvement appeared in the degree of inflammation at 10 dpi and 12 wpi to mostly moderate. These results are corresponding to Nassef *et al.* [39] who also showed that albendazole treatment for *Toxocara canis* infected mice caused slight improvment of pathological changes in lung and brain tissues at the 45th dpi demonstrating that albendazole drug affects the pathological changes after a long period of time. Similarly, Reis *et al*. [40] studied the effect of some plant extracts and albendazole on *T. canis* infected mice and concluded that they reduced inflammation in liver at 17th dpi. Inversely, in group IV (infected and treated with aminoguanidine), marked improvement of inflammatory changes was observed from 2 dpi then at 10 dpi until complete recovery occurred at 12 wpi where all mice (100%) were normal. This is corresponding to Nassef *et al.* [39] who tested the effect of aminoguanidine treatment on *Toxocara canis* infected mice and also found that there was marked improvement in the degree of inflammation in lung and brain tissues from the 2nd dpi until more recovery at the 45th dpi compared to albendazole treatment. Ferreira *et al.* [41] suggested that aminoguanidine had an immunomodulator role that triggers an increase in the microbicidal response of neutrophils mainly related to reactive oxygen species production by nitric oxide synthase. The study of Hafez *et al.* [42] suggested that inhibitors of iNOS as aminoguanidine have protective effect on methotrexate induced hepatotoxicity and nephrotoxicity. The protective effect of these agents was due to decrease of TNF-α, iNOS and caspase-3 expressions.

Regarding the results of masson's trichrome staining of the present study, it was observed hat in group II (infected non treated mice), collagen fiber accumulation started to increase only from the 10th dpi to mostly moderate degree then it slightly decreased at 12 wpi to mild degree. Wu *et al.* [26] demonstrated that abnormally persistent collagen accumulation may cause irreversible fibrotic injury in the *Toxocara* granulomatous hepatitis. Unexpectedly, albendazole treatment to the infected mice with *Toxocara canis* eggs in group III caused increase in collagen fiber accumulation in the tissue of the small intestine at the 10th dpi to a severe degree (80%) and marked degree (20%) and at 12 wpi where all mice (100%) showed a severe degree (Graph 2). In contrast to the current study, Hrckova *et al*. [43] described that after early albendazole treatment of infected mice with *Mesocestoides vogae tetrathyridia*, there was slightly decreased collagen deposition in the liver paraenchyma in comparison with the control group. Surprisingly, treatment of infected mice with aminoguanidine in group IV caused inhibition in collagen deposition by masson's trichrome staining at the 10th dpi to mild degree (90%) and moderated degree (10%) then at 12 wpi, it was noticed that there was no deposition of collagen at all (100% normal) with no fibrosis and this was statistically highly significant (Graph 2). Corresponding to the present study, Malaviya *et al.* [44] reported that inhibition of iNOS by aminoguanidine (50 mg/kg, 2times/day, 1-3 dpi) caused inhibition of collagen deposition and fibrogenesis in lungs of rats early days after induced lung injury by nitrogen mustard. During investigation of the role of NO in fibrotic granuloma development in the musculature of mice infected with *Toxocara canis* from 1dpi to 8 wpi using the NOS inhibitors, L-NIL (l-N 6-1-iminoethyl lysine), it was concluded that L-NIL treatment resulted in large, irregularly shaped granulomas with suppressed collagen contents at 4 wpi but not at 8 wpi. The suppressed collagen contents might have been related to decreased serum NO and Th2-type cytokine of interleukin-4 but not Th1-type cytokine of interferon-ã expression [45].

Transforming growth factor-beta1 is the most known potent profibrogenic growth factor identified to date [46]. Several mechanisms are involved in the profibrotic effects of TGF-β1, including the transcriptional stimulation of the expression of numerous genes involved in the fibrotic process such as various collagens, proteoglycans, fibronectin and other relevant genes that play important roles in various aspects of the fibrotic process. Furthermore, numerous studies have shown that the three TGF-β isoforms are potent inducers of myofibroblasts either through activation of quiescent fibroblasts, generation of myofibroblasts through endothelial to mesenchymal transition or through the phenotypic conversion of various cell types into activated myofibroblasts [14, 47, 48].

Concerning immunohistochemical results of the present study, it was found that in group II, the grade of expression of TGF-β1 was significantly higher at the 10th dpi to mostly moderately positive, mainly infiltrating

lymphocytes then slightly decreased at 12 wpi where all the mice (100%) were mildly positive (Table 4). The results of Fan *et al*. [21] are similar to the result of present study where it showed that immunohistochemical localization of TGF-β1 reactivity in the tissue of the small intestine of *Toxocara canis* infected mice revealed that most infiltrating cells, predominantly eosinophils and lymphocytes in the inflammatory lesions, were positive for TGF-β1 as observed from 1 to 28 dpi. Also, Wu *et al*. [26] detected TGF-β1 mainly in infiltrating leukocytes in hepatic lesions with strong expressions due to infection with *Toxocara canis* eggs from 4 to 16 wpi. Invasion of the mouse brain by larvae of *Toxocara canis* caused induction of TGF-β1 expression in brain tissue at 4 and 8 wpi by using RT- PCR technique [49].

Furthermore, in albendazole treated group (GIII), it was noticed at the 10th dpi, there was a marked increase in grade of TGF-β1 expression where most of the mice (80%) were diffusely positive (3+) and 20% were moderately positive (2+). Then, at 12 wpi, there was more increase in expression of TGF-β1 where all mice (100%) were diffusely positive stained with a highly statistically significant difference ($p<0.001$)** (Table 4) (graph 3). The study of Zeromski *et al*. [14] showed similar results as they reported that stronger iNOS immunostaining could be seen in almost all mononuclear cells surrounding encapsulated *Trichinella spiralis* larvae in muscles of infected mice after albendazole treatment. Another study performed by Refik *et al.* [50] stated that the total nitrites concentrations increased in a patient with cystic echinococcosis and treated with albendazsole at a dose of 400 mg twice daily for one week before and for one month after surgical operations.

This may be explained by that albendazole was a strong generator of reactive oxygen and nitrogen species including serum NO, superoxide dismutase, catalase, glutathione reductase and glutathione S-transferase in normal rat hepatocytes as reported by Claudriana *et al.* [51]. The study of Nassef *et al.* [39] revealed that there was high expression of iNOS enzyme in tissues of mice infected with *Toxocara canis* eggs and treated with albendazole for 5 consecutive days. Boczon *et al.* [52] reported that the strongest induction of iNOS was observed in high-dose infections in late muscular phase of experimental trichinellosis. It was evident that about 3 or 4 fold stimulation of iNOS-derived production both in muscles and serum, respectively in infected mice after administration of a single dose of albendazole. Albendazole may potentiate its action in trichinellosis, besides the inhibitory influence on parasite's tubulin, stimulating a nitrogen free radical-based defense mechanism of the host. Nitric oxide production may augment TGF-β1 activity by modifying a naturally occurring neutralizing peptide which is a latent form of TGF-β1 [15]. Inversely, Saura *et al.* [53] suggested that the endothelial NO pathway interferes with TGF-beta/Smad2 signaling by directing the proteasomal degradation of activated Smad. The present study is not matching with Pang *et al.* [54] who showed that human infection with *Ecchinococcus granulosus* caused increase in serum level of TGF-β1 by cytometric bead array method while it decreased after combined surgery and albendazole treatment. Also, Zhou *et al*. [55] studied the effect of albendazole treatment on mice infected intraabdomenaly by *Echinococcus multilocularis* and realized that serum level of TGF-β1 decreased in treated than the control groups. Expression of TGFβ-1 was suspected in influencing the efficacy of albendazole treatment in the patients with *Strongyloides stercoralis* infection [56]. While in group IV (infected and treated with aminoguanidine), it was noticed that there was a decrease in the grade of expression of TGF-β1 from 2 dpi where 50% showed negative staining (0) and the other 50% showed mildly positive staining (1+). At the 10th dpi, there was also decrease in the grade of expression where most of the mice (90%) showed mild grade (1+) and 10% showed moderate grade (2+). While at 12 wpi, it was noticed that there was no expression of TGF-β1 in all mice (100%) at all compared to GII and GIII with a highly statistically significant difference ($p<0.001$)** (Table 4) (Graph 3).

Titanium dioxide nanoparticles induced renal injury could be mitigated by iNOS inhibitor aminoguanidine which decreases expressions of TGF-β1 in human renal proximal tubular cells [57]. Furthermore, the study of Serban *et al.* [58] revealed that high concentrations of aminoguanidine treatment inhibit transcriptional level of TGF-β expression by gelatin zymography in human embryonic kidney cells. Similarly, treatment of nephritic rats with the inducible NO synthase–specific inhibitor L-N6-(1-iminoethyl)-lysine thereby limited TGF-β1 mediated profibrotic signaling [59].

Regarding correlation between the degree of inflammation and degree of fibrosis in the tissues of the small intestine, it was found that, there was no significant correlation between infected non treated and infected treated groups either with albendazole or aminoguanidine ($p>0.005$) (Graph 4). While, there was a highly significant positive correlation between the degree of inflammation and the grade of TGF-β1 staining (Graph 5) and also,

between degree of fibrosis and and the grade of TGF-β1 staining between treated and non treated groups ($p<0.001$)** (graph 6). This means that the underlying mechanism where aminoguanidine decreases fibrosis was through inhibiting TGF-β1secretion and this may be through inhibiting synthesis of iNOS enzyme from the tissue. Also, increase in the degree of fibrosis in albendazole treated groups may be through triggering TGF-β1secretion which may be due to increase in expression of iNOS enzyme which is a stimulator for TGF-β1 [15, 39, 52]. This explains why albendazole treatment caused increase in expression of TGF-β1 in infected tissues due to infection with *Toxocara canis* eggs. Further studies should be done to explain how albendazole increases expression of TGF-β1 in mice infected with *Toxocara canis* eggs.

CONCLUSIONS

In conclusion, many considerations should be taken during the use of albendazole drug in treatment of helminthic infections as it may cause dangerous side effects as severe fibrosis through triggering TGF-β1 in the tissue of the small intestine which may cause further complications as stenosis, stricture formation, intestinal obstruction, dysmotility, dysbiosis and gastrointestinal cancer. Inversely, aminoguanidine is suggested to be a better altenative antihelminthic, anti-inflammatory and antifibrotic agent through its effect on TGF-β1 in tissues due to infection with helminthes. Further studies should be done upon the effect of aminoguanidine drug in treatment of other helminthic infections.

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Conflict of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical Statement: The authors assert that all procedures contributing to this work comply with the ethical standards of the national and institutional guides on the care and use of laboratory animals. The research was approved by the Ethics and Welfare Committee of Faculty of Medicine, Menoufia University.

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