Food and Waterborne Parasitology 26 (2022) e00144

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Investigation of the effect of the calcium channel blocker, verapamil, on the parasite burden, inflammatory response and angiogenesis in experimental *Trichinella spiralis* infection in mice

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ARTICLE INFO

Keywords:
T. spiralis
Verapamil
Angiogenesis
Inflammation
VEGF
CD31

ABSTRACT

Trichinella spiralis larvae have very special characters that make them able to completely transform the function of the affected muscle cells towards a self-serving environment, offering them nourishment and protection via what is known as "nurse cells". This setting may be affected by drugs that are used for the treatment of co-morbidities and co-infections as calcium channel blockers, which are widely used in clinical practice. In the present study, the effects of verapamil, ivermectin (IVM), and their combined administration on the parasitic burden, immuno-pathology and angiogenesis were investigated during experimental trichinellosis. Estimation of intestinal adult parasitic stages and muscle larvae was done. VEGF gene expression and CD31 immunohistochemical local expression were measured to investigate angiogenesis, in addition to histopathological examination to explore the extent of inflammation. Although verapamil did not have an effect on the adult worm count during the intestinal phase, it induced an anti-inflammatory effect on intestinal pathology. During the muscle phase, it was very effective in reducing the larval count by 93.78%. IVM effectively reduced the worm count by 85.34%, and the muscle larval count by 97.84%, while combined verapamil and IVM administration resulted in a significant reduction in both adult parasites by 69.5% and larval stages by 99%. Both verapamil and IVM and their combination induced a potent decrease in local CD31 protein expression and VEGF gene expression. The important role of calcium and calcium channels during the pathology of

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https://doi.org/10.1016/j.fawpar.2022.e00144
Received 15 September 2021; Received in revised form 13 January 2022; Accepted 17 January 2022
Available online 22 January 2022
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trichinellosis, in addition to the pivotal role of calcium on biological processes such as immunity and angiogenesis, make calcium-channel blockers promising candidates for drug repurposing in the management of helminthic infection.

1. Introduction

Trichinellosis is a re-emerging parasitic zoonosis with a considerable global distribution (Bai et al., 2017). Human trichinellosis is caused mainly *by Trichinella spiralis* species (*T. spiralis*) (Ranque et al., 2000), following consumption of raw or undercooked meat of wild animals and pigs. The severity of the clinical picture depends on the parasite species, number of ingested living larvae, age, sex and immune status of the host (Pozio, 2007; Wang et al., 2017).

T. spiralis has a unique life cycle that distinguishes it from other nematodes. The nematode induces pathology both in its adult stage during the intestinal phase of infection, and in its larval stage during the muscular phase of the disease. Also, the adult worm exists as a multi-intracellular parasite in the enterocytes of the small intestine (Gottstein et al., 2009).

During the enteral phase, the affected patients may complain of fever, malaise, anorexia, nausea, vomiting, abdominal pain, diarrhea and dehydration in severe cases, which may be life-threatening. In the parenteral phase, mainly striated muscles are affected and the patients may present with myositis and myalgia. Complications such as dyspnea, respiratory failure, myocarditis, heart failure and diffuse encephalopathy have also been reported (Yu and Qi, 2015).

The most discernible feature of trichinellosis is the larval encystation inside skeletal muscle fibers, modifying it into nurse cells. These cells serve to maintain the metabolic requirements of the encysted larvae and provide protection against the host immune system (Gottstein et al., 2009). This sophisticated host-parasite relationship is established and maintained by the recruitment of new blood vessels through the initiation of angiogenesis around the collagen capsules (Ock et al., 2013; Patra and Sarkar, 2014). This angiogenic process takes place secondary to the induction of the vascular endothelial growth factor (VEGF). During the early stages of nurse cell formation, VEGF is induced by thymosin β 4, and fibroblast growth factor 1 (FGF-1), but later on, its production is up-regulated by hypoxia-inducible factor 1 (HIF-1) (Ock et al., 2013). Calcium signaling is required for endothelial proliferation and angiogenesis induced by VEGF (Faehling et al., 2002). Inhibitors of calcium influx have been found to exert an anti-angiogenic effect. In addition to their effect on new vessel formation, calcium ions play an important role in the synthesis and release of inflammatory chemical mediators (Lucas et al., 2003; Eteraf-Oskouei et al., 2017). Calcium channel blockers (CCB) were found to exert an anti-inflammatory effect due to the decreased production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Liu et al., 2011). One of the commonly used CCB is verapamil, which is used in cardiac diseases such as hypertension and arrhythmia. It is reported to have a significant anti-inflammatory and anti-angiogenesis effect (Eteraf-Oskouei et al., 2017).

Treatment modalities for trichinellosis principally target the control of tissue inflammation by glucocorticoids and the elimination of infection by anthelmintics. The most famous anthelmintics, the benzimidazole derivatives, cause structural damage to the parasite by affecting the parasite cuticle (Gottstein et al., 2009). Other targets for antiparasitic drugs include parasite ion channels. Macrocylic lactones such as ivermectin (IVM) bind to glutamate-gated chloride (GluCl) channels, leading to a state of permanent hyperpolarization and paralysis. These GluCl channels are specific for invertebrates, which favors the safety of IVM for the mammalian host at appropriate concentrations.

In the present study, the effects of verapamil, IVM monotherapy and their combined administration on the parasitic burden, pathological changes and angiogenesis were investigated during the intestinal and muscular phases of experimental trichinellosis using parasitological, histopathological, immunohistochemical and molecular parameters.

2. Methodology

2.1. Experimental animals

The present study was conducted on 60 male, laboratory-bred Swiss albino mice, pathogen-free, weighing 25 ± 5 g and 4–6 weeks of age, obtained from the Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. The mice were kept on a standard diet and under a temperature of 24 C^{o}. The study was approved by the ethical committee of the Faculty of Medicine for Girls, Al-Azhar University and by the ethical community of animal research at TBRI and was conducted in accordance with international guidelines.

2.2. Experimental design

The number of study animals was determined after conducting preliminary experiments to evaluate the effect of verapamil on the parasite burden in experimental murine trichinellosis, since we have not found previous studies exploring that effect. It was observed that verapamil significantly reduced the larval count in mice sacrificed during the muscle phase of infection, while it had no significant effect on the adult worm count during the intestinal phase of infection. Therefore, a greater number of experimental animals was chosen to be sacrificed during the muscle phase as compared to the intestinal phase.

The mice were divided into four groups, each group included 15 mice; six animals were sacrificed on day 5 PI to study the intestinal phase of infection, while 9 animals were sacrificed on day 35 PI to study the muscle phase of infection:

Group I (Con): the control infected non-treated group.

Group II (IVM): infected mice receiving IVM.
Group III (Ver): infected mice receiving verapamil.
Group IV (IVM-Ver): infected mice receiving both IVM and verapamil.

2.3. Experimental infection

Each mouse was infected orally with 250 ± 50 infective *T. spiralis* larvae which were obtained originally from the Cairo abattoir and grown in successive cycles at the TBRI. The infective dose was determined individually for each mouse according to its weight, where each mouse received 10 larvae per gram body weight. Muscles of infected sacrificed mice were dissected and incubated for 2 h in 1% pepsin and 1% HCl in distilled water at 37 °C. During incubation, intermittent agitation using an electric stirrer was performed. The digested product was sieved on a 50 mesh/in. sieve to remove coarse particles. Larvae were then collected on a 200 mesh/in. sieve, washed twice and subsequently suspended in 150 ml tap water in a conical flask. The suspension containing the larvae was left to sediment, and then the supernatant was discarded. Larvae were recovered and counted under the dissecting microscope to adjust the individual infective dose for each mouse) Denham, 1965; Gamble, 1996 and Guenther et al., 2008).

2.4. Drug administration

Ivermectin tablets (Iverzine 6 mg tablets, Uni Pharma, Egypt) and verapamil (Isopten 2.5 mg/ml, Abbott, Egypt) were dissolved in distilled water and doses were calculated by the extrapolation of human therapeutic doses to animal doses (Paget and Barnes, 1964). IVM was administered on days 1, 5, 15 and 35 post-infection (PI) at a dose of 4 μg /mouse/day orally (Basyoni and El-Sabaa, 2013), whereas verapamil was administered from day 1 till day 35 PI at a dose of 30 μg /mouse /day by intra-peritoneal injections (Gros et al., 2010).

2.5. Animal sacrifice

Animal sacrifice by cervical dislocation under anaesthesia was done according to Denham (1965) and Gamble (1996), on day 5 PI for the intestinal phase and day 35 PI for the muscular phase, to determine the impact of the used drugs on adults and larvae respectively.

2.6. Parasitological examination

Detection and counting of the number of adult worms per milliliter of intestinal fluid of mice was done microscopically on day 5 PI. The small intestines were opened longitudinally, washed with saline and cut into small pieces. Incubation at 37 °C was done in phosphate buffered saline (PBS) for 2 h. The adult worms were then collected and counted under a dissecting microscope (Fadl et al., 2020).

Detection and counting of encysted larvae was done on day 35 PI. The diaphragms of the infected mice were dissected, cut into small pieces and weighed. They were then compressed on microscopic slides and larvae were counted under the dissecting microscope. The larval count was calculated to be expressed per gram muscle tissue (Denham, 1965; Gamble, 1996).

2.7. Histopathological examination

Intestinal specimens (1 cm from the small intestine at the junction of the proximal 1/3 and distal 2/3) (Nassef et al., 2010) were taken from mice sacrificed on the 5th day PI, while skeletal muscle specimens were taken from mice sacrificed on 35th day PI (Zeromski et al., 2005; Monib et al., 2010). These specimens were fixed in 10% formalin, dehydrated, cleared and embedded in paraffin blocks. Paraffin sections were cut to be of 5 μm thickness, stained by hematoxylin and eosin then examined microscopically to detect histopathological changes (Shalaby et al., 2010). We examined the larval capsule, proliferating vessels, morphological changes, infected villous architecture, goblet cell distribution and inflammatory cellular infiltrate. The slides were evaluated by using Olympus light microscopy.

2.8. Immunohistochemical examination

The microvascular density (MVD) was assessed by measuring the average number of the endothelial cell marker PECAM-1 / CD31. Paraffin sections from the diaphragm of each group were mounted on adhesive-coated glass slides for immune staining. After being de-waxed and rehydrated, endogenous peroxide blockage was done by hydrogen peroxide 3%. Antigen retrieval was achieved by immersing the slides in a thermostatic bath containing pre-heated ethylene diamine tetra acetic acid (EDTA) for 30 min at 98 °C. Cooling down was then performed at room temperature for 20 min. The slides were incubated with CD31 monoclonal antibody at a 1:100 dilution (Cell Marque, Rocklin, CA, USA; clone JC70, mouse monoclonal) overnight at 4 °C. Visualization of the antigen-antibody complex was achieved with diaminobenzidine (DAB) (DAKO) reaction, and counterstained by Meyer's hematoxylin. The endothelial lining of intermediate-sized inter-muscular blood vessels was taken as an internal positive control. Negative control was obtained by omitting the primary antibody on a slide in the same run. The entire slides were subsequently scored by Olympus light microscopy. Positive CD31 reaction was noted by a brown membranous cytoplasmic stain; provided that the staining did not represent

a background or artifact. This included micro-vascular formation and inflammatory cellular infiltrate (as CD31 is expressed on endothelial cells, on the surface of platelets, monocytes, neutrophils and some types of T cells). The intensity and extent of the marker staining of inflammatory cells were subjectively graded as mild, moderate and marked. Three visual larvae with the highest number of stained vessels were identified as 'hot spots'. Microvascular density (MVD) was estimated by the average number of CD31 positive luminal structures. This was manually done by a single expert pathologist. Vessels were counted in each slide using a 200× magnification field. The total number was divided by three and expressed as mean per sample group (Weidner et al., 1991).

2.9. Molecular assay

2.9.1. Isolation of total RNA and reverse transcription

Separation of the total RNA from the diaphragm of mice was performed using the Thermo-scientific RNA Mini Kit at day 35 PI to evaluate angiogenesis during the muscular encystation phase of infection. Reverse transcription was performed on freshly extracted RNA samples using M-MLV Reverse Transcriptase RNAase H- (Solis BioDyne).

2.9.2. Measurement of VEGF gene expression levels during nurse cell formation by real-time PCR

Each reverse transcription product was subjected to Microfluidic LabChip real-time PCR using DNA and the intercalating dye; SYBR Green®, to detect PCR products non-specifically, using β-actin as an internal control. The primers used in real-time PCR for VEGF were 5′- CTTGCCTTGCTGCTCTACC 3′and 5′- CACACAGGATGGCTTGAAG 3′ and those for β-actin were 5′- GTGGGGCGCCCCAGGCACCAG 3′ and 5′- TCCTTAATGTCACGCACGATTTC 3′.

2.9.3. Expression calculation

Target concentration was expressed in relation to the concentration of the housekeeping gene.

Relative mediator expression = copy no of mediator /copy no of β-actin = concentration of mediator / concentration of β-actin (Suzuki et al., 2000).

2.10. Statistical analysis of collected data

Statistical analysis was performed by using the SPSS program (statistical package of social science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows. Median and range were calculated to measure the central tendency and dispersion of quantitative data. Comparison between groups was done using the Kruskal-Wallis test to determine the significance in the difference between two medians. The student *t*-test was employed to determine the significance in the difference between two means. The level of significance was calculated at a *P*-value of <0.05 and high significance was stated at P-value <0.01. The confidence level was determined at 95% using the Mann Whitney test.

3. Results

3.1. Parasitological results

3.1.1. Intestinal phase

The number of adult *T. spiralis* worms was determined at day 5 PI and was expressed as number of adults/ml of intestinal fluid. The median adult count in group I (infected non-treated control group) was found to be 250/ml. The administration of IVM alone (group II) resulted in a significant decrease in worm burden (40 adults/ml). Verapamil monotherapy (group III) on the other hand, had no effect on the intestinal adult count (250 adults/ml). The combined administration of IVM and verapamil (group IV) led to a significant decrease in intestinal worm count, though to a lesser extent than that noticed in IVM monotherapy (70 adults/ml) (Table 1). The difference between the adult count in groups II and IV was not statistically significant ($P = 0.249$).

Table 1
Median and range of the number of *T. spiralis* adults/ml in intestinal fluid of mice in infected non-treated group versus all treated groups.

Animal groups	Number of *T. spiralis* adults/ml in the intestinal fluid		Percentage of reduction from group I	*P* value
	Median	Range		
Group I (Con)	250	200–300		
Group II (IVM)	40	0–50	85.34%	0.004*
Group III (Ver)	250	200–300	0.00%	0.733
Group IV (IVM-Ver)	70	0–100	69.50%	0.004*

Con = Control infected non-treated group; IVM = IVM-treated group; Ver = Verapamil-treated group; IVM-Ver = Combined IVM and verapamil regimen group.

* Highly significant difference (*p* value <0.01).

3.1.2. Muscular phase

Thirty-five days after treatment, the median number of *T. spiralis* larvae/g of muscle tissue was determined.

The median larval count in group I (infected non-treated control group) was found to be 1150 larvae/g muscle tissue. The administration of IVM alone (group II) resulted in a significant decrease in larval burden (40 larvae/g). Verapamil monotherapy (group III) was also found to decrease the larval count as compared to the control group (67 larvae/g). The combined administration of IVM and verapamil (group IV) led to a more pronounced decrease in larval count (11 larvae/g), which was significantly lower than that found in the rest of the study groups (Table 2).

3.2. Histopathological results

3.2.1. Intestinal phase

Histopathological examination of sections of the small intestine of mice in the infected non-treated group (group I) showed intraluminal worms, marked villus atrophy and goblet cell depletion with dense mixed cellular inflammatory infiltrate (Fig. 1-I). Sections from IVM-treated mice (group II) revealed a prominent reduction in the inflammatory cellular infiltrate, with near villous architecture recovery and regaining of goblet cells (Fig. 1-II). Verapamil-treated mice (group III) showed focal goblet cell reappearance and some reduction in the cellular inflammatory response, as compared to group I (Fig. 1-III). Combined verapamil and IVM administration (group IV) also resulted in the reduction of the inflammatory cellular infiltrate along with focal recovery of villous (Fig. 1-IV).

3.2.2. Muscular phase

3.2.2.1. Histopathological results. Histopathological examination of sections of skeletal muscles of infected non-treated mice showed encysted larvae with intact thick capsules (Fig. 2-I), surrounded by cellular infiltrate, including neutrophils, eosinophils, macrophages and lymphocytes (inset of Fig. 2-I). Sections from the IVM-treated group, verapamil-treated group and combined IVM and verapamil group showed coagulative necrosis of the larvae, disruption of the capsules and reduction of the inflammatory cellular infiltrate (Figs. 2-II, 2-III, 2-IV).

3.2.2.2. Immunohistochemical results. Immunohistochemical staining of CD31 marker was performed in skeletal muscle sections. This was done to determine the microvascular density (MVD), which reflects the average number of CD31 positive luminal structures. The Infected non-treated group revealed a marked positive brown immunostaining demonstrating angiogenesis in addition a dense presence of inflammatory cells. The average number of MVD was 45/field, with a marked degree of inflammatory cellular infiltrate (Fig. 2-I). Sections from the IVM-treated group showed a prominent reduction in the level of angiogenesis and cellular inflammatory infiltration, were the average number of MVD was 3/field (Fig. 2-II). III- The verapamil-treated group showed a positive immunostaining of angiogenesis with an average number of MVD of 8/field, and moderate degree of inflammation (Fig. 2-III). IV- Combined IVM-Verapamil regimen group showed an average number of MVD of 5/field, with a mild degree of inflammation (Fig. 2-IV).

3.3. Molecular results

Vascular endothelial growth factor mRNA expression level was detected in the skeletal muscle tissues of the different study groups using real-time PCR during the encystation phase of infection at 35 PI. The mean number of VEGF mRNA was 1.68 ± 0.18 in the infected non-treated group. Samples from the IVM-treated group showed the lowest level of VEGF mRNA as compared to the control infected group (0.128 ± 0.0228; *P*-value<0.01). The reduction in VEGF mRNA expression was less pronounced in the verapamil-treated group (0.324 ± 0.062) and the combined IVM-verapamil (0.196 ± 0.024) group, though still statistically significant as compared to samples from the infected non-treated group (P-value<0.01) (Table 3).

Table 2
Median and range of the number of *T. spiralis* larvae/g of muscle of mice in infected non-treated group versus all treated groups.

Animal groups	Number of *T. spiralis* larvae/g muscle tissue		Percentage of reduction from group I	P value
	Median	Range		
Group I (Con)	1150	800–1300		
Group II (IVM)	40	0–50	96.52%	0.004*
Group III (Ver)	67	27–90	94.17%	0.004
Group IV (IVM-Ver)	11	0–14	99.04%	0.004*

Con = Control infected non-treated group; IVM = IVM-treated group; Ver = Verapamil-treated group; IVM-Ver = Combined IVM and verapamil regimen group.

* Highly significant difference (p value <0.01).

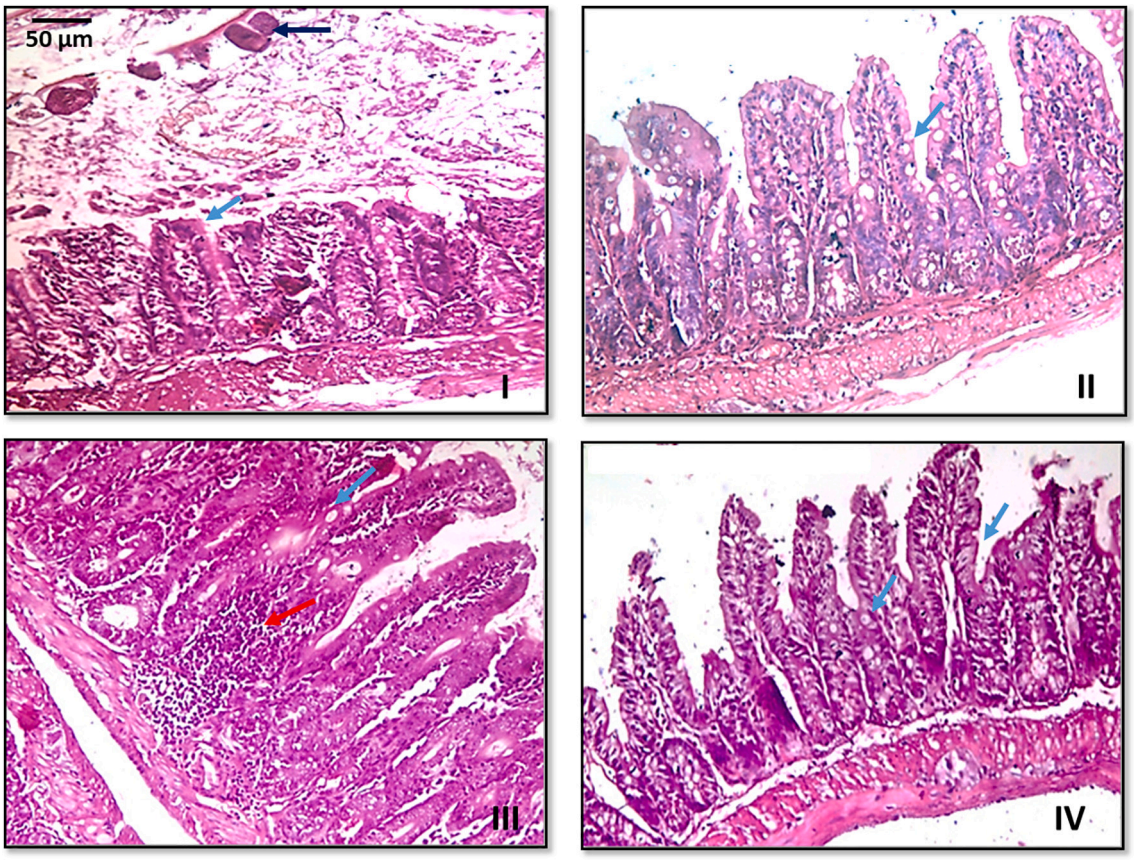

Fig. 1. Light microscopic pictures of histopathological sections in the small intestines of the different study groups stained with H&E (200×). I- Infected non-treated group shows intraluminal worms (black arrow). The intestinal mucosal villi show a marked villous atrophy (blue arrow) with dense cellular inflammatory infiltration. II- IVM-treated group infected treated group shows marked improvement, where most of the villi have returned to their normal pattern with goblet cell regaining (blue arrow) and prominent reduction in the inflammatory cellular infiltrate. III- Verapamil-treated group shows some cellular inflammatory infiltrate (red arrow) and villi regaining their length with few dispersed goblet cells (blue arrow). IV- Combined IVM-Verapamil regimen group shows improvement, where focal villi show broadening (blue arrows), some goblet cells and a reduction in the inflammatory cellular infiltrate.

4. Discussion

Trichinellosis is a serious disease that has a global widespread distribution with significant implications on medical, veterinary and economical fields, especially in developing countries (Attia et al., 2015).

T. spiralis larvae do not only occupy and develop inside the muscle cells, but they also transform them into nurse cells, which become surrounded by a vascular network. These complex apparatuses serve to nourish the parasite, act as a reliable waste disposal system, and protect the encysted larvae from the host immune response. *T. spiralis* has been reported to induce angiogenesis mainly by enhancing the expression of the vascular endothelial growth factor (VEGF) (Ock et al., 2013).

Anthelmintic choices for the treatment of trichinellosis include IVM as an alternative to benzimidazoles. It is a potent macrocyclic lactone that induces paralysis in nematodes and arthropods through the influx of chloride ions across the cell membrane (Basyoni and El-Sabaa, 2013). Ion channel blockers are well-recognized anti-parasitic agents. Since calcium is an important component in helminthic neuromuscular signal transduction, drugs targeting calcium signaling have been developed. Praziquantel exerts its effect by inhibiting voltage-gated calcium channels in flatworms (Jeziorski and Greenberg, 2006).

The biochemical environment in patients with encysted *Trichinella* larvae is affected by other drugs that may be used for the treatment of co-morbidities and co-infections. Among these drugs are calcium channel blockers (CCB), which are widely used in clinical practice (Lee et al., 1997). Calcium plays an important role in the physiology and pathophysiology of numerous cell types, including the gut smooth muscle cells, the enteric neurons, and the skeletal muscle fibers (Lee et al., 1997). CCBs have a direct skeletal muscle relaxant effect and reduce the force of contraction of the striated muscles by inhibiting calcium release from the sarcoplasmic reticulum (Zamponi et al., 2015). In addition, calcium plays an important role in blood vessel formation and contraction. It also plays a role in the calcification of nurse cells, and may thus compromise the environmental condition needed for the encystation of *Trichinella* (Lee et al., 1997).

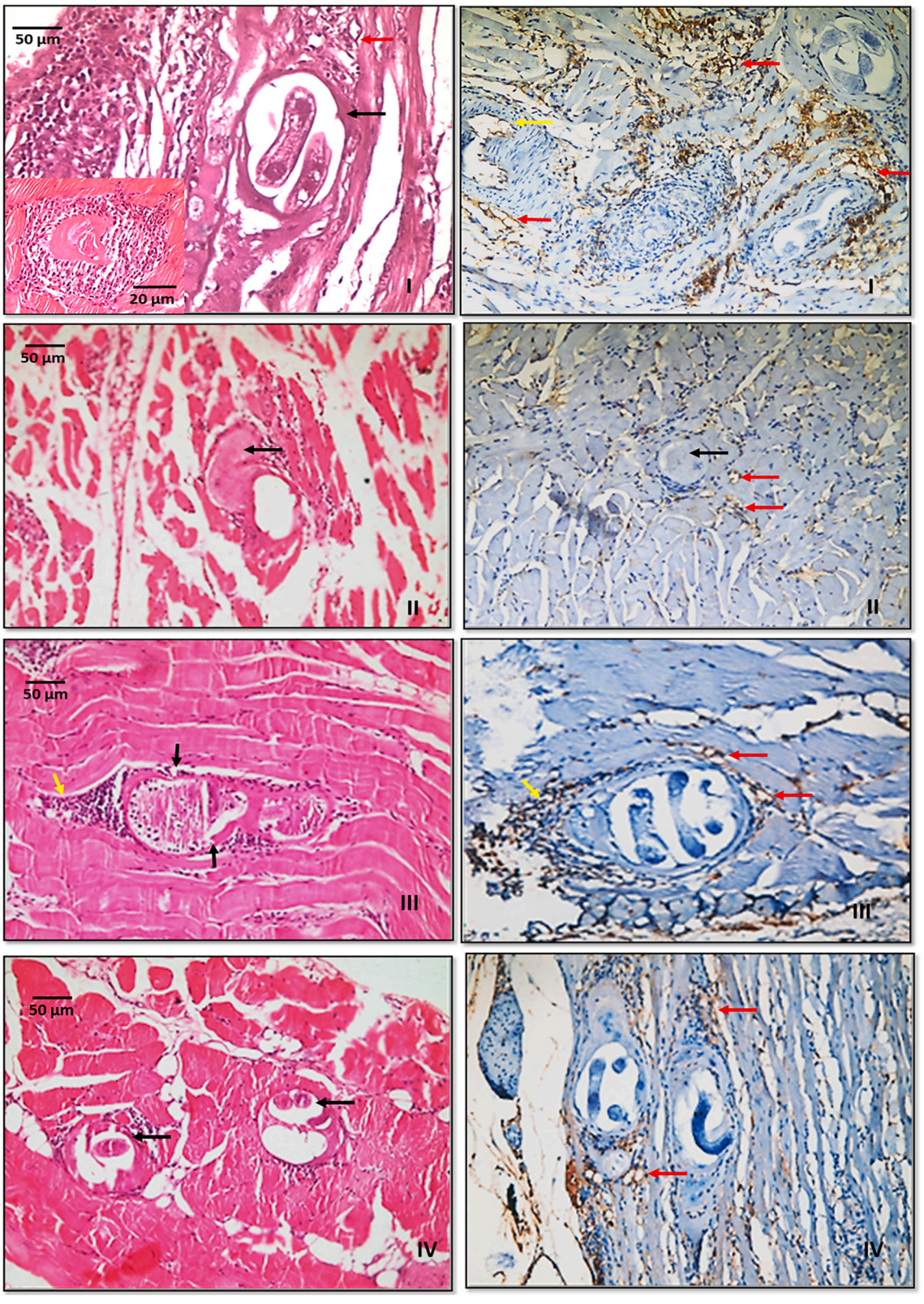

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Fig. 2. Microscopic pictures of histopathological skeletal muscles sections of the different studied groups stained with H & E, as well as comparative CD31 stained immunohistochemical pictures (H&E, and CD31 stain, 200×, scale bar: 50 μm).

I- Infected non-treated group showing encysted larvae. Viable larvae are seen coated with a thick complete collagenous capsule (black arrow). Marked inflammatory cellular infiltrate is seen, including lymphocytes, eosinophils, macrophages and some neutrophils (inset 400×). Surrounding dense vascular proliferation is noted, highlighted by positive brown CD31 (red arrows). An intermediate sized inter-muscular blood vessel with stained endothelial lining acted as an internal control (yellow arrow).

II- IVM-treated group shows prominent reduction in the intensity of inflammatory infiltrate and angiogenesis. Larvae show complete degeneration, capsular dissolution and coagulative necrosis with homogenized eosinophilia (black arrow). Minimal blood vessels are detected as luminal structures with positive CD31 stained endothelial wrapping (red arrows).

III- Verapamil-treated group shows a moderate reduction in the intensity of inflammatory cellular infiltrate (yellow arrow). Larvae show degeneration, with capsular breaks and disruption (black arrows). Some proliferating blood vessels are detected by positive brown CD31 staining (red arrows).

IV- Combined IVM-Verapamil regimen group shows reduction in the intensity of inflammatory infiltrate and angiogenesis. The larvae show coagulative necrosis, with partial capsular dissolution (black arrows). The proliferating blood vessels are detected by positive brown CD31 staining (red arrows).

Table 3
Mean and standared deviation of VEGF expression of muscle of mice in infected non-treated group versus all treated groups.

Animal groups	VEGF expression (RT-qPCR)			
	Mean	SD	t-test	P-value
Group I (Con)	1.68	0.18		
Group II (IVM)	0.128	0.0228	−25.662	<0.001*
Group III (Ver)	0.324	0.062	−21.368	<0.001*
Group IV(IVM-Ver)	0.196	0.024	−24.516	<0.001*

Con = Control infected non-treated group; IVM = IVM-treated group; Ver = Verapamil-treated group; IVM-Ver = Combined IVM and verapamil regimen group.
* Highly significant difference (p value <0.01).

In the present work, verapamil was selected to explore the impact of calcium channel blocking on both the intestinal and muscular phases of *T. spirals* infection with or without IVM administration.

Verapamil was found to have no effect on the adult count during the intestinal phase. In contrast, it was very effective in reducing the larval count during the muscle phase, where the reduction of *T. spirals* larvae/g of muscle was 93.78%. The absence of the effect of verapamil on the parasitic burden during the intestinal phase might be explained by a study performed by Lee et al. (1997), who revealed that verapamil significantly inhibits giant migrating contractions during inflammation and has a potential role in minimizing diarrhea that could prevent expulsion of adult worms from the intestine.

As for the effect of verapamil on *T. spirals* larvae in infected muscles, it could be attributed to its anti-inflammatory and anti-angiogenetic effect (Eteraf-Oskouei et al., 2017).

Verapamil was found to exert a parasiticidal effect on *Echinococcus multilocularis* in vivo and in vitro, as reported by Gao et al. (2021). This effect was associated with the down-regulation of calmodulin and calmodulin-dependant protein kinase II, which are part of the calcium signal transduction pathway. These findings emphasize the role of calcium-dependent biochemical processes during parasitic infections and their potential value as targets for anti-parasitic drugs. In a preceding study by Vaid et al. (2008), inhibition of Ca2/calmodulin-protein kinase B-like enzyme signaling was found to impede erythrocyte invasion by *Plasmodium falciparum*.

In this study, IVM effectively reduced the intestinal worm count by 85.34%, and the muscle larval count by 97.84%. In a study by Basyoni and El-Sabaa (2013), the effect of myrrh and ivermectin against *T. spiralis* infection in mice was evaluated. The reduction of the parasitic burden by ivermectin was reported to be 80% in intestinal adults and 54% in muscle larvae. Similar results were obtained by Song-Mingxin et al. (2002) who found that IVM was highly effective against the migrating and encysted larvae with a 75%-89% and 71%-82% reduction, respectively, but it was only 47% - 58% effective against the adult stages. The effect of IVM is attributed to its effect on glutamate-gated chloride channels. IVM acts also on γ-aminobutyric acid (GABA) receptors which block the signal between neurons and muscles. These receptors are more accessible in nematodes than in vertebrates (Bai and Ogbourne, 2016).

In the current study, the effect of combined verapamil and IVM administration on *T. spirals* adults in the intestine resulted in a significant reduction in the number of *T. spirals* adult/ml of intestinal fluid by 69.5%, whereas the percentage of reduction in the number *T. spirals* of larvae/g of muscle was 99%.

The effect of verapamil on the pharmacokinetics of IVM was studied by Molento et al. (2004). Animals receiving both drugs showed a higher peak of IVM blood concentration and plasma availability than those receiving IVM alone. Verapamil acts as an inhibitor for P-glycoprotein, a protein responsible for the transport of molecules from the bloodstream to the GIT, and can thus affect the blood concentration of IVM.

Histopathological examination of sections of the small intestine in the infected non-treated group showed intraluminal worms and villus atrophy with marked cellular inflammatory infiltrate. Sections from mice receiving IVM alone or in combination with verapamil

revealed a prominent reduction in the inflammatory cellular infiltrate and broadening of the villi. In comparison, sections from verapamil-treated mice showed a mild reduction in the cellular inflammatory response. The effect of infection on muscle pathology was evident in the form of inflammatory cellular infiltrate surrounding the encysted larvae. The larvae were coated by a thick complete collagenous capsule with intact larval morphology. Again treatment with IVM alone or in combination with verapamil resulted in a prominent reduction of inflammatory cellular infiltration, in addition to larval degeneration and necrosis, while verapamil alone resulted in a moderate reduction in inflammation and coagulative necrosis of the larvae. Similar results were obtained by a previous study that was done by Basyoni and El-Sabaa (2013), who found that treatment with IVM resulted in coagulative necrosis and marked degenerative changes of *Trichinella* larvae in muscle.

The anti-inflammatory effect of verapamil was also demonstrated by Gupta et al. (2013), who reported that verapamil improved the pathology of *Mycobacterium tuberculosis* in infected lungs. In addition, a study by Khaksari et al. (2004) reported a potent anti-inflammatory effect of verapamil in adrenal glands of rats after the induction of inflammation using carrageenan. They attributed these results to the inhibition of Ca2+ influx which plays a major role in the synthesis and release of inflammatory mediators. Reduction of Ca2+ leads to the inhibition of the activity of phospholipase A2 and phospholipase C, the enzymes responsible for the synthesis of leukotrienes and stabilization of the cell membrane integrity, thus preventing tissue injury and inflammation. In addition, Eteraf-Oskouei et al. (2017) found that verapamil had a significant anti-inflammatory effect in the air pouch model and explained that this effect may be due to the inhibitory effects of verapamil on the release of pro-inflammatory mediators such as TNF-α and IL-1β in the exudates which can play a role in decreasing leukocyte recruitment.

Nurse cell formation exploits the metabolic machinery of the host and induces the formation of a vascular network to support its maintenance. Angiogenesis can be assessed by the estimation of microvascular density through tissue staining if certain endothelial cell markers such as CD31, in addition to the detection of inducers of inducers of angiogenesis such as VEGF (El-Dardiry et al., 2021). In the current study, all drug regimens resulted in decreased CD31 immunohistochemical expression, and decreased gene expression of VEGF, as compared to the infected non-treated group. The anti-angiogenic effect was most pronounced in samples from mice receiving IVM alone, followed by samples from mice receiving both IVM and verapamil. Verapamil alone also displayed an anti-angiogenic effect, though less prominent than that observed in the other drug regimens. Capó et al. (1998) reported that VEGF mRNA and VEGF peptide increased in the muscle tissue of *Trichinella*- infected mice from day 7 PI and persisted for up to 16 months. They postulated that the induction of VEGF could be attributed to the generation of a hypoxic state secondary to infection. Kang et al. (2011) disputed this hypothesis, since they found that *T. spiralis* infection does not induce hypoxia in nurse cells, after localization of tissue hypoxia regions by the hypoxia marker pimonidazole. They thus concluded that the expression of angiogenic factors such as thymosin β4 and VEGF serves to support the existence of nurse cells. 'Infectious angiogenesis' is a well-recognized concept in the pathogenesis of infectious diseases, and offers to be a promising therapeutic target to attenuate pathogen-induced pathology, in synergism with antimicrobial therapy (Cerimele et al., 2003; El-Dardiry et al., 2021). Antiparasitic drugs that have an anti-angiogenic activity, in addition to their direct action on the parasite, merit special attention to be rediscovered and repurposed in the management of parasitic diseases (Crump, 2017). Siddiqui et al. (2021) demonstrated that IVM inhibited cancer cells of *Schistosoma*-associated bladder cancer by stable binding to VEGF receptor pockets. Hajighasemi and Mirshafiey (2015) also demonstrated that verapamil down-regulated the production of VEGF in human peripheral blood mononuclear cells. Eteraf-Oskouei et al. (2017) demonstrated in an air pouch model that verapamil acts as an anti-inflammatory and anti-angiogenic agent by attenuating the effects of IL-1β and VEGF.

5. Conclusion

The role of ion channel blockers in the treatment of parasitic infections is well-recognized. The pivotal role of calcium on both nematode physiology and host biological processes such as immunity and angiogenesis, make calcium-channel blockers promising candidates for drug repurposing in the management of helminthic infections. Verapamil was effective in reducing the *T. spiralis* larval count in infected muscles and showed a mild to moderate anti-inflammatory effect on both intestinal and muscular pathology. In addition, an anti-angiogenic effect was observed in infected muscle tissue. These observations recommend verapamil to be included in the anti-*Trichinella* regimen, especially during the encystation phase of the disease.

Declarations of Competing Interest

None.

Funding sources

None.

Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

References

Attia, R.A., Mahmoud, A.E., Farrag, H.M., Makboul, R., Mohamed, M.E., Ibraheim, Z., 2015. Effect of myrrh and thyme on Trichinella spiralis enteral and parenteral phases with inducible nitric oxide expression in mice. Mem. Inst. Oswaldo Cruz 110 (8), 1035–1041. https://doi.org/10.1590/0074-02760150295. Epub 2015 Dec 11. PMID: 26676322; PMCID: PMC4708024.

Bai, S.H., Ogbourne, S., 2016. Eco-toxicological effects of the avermectin family with a focus on abamectin and ivermectin. Chemosphere 154, 204–214.

Bai, X., Hu, X., Liu, X., Tang, B., Liu, M., 2017. Current research of trichinellosis in China. Front. J. Microbiol. 8, 1472–147.

Basyoni, M.M., El-Sabaa, A.A., 2013. Therapeutic potential of myrrh and ivermectin against experimental Trichinella spiralis infection in mice. Korean J Parasitol. 51 (3), 297–304. https://doi.org/10.3347/kjp.2013.51.3.297.

Capó, V., Despommier, D.D., Polvere, R.I., 1998. Trichinella spiralis: vascular endothelial growth factor is up-regulated within the nurse cell during the early phase of its formation. J. Parasitol. 84 (2), 209–214.

Cerimele, F., Brown, L.F., Bravo, F., Ihler, G.M., Kouadio, P., Arbiser, J.L., 2003. Infectious angiogenesis: Bartonella bacilliformis infection results in endothelial production of angiopoetin-2 and epidermal production of vascular endothelial growth factor. Am. J. Pathol. 163 (4), 1321–1327. https://doi.org/10.1016/S0002-9440(10)63491-8.

Crump, A., 2017. Ivermectin: enigmatic multifaceted 'wonder' drug continues to surprise and exceed expectations. J Antibiot 70, 495–505. https://doi.org/10.1038/ja.2017.11.

Denham, D.A., 1965. Studies with methyridine and Trichinella spiralis. I. Effect upon the intestinal phase in mice. Exp. Parasitol. 17, 10–14.

El-Dardiry, M.A., Abdel-Aal, A.A., Abdeltawab, M., El-Sherbini, M., Hassan, M.A., Abdel-Aal, A.A., Badawi, M., Anis, S.E., Khaled, B.A., Al-Antably, A.S., 2021. Effect of mast cell stabilization on angiogenesis in primary and secondary experimental Trichinella spiralis infection. Parasit. Vectors 14 (1), 567. https://doi.org/10.1186/s13071-021-05075-9.

Eteraf-Oskouei, T., Mikaily Mirak, S., Najafi, M., 2017. Anti-inflammatory and anti-angiogenesis effects of verapamil on rat air pouch inflammation model. Adv. Pharm. Bull. 7 (4), 585–591. https://doi.org/10.15171/apb.2017.070.

Fadl, H.O., Amin, N.M., Wanas, H., et al., 2020. The impact of L-arginine supplementation on the enteral phase of experimental Trichinella spiralis infection in treated and untreated mice. J. Parasit. Dis. 44, 737–747. https://doi.org/10.1007/s12639-020-01245-1.

Faehling, M., Kroll, J., Föhr, K.J., Fellbrich, G., Mayr, U., Trischler, G., Waltenberger, J., 2002. Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole. FASEB J. 16, 1–29. https://doi.org/10.1096/fj.01-0938fje.

Gamble, H.R., 1996. Detection of trichinellosis in pigs by artificial digestion and enzyme immunoassay. J. Food Prot. 59, 295–298.

Gao, H.J., Sun, X.D., Luo, Y.P., Pang, H.S., Ma, X.M., Zhang, T., Jing, T., Hu, W., Shen, Y.J., Cao, J.P., 2021. Anti-echinococcal effect of verapamil involving the regulation of the calcium/calmodulin-dependent protein kinase II response in vitro and in a murine infection model. Parasit. Vectors 14 (1), 108. https://doi.org/10.1186/s13071-021-04618-4.

Gottstein, B., Pozio, E., Nöckler, K., 2009. Epidemiology, diagnosis, treatment, and control of trichinellosis. Clin. Microbiol. Rev. 22, 127–145.

Gros, A., Puig, C., Guedan, S., Juan, J.R., Ramon, A., et al., 2010. Verapamil enhances the Antitumoral efficacy of oncolytic adenoviruses. Mol. Ther.. J. 18, (5) 903–911.

Guenther, S., Nöckler, K., von Nickisch, M., Landgraf, M., Ewers, C., 2008. Detection of T. spiralis T. britovi and T. pseudospiralis in muscle tissue with real-time PCR. J. Microbiol. Methods 75, 287–292.

Gupta, S., Tyagi, S., Almeida, D.V., Maiga, M.C., Ammerman, N.C., Bishai, W.R., 2013. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am. J. Respir. Crit. Care Med. 188 (5), 600–607. https://doi.org/10.1164/rccm.201304-0650OC. PMID: 23805786; PMCID: PMC3827702.

Hajighasemi, F., Mirshafiey, A., 2015. Effect of verapamil on vascular endothelial growth factor production in immunocompetent cells. Eur. Respir. J. 46, PA4011 https://doi.org/10.1183/13993003. congress-2015.PA4011.

Jeziorski, M.C., Greenberg, R.M., 2006. Voltage-gated calcium channel subunits from platyhelminths: potential role in praziquantel action. Int. J. Parasitol. 36 (6), 625–632.

Kang, Y.-J., Jo, J.-O., Cho, M.-K., Yu, H.-S., Ock, M.S., Cha, H.-J., 2011. Trichinella spiralis infection induces angiogenic factor thymosin β4 expression. Vet. Parasitol. 181 (2–4), 222–228. https://doi.org/10.1016/j.vetpar.2011.03.058.

Khaksari, M., Mahani, S.E., Mahmoodi, M., 2004. Calcium channel blockers reduce inflammatory edema in the rat. Involvement of the hypothalamus-pituitary-adrenal axis. Indian J. Pharm. 36, 351–354.

Lee, C.W., Sarna, S.K., Singaram, C., Casper, M.A., 1997. Ca2+ channel blockade by verapamil inhibits GMCs and diarrhea during small intestinal inflammation. Am. J. Phys. 273 (4), G785–G794. https://doi.org/10.1152/ajpgi.1997.273.4.G785 (PMID: 9357819).

Liu, Y., Lo, Y.C., Qian, L., Crews, F.T., Wilson, B., et al., 2011. Verapamil protects dopaminergic neuron damage through a novel anti-inflammatory mechanism by inhibition of microglial activation. Neuro.pharmacol.J. 60 (2), 373–380.

Lucas, R., Alves, M., Del Olmo, E., San, F.A., Paya, M., 2003. LAAE-14, a new in vitro inhibitor of intracellular calcium mobilization, modulates acute and chronic inflammation. Biochem. Pharmacol. 65 (9), 1539–1549. https://doi.org/10.1016/s0006-2952(03)00120-5 (PMID: 12732367).

Molento, M.B., Lifschitz, A., Sallovitz, J., Lanusse, C., Prichard, R., 2004. Influence of verapamil on the pharmacokinetics of the antiparasitic drugs ivermectin and moxidectin in sheep, 92(2), pp. 121–127. https://doi.org/10.1007/s00436-003-1022-3. Epub 2003 Nov 21. PMID: 14634800.

Monib, M.E.M., Shaheen, M.S., Galal, L.A., Farrag, H.M., 2010. Role of T. spiralis adult and larval antigens in immunomodulation of nitric. Assiut.Med. J. 34, 147–158.

Nassef, N.E., El-Sobky, M.M., Afifi, A.F., 2010. Worm and larval burden, histopathological and ultrastructural evaluation of T. spiralis vaccination using crude worms and/or larvae antigens: experimental studies. P.U.J. 3, 27–38.

Ock, M.S., Cha, H., Choi, Y.H., 2013. Verifiable hypotheses for Thymosin β4-dependent and independent angiogenic induction of Trichinella spiralis triggered nurse cell formation. Int. J. Mol. Sci. 14, 23492–23498.

Paget, G.E., Barnes, J.M., 1964. Evaluation of results: Qualitative application in different species. In: Laurence, D.R., Backarach, A.L. (Eds.), Evaluation of Drug Activities. Pharmacometrics, vol. 1. Academic press, London and New York, pp. 160–167.

Patra, C., Sarkar, S., 2014. Nurse cell Biology of Trichinella spiralis, I.J.O.A.R.T.4(3), pp. 2278–7763.

Pozio, E., 2007. World distribution of Trichinella spp. infections in animals and humans. Vet. Parasitol. 149, 3–21.

Ranque, S., Faugère, B., Pozio, E., La Rosa, G., Tamburrini, A., Pellissier, J., et al., 2000. Trichinella pseudospiralis Outbreak in France. Emerg. Infect. Dis. 6 (5), 543–547. https://doi.org/10.3201/eid0605.000517.

Shalaby, M.A., Moghazy, F.M., Shalaby, H.A., Nasr, S.M., 2010. Effect of methanolic extract of Balanites aegyptiaca fruits on enteral and parenteral stages of Trichinella spiralis in rats. Parasitol. Res. J. 107, 17–25.

Siddiqui, A.J., Khan, M.F., Hamadou, W.S., Goyal, M., Jahan, S., Jamal, A., Ashraf, S.A., Sharma, P., Sachidanandan, M., Badraoui, R., Chaubey, K.K., Snoussi, M., Adnan, M., 2021. Molecular docking and dynamics simulation revealed Ivermectin as potential drug against Schistosoma-associated bladder Cancer targeting protein signaling: computational drug repositioning approach. Medicina 57 (10), 1058. https://doi.org/10.3390/medicina57101058.

Song-Mingxin, L., Yixin Zhang, X., Hui, H., et al., 2002. Efficacy of ivermectin and albendazole against the various phases of 4 Trichinella isolates in treating mice. Chin. J. Zoon. 18, 89–91.

Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66 (11), 4605–4614. https://doi.org/10.1128/AEM.66.11.4605-4614.2000.

Vaid, A., Thomas, D.C., Sharma, P., 2008. Role of Ca2+/calmodulin-PfPKB signaling pathway in erythrocyte invasion by plasmodium falciparum. J. Biol. Chem. 283 (9), 5589–5597. https://doi.org/10.1074/jbc.M708465200. Epub 2007 Dec 28. PMID: 18165240.

Wang, M., Wu, L., Weng, R., Zheng, W., Wu, Z., 2017. Therapeutic potential of helminths in autoimmune diseases: helminth-derived immune-regulators and immune balance. Parasitol. Res. 116 (8), 2065–2074.

Weidner, N., Semple, J.P., Welch, W.R., Folkman, J., 1991. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med. 324, 1–8.

Yu, Y.R., Qi, Y.F., 2015. Progress in treatment and prevention of Trichinellosis. J Infect Dis Ther 3, 251. https://doi.org/10.4172/2332-0877.1000251.
Zamponi, Gerald W., Striessnig, Koschak, Dolphin, 2015. Pharmacology of Voltage-Gated Calcium Channels. Pharmacol. Rev. https://doi.org/10.1124/pr.114.009654.
Zeromski, J., Boczoń, K., Wandurska, N.E., Mozer, L., 2005. Effect of aminoguanidine and albendazole on inducible nitric oxide synthase (iNOS) activity in T. spiralis-infected mice muscles. Folia Histochem. Cytobiol. 43, 157–159.