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Therapeutic efficacy of Albendazole and Mefloquine alone or in combination against early and late stages of *Trichinella spiralis* infection in mice

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Summary

This study aimed to determine the effectiveness of mefloquine alone or combined with albendazole in reduced doses against *T. spiralis* infection. One hundred and twenty albino mice were orally infected with 200 *T. spiralis* larvae/mouse. Drugs were administered during the enteral phase on days 1 to 3 and on the chronic phase on days 35 to 37 post-infection, and mice were sacrificed, respectively, at days 7 or 48 post-infection to count mature intestinal worms or encysted muscle larvae. The effect of the treatment on the histology of the target organs of each phase, intestine and diaphragm, was also evaluated. A significant decrease in intestinal worms was found in all treated groups relative to the untreated control group at a peak of 93.7% in the combination albendazole-mefloquine group. Results in all treated groups demonstrated a significant decrease in muscle larvae relative to untreated control groups, achieving 86.2 % in the combined albendazole-mefloquine group. There was a marked improvement in the intestinal and muscular architecture in all treated groups compared to the non-treated control group. Notably, the albendazole-mefloquine group showed an almost complete recovery. The combined albendazole-mefloquine low dose regimen had the highest effect on reducing parasite burden and restoring normal histological architecture.

Keywords: Albendazole; Mefloquine; combined treatment; therapeutic efficacy; trichinellosis; parasite stages

Introduction

Trichinellosis is a severe meat-borne parasitic disease. It is broadly spread around the world with considerable significance in several developing countries (Bai *et al*., 2017). In Egypt, It has been documented in man (Abdel-Hafeez *et al*., 2015), in addition to, fresh and processed pork (Morsy *et al*., 2000; Youssef & Uga, 2014; Dyab, 2019). Human infection is caused by the ingestion of raw or insufficiently cooked meat from pigs or other *Trichinella*-infected animals. Despite the availability of effective and relatively safe drugs such as albendazole (ABZ) and mebendazole for the treatment of trichinellosis, these drugs have several drawbacks, such as the emergence of parasite drug resistance (Vercruysse *et al*., 2007) and poor absorption of drugs in the intestinal lumen due to their low solubility (Pozio *et al*., 2001; Kalaiselvan *et al*., 2007). Despite being successful against the enteric stages of *T. Spiralis,* they're with limited activity against encapsulated parasite larval stages (Pozio *et al*., 2001; Kalaiselvan *et al*., 2007) and the newborn larvae (Yadav & Temjenmongla, 2012; Saad *et al*., 2016). They are also contraindicated in children under three years of age and during pregnancy (Yadav & Temjenmongla, 2012). Subsequently, an urgent need for safe, efficient and nontoxic anti-Trich-

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inellosis has emerged. Mefloquine (MQ) is a synthetic antimalarial drug that is effective against *Plasmodium* (Wong *et al*., 2017), *Echinococcus multilocularis* (Küster *et al*., 2011; Rufener *et al*., 2018), Schistosomiasis (Xiao, 2013; Abou-Shady *et al*., 2016), and cancer cells (Sharma *et al*., 2012; Liu *et al*., 2016).

To preserve the efficacy of current drugs and slow down the spread of resistance, several strategies were used, including the use of alternative drugs, rotation of drugs from different chemical groups, and use of drug combinations (Waller, 1997; Sangster, 2001; Singh & Yeh, 2017). The latter is considered the most effective approach to delaying resistance (Barnes *et al*., 1995; Sangster, 2001). Therefore, the purpose of this study was to evaluate ABZ and MQ efficacy alone or in a low dose combination in *T. spiralis* infected mice, analyzing parasite burden and pathological changes in the intestine and muscles of these animals.

Materials and Methods

Mice

The study was carried out on one hundred and twenty male CD1 Swiss albino mice (20 ± 2 gm). The mice were bred and maintained at the Schistosome Biology Supply Center (SBSC) of Theodor Bilharz Research Institute, Giza, Egypt. Mice handling and treatment were conducted according to internationally valid guidelines and ethical conditions.

Parasite and Infection

T. spiralis larvae were originally isolated from the diaphragms of infected pigs obtained from El-Bassatine local Abattoir, Cairo, Egypt. The larvae were routinely maintained in our laboratory by a consecutive passage in mice. Muscles of mice heavily infected with *T.spiralis* were cut and digested in a solution formed of 1 % pepsin and 1 % concentrated hydrochloric acid in warm tap water as described by Dunn and Wright (1985). After overnight incubation at 37 °C, larvae were extracted using the sedimentation technique, washed several times with saline, and the number of larvae/ml was counted using a hemocytometer. The 12-hour fasting mice were infected orally with 200 larvae using a tuberculin syringe according to Wassom *et al.* (1988).

Experimental design

ABZ (Alzental) suspension was purchased from the Egyptian International Pharmaceutical Industries Co. (EIPICO) as 20mg/ml and was orally given in a dose of 50mg/kg/day. MQ (mephaquin) (Mepha Ltd., Aesch-Basel, Switzerland) was orally administered in one dose of 400 mg/kg/day which was freshly suspended in 7 %

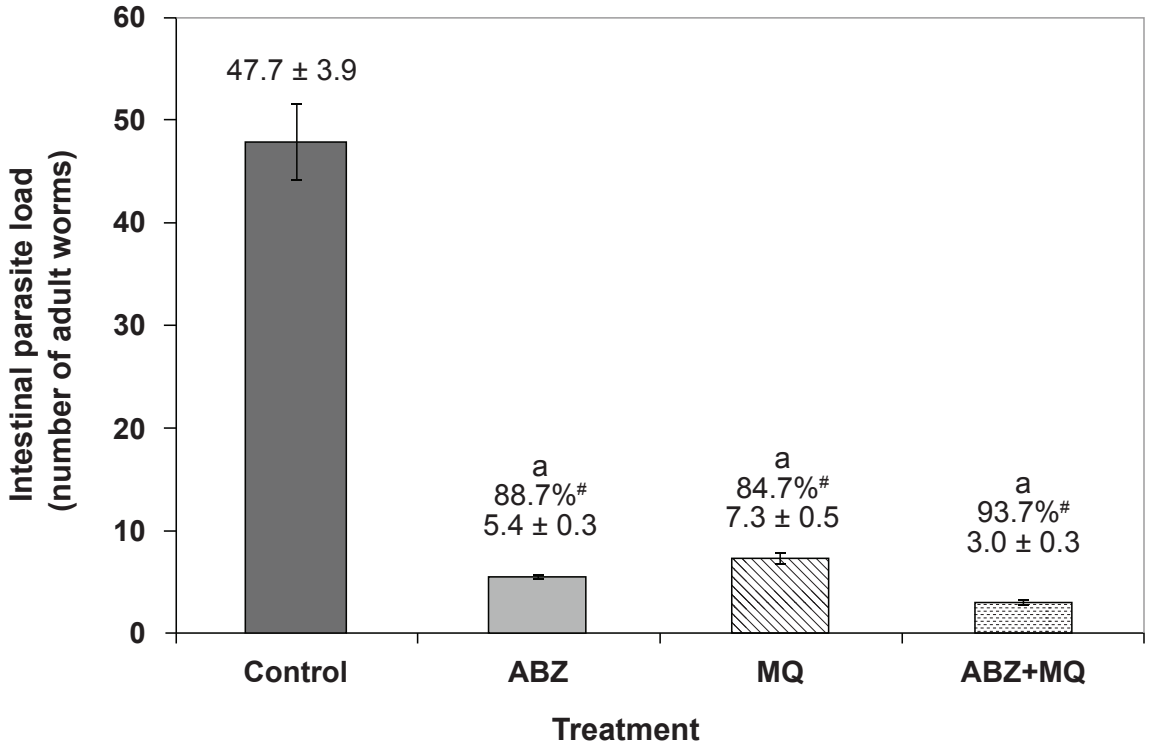

Fig. 1. Intestinal parasitic burden in CD1 Swiss albino male mice infected with *T. spiralis* L1 larvae and treated with Albendazol, Mefloquine, or both, in the acute phase of infection.

Description: The number of worms recovered from the intestine estimates the intestinal parasitic burden (see M&M). Each bar represents the mean ± SEM of 10 mice. Differences among treatment groups were analyzed with ANOVA; comparisons between treatments were done with LSD post-test. Differences were considered significant if $P < 0.05$. a: significantly different compared with the control group. #: percent change from the control group.

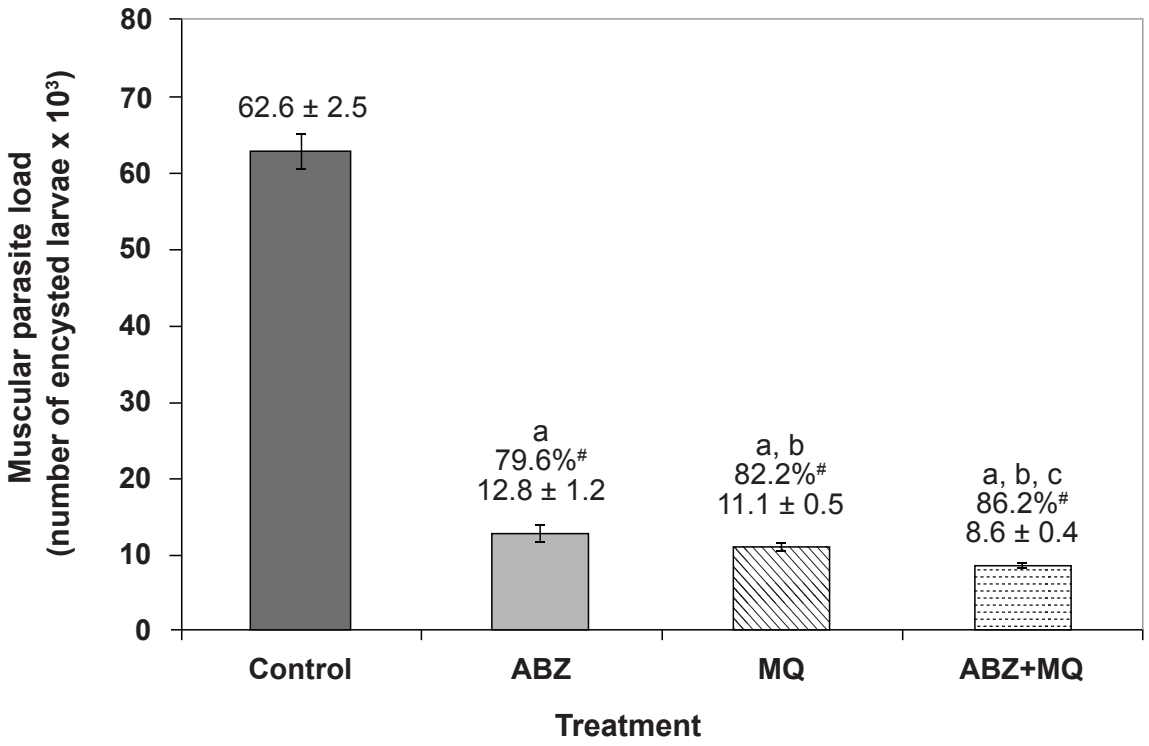

Fig. 2. Muscular parasitic load in CD1 Swiss albino male mice infected with *T. spiralis* L1 larvae and treated with Albendazol, Mefloquine, or both, in the chronic phase of infection.
Description: The number of encysted larvae recovered from the muscles estimates the muscular parasite load (see M&M). Each bar represents the mean ± SEM of 10 mice. Differences among treatment groups were analyzed with ANOVA; comparisons between treatments were done with LSD post-test.
Differences were considered significant if $P < 0.05$. a: significantly different compared with the control group. b: significantly different compared with ABZ group.
#: percent change from the control group.

(v/v) Tween-80, 3 % (v/v) ethanol and distilled water (Keiser *et al*., 2009; Fakahany *et al*., 2014). A combination of ~ 1/3 doses of both drugs [ABZ (20 mg/kg/day) and MQ (140 mg/kg)] both were prepared separately, then orally administered one after the other. All mice were infected with 200 *T. spiralis* larvae and randomly divided into two main experimental groups according to the time of initiation of the treatment: group I, treated in the acute phase of infection (from day 1 post-infection); group II, treated in the chronic phase (from day 35 post-infection). Each group was further divided into 4 subgroups (n = 15), non-treated control (group C); ABZ-treated (50 mg/kg/day for 3 consecutive days) (group ABZ); MQ-treated (a single 400 mg/kg dose) (group MQ); ABZ (20 mg/kg/day for 3 consecutive days) and MQ (140 mg/kg)-treated (group ABZ+MQ). Mice from group I were sacrificed on day 7 post-infection to analyze intestinal worm burden (n = 10) and histopathological changes in the small intestine (n = 5). Mice from group II were sacrificed on day 48 post-infection to determine larval muscle burden (n = 10) and histopathological changes in the diaphragm (n = 5).

Isolation of adult worms and muscle larvae

Adult worms were collected using the method of Wranicz *et al.* (1998). The worms recovered from the small intestine were counted, and the intestinal worm load was expressed as the total number of intestinal worms per mouse. Muscle larvae were recovered from infected mice's carcasses by artificial digestion according to standard procedures (Jiang *et al*., 2012). Muscle larval load was determined by counting all the larvae present in a carcass digest aliquot; it was expressed as a total number of encysted larvae per mouse.

Histopathological examination

Small intestine samples were obtained from mice sacrificed in the acute phase of infection (group I, subgroups C, ABZ, MQ and ABZ + MQ) according to the method described by Nasseff *et al.* (2018). The diaphragm was removed after euthanasia from mice treated in the chronic phase (group II, subgroups C, ABZ, MQ and ABZ + MQ). The specimens were fixed in 10 % buffered formalin solution, dehydrated, cleared, and embedded in paraffin blocks. Five µM-thickness paraffin sections were processed, mounted on glass slides, and stained with hematoxylin-eosin for histopathological examination.

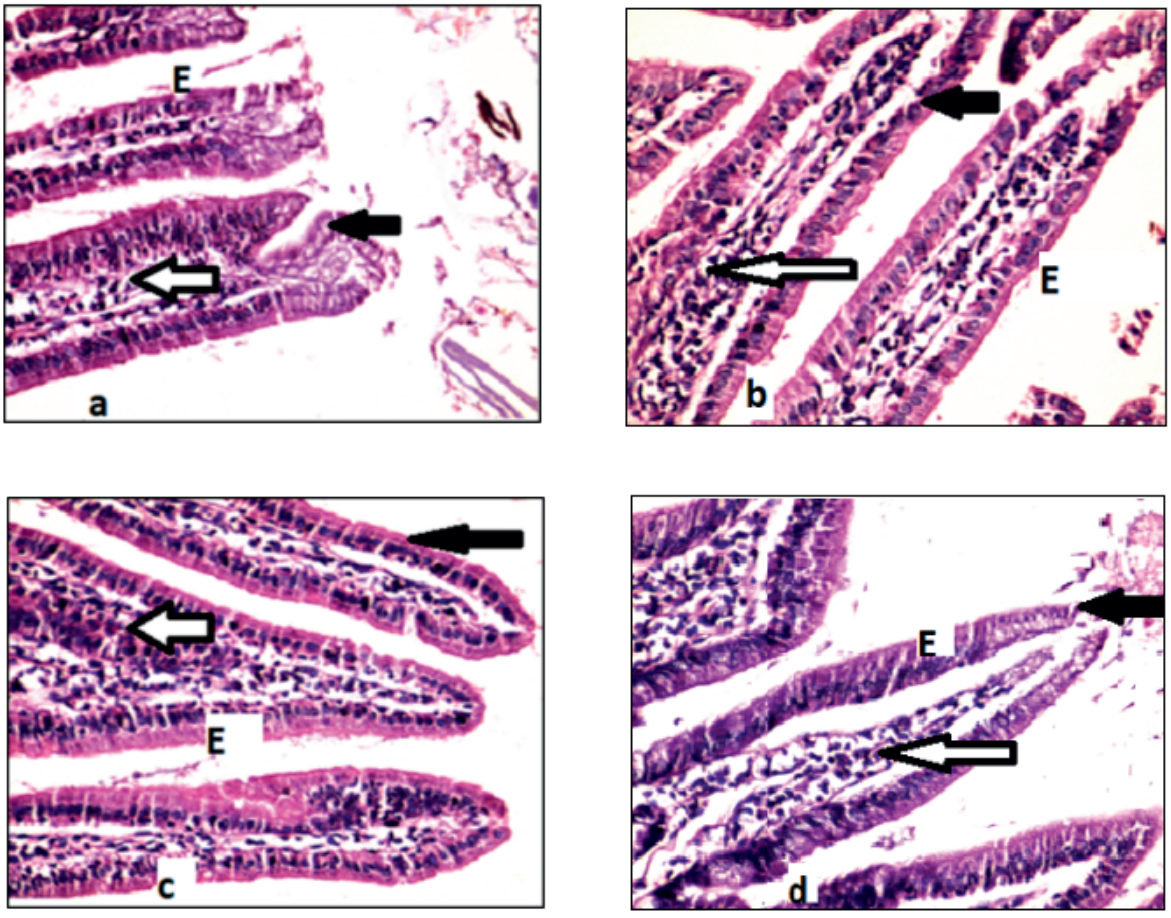

Fig.3. Representative micrographs illustrating the histology of the small intestine of mice infected with *T. spiralis* and treated with antiparasitics in the acute stage of infection.

Description: Tissue samples were obtained on day 7 post-infection. (a) non-treated control, showing dense inflammatory cellular infiltrate (white arrow), mainly in the core of the villi, shedding of the epithelial lining (E), and flattening and hyperplasia of the crypts of the villi (black arrow). H&E staining, magnification 200X. (b) ABZ-treated, showing mild inflammatory infiltrates, mainly within the core of the villi (white arrow) with apparently intact epithelial lining (E) with finger-like villi (black arrow). H&E, X400. (c) MQ-treated, showing intact epithelium lining (white arrow), intact epithelium lining (E), and finger-like villi (black arrow). H&E, X400. (d) ABZ+MQ-treated, showing more reduction in the intensity of the inflammatory infiltrate (line) with intact lining epithelium (white arrow) (E) and finger-like villi (black arrow). H&E, X400.

Statistical analysis

Results are expressed as mean ± standard error. Test of normality, Kolmogorov-Smirnov test, was used to measure the distribution of data. So, a comparison between variables was performed using one way ANOVA followed by LSD test as a post-hoc test if the significant result was recorded. Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. P-value ≤ 0.05 was considered significant.

Ethical Approval and/or Informed Consent

All the experiments on animals were carried out according to the internationally valid guidelines after the approval of the Institutional Ethical Committee of Theodor Bilharz Research Institute (TBRI-REC), the serial number of the protocol: PT (527).

Results

Adult T. spiralis worm count in the small intestine of mice sacrificed at the 7thday post-infection

There is a statistically significant difference in the mean value of worm count between the four studied subgroups (F= 118.199; p= 0.001). The adult *T. spiralis* worm count is significantly decreased in all *T. spiralis*-treated groups (p= 0.001) compared with its corresponding value in (*Group C)*. However, (group ABZ+MQ) recorded the highest percentage reduction in worm count (93.7 %) followed by (group ABZ) (88.7 %) and finally (group MQ) (84.7 %) (Fig. 1).

T. spiralis larvae count in the muscles of mice sacrificed 48 days post-infection

The mean value of larvae count had a statistically significant differ-

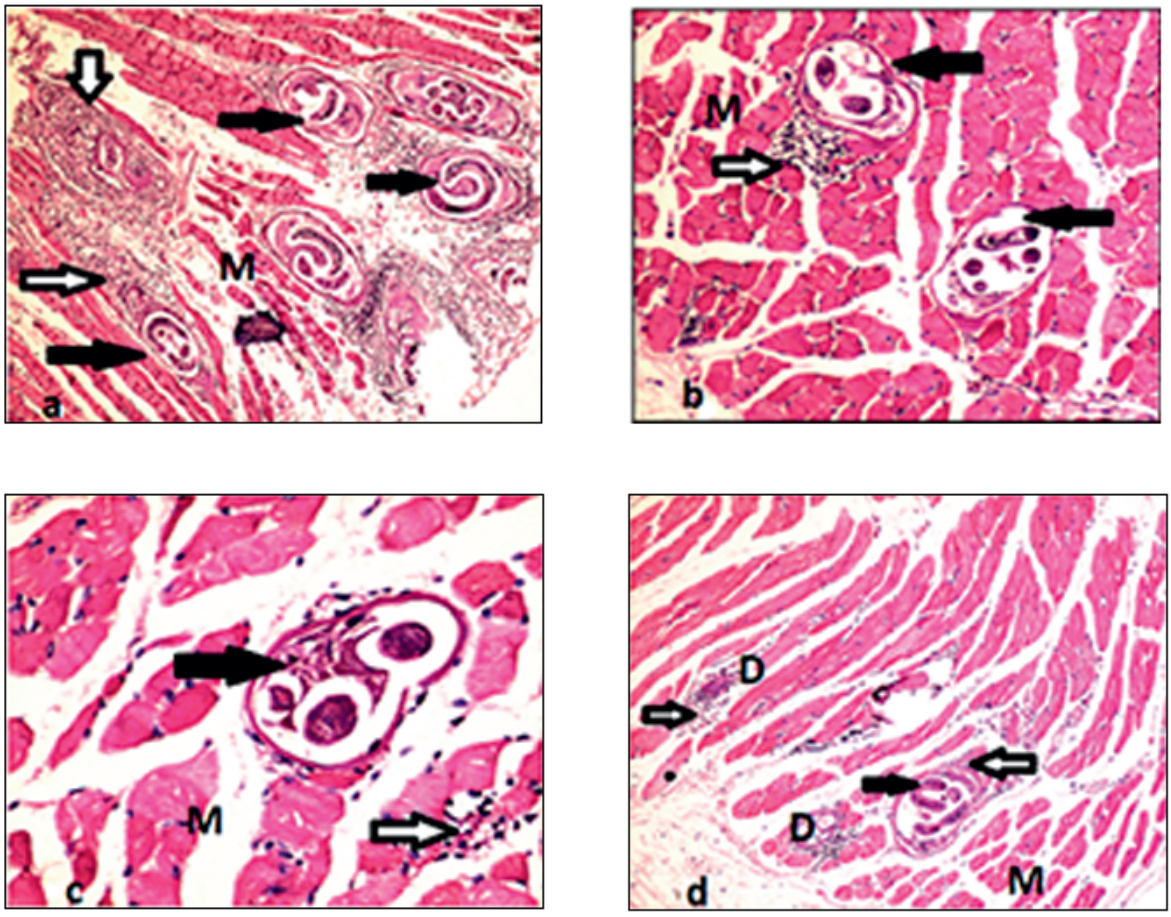

Fig.4. Representative micrographs illustrating the histology of the diaphragm of mice infected with *T. spiralis* and treated with antiparasitics in the chronic stage of infection.

Description: Tissue samples were obtained on day 7 post-infection. (a) non-treated control, showing heavy larvae embedded in the musculature of the diaphragm (black arrow) surrounded by intense inflammatory reaction (white arrow), atrophy, distancing, and tearing of muscle (M). H&E, X200 (b) ABZ-treated, showing larval deposition (black arrow) surrounded by mild muscle inflammation (white arrow). H&E, X200 (c) MQ-treated, showing minimal cellular inflammation (white arrow) and single larva deposition (black arrow). H&E, X400 (d) ABZ+MQ-treated, showing degenerated larvae (D) with broken down incomplete capsule which is completely invaded and surrounded by inflammatory cells (white arrow), inflamed skeletal muscle bundles (M), and single larval deposition (black arrow). H&E, X200.

ence between the four studied subgroups (F= 333.881; p= 0.001). The *T. spiralis* larvae count significantly decreased in all infected-treated groups (p= 0.001), in comparison with its corresponding value in (group C). The total larval counts in (ABZ+MQ group) were significantly decreased in relation to its corresponding value in (ABZ group) (p=0.045). The combined treatment (ABZ+MQ) received on the 35th day post infection showed a drug efficacy of (86.2 %) on encysted *T. spiralis* larvae followed by (MQ group) (82.2 %) and finally (ABZ group) (79.6 %) (Fig. 2).

Histopathological study

The histological analysis of small intestine sections from *T. spiralis*-infected non-treated mice showed inflammatory cells infiltrating both the mucosa and submucosa, in addition to, shedding of the epithelial lining, flattening of the villi and hyperplasia of the crypts of Lieberkühn (Fig. 3a). The infiltrate was composed mainly of lymphocytes and plasma cells, with few neutrophils, eosinophils, and fibroblasts. Intestinal sections of both ABZ- and MQ-treated groups showed a reduction in the inflammatory infiltrate, apparently intact epithelial lining and finger-like villi, and a similar recovery to its normal shape (Figs. 3b and 3c). In the ABZ-MQ-treated group, this reduction reached its maximum, accompanied by a marked decrease in all cellular infiltrates (Fig. 3d).

The diaphragms of non-treated mice showed a massive presence of diffused *T. spiralis* larvae (Fig. 4a). Each larva was limited by a collagenous capsule and heavy inflammatory cellular infiltration with atrophy, distancing, and muscle tearing. In contrast, diaphragm tissue samples of treated animals showed fewer encysted larvae and minimal inflammatory cellular infiltration surrounding them (Figs. 4b and 4c). Furthermore, the diaphragm sections

of the mice that received the combined treatment ABZ+MQ had much fewer encysted larvae, most showing degenerative changes in their contents and capsules (Fig 4d).

Discussion

To date, the drugs against *T. spiralis* are restricted all over the world. The broad-spectrum drug, ABZ, has many adverse drug responses such as encephalitis, epilepsy, extreme medicate eruptions, and even death (Shalaby *et al.,* 2010; Yadav & Temjenmongla, 2012; Matadamas-Martínez *et al.,* 2013). Moreover, it shows diminished effectiveness against *T. spiralis*- encysted larvae (Pozio *et al*., 2001; Siriyasatien *et al.,* 2003; Kalaiselvan *et al*., 2007). Thus the search for novel, safe, and effective anthelminthic agent against the encapsulated muscle larvae of *T. spiralis* is a main objective in medical research.

Repurposing drugs to achieve antihelminthic efficacy is a matter of the utmost importance. This approach seems to result in lower costs, a lower hazard of failure, more safety, and speedier time during the drug development process (Andrews *et al.,* 2014; Panic *et al.,* 2014). MQ has been repurposed against a wide variety of infectious agents (Rodrigues-Junior *et al.,* 2016; Aly *et al.,* 2017; Balasubramanian *et al.,* 2017) as well as helminths like Schistosomiasis (Keiser *et al.,* 2009, 2010; Keiser & Utzinger, 2012) and Echinococcosis (Manneck *et al.,* 2010; Fakahany *et al.,* 2014; Rufener *et al.,* 2018). The results of this study revealed a significant reduction in both *T. spiralis* intestinal worm and muscular larvae count in all treated groups compared to their corresponding *T. spiralis* infected control groups. This decrease was statistically comparable in the case of intestinal worms reaching its maximum peak in the combined ABZ-MQ-treatment (93.7 %). Moreover, the muscular larvae count recorded a significant maximal decline in the combined ABZ-MQ-treated group (86.2 %) compared with the ABZ-treated group (79.6 %) and at the same time MQ alone gave a better reduction (82.2 %) than ABZ, yet still insignificant. It was found that the efficacy of ABZ against *Trichinella* infection is more effective against the intestinal stage than against the muscular stage with a reduction percent extended from 62 % to 100 %, and its effectiveness against muscle larvae ranged from 26 to 91 reduction percent (Shoheib *et al.,* 2006; Shalaby *et al.,* 2010; Attia *et al.,* 2015; Nada *et al.,* 2018). The differences in the effectiveness of ABZ against both the intestinal and muscular stages depend on the dosage, time, and duration of treatment (Siriyasatien *et al*., 2003; Codina *et al.,* 2015).

Ordinarily, MQ intervenes in the killing of malaria parasites by suppressing parasite protein synthesis (Wong *et al.,* 2017). *In vitro* studies reported that MQ kills helminths by making different alterations in their tegument including muscular contraction, mechanical devastation, and metabolic disorders (Abdel-fattah and Ahmed, 2011; Küster *et al.,* 2011; Fakahany *et al.,* 2014). Furthermore, it has anti-angiogenic properties against cancer cells (Kamili *et al.,* 2017) raising its activity of decreasing the muscle larval counts by making the skeletal muscle cells less hospitable to *Trichinella* larvae, depriving the larvae of their nourishment with the accumulation of their wastes triggering their death. The effectiveness of MQ against *T.spiralis* infection is related to its oxidative, apoptotic, and inflammatory properties (Elmehy *et al.*, 2021). Interestingly, the combined treatment shows a tendency to have better therapeutic efficacy than the single drugs. It produces a maximal diminishment in both intestinal and muscular forms (93.7 % and 86.2 %) respectively. This efficacy is achieved with doses one-third lower than those prescribed for each pharmaceutical, which, would indicate synergy between both drugs. Recommendations for medication are commonly for the lowest doses that achieve acceptable outcomes. The results of the present study confirmed that ABZ-MQ-combined treatment is an important option that combines the efficacy of parasite reduction and a low side effect profile with appropriate dosing. The impressive parasiticidal effects are likely to be attributed to the dual action of both ABZ and MQ against *T. spiralis* adult worms and muscle larvae. Elimination of the intestinal forms of *T. spiralis* is significant for early and effective treatment, which is the essential objective of anthelmintic treatment within the first 3 days following *T. spiralis* infection. The synergistic combination of ABZ and MQ possess many advantages against *T. spiralis* due to their accessibility in the markets, broadly characterized in terms of bioavailability, pharmacokinetics and wide therapeutic window (Looareesuwan *et al.,* 1987; Dayan, 2003; Gutman *et al.,* 2009; Ceballos *et al.,* 2018).

Herein, tissue damage and severe inflammations in the histopathological sections of both intestinal and skeletal muscles of *T. spiralis* infected mice was attributed to the increased levels of reactive oxygen species (ROS), superoxide dismutase (SOD), inducible nitric oxide synthase (iNOS), and the overexpression of COX-2, anti-apoptotic particles and inflammatory cells (Othman & Shoheib, 2016). These molecules have been produced not only by the parasite but also by the host cells, increasing tissue damage and reducing apoptotic processes during their defensive response. The parasite triggers angiogenesis through secretion of the panel of immunological molecules like vascular endothelial growth factor (VEGF) (Capo *et al.,* 1998), fibroblast growth factor (FGF)-1, FGF-2, and insulin-like growth factor (IGF-1) (Kang *et al.,* 2011) which in turn results in the development of blood vessels which are essential for larvae survival (Akiho *et al.,* 2005; Serna *et al.,* 2006; Romero *et al.,* 2008; Othman *et al.,* 2016).

Both the intestinal and skeletal muscles of the treated groups had mild inflammatory infiltrates and minimal cellular inflammation. The maximum reduction in these cells of the immune system was found in the combined ABZ-MQ-treated groups especially in muscles of the diaphragm surrounding the encysted larvae. As a result of the diminished number of both worms and larvae, restoration of the normal appearance of host cells was noted accompanied by an obvious diminishment in inflammatory cells (Attia *et al*., 2015; Abou Rayia *et al.,* 2017; Elgendy *et al.,* 2020) particularly in combined ABZ-MQ treated group. The confirmed parasiticidal

effects of MQ in this study may be due to its involvement in ROS generation (Elmehy *et al.,*2021) in addition to its anti-angiogenic and apoptotic properties against the parasites in hosted tissues (Kamili *et al.,* 2017).

In conclusion, the use of combined ABZ-MQ-treatment in reduced regimens has the maximum effect not only on reducing the two phases of parasite burden but also on the restoration of normal tissue architecture. MQ treatment is as successful as ABZ treatment in killing both phases of *T. spiralis* and consequently restoring the normal tissue architecture of the host. The synergistic activity of the two drugs overcomes their side effects and increases their biological activity. Besides, the chemical complexity of the mixtures reduces the risk of drug resistance.

Conflict of Interest

The authors state no conflict of interest.

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