Original Article

Evaluation of the therapeutic efficacy of metformin with or without albendazole on experimental muscular trichinosis

Shaimaa A Sharaf-El-Deen[1], Mona M El-Sobky[1], Gehan S Sadek[1,3], Bahaa E El-Aswad[1], Amira M Atallah[1], Rania I Yassien[2], Amany I Ammar[1]

Departments of Medical Parasitology[1] and Histopathology[2], Faculty of Medicine, Menoufia University[1,2], Egypt, and King Salman International University, KSA[3]

ABSTRACT

Background: Most drugs used for the treatment of trichinosis showed a limited bioavailability, and induced adverse side effects on administrating high doses. Therefore, there is a need to develop new agents to improve the bioavailability of these drugs.
Objective: To assess the efficacy of metformin (MTF) administration as a co-drug with ALZ to treat muscular trichinosis.
Material and Methods: A total of 25 mice were divided into five equal groups, categorized as negative control group, and four *T. spiralis*-infected groups: positive control, MTF-treated group, ALZ-treated group, and combined MTF-ALZ-treated group. Larval burden, muscle histopathology, new vascular formation by measuring CD34, and expression of the gene encoding myogenin (Myog) by real time PCR were investigated at the end of the experiment 35th day post-infection (dpi).
Results: The results demonstrated that the group treated with MTF plus ALZ had the best outcome among all parameters, followed by the group treated with MTF, and finally the group treated with ALZ. Both MTF with ALZ proved to be a promising combination for the treatment of muscular trichinosis in experimentally-infected mice.
Conclusion: It was concluded that MTF reduced the pathology of muscular phase of trichinosis. Further studies are recommended to better understand MTF pharmacokinetics.

Keywords: albendazole; metformin; muscular vasculature; myogenin; nurse cells; trichinosis.

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Corresponding Author: Shaimaa A. Sharaf-El-Deen; **Tel.:** +20 1001118501**; Email:** shaymaa.sharafeldeen@med.menofia.edu.eg

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INTRODUCTION

Trichinella species are worldwide foodborne nematodes that cause trichinosis in many species of mammals, reptiles, and birds. Human infections are often caused by *T. spiralis* after ingesting undercooked pork[1]. Approximately 11 million patients worldwide are chronically infected with *T. spiralis*[2]. Trichinosis in animals adds to the economic burden on the livestock industry and trade[2,3]. Clinical manifestations vary according to the number of ingested live larvae, age, gender, health status and cultural group of the host. It often starts with nonspecific intestinal manifestations which relate to the presence of adult worms in the small intestine. Once the larvae are laid and find their way to the circulation, this short enteral phase ends and the parenteral phase begins. The most common manifestations of the parenteral phase are myalgia, and periorbital oedema, and it ends when the larvae reach the skeletal muscles of the host[4,5]. Muscles have a strong capacity for regeneration under normal circumstances, allowing them to heal themselves after injury. During muscle regeneration process, the satellite muscle progenitors cells exit their normal quiescent state to start proliferating. After several rounds of proliferation, most of the satellite cells differentiate and fuse to form new myofibers to repair damaged ones[6].

Injury of the skeletal muscles by the invading *Trichinella* larvae initiates a process of repair, as after any trauma. However, *Trichinella* borrows only the initial part of this repair process to construct its own home for a long-lasting infection in skeletal muscles of the hosts without killing them[7]. A septum is formed between the injured and intact fibers and differentiation of satellite cells is stimulated. Unlike other causes of muscle injury, the satellite cells mis-differentiate into the nurse cells. The infected muscle cells arrest or stop their cell cycle progression. This arrest occurs before satellite cells' differentiation that are responsible for muscle regeneration and repair. Instead, they undergo a process called de-differentiation to form specialized cells known as nurse cells[8]. Formation of nurse cells occurs through

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a finely orchestrated set of cellular responses, forming a well-innervated, fully vascularized, and contractile muscle apparatus after about 20-30 dpi[6,9,10].

To avoid muscle injury, treatment must be initiated during the first few days after trichinosis to prevent onset of the parenteral phase of the disease. However, treatment is usually started only after the larvae reach the host muscles and start to cause muscle pain. Unfortunately, neither mebendazole nor ALZ are fully effective in the treatment of invading larvae. However, because no major side effects are detected with ALZ treatment, it is usually used to treat symptomatic patients. The intestinal absorption rate of oral ALZ is ~1–5%. Using higher doses or prolongation of treatment, to compensate for this low absorption, can lead to toxicity; for example, neutropenia due to myelosuppression[11]. Till now, no anthelminthic drug has shown total efficacy against the newborn larvae or the maturing first-stage larva[12].

The vascular network that surrounds the nurse cell is a vital structure that regulates proper transport of nutrients and wastes for maintenance of larvae metabolism. Therefore, a drug that can effectively suppress angiogenesis around the encysted larvae can be a good candidate for treatment of muscular trichinosis[6,13,14]. Many proteins are involved in the regulation of neo-angiogenesis, among them is CD34. Notably, the latter is a type I transmembrane phosphorylated glycoprotein expressed on the surface of hematopoietic progenitor cells that can differentiate into new blood vessels. It is expressed on the mucosal dendritic cells, mast cells, eosinophils, and other immune cells[15]. Besides, CD34 is extensively involved in cell adhesion, inflammatory cell chemotaxis, cell proliferation and differentiation, and enhancement of the inflammatory response[16].

On the other hand, MTF, the first-line treatment for type 2 diabetic mellitus, exhibited regulatory effects in the process of angiogenesis. Its anti-angiogenesis properties prove to inhibit the occurrence of tumor metastasis and alleviated patients' symptoms with polycystic ovary syndrome[17]. It was observed that numerous genes and signaling pathways are involved in the production of nurse cells. Among the myogenic regulatory factors, MyoD and MyoG crucial for myogenesis and muscle regeneration, were overexpressed in infected muscle tissues during trichinosis[6]. Therefore, analyzing the expression of *myoG* gene and its regulatory factors in muscular tissues by real time PCR would assist in evaluating MTF efficacy.

In view of the foregoing literature, the current work was performed aiming to assess the efficacy of MTF administration as a co-drug with ALZ for treating muscular *T. spiralis* in experimentally infected mice.

MATERIAL AND METHODS

This experimental study was conducted at the Theodor Bilharz Research Institute (TBRI) and Parasitology Department, Faculty of Medicine, Menofia University during the period from April to June, 2020.

Study design: The study was designed to compare MTF-ALZ combined treatment versus single drug treatment in muscular trichinosis. Parameters used included larval burden, muscle histopathology, CD34 expression, and measurement of *myoG* gene expression.

Animals and study groups: A total of 25 inbred pathogen-free male Swiss albino mice (6–8 w, 18–20 gm) were randomly categorized into five groups. Each group included 5 mice. Group I (GI): non-infected negative control group; group II (GII): *T. spiralis*-infected positive control group; group III (GIII): MTF-treated group; group IV (GIV): ALZ-treated group; and group V (GV): MTF-ALZ-treated group.

Mice infection: Mice of groups II-V were infected with a dose of 200±10 *T. spiralis* larvae/mouse using an intra-esophageal tube as previously described[18].

Treatment schedule and dose: Mice received oral MTF (Minapharm, Egypt) at a dose of 50 mg/kg/day dissolved in distilled water[19], and ALZ at a dose of 50 mg/kg/d[20]. Administration of both drugs was performed daily starting on the 12th dpi till the end of the experiment.

Sample collection: Mice were euthanized by decapitation 35 dpi. Diaphragms and thigh muscles were dissected and divided into three parts. The first part was digested in 1% pepsin, and 1% concentrated HCL in distilled water[21] for larval counting. The second part was preserved in 10% formalin for further histopathological studies. The third part was frozen for real-time PCR to quantify *myoG* gene expression.

Muscle larval burden: After muscle digestion, the supernatant fluid was discarded, and the larvae in the sediment were counted microscopically using a McMaster counting chamber[19]. Total number of living larvae/cm^3 = mean number of living larvae in both sides of the chamber/0.001.

Muscle histopathology: Formalin-fixed muscles were processed and stained with H&E following the standard process. Specimens were randomized, coded, and blindly assessed for the degree of pathology[22].

Immunohistochemical staining[23]: Staining of the CD34 endothelial progenitor cells in paraffinized muscle tissues was performed using mouse anti-CD34 antibodies (ab 185732, Abcam, USA). Positivity was identified when the cell membrane alone or

together with the cytoplasm showed brown staining. Immunohistochemical grading of CD34 staining was calculated by histoscore (H-score). Intensity of membrane staining was given a number from 0 (not stained), to 1+, 2+ and 3+. The percentage of stained cells in each tissue was multiplied by the intensity of staining. A score of 0-300 was given for muscle tissue [1 x (% cells 1+) + 2 x (% cells 2+) + 3 x (% cells 3+)][24].

Evaluation of *myoG* gene expression in muscular tissue by real time PCR[25,26]: Total RNA was extracted from frozen muscular tissue samples using miRNeasy Micro Kit (Cat. No. 217084, Qiagen, Germany) according to the manufacturer's protocol. The purified RNA was used as a template to produce a complementary DNA (cDNA) using reversed transcriptase (RT) enzyme by QuantiTect Rev. (Cat. No. 205311, Qiagen, Germany) following the supplier's recommendations. Relative quantitative real-time PCR was performed in an Applied Biosystems StepOnePlus Real-Time PCR System (ThermoFisher Scientific, USA), using the QuantiTect SYBR Green PCR kit (Cat. 204143, Qiagen, Germany) according to manufacturer's instructions. The *myoG* gene expression was quantified relative to the housekeeping gene (*β-actin*). The primers used in real-time PCR for *myoG* gene were 5′-TCTACCGGAGCCCCACTTC-3′ (forward primer), and 5′-CATCAGGACAGCCCCACTTA-3′ (reverse primer)[25], and those for *β-actin* were 5′-TTGGGTATGGAATCCTGTGG-3′ (forward primer), and 5′-GGTGTAAAACGCAGCTCAGT -3′ (reverse primer)[25].

Statistical analysis: Data collected were tabulated and analyzed by SPSS version 22.0 on IBM compatible computer. Quantitative values of the measured parameters were ex-pressed as mean ± standard deviation (SD). Mann-Whitney test and chi-square (χ^2) tests were used. Significance is considered when $P<0.05$.

Ethical consideration: Mice were bred under standard breeding conditions at the animal house of Theodor Bilharz Research Institute (TBRI) Giza, Egypt. The breeding room was air-conditioned at 20-22°C, and mice were fed commercial pellet food, and provided with water *ad libitum*. All research procedures were performed in accordance with the international ethical guidelines approved by the institutional ethical committee (IRP: 191219PARA9).

RESULTS

Larval counts: Group V that received the combined therapy had the highest percentage of larval count reduction (7380±1965.2; 83.2%). The MTF-treated group (GIII) also showed a marked reduction of larval count (10800±1095.4; 75.5%) ranking second to GV, with a statistically significant difference between both groups (P<0.05). The lowest percentage of reduction was recorded in the ALZ-treated group (22000±1369.3; 50.01%) with a statistically significant difference compared to GIII and GV ($P<0.001$) (Fig. 1).

Muscle histopathology: The degree of inflammatory reaction in the surrounding muscle tissue was the lowest in GV that received the combined therapy (100% mild inflammation). The MTF-treated group (GIII) also showed marked reduction of inflammation ranking second to GV, (80% of the mice showed mild and the other 20% revealed moderate inflammation). The ALZ-treated group (GIV) ranked last (40% of the mice showed mild and 60% had moderate inflammation) (Figs. 2 and 3).

Immunohistochemical staining of CD34 expression: The expression of the hematopoietic progenitor cell (HPC) marker, CD34, was significantly reduced in the combined therapy group (GV), with a mean H-score of 28±21.7, followed by GIII, MTF-treated group (130±27.4), with statistically significant differences between both groups ($P<0.001$). The GIV, ALZ-treated mice, demonstrated the highest CD34 expression (mean H-score=218±10.9) compared to the other treated groups. However, it remained lower than the infected control (mean H-score=288±10.9; $P<0.001$) (Figs. 4 and 5).

Gene expression: Expression levels increased significantly ($P<0.001$) in the infected non-treated group (GII) (96±5.5) compared to the negative control group (GI). The GV mice treated with both MTF and ALZ, had the lowest Myog expression value (7.0±2.7). There were statistically significant differences between GV and all other studied groups ($P<0.001$) (Fig. 6).

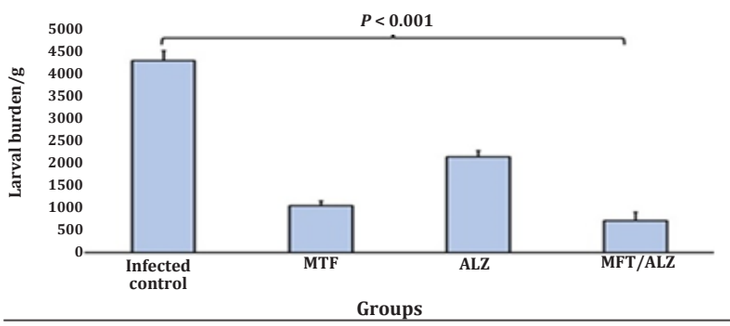

Fig. 1. Larval burden in the study groups. The lowest numbers of larvae were detected in MTF-ALZ-treated group, with statistically significant differences compared to other groups.

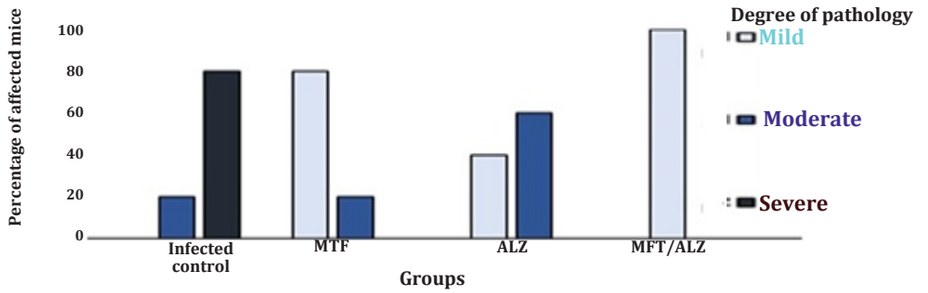

Fig. 2. Degree of pathology in the study groups. The lowest degree of pathology was detected in MTF-ALZ group (100% mild inflammation).

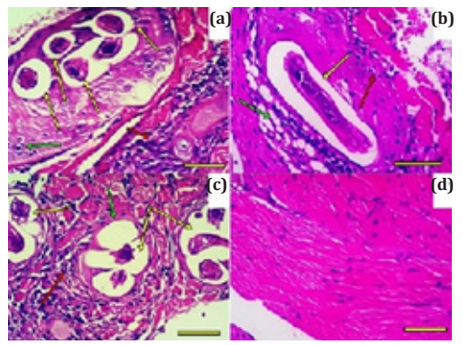

Fig. 3. Stained muscle sections of different study groups. **(a)** infected control group showing transverse sections of multiple *T. spiralis* larvae (yellow arrows) surrounded by a capsule with hypertrophied nurse cell nuclei (green arrow) and severe inflammatory reaction (red arrow). **(b)** MTF-treated group showing single encapsulated larva (yellow arrow) surrounded by mild inflammatory reaction (red arrow) and fatty necrosis (green arrow) of few muscle fibers. **(c)** ALZ-treated group revealing moderate number of encapsulated larvae (yellow arrow) with thin capsular wall (green arrow) surrounded by moderate inflammatory reaction (red arrow). **(d)** MTF-ALZ-treated group with total absence of larvae and inflammation. Muscle fibers are apparently normal (x200; scale bar = 100 µm).

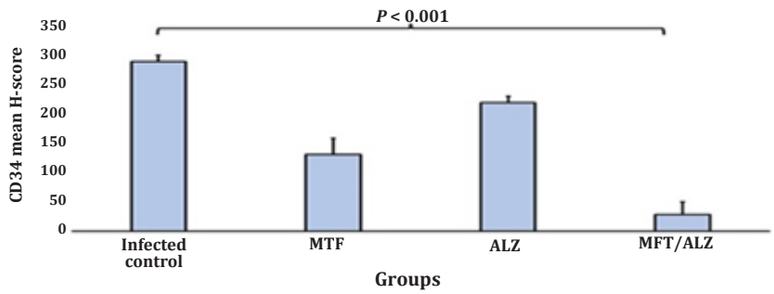

Fig. 4. Mean H-scores of the study groups. The lowest mean H-score was detected in MTF-ALZ-treated group with statistically significant differences compared to other groups of the study.

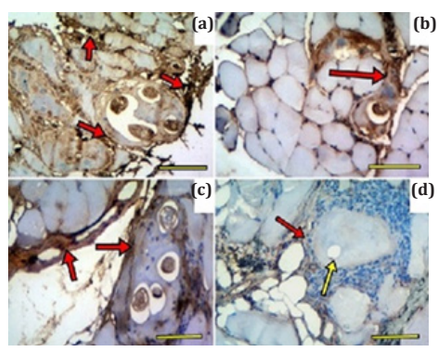

Fig. 5. Immunohistochemical CD34 antibody staining of muscle tissue sections of different study groups. **(a)** infected control group showing strong expression of CD34 (red arrow). **(b)** MTF-treated group showing mild expression of CD34 (red arrow). **(c)** ALZ-treated group showing moderate expression of CD34 (red arrow). **(d)** MTF-ALZ-treated group showing weak expression of CD34 (red arrow) around an empty capsule (yellow arrow). [x200; scale bar = 100 µm].

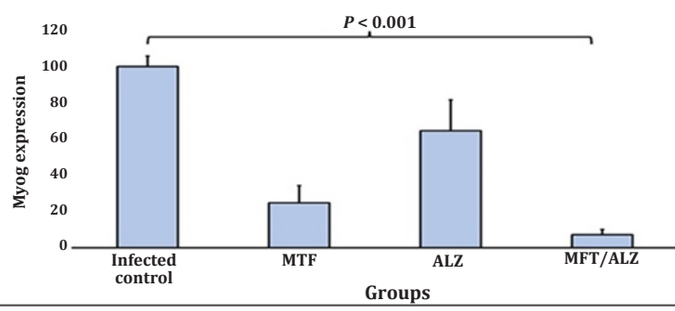

Fig. 6. Myog expression of the study groups. The lowest Myog expression was detected in MTF-ALZ-treated group V with statistically significant differences compared to other study groups.

DISCUSSION

The current work evaluated MTF as a potential co-drug that can potentiate the therapeutic efficacy of the main anti-*T. spiralis* drug, ALZ, for treatment of muscular trichinosis. Despite its satisfying effects against intestinal adults, the efficacy of ALZ against muscle encysted larvae is weak because of its low bioavailability[11]. The encysted larvae can survive for years because they utilize the initial steps of the muscle repairing process to construct their own nurse cells. These nurse cells protect them from being destroyed by the host humoral and cellular immune response. Nurse cells are surrounded by a vascular network which originates from neighboring arterioles. This network of blood vessels allows the nutrients and wastes to be evenly transported to meet the needs for larvae's metabolism. Therefore, a drug that can effectively inhibit angiogenesis during the encystment is a good candidate to suppress the development of *T. spiralis* muscle larvae[6,13,14].

The anti-angiogenesis properties of MTF were documented in previous studies[17]. Its prophylactic effect in muscular trichinosis was reported by Othman *et al*.[19] and was explained by its ability to reduce the oxidative stress and the expression of vascular endothelial growth factor (VEGF) in muscular trichinosis, although it failed to reduce the number of encysted larvae. This was the reason for studying MTF's therapeutic properties as a co-therapeutic agent with ALZ in treatment of muscular trichinosis. Metformin was started at day 12 pi to allow for the development of encysted muscle larvae. This allowed us to assess the therapeutic, not the preventative, properties of the drug since at 12 dpi, a noticeable pathology would have appeared[27]. In real infections in humans, most of the patients have nonspecific intestinal manifestations early in the infection and are diagnosed during the parenteral phase.

Results of larval count were in accordance with Othman *et al*.[19] who showed that there was a significant decrease in total larval counts in the group treated with MTF. Loos *et al*.[28] demonstrated that the combination therapy of MTF with ALZ offered an alternative to improve the efficacy and reduce the toxicity of the high-dose ALZ monotherapy currently employed for alveolar ecchinococosis.

The statistically significant reduction of muscle pathology detected in both MTF-treated groups suggests a pathology reducing criterion of MTF. The beneficial effect of MTF in reduction of muscle injury was recorded in previous studies with different explanations of the underlying mechanisms. Langone *et al*.[29] attributed this effect to regulation of the intracellular calcium levels which increase the muscle resistance to injury. In addition, Yang *et al*.[30] reported an anti-muscle dystrophic action of MTF due to regulation of the lipid deposition and glucose utilization by muscles. After muscle injury, neovascularization is required to supply nutrition and to create a proper environment for tissue regeneration. This process is carried out by the endothelial progenitor cells (EPCs) that can differentiate into endothelial cells and contribute directly to the formation of new blood vessels. The EPCs usually express both primitive hematopoietic progenitor markers (CD34 or CD133), and endothelial markers (CD31, VEGF receptor 2, and vascular endothelial cadherin)[31]. In the current study, we detected a statistically significant reduction in CD34 expression around the encapsulated larvae in the MTF-treated groups. Lower vascularization may result in starvation of the treated larvae with subsequent weakness of their tissue-destructive ability, so they could be easily removed by the immune system of the host[13]. This may explain the detected reduction of pathology and larval count in MTF-treated groups. The high reduction detected in combined-therapy group may be because ALZ killed the unarmed starving parasite. Reduction of vascularization by MTF was similarly reported by Othman *et al*.[19]. They explained their results by the suppressive effect of MTF on VEGF. The regulatory effect of MTF on blood vessel proliferation was described in various studies and different mechanisms of action were reported e.g., CD34 modulation[32] and up-regulating the expression of peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β)[33].

Additionally, the partial effects of ALZ against encysted larvae can be explained by its anti-angiogenic effects that appeared in ALZ-treated group. Additive effects can explain the very high degree of protection against muscular trichinosis when combined with another antiangiogenic agent, MTF. This action was similarly reported by El-Dardiry *et al*.[34] who attributed ALZ antiangiogenic action to its downregulating effect on VEGF. This can explain the associated reduction of the endothelial growth factor (CD34).

Myogenic regulatory factors (MyoD and MyoG) are overexpressed in infected muscle tissues and are known to be essential for myogenesis and muscle regeneration[24], so they could be a parameter for assessing MTF efficacy. Besides, MyoG is one of the more thoroughly researched tissue-specific molecules exclusive to skeletal muscle and is a crucial developmental regulator for the creation of skeletal muscle[35]. It causes the synthesis of proteins necessary for cell cycle termination, signaling myogenic cells to stop growing and the satellite cells otherwise de-differentiate to form the nurse cells serving the parasite benefits[36]. This could explain why Myog expression was increased in *Trichinella* infected control group (GII). Accordingly, reduced MyoG

expression n MTF-treated group (GIII) indicated that MTF influenced MyoG expression moving or forcing the muscle cell back on the right path. Meanwhile, combined treatment exhibited a stronger impact on lowering gene expression, and provided a novel promising approach to treat muscular trichinosis.

In conclusion, MTF reduced the pathology of muscular trichinosis due to reduced vascularization, prevention of proper nurse cell formation, and downregulation of Myog expression.

Author contributions: Sharaf-El-Deen SA, El-Sobky MM, Sadek GS, El-Aswad BE, Atallah AM, Ammar AI designed the research topic. Sharaf-El-Deen SA, Atallah AM, Ammar AI were responsible for acquisition, analysis, and interpretation of the research data. Sharaf-El-Deen SA and Ammar AI wrote the manuscript draft. Yassien RI was responsible for the histopathological data. El-Sobky MM, Sadek GS, and El-Aswad BE revised the article for publication. All authors approved the final revision before publication.

Conflict of interest: The authors declare that they have neither competing interests with any organization that could influence this work, nor potential disagreement with respect to the authorship.

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