Role Of α- chymotrypsin And Colchicine As Adjuvant Therapy In Experimental Muscular Trichinellosis: Parasitological, Biochemical & Immunohistochemical Study

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BACKGROUND AND OBJECTIVE: Muscular trichinellosis is a crippling disease, and it is not satisfactorily treated by the anti-parasitic drug albendazole. Trichinella spiralis larvae can survive for long time in muscle phase by formation of fibrous (collagenous) capsule around it, for their protection from host immune system. Colchicine, is a well known antifibrotic that is used long time ago in different fibrotic diseases, and proved recently to be safe for use even in long durations. Also, α-chymotrypsin is one member of proteinase enzymes that were proved to cause degradation of extracellular matrix components. So, this study aimed to investigate the effect of adding these antifibrotic agents to albendazole during treatment of muscular trichinellosis. MATERIAL AND METHODS: The study was performed on 175 albino mice comprising 7 equal groups 25 mice each. Mice from groups I to VI were infected by T.spiralis larvae orally (200larvae / mouse), group VII served as the healthy non infected group for comparison of biochemical and immunohistochemical results. Group II was treated by albendazole alone orally for three successive days, groups III,IV were given α-chymotrypsin intramuscularly and colchicine orally respectively, for four weeks. Groups V,VI were given combined therapy of albendazole and α-chymotrypsin & albendazole and colchicine respectively for four weeks. Assessment of results was achieved through; parasitological, biochemical and immunohistochemical studies. Total larval count was done to all studied infected groups, measurement of tissue markers of fibrosis'' muscle hydroxyproline and (MMP-2)'',and also immunohistochemical staining of collagen type IV and fibronectin was done to both infected groups and the non infected one for comparison. Statistical analysis was performed to determine differences between studied groups in different measured parameters. RESULTS: In groups treated with combination therapy (albendazole and α-chymotrypsin, ''group V'' & albendazole and colchicine'' group VI''), there was a marked reduction in total larval count, reduction of muscular hydroxyproline levels ,elevation of muscular MMP-2 when compared to the group treated with albendazole alone ''group II'', the differences were found to be statistically significant (p<0.05). At the same time , the differences in the previously mentioned parameters were proved to be non significant statistically between group V and group VI. There was also a decrease in staining intensity of collagen type IV and fibronectin in groups received the antifibrotics(groups III to VI)in comparison to both groups I and II and the , there was a statistical positive correlation between hydroxyproline biochemical mean value and staining intensity(p<0.05).CONCLUSION: Addition of antifibrotic drugs (α- chymotrypsin and colchicine) as adjuvant therapy to antiparasitic drugs in treatment of muscular trichinellosis could be a beneficial measure to improve treatment outcome by decreasing both numbers of larvae in the muscle, and fibrous tissue formation, which form together the backbone of pathology and crippling to the patient.

INTRODUCTION

Trichinella spiralis, the causative agent of trichinellosis, is one of the most interesting animal parasitic nematodes as it occupies two distinct intracellular niches within the same host i.e. intestinal epithelium " during intestinal phase " and skeletal muscle cells " during muscle phase". However, the skeletal muscle cells are the only cell type that can support growth, development, and long–term survival of the Trichinella larvae. Residence of larvae in skeletal muscle cell leads to myalgia, which is an outstanding clinical sign of trichinellosis, it was reported in 85%of patients in acute illness and 72% in chronic phase. Muscle phase of the infection is accompanied by severe myositis reaction with muscle pain, weakness and easy fatigability together with reduction in mechanical properties of the affected muscle. All these changes are related to number of larvae in the muscle. Survival of the larva inside the skeletal muscle myotube depends on the creation of the so called "encapsulated Nurse cell" around it, this could be achieved through the effect of tyvelosylated excretory / secretory proteins of the larva. From its name the Nurse cell is responsible for both;
meeting the parasite's metabolic nutritional demands (10) and protection of the parasite from the host's defense mechanisms. (11) The source of collagen capsule is speculated; it may be originated from modified muscle fibers, or the fibroblasts outside the altered muscle fibers. (12) The inner layer of the capsule is formed of glycoproteins, proteoglycans, lammin, fibronectin, and collagen. The outer layer is composed of fibronectin, type IV collagen and probably type III collagen. (13,14) Fibronectin is a dimeric glycoprotein which is present in cells, extracellular matrix, and blood. It is involved in cell adhesion, tissue organization, and wound healing. Collagen IV is a major constituent of the basement membranes along with laminins and enactins. It can form insoluble fibers with high tensile strength. (14) The capsule is surrounded by intense inflammatory infiltrate composed of mainly macrophages, lymphocytes, eosinophils, plasma cells, fibroblasts and neutrophils mainly polar in distribution. (15)

It is to be mentioned here that, collagen which is one of the major structural components of the extra-cellular matrix exhibits resistance to proteolytic cleavage by endogenous and exogenous proteinases except for matrix metalloproteinases (MMPs) that are secreted by many types of cells including fibroblasts and macrophages (16) as inactive proenzymes requiring the cleavage of a propeptide for activation "that could be mediated by antifibrotic drugs". (17) It was proved that matrix metalloproteinase-2 (MMP-2), which belongs to this family, is capable of disintegrating extra-cellular matrix components especially fibronectin as well as collagen types IV and V. (18)

The expression and activity of these MMPs are regulated by large variety of modulators, inflammatory cytokines and drugs; including tissue inhibitors of MMPs (TIMPs), transforming growth factor-β1 (TGF-β1) and alpha2-macroglobulin. So, because metalloproteinases and their tissue inhibitors (MMPs / TIMPs) are key regulators of collagen metabolism and the physiopathology of fibrosis (19), they play a very important role in pathogenesis of many diseases, and hence their drug-induced regulation may be critical in treatment of many fibrotic diseases. (20,21)

Albendazole is a newer benzimidazole carbamate, that is used worldwide against a wide variety of human helminthic infections. (22) It was found to be active against not only a wide variety of intestinal and tissue nematodes as; ascariasis, hookworm and also lymphatic and tissue filariasis (23,24), but also proved to be effective against some cestodes as, cysticercosis (25) and cystic hydatid disease. (26)

Inspite that, albendazole is still the available drug of choice used for trichinellosis treatment (27), it was proved that although benzimidazole compounds are highly active against the intestinal stages of Trichinella spiralis in human beings, they are marginally effective against larval stages in tissues. (28)

So, trichinelllosis which is a re-emerging zoonosis, needs more comprehensive approach to its treatment especially of the muscle phase at which the patient mostly seeked medical advice. (29,30) Regarding that the target point in treatment of muscle phase of Trichinillosis may be directed towards the encapsulated-Nurse cell. This could be accomplished by using antifibrotic drugs either preventing Nurse cell collagen formation or targeting its breakdown. Introduction of different antifibrotic agents as adjuvant therapy to the specific treatment was widely studied in the last few years, either on models of parasitic infections as hepatic schistosomiasis mansoni (31), or non parasitic injury of skeletal muscle. (32)

Colchicine is an alkaloid that has been used for centuries in gouty arthritis. In the last 50yr it has been employed for an increasing number of diseases including familial Mediterranean fever, Behcet’s syndrome, scleroderma, amyloidosis. (33) Also, because of its antifibrotic and anti-inflammatory effects, colchicine has been proposed as a treatment for hepatic fibrosis of various etiologies. (34) The exact mechanism of colchicine action is not fully understood. Most of its pharmacological effects appear to be related to inhibition of microtubule self-assembly by binding β-tubulin, thus, inhibiting movement of intercellular granules and secretion of various substances. (35) These complex actions, form the basis for the prophylactic or therapeutic application of colchicine in a whole range of many diseases that have so far been unsatisfactorily controlled by other treatments. Fibrotic and
inflammatory systemic diseases seem to be particularly predestined for this.\(^{(36)}\)

It has been reported that proteinase therapy (including \(\alpha\)-chymotrypsin) may have beneficial effects in treatment of fibrosis and certain cancers.\(^{(37)}\) The mode of action of such preparations was not clear. However, the results suggested that these polypolymerase proteinases were proved to cause degradation of the extra-cellular matrix (ECM) components.\(^{(38)}\) So, the application of \(\alpha\)-chymotrypsin as an antifibrotic therapy could be suggested.

The aim of this study was to assess the role of "colchicine" and "\(\alpha\)-chymotrypsin" as antifibrotic drugs when applied as adjuvant therapy to the antiparasitic drug "albendazole" in muscle phase of experimental trichinellosis in mice.

**MATERIAL & METHODS**

**Parasite:**

The strain of *Trichinella spiralis* was obtained from infected pig in Cairo abattoir and maintained in albino mice and rats in Parasitology Department laboratory, Tanta University.

**Animals:**

One hundred and seventy five Swiss albino mice, weighing 15–20 gm each, and aging eight weeks were used in this study after being proved to be parasite-free. All animals were supplied from Abo–Rawash, Giza Governorate. They were maintained ad libitum on ordinary laboratory diet throughout the duration of the experiment. Animals were divided into seven equal groups.

**Drugs and Dosage:**

- **Albendazole:** was supplied as suspension, 100mg /5ml, (SIGMA pharmaceutical Co.). It was given by oral gavage daily in a dose of 50mg /kg \(^{(39)}\) for three successive days.
- **\(\alpha\)-chymotrypsin:** was supplied as vials, 5mg each, (Amoun Pharmaceuticals). It was given by daily I.M. injection of 1.3mg /kg \(^{(40)}\) for four weeks.
- **Colchicine:** was supplied as tablets, 500\(\mu\)g each, (ADWIC pharmaceuticals Co.). It was dissolved in distilled water and given by oral gavage daily in a dose of 200\(\mu\)g /kg \(^{(41)}\) for four weeks.

**Animal groups:**

The animal groups of experimental model of trichinellosis (150 mice) were infected orally by living *Trichinella spiralis* larvae, in a dose of 200 larvae / mouse.\(^{(42)}\) Twenty days post infection ,drug administration was started according to the following regimen:

**Group I:** Served as infected non-treated control group.

**Group II:** Received albendazole therapy for three successive days.

**Group III:** Received \(\alpha\)-chymotrypsin alone for four weeks.

**Group IV:** Received colchicine alone for four weeks.

**Group V:** Received combined therapy of albendazole and \(\alpha\)-chymotrypsin for four weeks.

**Group VI:** Received combined therapy of albendazole and colchicine for four weeks.

**Group VII:** Normal mice served as healthy control group.

The day following the last dose of the antifibrotic drugs administration, all the animals were sacrificed, skinned and eviscerated. A part of the skeletal muscle from each mouse was removed, and divided into two portions; one for histo-pathological and immuno-histochemical study and the other for measurement of tissue hydroxyproline and matrix metalloproteinase-2(MMP-2) , as tissue markers of increased extra-cellular matrix formation.

*Total larval count:* The remaining carcasses of each mouse were processed to free the muscle larvae using pepsin / hydrochloric acid digestive solution. Total larval count (number of larvae/mouse) was then carried out.\(^{(42)}\)

*Hydroxyproline assay:* Hydroxyproline, which is directly proportional to type IV collagen content, was measured by colorimetric method.\(^{(43)}\) Briefly, the skeletal muscle specimen was hydrolysed in 2 ml of 6 N HCl for 18 hours at 110°C. Then the reaction mixture was neutralized with sodium hydroxide titration to pH 7, and centrifuged at 1500 rpm. Hydroxyproline in the samples was reacted with oxidant (1 ml of 0.6 mol/L chloramines-T in acetate-citric acid buffer; Sigma) for 30 minutes and Ehrlich’s reagent (7.5% p-dimethylaminobenzaldehyde; Sigma) in 60% perchloric acid (Fisher Chemical, Fair Lawn, NJ) at 65°C for 15 min, and hydroxyproline content was determined by spectrophotometer at 560 nm. Muscle
hydroxyproline was quantitated against a standard curve set up with purified hydroxyproline (Sigma).

*Assay of MMP-2:* 0.5 gm of the muscle was homogenized with potter-Elvenhjem tissue homogenizer in phosphate buffer saline 50mM, pH 7.4. The crude homogenate was centrifuged at 7000 xg for 30 minutes at 4°C, the resultant supernatant was assayed for protein content.*(44) Aliquots containing 1mg/ml were assayed for MMP-2. Their levels were measured by sandwich enzyme-linked immunosorbent assay using Quantikine ELISA kits with sensitivity levels 4.6pg/mL.

*Histopathology:* Examination of formalin fixed paraffin embedded tissue sections representing tissues obtained from hind limb skeletal muscles and diaphragmatic muscles from all studied groups, using ordinary H&E staining method was done. The examination considered number of larvae/low power field "semi-quantitatively", fibrous capsule integrity and thickness "semi-quantitatively"*(45)*, larval appearance and affection, and adjacent cellular inflammatory infiltrates.

*Immunohistochemistry:* Immunohistochemistry study was performed on 10 selected specimens from each group. Five(5) microns thickness tissue sections cut on positively charged slides(Biogenex) were immersed in xylene for 1.5 hours, into descending grades of alcohols, then into distilled water. Epitope retrieval for collagen IV was done using microwave treatment in 0.1M citrate buffer PH 6.0 for 20 minutes. Epitope retrieval for fibronectin was done using proteinase K solution in humid chamber for 20 minutes at 37 C°. Serum blocking for both antibodies was done by normal goat serum blocking solution for 30 minutes. Primary monoclonal rabbit anti-mouse antibody for collagen IV Ab-3(LAB VISION), as well as primary monoclonal anti-mouse antibody for fibronectin (clone FBN11- LAB VISION) were incubated with tissue sections in a ready-to-used dilution overnight in humid chamber (refrigerator). Endogenous peroxidase was blocked by peroxidase blocking solution for 10 minutes, then application of biotinylated secondary antibody for 30 minutes. The tissue sections were incubated with streptavidin-HRP detection system solution(Vector laboratories). Each step was followed by wash with phosphate buffered saline (PBS). Color development was done using 3'3 diaminobenedene (DAB) as a substrate with Mayer’s hematoxylin as a counter stain.

**Interpretation of immunostaining:** Brownish coloration of T.spiralis capsule was observed and considered as positive reaction. Intensity of staining as well as visually determined capsular thickness in studied groups were determined semi-quantitatively.*(49) Staining of the basal lamina of uninfected muscles was used as an internal positive control.(12)

**Statistics:** Values of the measured parameters in this study were expressed as mean±SD. The difference between each two groups was determined using student’s t-test. It was considered statistically significant at p<0.05, p<0.01 or p<0.001. The percentage change (%) in larval count was also calculated. The statistical analyses were processed according to the conventional procedures*(46)* using Statistical Program of Social Sciences (SPSS) software for windows, version10.0.

**RESULTS**

Results of total larval count and their percentage change, are demonstrated in table (1) & fig. (1). The highest mean larval count value was recorded in group I (31605± 745 larvae / mouse), whereas the least count was found in group VI, with mean value of (7925± 426 larvae / mouse). The mean larval counts of the different treated groups were found to be significantly reduced when compared to the control "infected non- treated " group [ 10995 ± 592 (group II),11276± 430 (group III),23254 ± 70 (group IV), 8018± 375 (group V) and 7925± 426 (group VI); respectively vs 31605± 745 (group I) ] with percentage reduction of 65.2%,64.3%, 26.4%,74.6% and 74.9%;in the treated groups respectively. Compared to group II treated with albendazole alone, the combined therapy with α-chymotrypsin (group V) and colchicine (group VI) showed significant reduction in mean values of total larval count (8018 ± 375 and 7925± 426 ;respectively vs 10995 ± 592). On the other hand, the difference between groups V and VI as regards the total larval counts was found to be statistically non–significant , with percentage reduction of 74.6% and 74.9% respectively.
Table (1): Comparison between the different studied groups for total larval count (mean±SD)

<table>
<thead>
<tr>
<th>Groups (n=25)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval count (Larvae/Mouse)</td>
<td>31605±745</td>
<td>10995±592</td>
<td>11276±430</td>
<td>23254±704</td>
<td>8018±375</td>
<td>7925±426</td>
</tr>
<tr>
<td>P value</td>
<td>p1&lt;0.001</td>
<td>p1&lt;0.001</td>
<td>p1&lt;0.001</td>
<td>p1&lt;0.001</td>
<td>p1&lt;0.001</td>
<td>p2&lt;0.001</td>
</tr>
<tr>
<td>Percentage reduction (%)</td>
<td>65.2%</td>
<td>64.3%</td>
<td>26.4%</td>
<td>74.6%</td>
<td>74.9%</td>
<td></td>
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</tbody>
</table>

n: number

p1: group II (albendazole treated), group III (received α-chymotrypsin), group IV (received colchicine), group V (combined albendazole & α-chymotrypsin therapy), group VI (combined albendazole & colchicines therapy) vs group I (infected non-treated).

p2: group V and group VI vs group II.

p3: group VI vs group V.

The results of mean levels of muscle hydroxyproline (µg/g) are demonstrated in Table (2). Infected non-treated group I showed significant increase in comparison to the normal control group (1.40±0.58 vs 0.70±0.19). Compared to group I, group II treated with albendazole alone showed non significant change in muscle hydroxyproline level (1.15±0.48 vs 1.40±0.58), while the other treated groups showed significant reduction [0.72±0.24 (group III), 0.94±0.41 (group IV), 0.92±0.29 (group V) and 0.88±0.27 (group VI); respectively vs 1.40±0.58 (group I)]. Combined therapy with α-chymotrypsin (group V) and colchicine (group VI) showed significant reduction in comparison to group II treated with albendazole alone (0.92±0.29 and 0.88±0.27 vs 1.15±0.48). On the other hand, the difference in muscle hydroxyproline levels between groups V and VI was statistically non significant.
Table (2): Comparison between the different studied groups for muscle hydroxyproline

<table>
<thead>
<tr>
<th>Groups (n=25)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
<th>Group VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline(µg/g tissue)</td>
<td>1.40±0.58</td>
<td>1.15±0.48</td>
<td>0.72±0.24</td>
<td>0.94±0.41</td>
<td>0.92±0.29</td>
<td>0.88±0.27</td>
<td>0.70±0.19</td>
</tr>
<tr>
<td>P value</td>
<td>p1&lt;0.001</td>
<td>p2&gt;0.05</td>
<td>p2&lt;0.001</td>
<td>p2&lt;0.01</td>
<td>p2&lt;0.001</td>
<td>p2&lt;0.05</td>
<td>p2&gt;0.05</td>
</tr>
</tbody>
</table>

n : number
p1 : group I (infected non-treated) vs group VII (normal control).
p2 : group II (albendazole treated), group III (received α-chymotrypsin), group IV (received colchicine), group V (combined albendazole & α-chymotrypsin therapy), group VI (combined albendazole & colchicine therapy) vs group I (infected non-treated).
p3 : group V and group VI vs group II.
p4 : group VI vs group V.

Table (3): Comparison between the different studied groups for muscle MMP-2 levels (mean±SD).

<table>
<thead>
<tr>
<th>Groups (n=25)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
<th>Group VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>p1&lt;0.001</td>
<td>p2&gt;0.05</td>
<td>p2&lt;0.001</td>
<td>p2&lt;0.01</td>
<td>p2&lt;0.001</td>
<td>p3&lt;0.001</td>
<td>p2&lt;0.05</td>
</tr>
</tbody>
</table>

n : number
p1 : group I (infected non-treated) vs group VII (normal control).
p2 : group II (albendazole treated), group III (received α-chymotrypsin), group IV (received colchicine), group V (combined albendazole & α-chymotrypsin therapy), group VI (combined albendazole & colchicine therapy) vs group I (infected non-treated).
p3 : group V and group VI vs group II.
p4 : group VI vs group V.
- **Histopathology results:**

**Group I**: Tissue sections revealed coiled *T. spiralis* larvae within nurse cells surrounded by thick capsule with intense inflammatory infiltrate formed of macrophages, plasma cells, and lymphocytes fig(2). Low power examination showed many encysted larvae in infected muscles fig(3).

**Group II**: There was lesser number of larvae by low power field examination than group I. The capsule in most of the larvae appeared thick and complete fig (4).

**Group III, IV**: No visual difference between the two groups was noticed, and larval capsules showed areas of thinning, and breakdown.

**Group V, VI**: In the two groups there was marked reduction in numbers of larvae by low power field examination. Most of the larvae showed fragmentation, degeneration, homogenization and invasion by inflammatory cellular infiltrate. The capsules around most of the larvae showed thin areas, splitting, vacuolation, and areas of breakdown fig(5), fig(6), and fig(7).

- **Immunohistochemistry results:**

Approximate results for both collagen type IV and fibronectin were noticed in studied groups. The areas of positive staining showed outer layer distribution of both collagen and fibronectin in the capsule.

**Group I**: Larval capsule showed intense staining for both antibodies specially in the outer layer of the capsule fig(8).

**Group II**: The capsule in most of the larvae was completely and intensely stained for both antibodies fig(9).

**Group III, IV**: Areas of decreased staining intensity, and incomplete staining as a result of capsular breakdown fig(10), and fig(11) were noticed, without difference between the two groups.

**Group V, VI**: Most of larval capsules were faintly and even negatively stained fig(12). There was also incomplete staining as a result of capsular breakdown with disappearance of many larvae fig(13). No difference between the two groups was noticed.

By using a semi-quantitative assessment of (1-3) score for staining intensity, for statistical analysis, Pearson correlation coefficient showed that intensity of staining of the capsule for collagen and fibronectin antibodies was positively correlated to the mean value of muscular hydroxyproline measurements i.e (P<0.05).
Figure (2): section from skeletal muscle of an untreated specimen showing *T. spiralis* larva surrounded by thick capsule and intense inflammatory cellular infiltrate; lymphocytes, plasma cells, macrophages, and neutrophils (H&E x 400).

Figure (3): section from diaphragm of an untreated specimen showing many encysted larvae surrounded by intense inflammatory infiltrate (H&E x 40).

Figure (4): section from skeletal muscle of a specimen under "albendazole" treatment showing that most of the larvae have intact capsule but some are dead and homogenized "arrow" (H&Ex100).

Figure (5): section from diaphragm of a specimen under combined therapy (α-chymotrypsin + albendazole) showing absence of the capsule around dead homogenized larva with invasion by inflammatory cells "thin arrow", vacuolation and splitting of the capsule "thick arrow" (H&Ex200).

Figure (6): section from skeletal muscle of a specimen under combined therapy (α-chymotrypsin + albendazole) showing splitting of the capsule into thin layers "arrows" with vacuolation of the capsule and larva (H&Ex200).

Figure (7): section from diaphragm of a specimen under combined therapy (colchicine + albendazole) showing dead homogenized larvae "thin arrows", vacuolated damaged capsule "arrow head", and broken down incomplete capsule "thick arrow" (H&Ex200).
Figure (8): Section from skeletal muscles of an untreated specimen showing complete intense staining of the outer layer of *T. spiralis* capsule by fibronectin antibody (Streptavidin-biotin-DABx200).

Figure (9): Section from skeletal muscle of a specimen under "albendazole" treatment showing dead homogenized larva surrounded by completely intensely stained capsule for collagen type IV antibody, while other larva is intact with complete capsule (Streptavidin-biotin-DABx200).

Figure (10): Section from skeletal muscle of a specimen under "α-chymotrypsin" treatment showing incomplete faint staining for collagen type IV with intact larva (Streptavidin-biotin-DABx200).

Figure (11): Section from skeletal muscles of a specimen under "colchicine" treatment showing incomplete faint staining of the capsule for fibronectin antibody with intact larva (Streptavidin-biotin-DABx200).

Figure (12): Section from diaphragm of a specimen under (α-chymotrypsin + albendazole) treatment showing negative staining for fibronectin antibody in the capsule with capsular vacuolation and disappearance of larva from some cysts (Streptavidin-biotin-DABx200).

Figure (13): Section from skeletal muscle of a specimen under (colchicine + albendazole) treatment showing incomplete staining of collagen type IV due to capsular breakdown "arrow", there is also disappearance of larva (Streptavidin-biotin-DABx200).
DISCUSSION

Trichinellosis is a serious parasitic zoonosis, which is widely distributed all over the world. Undercooked pork is still the predominant source of human infection. (47,48) Trichinella species "predominantly Trichinella spiralis" is the causative parasite of this disease. (62) While Trichinella spiralis infection causes both intestinal and skeletal muscle pathology in the same host, in contrast to the intestinal phase which is transitory and usually pass unnoticed, the encystment phase is prolonged, accompanied by serious symptoms and difficult to be controlled. (49,50)

Over a period of 15-20 days after skeletal muscle cell invasion by Trichinella spiralis larva, a number of changes occur that culminate in the formation of an intimate host–parasite complex called the "Nurse-cell" that is surrounded by collagen capsule. (15,51) The newly formed encapsulated, Nurse–cell, is designed to support the parasite life inside its muscle niche for long times otherwise it will die. (62)

Albendazole is still the available drug of choice in trichinellosis treatment. (67) Its efficacy is attributed to its active metabolite; albendazole–sulphoxide that is formed rapidly after oral administration of the drug. (63) The primary action in susceptible nematodes, is the selective inhibition of the parasite microtubule assembly and polymerization, without effect on human cells, by specific high-affinity binding to the parasite β–tubulin, with consequent inhibition of microtubule-dependent uptake of glucose. Moreover, albendazole was proved to produce many biochemical changes in the target nematode as; inhibition of mitochondrial fumarate reductase, reduction of glucose transport and uncoupling of oxidative phosphorylation. (28,54,55)

In the present study, treatment with albendazole alone caused relatively low percentage reduction of total larval count (65.2%), that was concurrent with its non significant effect on the tissue fibrogenic markers (hydroxyproline and MMP-2) and non significant histopathological changes when compared to infected non-treated group. This result is in agreement with those of many recent studies carried out to evaluate efficacy of albendazole therapy against different phases of Trichinella spiralis infection. Comparing percentage reduction of larvae, revealed high efficacy of albendazole in the intestinal phase (100% reduction), with only a rate of reduction ranged between 45.4% (56) and 71% (57) in the muscle phase. Moreover, it was proved that in contrast to the adult stage of Trichinella spiralis which responds very well to albendazole therapy (reduction rate of 96.5%), the encysted muscle larvae were found to be less sensitive to the drug, where a reduction rate of (94.7%) was only reached when the therapeutic dose increased by ten times. (59) This observation could be explained by the extreme variation in plasma concentrations of the active metabolite albendazole–sulphoxide probably because of variable absorption of the drug from the intestine after oral administration. (28) Also, the lack of effects of albendazole on fibrogenic markers could be contributed to its selective inhibition of the parasite's microtubule assembly and polymerization, without effect on human cells (65), where, microtubules were proved to control both the morphological organization in fibroblasts and the extracellular matrix structures. (58)

So, it appeared that benzimidazole compounds which have been used in the chemotherapy of trichinellosis, were unable to control efficiently muscle phase of the disease. Hence, better approaches and modifications of this phase treatment should be researched. (59)

Because the issue here is how to overcome the obstacle of the fibrotic encapsulated, Nurse–cell that protects the parasite and prevents the anthelmintic drug "albendazole" to be in direct access to it; so, introduction of different antifibrotic agents could be suggested as adjuvant therapy to achieve better results during treatment of muscular trichinellosis.

In the current study, results of total larval count pointed to beneficial effect of adding antifibrotic agent either, " α-chymotrypsin or colchicine " to the standard anthelmintic drug "albendazole" during treatment of muscle phase of Trichinella spiralis infection. Comparison between mean value of total larval count of group II (10995±592) and those of either group V (8018±375) or group VI (7925±426), was found to be statistically significant. Hand in hand with this result, is the result of the percentage reduction of total larval count of...
the same groups; where this percentage was found to be (65.2%, 74.6% and 74.9%) in groups II,V and VI respectively. This result also was in coincidence with that of fibrogenic markers, where the tissue levels of hydroxyproline were significantly decreased and tissue levels of MMP-2 were significantly increased in the groups received combined therapy in comparison to those received albendazole alone. These results are in agreement with those of many other workers who proved that combined therapy of the antifibrotic agent "beta–aminoprorionitrile" and the standard anti-schistosomal drug "praziquantel" leads to marked reduction in hepatic and intestinal tissue egg loads in comparison to the groups received praziquantel alone. Moreover marked decrease in hepatic granuloma size was reported. Also, these results are in agreement with those of many studies that were designed to evaluate efficacy of colchicine as an antifibrotic agent in other models of parasitic infections; colchicine was proved to have a beneficial effect on schistosomal hepatic fibrosis with significant reduction in collagen fibrils deposition around hepatocytes together with better recovery of liver cells. Another study was carried out on schistosomal renal amyloidosis, where colchicine caused significant reduction in renal amyloid deposits when combined with the anti-schistosomal therapy. As regards results of the effect of combined antifibrotic and anti-parasitic therapy on tissue markers of fibrosis was also proved recently in model of hepatic schistosomiasis mansoni, where liver hydroxyproline was found to decrease by a percentage of 34%, this was explained by suppressing effect of the antifibrotic drug on TGF-B1 and TIMP-1.

Up to our knowledge, there are no available literatures about use of colchicine or α-chymotrypsin in the same model of muscular trichinellosis. Meanwhile, there are many studies that demonstrate their mechanisms of action as beneficial antifibrotic agents in many other disorders where fibrosis constitutes the main pathogenic feature, and so, they support the findings obtained in the current study.

The ancient drug colchicine has repeatedly been proposed as a novel drug for therapy of fibrosis in many diseases including; idiopathic pulmonary fibrosis, bleomycin-induced pulmonary fibrosis, and also pancreatic fibrosis following acute pancreatitis. In all these studies, colchicine was proved to have antifibrotic role expressed as reduction in levels of fibronectin, collagen deposition and hydroxyproline contents. Moreover it was shown that colchicine, in doses that could be used in humans, protected renal function by about 25% and reduced interstitial fibrosis in a model of renal glomerulosclerosis. Regarding safety, colchicine has been found to be safe at its antifibrotic doses of 1 to 2 mg per day, even when given continuously over long durations.

The exact mechanisms of colchicine action is not fully understood. Based upon current knowledge, two mechanisms are proposed. The first mechanism is through its direct pure mechanistic (physical) interaction with β-tubulin or with whole microtubules leading to their conformational changes and destabilization. The second mechanism is mediated by indirect alteration of expression of many genes such as fibronectin gene, through disruption of the cytoskeletal infrastructure with consequent inability of the light chain 3 protein (a microtubule-associated protein), in the absence of microtubules, to influence the sorting of fibronectin mRNA onto the polysomes, and this may explain how colchicine averts the assembly and deposition of fibers. This possibility may provide a wide implication of colchicine in other conditions of fiber deposition. These mechanisms could be confirmed by the fact that microtubular system was known previously to control the morphological organization in fibroblasts, intracellular cytoskeletal system, and also extra-cellular matrix structures "including fibronectin". On the contrary, other workers demonstrated that collagen inhibition is more likely an inhibition of cellular collagen secretion rather than a switch off of collagen mRNA transcription.

Regarding its effect on MMP-2, colchicine was found to up-regulate expression of matrix metalloproteinases (MMPs) in a time-dependent manner. Moreover, this drug was proved to increase activity of endogenous MMP-2. Transforming growth factor-β1 (TGF-β1) has been implicated as a major stimulator of
tissue fibrosis through several lines of action as; stimulation of fibroblasts proliferation with consequent increase in extra-cellular matrix production, potent induction of TIMP which inhibits the proteolytic activity of MMP by binding to its highly conserved zinc active site to form a stable complex in an equimolar fashion, and lastly through reduction of both (MMP) expression and extra-cellular matrix degradation. So, antifibrotic action of colchicine could be partially attributed to its significant suppressing effect on TGF-β1 mRNA, resulting in inhibitory effect on the excretion of extra-cellular matrix as collagen IV in fibroblasts.

Chymotrypsin, is among the best characterized members of proteases that act by a mechanism probably involves degradation of extra-cellular matrix components. So, its administration could be suggested to have a role against fibrosis. Extensive researches were accomplished to study effect of proteases on fibronectin "one of major components of extra-cellular matrix"; it was proved that various degrees of inhibition of fibronectin-mediated cell adhesion was observed after treatment with chymotrypsin, this inhibition was estimated to be approximately 30% and this treatment was proved to be non toxic. In addition, destruction of fibronectin by chymotrypsin was proved to produce collagen-binding fragments that were found to still bound to the native collagen, delaying the process of fiber formation.

Recently, the antifibrotic mechanism of chymotrypsin could be contributed to the observation that diverse proteinases, including alpha-chymotrypsin, was found to abolish the transforming growth factor-beta1 (TGF-β1) effect on fibroblasts in cell culture as they conferred binding of TGF-beta with alpha2-macroglobulin, and by this process high concentrations of TGF-beta might be reduced via enhanced clearance of alpha2-macroglobulin-TGF-beta complexes. Thus, proteinase therapy may have beneficial effects in treatment of fibrosis and certain cancers that accompanied by excessively high TGF-beta1 concentrations. Also, proteolytic enzymes was found to be able to convert alpha2-macroglobulin from the "slow" form to the "fast" form, whereby the "fast" form binds and inactivates TGF-beta1 irreversibly, especially in cases with high levels of TGF-beta. This antifibrotic effects of α-chymotrypsin could be confirmed by the recent findings proved that proteolytic enzymes derived from green-bottle larva Lucilia sericata, produced degradation of the extra-cellular matrix (ECM) components including fibronectin, and collagen types I & III through chymotrypsin-like activities.

Regarding the effect of α-chymotrypsin on matrix metalloproteinases (MMPs), conversion of proMMPs to their active forms is a crucial step in the destruction and remodeling of the extra-cellular matrix (ECM), where this activation can be achieved in vitro by endogenous proteinases such as trypsin, chymotrypsin. However, the mechanism of modulation of MMPs and extra-cellular matrix by exogenous proteinases such as α-chymotrypsin still remains obscure.

In the present study it was found that there is pathological differences between infected untreated group and the group under both albendazole therapy and combination therapy in the form of semi-quantitative decrease in larval count per low power field in the group under albendazole treatment as well as death of some larvae. In the group under combination therapy there was larval count decrease, splitting, vacuolation and damage to the capsule. Comparatively, it was noticed a pathological impression of decrease of fibrosis between fibrotic model rats and rats under interferon therapy given to decrease induced liver fibrosis, using ordinary H&E staining method. Immunohistochemical observations supported histopathological evaluation; i.e there was a difference in staining intensity of larval capsule between both untreated group, group under albendazole treatment and between groups under antifibrotic drugs as single or in combination with albendazole. These results are in agreement with other study used Sirius red stain histochemical method to stain collagen as well as anti collagen IV monoclonal antibody to compare amount of fibrosis between fibrous model and rats under interferon therapy reporting a semi-quantitative difference in degree of staining. Also, immunohistochemical evaluation of collagen I and fibronectin after antifibrotic treatment of rat liver fibrosis showed decrease in staining of both
In this study level of hydroxyproline was positively correlated to immunohistochemical staining intensity. This finding is in agreement with other workers who reported that hydroxyproline liver content is correlated to histological signs of liver fibrosis.\(^{(84)}\) The histopathological and immunohistochemical evaluation of fibronectin and collagen type IV in muscular trichinellosis after addition of antifibrotic drugs needs further study.

This study showed the beneficial effect gained from adding an antifibrotic agent (\(\alpha\)-chymotrypsin or colchicine) to the standard anti-parasitic drug (albendazole). This result depends on the statistically significant decrease in mean values of total larval count in groups received combined therapy when compared to the group received the anti-parasitic alone, moreover levels of tissue markers of fibrosis (hydroxyproline and MMP-2) were proved to be significantly improved with combination therapy as regards single anti-parasitic one. However, there was no significant difference between the two groups received the combination therapy as regards all measured parameters in the experiment. So, \(\alpha\)-chymotrypsin and colchicine seem to have an equieffective antifirotic action when introduced as novel adjuvant therapy to albendazole for treatment of muscular trichinellosis. As regards antifibrotic mechanisms of these antifibrotic drugs, they still need further researches to be clearly determined with evaluation of the beneficial versus adverse effects. Regarding that low levels of MMP-2 were found to be concurrent with increased extra-cellular matrix formation, successful treatment of various fibrotic diseases may be directed towards investigations of new drugs that activate MMPs or increase their expression.

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إن إصابة العضلات بيرقات طفيفة الدودة الشعرية الحزازية تعتبر من الإصابات الخطرة والتي تسبب نوع من الإعانة للمصاب، وحتى الآن فان النتائج التي يتم الوصول إليها باستخدام عقار "الإلينبندازول" في علاج تلك الإصابة تعتبر غير مرضية.

لذا فقد حددت تلك الدراسة إلى محاولة تحصين نتائج العلاج بإضافة عقار ميثيل للإلينبندازول. إذاً، ان فان دلالات تليف (الإلفاكيوميريسين، الكولبيسين) إلى الإلينبندازول حيث أن هذه الإضافات هو درجة ثقيلة داخل خلايا العضلات تعود بصورة أساسية على تكوين حويصلة من النسيج الليفي والكولاجين حول اليرقات لحمايتها من جهاز المناعة.

و قد تم استخدام (175) فار، مايس أبيض في هذه الدراسة مجموعتين إلى (2) مجموعات متساوية؛ تم إعطاء (1) مجموعات بيرقات الدودة الشعرية الحزازية عن طريق الفم (يرقة، فار) وقد قسمت مجموعات الفران على النحو التالي:

- مجموعة (1): مجموعة قياسية ضابطة "تعد عددها و لا تتناول اي علاج".
- مجموعة (2): تم علاجها لمدة ثلاثة أيام متتالية بعقار "الإلينبندازول" فقط عن طريق الفم بعد وصول اليرقات إلى العضلات.
- مجموعة (3): تم إعطاءها عقار "الإلفاكيوميريسين" بمفرده لمدة أربعة أسابيع عن طريق الحقن العصلي.
- مجموعة (4): تم إعطاءها عقار "الكولبيسين" بمفرده لمدة أربعة أسابيع عن طريق الفم.
- مجموعة (5): تم علاجها بعقاري "الإلينبندازول" و "الإلفاكيوميريسين".
- مجموعة (6): تم علاجها بعقاري "الكولبيسين" و "الإلينبندازول".
- مجموعة (7): مجموعة ضابطة قياسية ضعيفة مقارنة النتائج الكيميائية والبيولوجية.

بعد انتهاء إعطاء الإضافات الميثيل للإلينبندازول ذهب الفران لتقييم النتائج و تم عمل:

(1) عد كامل لليرقات في الفران المعدية في جميع المجموعات.
(2) قياس نسبة دلالات التليف في الأنسجة "الهيبروكسيبوليون و انزيم الماتريكس ميتالوبروتيناز-2".
(3) فحص الأنسجة العضوية بностью و هستوكيومانيا حيث تم صباغة الكولاجين (4) و الفيبرونکتين.

و قد اتضح من هذه الدراسة أن المجموعتين الثالثتين عملا إعاقة عقار ميثيل للإلينبندازول إلى عقار الإلينبندازول كان عدد اليرقات فيما أقل بنسبة ذات دالة إحصائية من المجموعة التي تم معالجتها بعقار الإلينبندازول بمفرده. وكذلك فان دلالات ليف الأنسجة كانت في هاتين المجموعتين أقرب للفتيات في المجموعة الضابطة السليمة، و هذه القياسات الكيميائية تتماشى مع نتائج صباغة الأنسجة هستوكيومانيا في نفس المجموعات.

و بذلك نخلص إلى أن إضافة عقار ميثيل للإلينبندازول في علاج الإصابة بطفيل الدودة الشعرية الحزازية في العضلات كان ذو فائدة ملموسة في تحسين نتائج العلاج و من ثم فان تلك الإضافة لها دور كبير في تحسين حالة العضلات المصابة و الحد من معاناة المرض.