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THE AMELIORATING EFFECT OF MENTOFIN® ON RESPIRATORY SYSTEM OF BROILER CHICKENS CHALLENGED WITH *MYCOPLASMA GALLISEPTICUM* STRAIN IN ASSIUT GOVERNORATE

AL SHIMAA, R.SAYED [1]; MARWA, A.AHMED [2]; MOSTAFA, A. SHEHATA [3]; MOEMEN, A. MOHAMED [3]; SABRY, I. EISSA [4] AND MARWA, M. SAFWAT [3]

[1] Animal Health Research Institute, Asiut.

[2] Department of Pathology, Faculty of Veterinary Medicine, Aswan University, Egypt.

[3] Faculty of Veterinary Medicine, Assiut University.

[4] Chief Researcher of Mycoplasma Dept. Animal Health Research Institute Agriculture, Research Center, Doki, Giza.

Received: 31 December 2019; **Accepted:** 31 January 2020

ABSTRACT

The current study was conducted to investigate the impact of Mentofin® in protection of the respiratory system of broiler chickens challenged with *Mycoplasma gallisepticum* (*MG*) strain. One hundred and five – one day old chicks were randomly divided into three equal groups (35 chicks /group) and reared in isolated rooms. Group (A) *MG* challenged, Group (B) was treated with Mentofin® and *MG* challenged and Group (C) control –ve group (not challenged with *MG* and not treated with Mentofin®). At 1st week of age, an intra-tracheal challenge of the birds with *MG* strain (containing 1 x 10^6cfu/ mL/bird) was given to A and B. Mentofin® was administered orally for 6 days, beginning from 8th day of ages to 13th day of ages. Tracheal and lung samples for histopathological examination were collected from chicken at 9th day to 14th day of age and at 28th day of age. In *MG*-challenged birds (group A) histopathological lesions included mucosal hyperplasia, mucus accumulation, tracheal deciliation, inflammatory cells infiltration and goblet cell hyperplasia in tracheal tissues, moreover, congestion and pneumonic foci in lung tissues. The histopathological lesions of birds treated with Mentofin® and *MG* challenged (group B) revealed a significant reduction in histopathological lesions in tracheal and lung tissues. Mentofin® was able to reducing respiratory problems, and recommended to be used as a prophylaxis treatment and supportive treatment with antibiotic in cases of *mycoplasma gallisepticum* infection.

Keywords: *Mycoplasma Gallisepticum* isolation, PCR, Mentofin®, Histopathological examination.

INTRODUCTION

Mycoplasma gallisepticum (*MG*) is the most pathogenic avian mycoplasmas that cause respiratory infection in poultry. *M. gallisepticum* causes chronic respiratory disease (CRD) in chickens and have been reported to cause serious economic losses (Osman *et al*., 2009). The clinical signs of avian mycoplasmosis are sneezing, coughing, and respiratory rales; ocular and nasal discharge; decreased feed intake and increased morbidity. Increasing mortality in birds with *MG* infection caused by concurrent bacterial and/or viral infection has been widely reported (Raviv and Kleven, 2009).

Mycoplasma gallisepticum infection has tropism primarily for mucosal membranes of the respiratory tract, conjunctiva and sinuses (Levisohn and Kleven, 2000).The organism usually enters the host via the respiratory tract; upper airways and trachea are the preferred sites of infection for most of the strains of *MG* (Kleven, 1997).

Mentofin®, a natural herbal product consisting of some essential volatile fatty acids and natural herbal essences (10%) eucalyptus oil, 10% menthol, 33% liquid builders, and 47% saponins) Rehman *et al*. (2013). The essential oils of *Eucalyptus* species possess important biological activities including antibacterial, anti-inflammatory, diaphoretic, antiseptic, analgesic effects (Cimanga *et al*., 2002) and antioxidant properties (Damjanović-Vratnica *et al*., 2011), and safely used in broiler and layer chicken production, the product was able to help in preventing respiratory disease complexes (Barbour *et al*., 2005), increasing performance and strengthening the immune system (Bragg, (2004 and 2006); Carli *et al*., 2008).

The aim of this study was to estimate the Mentofin® effect on respiratory system of Mentofin® treated groups following a challenge with *Mycoplasma*

Corresponding author: Dr. Marwa, M. Safwat
E-mail address: merovet84@gmail.com
Present address: Faculty of Veterinary Medicine, Assiut University.

gallisepticum through gross and histopathological evaluation.

MATERIALS AND METHODS

I-Isolation and Identification of the *Mycoplsama* from birds suspected to be infected:

A) Sampling:
One hundred and twelve (112) samples including lung and trachea were collected from 112 freshly dead and slaughtered broiler chickens showing respiratory manifestation (watery to mucoid nasal discharge, sneezing, gasping, tracheal rales and conjunctivitis) were collected from different localities in Assiut with ages ranged from (20-35 days), weren't vaccinated against *Mycoplasma gallisepticum*.

B) Procedures for isolation of *Mycoplasma* (Sabry and Ahmed, 1975):
Samples were placed on PPLO broth and brought to the laboratory of avian and rabbit diseases Department, Assiut University for subsequent culturing on PPLO media that containing horse serum, dextrose , yeast extract and thallium acetate with penicillin. Broth bottles were incubated at 37˚C for 3-4 weeks before being discarded as negative samples; however the agar plates were incubated under reduced oxygen tension in humidified candle treated with Mentofin® (EWABO - Germany) and *MG* challenged, Group C (control –ve group not challenged with *MG* and not treated with Mentofin®). At 1st week of age, an intra-tracheal challenge of the birds with *MG* strain (containing 1 x 10^6cfu / mL/bird**)** was given to (A and B) groups. Mentofin® was given to (B) groups.

Essential oils of Mentofin® were administered for 6 days, beginning from 8th day to 13th day of age.

Mentofin® was diluted in V/V of 0.025 mL/100 mL of drinking water. Each treated bird started receiving the diluted Mentofin®, twice a day (morning and evening), in a volume of 1mL/bird/time in the drinking water. Group C (control group not challenged with *MG* and not treated with Mentofin®).

Histopathological examination
Tracheal and lung samples for histopathological examination were collected from chicken at 9th day to 14th day of age and at 28th day of age. Three birds were slaughtered from each group and trachea and lung were collected and fixed in 10% buffered neutral formalin. The fixed tissues were embedded in paraffin, sectioned at 4 *µm* thick and stained with haematoxylin and eosin H&E (Bancroft and Stevans, 1993). The slides were examined under light microscope.

jar then examined microscopically for *Mycoplasma* colonies after 48 hrs. and daily up to 7-10 days for "fried-egg shape colonies", then subjected to arginine deamination and glucose fermentation tests for biochemical characterization according to (Enro and stipkovits, 1973) .

C) Polymerase Chain Reaction (PCR) for detection of *Mycoplasma gallisepticum*:
The extraction of bacterial DNA was performed using QIAamp manual kit according to manufacturer's instructions. Two specific oligonucleotide primers for identification of *M.gallisepticum* were used for detection of *MGc2* specific gene for *M.gallisepticum*. The sequence of primer forward (F) was (5'- CGC AATTTG GTC CTA ATC CCC AAC A-3').The sequence of primer reverse(R) was (5'-TAAACC CAC CTC CAG CTT TAT TTC C-3') (Garcia *et al*., 2005). Amplification was performed by heating the sample for 4 minutes at 94ºC for initial denaturation. After this step forty cycles were performed as follows: Denaturation for 20 sec. at 94ºC, annealing for 40 sec. at 58ºC and extension for 1 minute at 72ºC with the exception of final extension step was held for 7 minutes for final extension. The analysis of PCR amplified products was done by using 8µl of amplified PCR product, mixed with 2µl loading dye and electrophoresed through 1% agarose gel and DNA was visualized by UV fluorescence after ethidium bromide staining.

II- The evaluating effect of Mentofin® on the experimentally challenged birds with isolated *M. gallisepticum*:
Birds: One hundred five–one day old broiler chicks Ross (308) from breeder farm were used in the present study, with an average body weight of 37-40 gm, obtained from El Qusia Assiut. Chicks were housed in a well-isolated floor pens under complete hygienic conditions. Chicks were reared in a complete block design provided with wood shaving litter, plastic feeders and waters. Chicks were fed adlibtum on commercial broiler ration.

Preparation of Challenge Strain:
Isolated field *M. gallisepticum* strain was morphologically, molecularly characterized and subjected to sequencing for detection of partial *16s rRNA gene* with accession number in GenBank (15mgAsyut-Egypt-2016) used in preliminary study after counting using colony forming unit method according to Jett *et al*. (1997) for detection of the pathogenicity and evaluation of Mentofin® as a reducing agent of histopathological lesions which caused by *MG* infection.

Experimental design:
One hundred and five– one day old chicks were randomly divided into three equal groups (35 in each group).Group (A) *MG* challenged, Group (B) was

RESULTS

From 112 trachea and lung pooled samples 6 were suspected to be positive for *Mycoplasma gallisepticum* isolation with incidence rate (5.36%). Microscopically the shape of colony was fried egg appearance as showing in (fig.1), biochemically glucose fermentation test was positive and negative to arginine. *M. gallisepticum* strain detected by PCR using *MG*c2 gene gave a characteristic common band at 300 bp fragment (fig. 2).

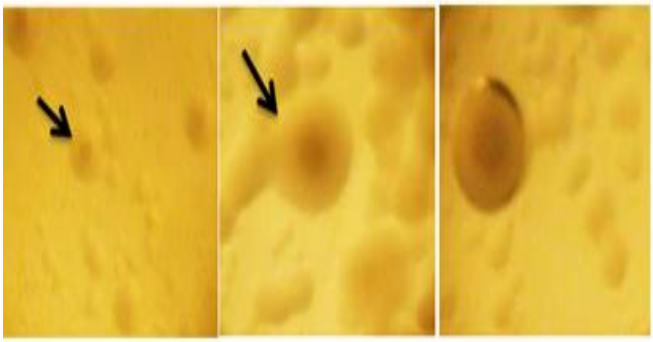

Fig. (1): colonial appearance of *Mycoplasma gallisepticum* isolates by dissecting microscope (fried egg appearance).

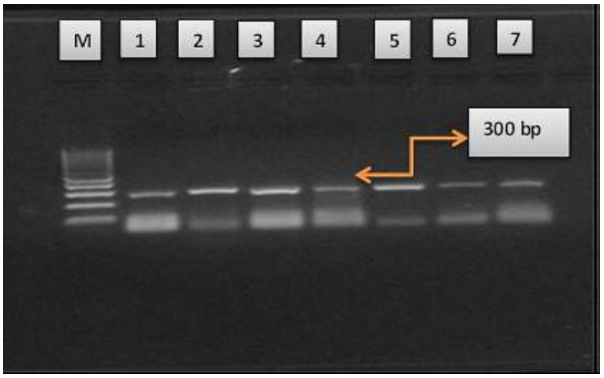

Fig. (2): *Mycoplasma gallisepticum* detected by PCR using *mgc2* gene Positive samples produce band (300 bp), Lane M: 100 bp DNA
Ladder, Lane 1, 2, 3, 4, 5, 6 and 7 were positive samples
Produce band (300 bp).

Results of experimental infection

Clinical findings in infected group (A) (challenged group), Chicks in infected group were dull, depressed with ruffled feather, coughing and sneezing, nasal discharges and open mouth breathing with moist rales.

Gross Pathology of infected group (A) , trachea showed the evidence of congestion and hemorrhages. Lung revealed dark red color appearance and congestion (fig.3), air sacculitis was observed in air sacs, these become cloudy thickened and covered with caseous exudates. Gross pathology of group (B), trachea showed no evidence of congestion and hemorrhages. Lung revealed normal color and no congestion. Air sacs appear normal as thin-walled and no cloudness.

Fig. (3): showing gross pathology of *Mycoplasma gallisepticum* on trachea and lung which represented in congestion and hemorrhages on both tissues

Histopathological evaluation:

The histopathology of the tracheal sections in birds of the challenged group (A) showed mucosal hyperplasia. This microscopic lesion of mucosal hyperplasia was reduced in group (B) Mentofin® treated and *MG* challenged as shown in (fig 4a-5a).

Tracheal sections with mucus accumulation in bird challenged group (A) versus clear tissue in tracheal sections of birds treated with Mentofin® and *MG* challenged group (B) are shown in (figs 4b-5b).

Tracheal deciliated tissue from a *MG* challenged bird deprived of Mentofin® treatment in group (A). There was an apparent reduction in tracheal deciliation in *MG* challenged and Mentofin® treated (group B) as shown in (fig 4c-5c).

The inflammatory cells infiltration in lamina propria of tracheal mucosa was reduced in *MG* challenged and Mentofin® treated group (B) in comparison to group A- challenged bird deprived of Mentofin® treatment as shown in (fig 4d-5d).

Trachea with goblet cell-hyperplasia was observed in group A, *(MG* challenged and deprived of Mentofin®) which was absent in Group B, (*MG* challenged and Mentofin® treated) as trachea appeared normal with normal goblet cells (fig 4e-5e).

Normal tracheal tissue was showed in group C (control group, not challenged and not treated).

Histopathological examination of the lung revealed marked congestion and pneumonic foci in birds of the challenged group (A), Mentofin® caused relieve somewhat histological lesions in lung from mild to no congestion and no pneumonic foci in *MG* challenged and Mentofin® treatment group (B) as in (fig 4f-5f).

Normal lung tissue was showed in group C (control group, not challenged and not treated)

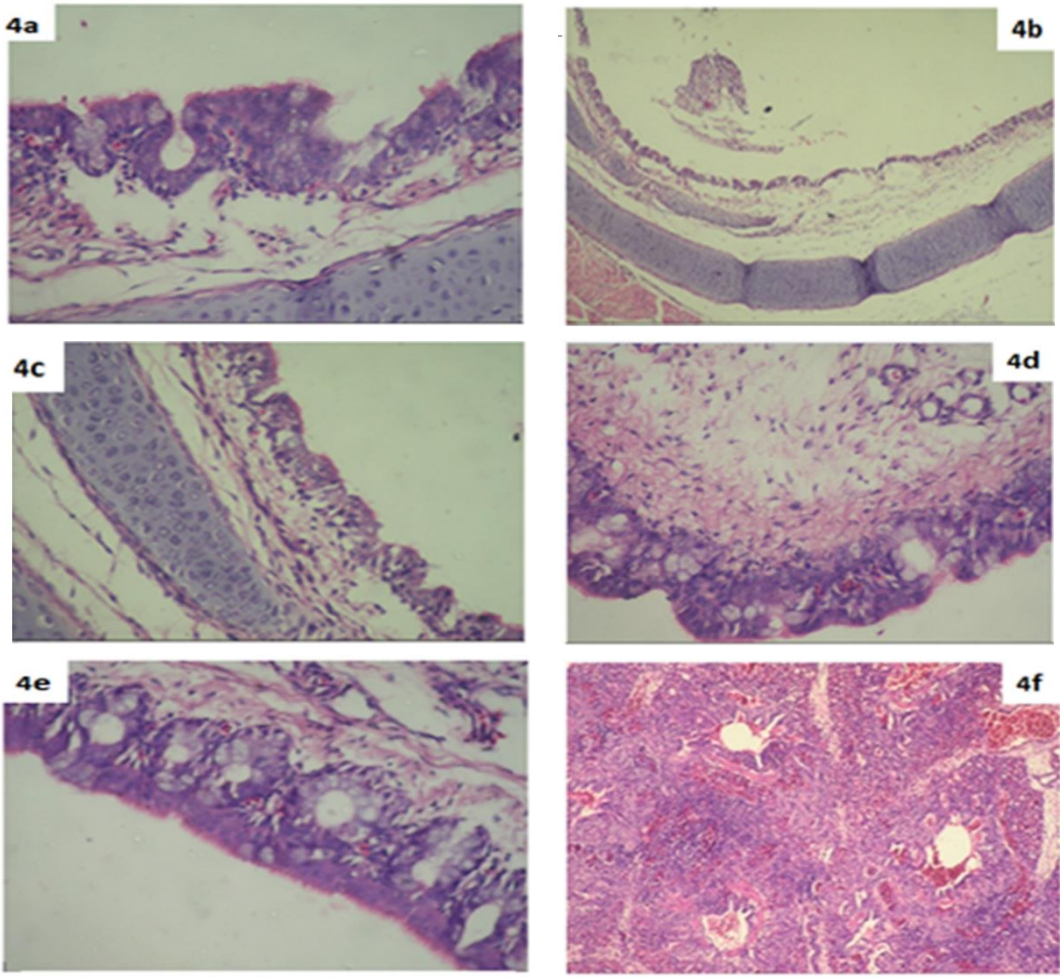

Fig. (4a): Tracheal section showing mucosal hyperplasia in group A (*MG* challenged and Mentofin deprived).
Fig. (4b): Tracheal section showing accumulations of mucus in tracheal lumen in group A (*MG* challenged and Mentofin deprived).
Fig. (4c): Tracheal section showing deciliation in group A (*MG* challenged and Mentofin deprived).
Fig. (4d): Tracheal section showing high inflammatory cells infiltration in group A (*MG* challenged and Mentofin deprived).
Fig. (4e): Tracheal section showing hyperplasia in goblet cells in group A (*MG* challenged and Mentofin deprived).
Fig (4f): Lung section showing multiple pneumonic foci and congestion in group A (*MG* challenged and Mentofin deprived).

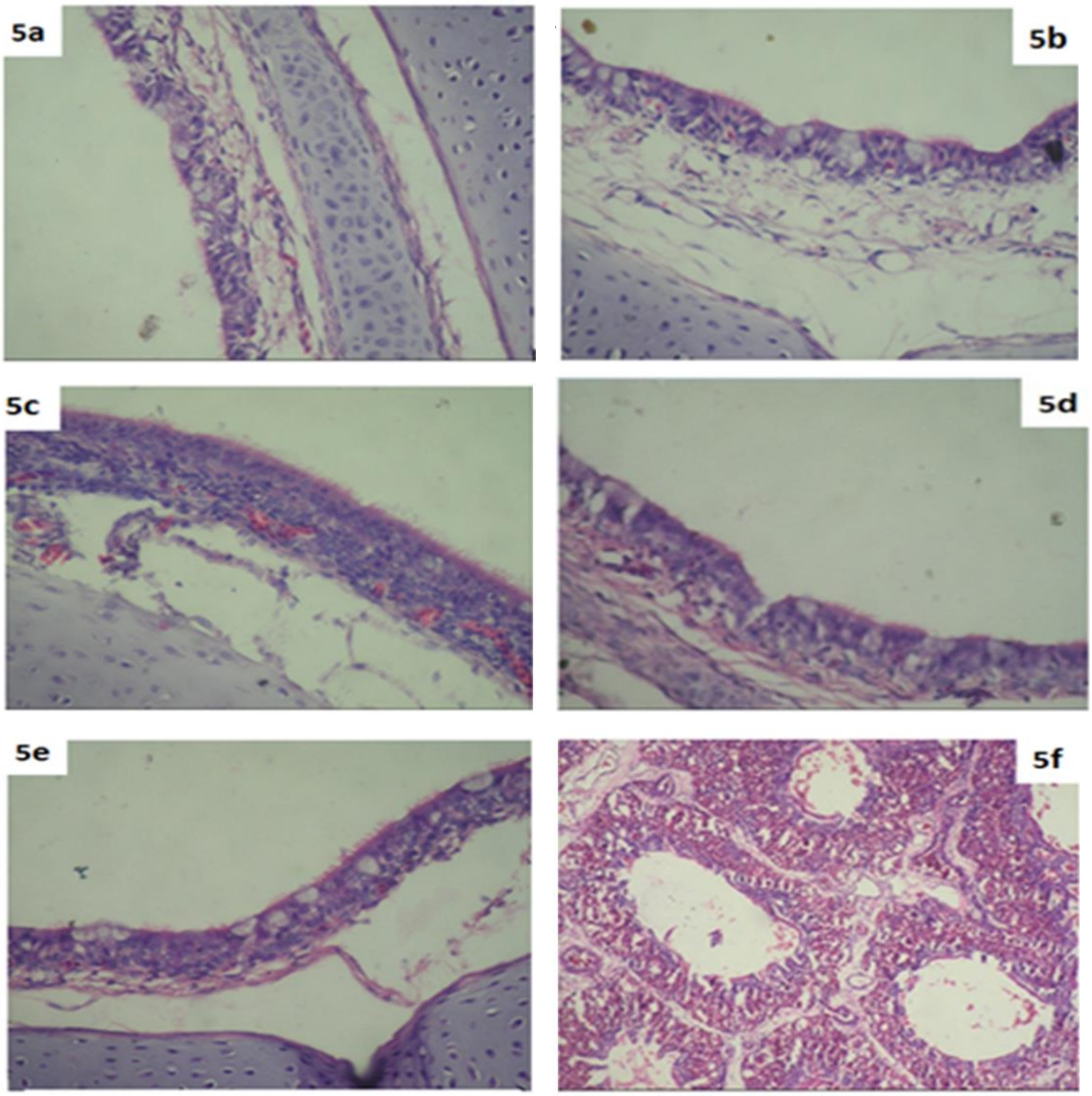

Fig. (5a): Tracheal section showing absence of mucosal hyperplasia in group B (MG challenged and Mentofin treated).
Fig. (5b): Tracheal section showing clear of mucus in tracheal lumen in group B (MG challenged and Mentofin treated).
Fig. (5c): Tracheal section showing normal ciliated in group B (MG challenged and Mentofin treated).
Fig. (5d): Tracheal section showing low inflammatory cells infiltration in group B (MG challenged and Mentofin treated).
Fig. (5e): Tracheal section showing goblet cells within normal in group B (MG challenged and Mentofin treated).
Fig. (5f): Lung section showing no pneumonic foci and no to mild congestion in group B (MG challenged and Mentofin treated)

DISCUSSION

In this study the percentage of isolation and identification of *Mycoplasma gallisepticum* of collected samples (112 lung and trachea) was 5.36 % (6 out of 112samples). These results accepted with those results of Shaker (1995) who found that the overall isolation rate was 4.04% for *M.gallisepticum*, On the other hand our results do not go hand by hand with those of Stalkencht *et al*. (1998) they could isolate *M.gallisepticum* from naturally infected chickens through the classical methods at rate of 30%. This confusion may be due to several causes such as number of birds, media used, cultivation and age of birds.

Molecular techniques, such as polymerase chain reaction (PCR), are established and are sensitive, fast and highly specific methods for the detection of *Mycoplasma* (Garcia *et al*., 2005). Several PCR primers targeting different genes of *MG* were

described previously (Collett *et al.,* 2005) in which *MGc2* one is preferable.

The percentage of samples detected by using *MGc2*gene was (46.88%) 15 out of 32 samples. This result in agreement extent with Gondal *et al*. (2015) who detected of the nucleic acid of *MG* by using PCR from tracheal tissue 42.47% by using specific primer of *MG*. On other hand this result confuses with Eissa *et al*. (2008) who used PCR identification of the obtained isolates of 5 *MG* isolates from broiler chickens (14.25%).

In an examined trial for testing the effect of Mentofin® in reduction of the lesions of *MG* infection, the challenged group (A) exhibited dullness, depression with ruffled feather and various respiratory signs and there were serious involvement of trachea, lungs, air sacs in postmortem examination in opposite to the control group C (not challenged and not treated) that did not exhibit any clinical and pathological changes and similar results reported by Islam *et al*. (2011).

The histopathological changes of trachea in the challenged (group A), were in the form of deciliation, mucosal and goblet Cell hyperplasia, mucus accumulation, and inflammatory cells infiltration. Our results are in concordance with Barbour *et al*. (2006); Khairy *et al*. (2012) and Stipkovits *et al*. (2012), and the pulmonary histopathological changes, were in the form of congestion, pneumonic areas, the obtained results are in agreement with those mentioned by Eissa, (2001)*;* Dardeer *et al.* (2004) and Ahmed (2005). Furthermore, Stipkovits, (1995) mentioned that the *Mycoplasma* enters the respiratory tract and attaches to the cilia and the surface of the epithelial cell lining of the respiratory tract. The *Mycoplasma* produces various toxic metabolites, besides the depletion of the amino acids, fatty acids and DNA precursors. Such products disturb the normal function of the epithelial cells of the mucous membrane of the respiratory tract. A decrease of the excretion of mucus and motility of the cilia or even their destruction can be observed. These factors help the *Mycoplasma* to move down to the lungs and air sacs causing their damage and Volatile oils that existed in Mentofin could be recommended as an alternative natural safe way to the drugs in controlling MG infection to overcome the problems of drug resistance and the common drug tissue residues under our field condition (El-Ghany, 2008).

The mechanism by which the volatile oils can relief the respiratory sings was explained by Page, (2004); Zakay-Rones *et al.* (2004) and Salari *et al.* (2006). Those authors suggested that the active ingredients of *Eucalyptus* spp can protect the first line of defense in the poultry host through the thinning of the mucus in the respiratory tract which could help in its outward flow, pushing with it the microorganisms, preventing their colonization, and thus protecting the cilia from consequent damage.

Concerning to the histopathological findings in group B (which challenged and treated with Mentofin®), revealed the effectiveness of Mentofin® in comparison to group A. Improving in cilia and decreasing hyperplasia in mucosa and goblet cell and clear mucus and low inflammatory cells infiltration were observed of trachea. These results are in accordance with Barbour *et al.* (2006). Who evaluated the histopathological changes of eucalyptus and peppermint oils treated versus deprived broilers subjected to three different natures of challenges (MG, H9N2, and a combination of MG/H9N2) they found that this treatment resulted in significant decrease in tracheal deciliation in MG and MG/H9N2-challenged birds, significant decrease in tracheal goblet cells degeneration in MG-and MG/H9N2-challenged birds, significant decrease in tracheal mucus accumulation in MG-challenged birds, and significant decrease in heterophil infiltration in MG/H9N2- challenged birds. The previously mentioned authors detected that there is synergism among the active ingredients of *Eucalyptus* spp. And peppermint for providing protection of the goblet cells in the upper respiratory system.

The results concerning to group B in comparison to group A may be presumed due to the action of volatile oils in liquefaction and loosening of the respiratory thick sticky exudates which could be expelled by the birds and consequently reduced the hypoxia, improved breathing and finally increased the feed intake and body weights. In the present study we found that Mentofin® in group B caused relieve to some extent to histopathological lesion in lung.

ACKNOWLEDGMENT

The authors would like to thank Prof. Dr/ Awad Abd El-Hafez Ibrahim, Department of Avian and Rabbit Diseases, Faculty of Veterinary Medicine, Assiut University, for his sincere revision of the paper.

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التأثير المحسن للمنتوفين على الجهاز التنفسي للدجاج اللاحم الذي يواجه تحديا مع سلالة الميكوبلازما غاليسبتيكوم في محافظة أسيوط

الشيماء رفيق سيد ، مروة احمد احمد ، مصطفي عبد المطلب شحاتة ، مؤمن عبد العظيم ، صبري اسماعيل عيسي ، مروة محمد صفوت

E-mail: merovet84@gmail.com Assiut University web-site: www.aun.edu.eg

أجريت هذه الدراسة لبيان تاثير مركب المنتوفين في حماية الجهاز التنفسي لبداري التسمين تحت عدوتها بيمكروب الميكوبلازما جالسبتيكم. تم استخدام ١٠٥ كتكوت تسمين عمر يوم واحد من سلالة روس ٣٠٨ من انتاج شركة اسيوط للاستثمار وقد قسمت الطيور الي ٣ مجموعات المجموعة الاولي (A) تمت عدوتها بالميكوبلازما المجموعة الثانية (B) عولجت بالمنتوفين وتمت عدوتها بالميكوبلازما المجموعة الثالثة (C) تركت كمجموعة ضابطة بدون عدوي او علاج وبعمل الفحوصات الباثولوجية النسيجية للمجموعة الاولي (A) التي تمت عدوتها بالميكوبلازما وجد تراكم المخاط بالقصبة الهوائية مع تدمير اهدابها وحدوث زيادة فى عدد الخلايا الكاسية وهى الخلايا المسئولة عن افراز المخاط مع ظهور التهابات بالقصبة الهوائية بالاضافة الى حدوث زيادة فى عدد خلايا الغشاء المخاطى المبطن للقصبة الهوائية علاوة على ذلك وجدنا التهاب واحتقان في أنسجة الرئة. اما بالنسبة للمجموعة الثانية (B) التى تم علاجها بالمنتوفين بعد اصابتها معمليا بالميكوبلازما جاليسبتيكم وجد انخفاض كبير فى الافات المرضية فى انسجة القصبة الهوائية والرئة.