Therapeutic potentials of aivlosin and/or zinc oxide nanoparticles against *Mycoplasma gallisepticum* and/or *Ornithobacterium rhinotracheale* with a special reference to the effect of zinc oxide nanoparticles on aivlosin tissue residues: an *in vivo* approach

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ABSTRACT Respiratory diseases inflicted by *Mycoplasma gallisepticum* (MG) and *Ornithobacterium rhinotracheale* (ORT) cause severe economic losses and great burden to the poultry industry worldwide. Therefore, the current study was planned to assess the efficacy of aivlosin alone or in combination with zinc oxide nanoparticles (ZnO-NPs) in the treatment of experimental MG and/or ORT infections in broilers. Moreover, we also aimed to evaluate the role of ZnO-NPs on aivlosin residues in broiler tissues. A total of 1,440 Cobb chicks were allocated into 6 groups. At 14 d of age, chickens of groups 1 and 3 were experimentally infected with MG intratracheally and 6 d later, chickens of groups 2 and 3 were infected occulonasaly with ORT. Groups 1, 2, and 3 were divided into 4 subgroups; birds in subgroups 1, 2, and 3 were treated with aivlosin (A), ZnO-NPs (Z), and A/Z, respectively, while those in subgroups 4 was left without treatments. Moreover, groups 4 and 5 were kept noninfected and treated with aivlosin alone or in combination with ZnO-NPs, respectively. Finally, group 6 was kept as a negative control. The current results showed that the recovery from respiratory diseases caused by MG and/or ORT infections was most successful after treatment with A/Z in combination. Consequently, clinical signs, mortality rates, postmortem lesions of the respiratory organs, histopathological lesions of liver, trachea and lung and tracheal MG and ORT counts were significantly ($P < 0.05$) reduced following A/Z treatment. Taken together, high performance liquid chromatography analysis revealed that ZnO-NPs decreased the aivlosin residues in liver, muscle and skin of healthy and infected chickens. Based on these results, it could be concluded that aivlosin/ZnO-NPs therapy is a valuable approach for controlling MG and/or ORT infections in boilers.

Key words: Broiler chickens, *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale*, zinc oxide nanoparticles, aivlosin tissue residues

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INTRODUCTION

Respiratory diseases are considered the most serious problems facing the poultry production worldwide (Murthy et al., 2008). Several bacterial pathogens are incriminated as possible etiological agents of respiratory

tract infections either alone or in combination with other microorganisms. *Mycoplasma gallisepticum* (MG) and *Ornithobacterium rhinotracheale* (ORT) have been increasingly recognized as major respiratory pathogens in broilers. Both bacteria cause reduction in the weight gain and increased mortality, condemnation rates and medication costs, which result in significant economic losses in the poultry industry (Nascimento and Pereira, 2009). Recently, only few experimental studies demonstrated their pathogenic synergism in turkeys (Marien, 2007), layers (Sivaseelan et al., 2015), and broilers (Ellakany et al., 2019a).

Hitherto, macrolides have been commonly used in the treatment of respiratory diseases associated with MG and ORT in poultry flocks for many years (Giguère, 2013). Aivlosin, a novel second generation of macrolide, offers an excellent solution to the challenge of treating these infections in the clinical veterinary practice (Cerdá et al., 2010; Anne Forrester et al., 2011). Inappropriate use of macrolides or insufficient withdrawal time post treatment can lead to the presence of drug residues in poultry tissues, which subsequently increases the potential risk to the consumers. Long-term exposure of human to maximum levels of antibiotic residues from poultry products is associated with various adverse public health effects including gastrointestinal disturbance, tissue damage, hypersensitivity and neurological disorders (Babapour et al., 2012). Therefore, the information concerning depletion of aivlosin from edible tissues is needed to protect consumers. The Committee for Veterinary Medicinal product (CVMP) of the European Union (EU) established the Maximal Residual Limit for aivlosin in poultry tissues to be 50 μg/kg (EMEA, 2005). Besides, the misuse use of antibiotics in developing countries has been linked to the increased emergence of resistant strains that could potentially lead to clinical failures of antibiotic therapy (Abd El−Hamid et al., 2015, 2019a; Ammar et al., 2015, 2016). Therefore, there is an urgent need for new alternative antimicrobial agents.

Recently, nanotechnology has attracted a great attention in the pharmaceutical areas due to its ability to fight the microbial infections through the production of nanomaterials (Luo et al., 2007; Bendary et al., 2021; El-Sheikh et al., 2021; Ibrahim et al., 2021). The metal based nanoparticles such as Zinc oxide (ZnO) have pronounced antimicrobial activities suggesting their potential therapeutic applications (Sobha et al., 2010). The effective antibacterial activities of ZnO nanoparticles (ZnO-NPs) are owing to their small size (less than 100 nm) and their high surface-to-volume ratio, which enable the intimate interaction with bacterial membranes (Allaker, 2010). Moreover, ZnO is registered as "generally recognized as safe" by the U.S. Food and Drug Administration. However, its application into the nanoforms is accompanied with major problems including their toxicity and adverse environmental impact.

Despite the above mentioned antibacterial properties of aivlosin and ZnO-NPs, the actual *in vivo* efficacy of these antimicrobials for the treatment of avian respiratory diseases has been poorly investigated (Stipkovits and Mockett, 2007; Daghdari et al., 2017).

To gain more insights on the pathogenesis and control of single and dual respiratory infections with MG and ORT in broiler chickens, the present research attempted to establish *in vivo* experimental infection models using these bacterial agents and consequently evaluate the clinical effectiveness of aivlosin and ZnO-NPs against the induced infections. Detection of aivlosin residues is highly important for protection of the consumer health. To reinforce the control of aivlosin residues, strict measures must be taken for decreasing these residues in poultry tissues. Despite this interest, there are few available data, to the best of our knowledge, on the control of aivlosin residue levels in poultry tissues in Egypt. Therefore, the present wok was also planned to study the effect of ZnO-NPs on the concentrations of aivlosin residues in broilers' tissues following their oral administration. Finally, limited information is available about the *in vivo* toxicity of ZnO-NPs; therefore, we offered new insights into the accumulation of ZnO-NPs in chickens target organs.

MATERIALS AND METHODS

Birds

A total of 1,440 one-day-old specific pathogen free (SPF) Cobb chicks were purchased from Nile SPF Farm, Kom Oshiem, Fayom, Egypt. They were tested to be free from both ORT and MG using standard bacteriological and PCR techniques (van Empel and Hafez, 1999; García et al., 2005). All birds were reared on a floor based system and fed commercial balanced ration. They were housed in isolators at the Experimental Animals Research Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt with food and water ad-libitum.

Aivlosin and ZnO-NPs

Avilosin^{Tm} antibiotic is manufactured by the ECO Animal health, London, UK. It is dispended as a water soluble powder. Each 1 g contains 625 mg tylvalosin tartrate. Commercially available ZnO-NPs with an average size < 100 nm and surface area of 10-25 m2/g was supplied by Sigma-Aldrich, St. Louis, MO (Cat. No. 544906) with high chemical stability, photostability and high electrochemical coupling coefficient.

Bacterial Field Strains

Mycoplasma gallisepticum and *Ornithobacterium rhinotracheale* field strains were recently isolated from broiler chickens suffering from respiratory manifestations. *Mycoplasma gallisepticum* strain was grown into pleuro pneumonia like organism broth and agar media (Oxoid, Basingstoke, Hampshire, England, UK) supplemented with 20% (v/v) heat-inactivated horse serum (General Egyptian Organization for Biological Products

and Vaccines, Agouza, Dokki, Giza) under humid microaerophilic conditions (10% CO_2) at 37°C for 21 days. *Ornithobacterium rhinotracheale* strain was cultured onto 5% sheep blood agar medium at 37°C in 5% CO_2 incubator for 48 h. To reproduce pathopoiesis, both strains were rejuvenated for inoculation in chickens and after the appearance of the characteristic respiratory signs, the pathogens were re-isolated from trachea, lungs and air sacs. For assessing the *in vitro* antimicrobial activities of aivlosin and ZnO-NPs, stock suspensions of both compounds were prepared by dissolving in double-distilled water and then the suspensions were subjected to vigorous vortex shaking. The minimum inhibitory concentrations (MICs) of aivlosin and ZnO-NPs were then determined against both bacterial strains using broth microdilution method (Anonymous, 1998; Hannan, 2000). For evaluating the *in vitro* combination of aivlosin and ZnO-NPs, a checkerboard method was set up, in triplicate. The interaction between the investigated compounds was assessed via calculating the fractional inhibitory concentration index (FICI) according to the following equation: FICI = MIC of aivlosin in combination/MIC of aivlosin alone + MIC of ZnO-NPs in combination/MIC of ZnO-NPs alone. The results were interpreted as following: synergism (FICI $\leq$ 0.5), additivity (0.5 < FICI $\leq$ 1), indifference (1 < FICI $\leq$ 4) and antagonism (FICI > 4) (Fadwa et al., 2021a, 2021b).

Experimental Protocol

At two weeks of age, 1,440 chicks were marked and randomly allocated into six main groups (240 chicks/each) (Figure 1). At the same time, each bird of groups 1 (G1) and 3 (G3) was inoculated intratracheally with 0.2 mL of MG culture containing 10^9 color changing units /mL viable organisms (Awad et al., 2019). At 20 d of age, chickens of groups 2 (G2) and 3 were infected occulonasaly with ORT (10^9 colony forming units /mL) (Ellakany et al., 2019b). The bacterial infections were confirmed through observing the clinical signs and re-isolation and identification of the infecting strains using bacteriological and molecular methods. Chickens of groups 1, 2, and 3 were divided into 4 subgroups (60 birds/each). The birds in subgroups 1, 2, and 3 were treated with aivlosin (A) at a dose of 30 mg/kg bodyweight according to the manufacturer's recommendations, ZnO-NPs (Z) at a dose of 30 mg/kg bodyweight (Hesham and Eissa, 2014) and A/Z, respectively, while those in subgroups 4 were left as positive controls (infected non treated). All treatments were administered to the birds immediately after the appearance of the clinical signs (at 21 d of age) and continued once daily in the drinking water for 7 d. Apparently healthy chickens in groups 4 (G4) and 5 (G5) were kept noninfected and treated with A alone or in combination with Z as previously mentioned, respectively. During the treatment period, the water consumption of the birds was monitored. Groups 6 (G6) was kept as a negative control group (noninfected nontreated).

Ethical Statement

All the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Veterinary Medicine, Zagazig University under the reference number (ZU-IACUC/2/F/2021).

Evaluation of the Treatment Efficacy

All birds were daily observed for clinical signs and mortalities just after bacterial infections until 35 d of age (the end of the experiment). The mortality rates per each subgroup were recorded weekly and then the cumulative mortality rates were calculated at the end of the experimental period. The body weights of the birds in each group were detected at 21, 28 ,and 35 d of age. At 3, 5, and 7 d after the beginning of the treatment (24, 26, and 28 d of age), clinical signs' scores were recorded for 3 birds from each subgroup and then those birds were sacrificed and the gross lesions were scored after the postmortem (PM) examination. At the same intervals, counting of both ORT and MG in trachea was determined by quantitative real time polymerase chain reaction (qRT-PCR) assay. At 3 and 7 days after the beginning of the treatment, three sets of tissues (trachea, lung, and liver) were then subjected for histopathological examination. The parameters for evaluating the treatment efficacy in the experimental groups were illustrated as following:

Clinical Signs

During clinical examination of the birds in all groups, the respiratory signs of ORT and MG infections were scored as previously described (Kempf et al., 1998). The severity of respiratory symptoms was assessed and classified as followings: 1 = no symptoms, 2 = slight signs (sneezing), 3 = moderate symptoms (sneezing and mild tracheal rales) and 4 = severe symptoms (sneezing and frequent tracheal rales).

Postmortem Lesions

At necropsy, the gross lesions of the sacrificed birds were monitored and scored according to the score systems described previously (Nunoya et al., 1987) as following:

Air sacs: 0 = the air sacs were completely clear with no gross alterations, 1 = slight increase in thickness and marked accumulation of catarrhal exudate and 2 = severe increase in thickness and large accumulation of caseous exudate (fibrin deposition).

Trachea: 0 = no significant abnormalities, 1 = catarrhal exudate in the tracheal lumen and 2 = lumen of the trachea filled with caseous exudate.

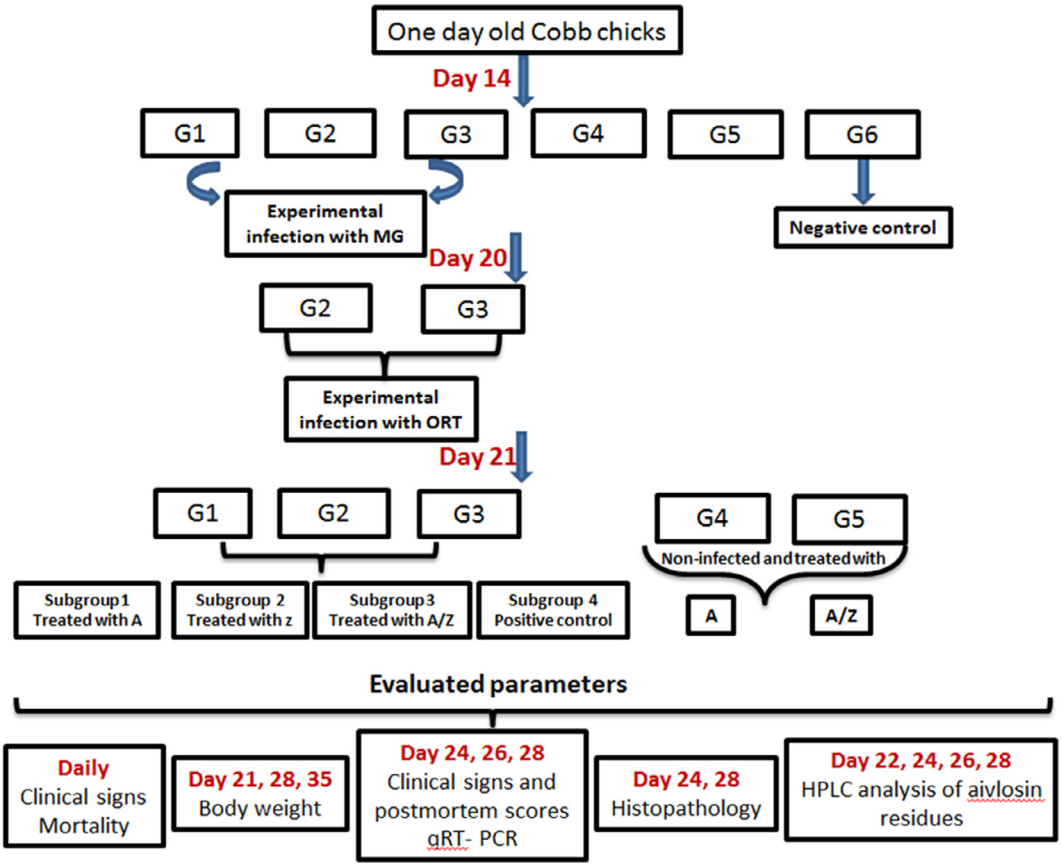

Figure 1. A flow chart illustrating the experimental design and evaluated parameters. A, aivlosin; G, group; HPLC: high performance liquid chromatography; MG, *Mycoplasma gallisepticum*; ORT, *Ornithobacterium rhinotracheale*; qRT-PCR: quantitative real time polymerase chain reaction; Z, zinc oxide nanoparticles.

Lungs: 0 = no alterations, 1 = unilateral pneumonia and 2 = bilateral pneumonia.

Histopathological Changes

Specimens from trachea, lung, and liver were fixed in 10% buffered neutral formalin solution. The tissues were dehydrated, embedded in paraffin and cut at 5 μm thicknesses. The tissue sections were then mounted onto glass slides and stained with Hematoxlin and Eosin for histopathological examinations (Bancroft and Layton, 2013).

Quantification of ORT and MG by QRT-PCR

Total DNA extraction from trachea was performed using a commercial QIAamp DNA Mini Kit (Qiagen GmbH, Germany) following the manufacturer's directions. The qRT-PCR assay was conducted using species-specific primers targeting *16S rRNA* and *mgc2* genes of ORT and MG, respectively as reported previously (van Empel and Hafez, 1999; García et al., 2005). Standard curves were prepared using DNAs extracted from different concentrations (10^2–10^6) of ORT and MG. The reaction was performed in a Stratagene MX3005P real-time PCR machine in a 25 μL reaction volume containing 12.5 μL of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen GmbH, Germany), 0.5 μL of each primer (20 pmoL), 6 μL of DNA template and 5.5 μL of water under the cycling conditions previously described (van Empel and Hafez, 1999; García et al., 2005). The sequences of the primers used to amplify *16S rRNA* and *mgc2* genes of ORT and MG are as following; 16S-F GAGAATTAATTTACGGATTAAG and 16S-R TTC GCTTGGTCTCCGAAGAT and F-CGCAATTTG GTCCTAATCCCCAACA and R-TAAACCCACCTC-CAGCTTTATTTCC, respectively. Results were expressed as $\log_{10}$ colony forming unit (CFU) per gram of examined tissues.

High Performance Liquid Chromatography Analysis of Aivlosin in Tissue Samples

At 1, 3, 5, and 7 d after the end of the treatment, 3 birds from groups 4 and 5 and all infected subgroups treated with A and A/Z were slaughtered. Tissue samples (liver, muscle, and skin) were collected and stored at −20°C until assayed. The analysis of aivlosin residues was done using the high performance liquid chromatography (HPLC) assay. All chemicals used in HPLC were supplied from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO).

Extraction of Aivlosin from Tissue Samples

Tissue samples were prepared according to the methods described previously (Lina, 2008). In brief, tissue

samples were extracted with acetonitrile and the extraction was adjusted with 0.1 M KH2PO4 (pH 4.5 ± 0.1) and defatted with hexane. The aqueous phase was set for further solid-phase clean-up using the solid phase extraction column (Bond Elute C18, 6 mL, 500 mg; Varian, Palo Alto, CA). The elute was evaporated to dryness under a gentle stream of nitrogen gas. The residue was dissolved in mobile phase and 50 μL of the sample was injected into the HPLC column (Sigma-Aldrich).

Liquid Chromatography Operating Conditions

The HPLC Agilent Series 1200 quaternary gradient pump, Series 1200 UV VIS detector and Series 1200 autosampler were used in HPLC analyses. The data were processed with HPLC 2D Chemstation software (Hewlett−Packard, France). Agilent C18 (5 μm, 4.6 × 250 mm) was used as a column. Column temperature was adjusted at 40°C by column thermostat. The mobile phase consisted of the acetonitrile and ammonium acetate buffer (0.15 M) (49:51, v/v) solution. The flow rate was 1.0 mL/min and the retention time was 7.2 min.

Quantification of Aivlosin in Tissue Samples and Validation of HPLC Analytical Method

The calibration curves of peak area against aivlosin concentration were plotted utilizing data from different concentrations (0.025−5 μg/g tissue). The optimized assay using HPLC was validated with respect to the following parameters; linearity, LOD (limit of detection), LOQ (limit of quantification), accuracy (recovery), and intra- and inter-day precision according to the International Conference on Harmonisation (ICH) guidelines Q2B for validation of analytical procedures (ICH, 1996). Linearity was evaluated by the coefficients of determination (suggest that their synergistic effect). Precision was expressed as relative standard deviation (RSD, %).

Analysis of ZnO-NPs by Flame Atomic Absorption Spectrophotometry

At 1, 3, 5, and 7 d after the end of the treatment, 3 birds from group 6 (negative control) and all infected subgroups treated with Z and A/Z were slaughtered. The tissue distribution of ZnO-NPs was detected in the liver, lung, spleen and kidney of slaughtered birds via measuring the total zinc levels by microwave digestion and flame atomic absorption spectrophotometry (FAAS) using an oxidizing air- acetylene flame at the 213.9 nm wavelength (AOAC, 2003).

Statistical Analyses and Data Visualization

At all-time points, student t-test (unpaired, 2-tailed) was used to detect if there were significant differences between investigated parameters of negative control group and the positive controls. The same assay was employed to determine if there were significant differences in aivlosin concentration in birds' tissues under different treatment conditions. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine if there were significant differences among different treatments in birds' body weights, clinical scores, PM lesions and bacterial counts. Percentages of survivability after each treatment and in positive controls were visualized using Kaplan-Meier plot and significance of differences among survival curves was determined using Log-rank (Mantel-Cox) test, where live and dead birds were recorded as 0 and 1, respectively. In all tests, P value of 0.05 was considered the cutoff value. Significance levels were *: < 0.05, **: < 0.01, ***: < 0.001. All analyses and plotting were done using GraphPad prism software (version 8.0.2) for Windows, GraphPad Software, San Diego, CA, www.graphpad.com.

RESULTS

Antimicrobial Activities of ZnO-NPs and Aivlosin and their Interaction

Field MG and ORT strains were highly sensitive to aivlosin with MIC values of 0.062 and 0.016 μg/mL, respectively (Supplementary Figure 1A). Moreover, ZnO-NPs exhibited potent antibacterial activities against MG and ORT isolates with MIC values of 0.5 and 0.125 μg/mL, respectively (Supplementary Figure 1B). According to the results of the checkerboard method, the combination of ZnO-NPs and aivlosin exhibited synergistic interactions on both MG and ORT strains with FICI values of 0.3 and 0.1, respectively.

Clinical Observations

Figure 2 illustrated the effects of different treatments on the body weights of birds (n = 3) in all experimental groups. At 21 and 28 d of age, there were significant (P < 0.05) differences in the body weights between the negative control group and the positive controls. Interestingly, significant (P < 0.05) improvements in the body weights were observed in MG and MG/ORT infected subgroups treated with A/Z comparing with those in the positive controls at 28 and 35 d of age, respectively. At the previous two intervals, the birds in the MG/ORT infected subgroup treated with A/Z had approximately 300 g increase in average body weight compared with the positive control; however, this difference was only statistically significant (P = 0.03) at 35 d of age.

The effects of using different treatments on the clinical scores of birds (n = 3) in all experimental groups are shown in Figure 3. None of the birds of the negative control group (G6) showed respiratory signs along the whole experimental period (clinical score = 1). At 24 days of age, sneezing became evident in the infected non treated subgroups (positive controls). The severity

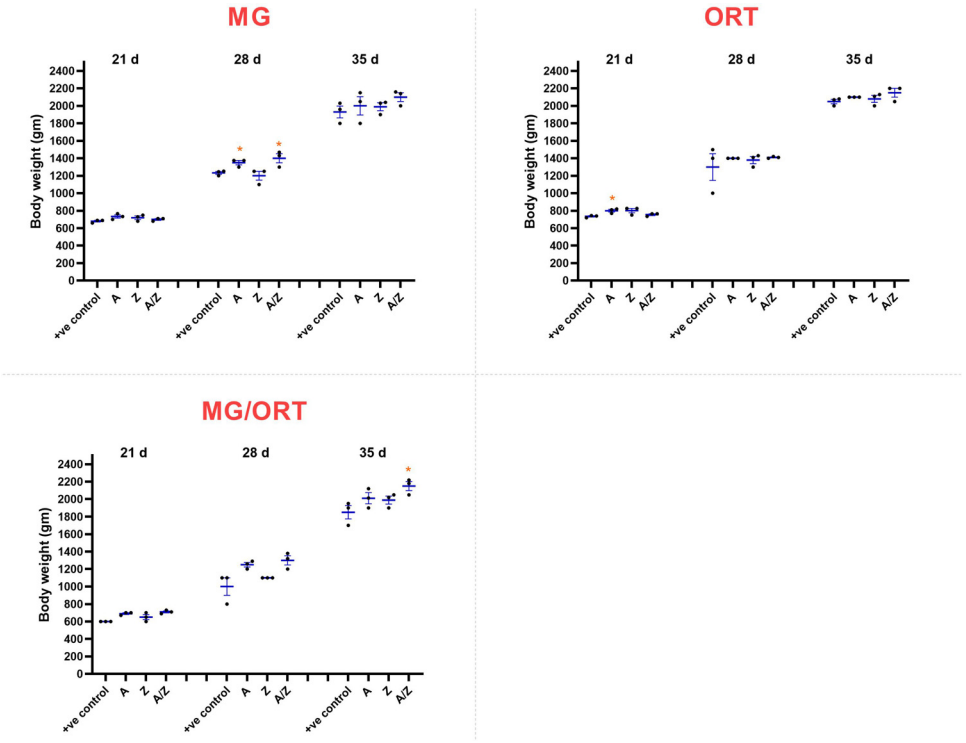

Figure 2. The effects of aivlosin (A), ZnO-NPs (Z), and A/Z (x-axis) treatments on the body weights (in grams) (y-axis) of birds (n = 3/each subgroup) experimentally infected with *Mycoplasma gallisepticum* (MG), *Ornithobacterium rhinotracheale* (ORT) and MG/ORT at 21, 28, and 35 d (d) of age. Each dot refers to the body weight of one bird. Stars refer to statistical differences in the birds body weights between infected treated subgroups and positive (+ve) controls. Statistical significance was calculated using One-way ANOVA with Bonferroni's Multiple Comparison Test at a cutoff level of 0.05. Significance levels; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

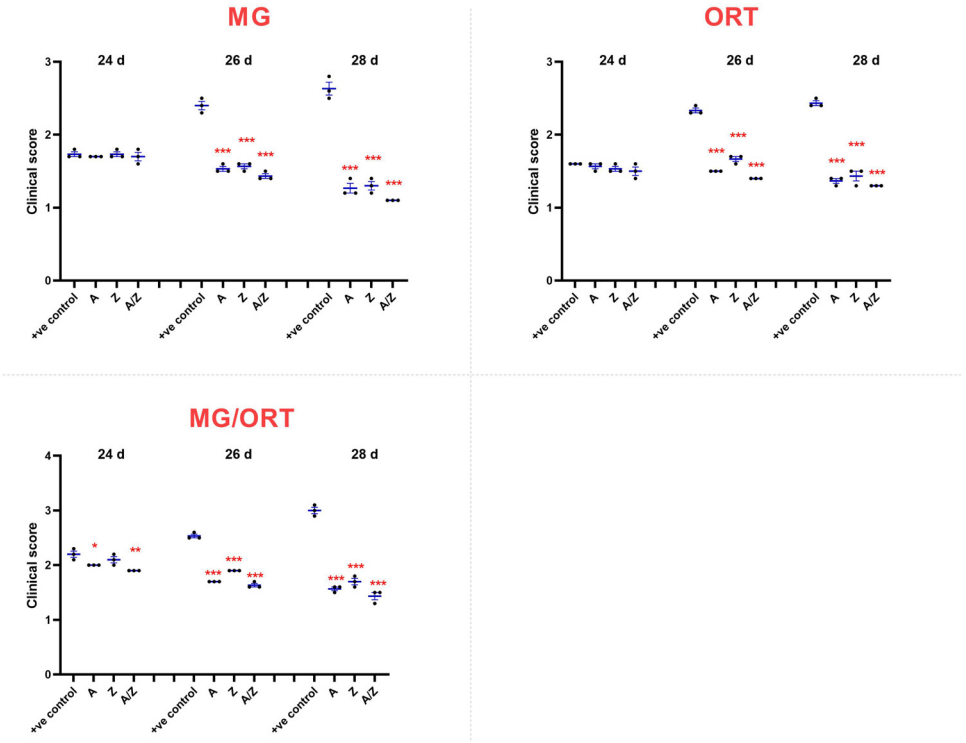

Figure 3. The effects of aivlosin (A), ZnO-NPs (Z) and A/Z (x-axis) treatments on the clinical scores (y-axis) of birds (n = 3/each subgroup) experimentally infected with *Mycoplasma gallisepticum* (MG), *Ornithobacterium rhinotracheale* (ORT) and MG/ORT at 24, 26 and 28 days (d) of age. Each dot refers to the clinical score of one bird. Stars refer to statistical differences in the clinical scores between infected treated subgroups and positive (+ve) controls. Statistical significance was calculated using One-way ANOVA with Bonferroni's Multiple Comparison Test at a cutoff level of 0.05. Significance levels; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

of the clinical signs was gradually increased until it reached the greatest values (up to 2.8, 2.5, and 3.1) at 28 d of age in the nontreated subgroups and infected with MG, ORT, and MG/ORT, respectively. All the treatments in our study succeeded in reliving the respiratory signs in all infected treated subgroups. At 26 and 28 d of age, the administration of all treatment regimens resulted in a significant reduction in the clinical scores of the birds in the infected subgroups comparing with the positive controls ($P < 0.001$). Birds treated with A/Z showed the least clinical scores (up to 1.1, 1.3, and 1.3) at 28 days of age in the infected subgroups of G1, G2, and G3, respectively.

Our results illustrated in Figure 4 revealed the effects of various treatments on the survivability of birds in all experimental groups. It has been showed that there were no mortalities in both negative control and ORT infected groups along the whole experimental period. Meanwhile, the percentages of the cumulative mortalities of birds in MG and MG/ORT infected non treated subgroups were 25% (15/60) and 40% (24/60), respectively. Overall, birds treated with A/Z showed the least significant ($P < 0.05$) cumulative mortality rates in MG (5%) and MG/ORT (6.7%) infected treated subgroups. Moreover, the cumulative mortality rates' in aivlosin and ZnO-NPs treated subgroups were 8.3 and 13.3% in G1 and 11.7 and 13.3% in G3, respectively. At the last week of the observation period, there were no mortalities in G1, G2 and G3 treated subgroups. Interestingly, there were significant differences in the survivability percentages among different treatments ($P < 0.0001$) at 22 to 28 and 29 to 35 days of age in both MG and MG/ORT infected treated subgroups.

The gross lesion scores of birds (n = 3) in all experimental groups are shown in Supplementary Figures 2 to 4. Along the whole experimental period, no marked macroscopic lesions in the air sacs, trachea and lung of sacrificed birds were observed in the negative control group (gross lesion scores = 0). Meanwhile, sacrificed birds from positive controls showed the highest gross lesion scores in all examined tissues. Interestingly, the MG/ORT infected subgroup exhibited sever macroscopic lesions in air sac, trachea and lung with gross lesion scores up to 2, 1.9, and 3, respectively at 28 d of age. Overall, a reduction in the lesion scores of air sac, trachea and lung was observed in all infected treated subgroups comparing with the positive controls at 26 and 28 d of age. At the previous intervals, the lowest significant ($P < 0.05$) lesion scores of air sac, trachea, and lung were achieved in all infected birds treated with A/Z.

Histopathological Findings

The trachea, lung and liver of chickens in the negative control group presented no marked microscopic changes. Chickens in positive controls showed the most evident changes in the examined tissues. At 24 d of age (10 and 4 d post MG and ORT infections, respectively), the

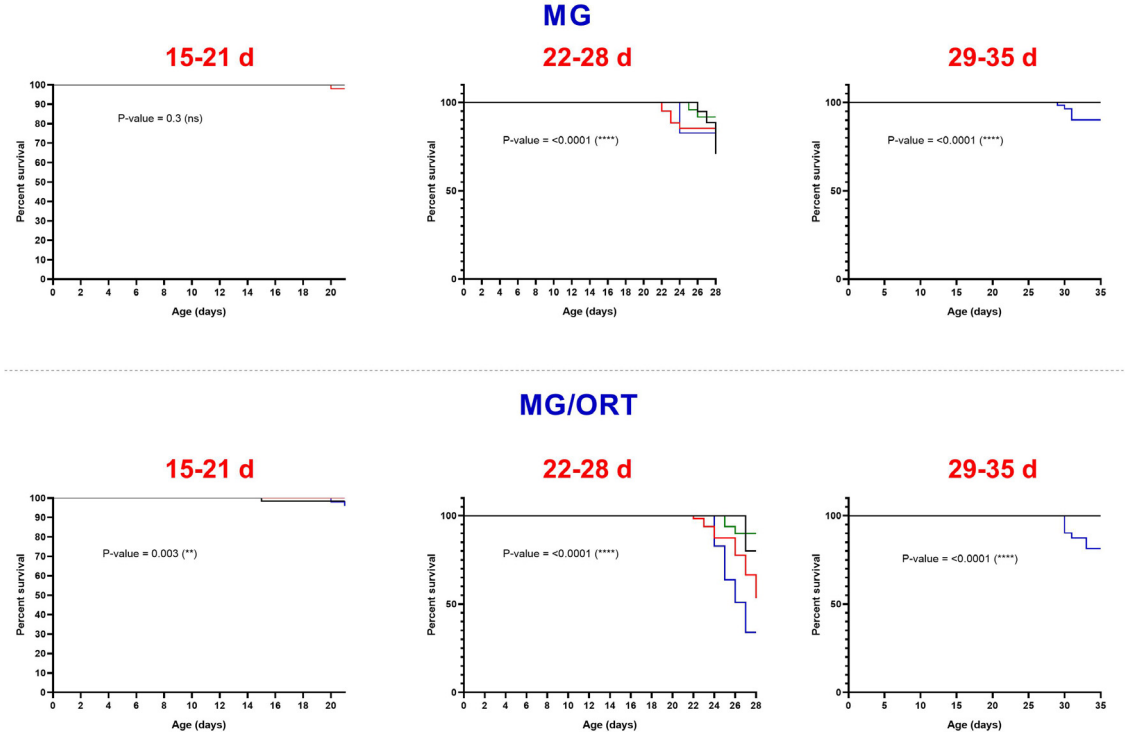

Figure 4. Kaplan Meier plot showing the survivability (y-axis) of birds experimentally infected with *Mycoplasma gallisepticum* (MG) and MG/*Ornithobacterium rhinotracheale* and treated with aivlosin (A), ZnO-NPs (Z) and A/Z treatments at 15−21, 22−28, and 29−35 days (d) of age (x-axis). Survival curves were color coded; black line: A, red line: Z, green line: A/Z and blue line: positive control. The time range written in red text above each curve indicates the age of birds during which the mortality was recorded considering that the bacterial infection started at 15 d of age. Stars refer to statistical difference among survival curves using Log-rank (Mantel-Cox) test at a *P*-value cutoff = 0.05. Significance levels; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

trachea of birds in MG and ORT infected non treated subgroups showed eroded surface with inflammatory cells' aggregations (Figure 5Ia) and desquamated epithelial and inflammatory cells in the lumen with destructed wall (Figure 5IIa), respectively. At the same interval, the mixed infected non treated birds exhibited more severe histopathological lesions in the trachea than those in the single infected non treated ones. These lesions were in the form of ulceration and erosions of the surface with sub epithelial edema mixed with inflammatory cells (Figure 5IIIa). Tracheal epithelial lining at 28 d of age showed marked thickening represented by aggregation of inflammatory cells in all infected non treated subgroups with mucoid shreds in the lumen, sub-epithelial edema and congested blood capillaries and hemorrhages and congested blood vessels in MG (Figure 5Ie), ORT (Figure 5IIe) and MG/ORT (Figure 5IIIe) infected non treated subgroups, respectively. Following ZnO-NPs and aivlosin treatments, the tracheal histopathological picture was improved in MG/ORT infected treated subgroups with mild intraepithelial inflammatory cells' infiltrations (Figure 5IIIf) and visible restoring in the epithelial lining due to regeneration attempts (Figure 5IIIg), at 28 d of age, respectively. At the same interval, their administration singly resulted in a promotion in the tracheal architecture as the trachea showed a near normal appearance in MG infected treated subgroups (Figure 5If and Ig). All the treatment trials succeeded to improve the tracheal histopathological architecture at 24 d of age in ORT infected treated subgroups (Figure 5IIb, IIc, and IId). Finally, treatment with A/Z resulted in no histopathological evidence of tracheal deterioration in all infected treated subgroups as the tracheal architecture showed apparently normal tracheal wall and free lumen at both studied intervals.

At both intervals, lungs of single infected non treated birds showed various lesions in the form of thickening of pulmonary tissues and intravascular septa, moderate engorged blood vessels and perivascular edema (Figure 6Ia, Ie, IIa, and IIe). Meanwhile, those of mixed infected non treated birds exhibited extensive lesions consisting of multifocal hemorrhages and marked congested blood vessels with lymphocytosis (Figure 6IIIa and IIIe). Pulmonary tissues of MG and ORT infected birds following treatment with aivlosin showed the least histopathological deterioration at 24 d of age (Figure 6 Ic and IIc) till they exhibited a nearly normal pulmonary architecture 4 d later (at the end of the treatment

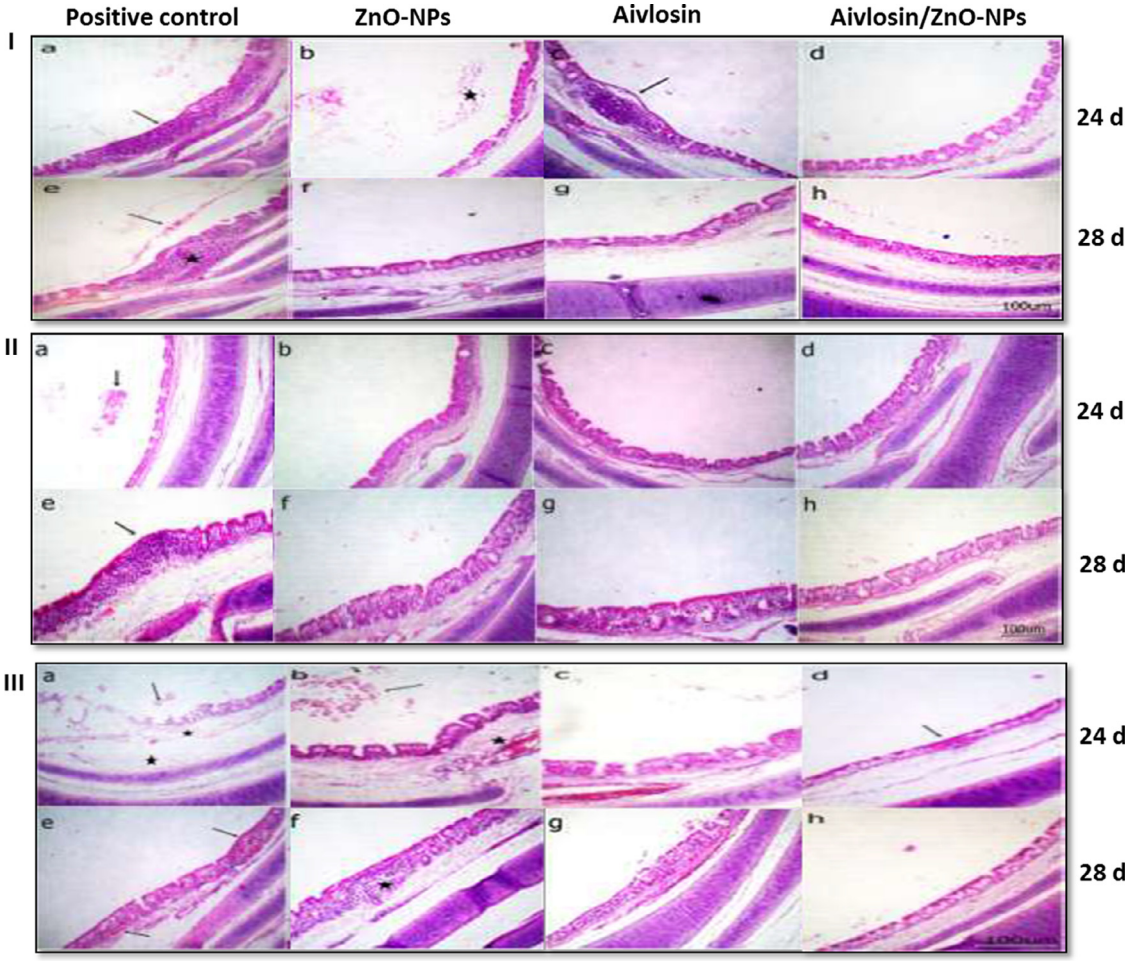

Figure 5. Histopathological sections of trachea of *Mycoplasma gallisepticum* (I), *Ornithobacterium rhinotracheale* (II) and *Mycoplasma gallisepticum*/ *Ornithobacterium rhinotracheale* (III) experimentally infected chickens without treatments (a and e) and post treatment with ZnO-NPs (b and f), aivlosin (c and g) and aivlosin/ZnO-NPs (d and h) at 24 days (a, b, c, and d) and 28 d (e, f, g, and h) of age (H&E stain, magnification = X400, Scale bar= 100 μm).

period) (Figure 6Ig and IIg). At 24 d of age, a better improvement of pulmonary histopathological picture was achieved in MG following treatment with A/Z (Figure 6Id) and ORT (Figure 6IId) infected treated subgroups; meanwhile, slight congested blood vessels were still present in MG/ORT infected treated subgroups (Figure 6IIId).

In the liver of MG or ORT infected non treated birds, interstitial inflammatory cells' aggregations with mild extravasated erythrocytes or mild congested blood vessels were observed at 24 d of age (Figure 7Ia and IIa) and peribiliary or multifocal inflammatory cells' aggregations were noticed 4 days later (at 28 d of age) (Figure 7Ie and IIe). Meanwhile, mixed (MG/ORT) infection aggravated the liver histopathological lesions in comparison with the single ones. These lesions were in the form of marked necrosis in the hepatic tissues at 24 d of age (Figure 7IIIa) and interstitial inflammatory cells' aggregation with congested blood vessels and peribiliary fibrosis at 28 d of age (Fig. 7IIIe). Treatment with A/Z resulted in ameliorating the liver histopathological picture at 24 d of age in ORT (Fig. 7IId) infected treated birds and at 28 d of age in MG (Figure 7Ih) and MG/ORT (Figure 7IIIh) infected treated birds. Moreover, aivlosin treatment led also to a promotion in the hepatic structure in MG (Figure 7Ig) and ORT (Figure 7IIg) infected treated birds at 28 d of age.

Quantitation of Tracheal ORT and MG

The effects of various treatments on the viable tracheal ORT and MG counts (log10) determined by qRT-PCR assay are illustrated in Figure 8. At 24, 26, and 28 d of age, the viable ORT and MG counts ($\log_{10}$) in the mixed infected non treated subgroups were consistently higher compared with those in the single infected non treated ones. In all infected non treated subgroups, the peak viable bacterial counts were attained at 26 d of age (12 and 6 d after MG and ORT infections, respectively). At that interval, it was observed that the highest MG and ORT counts were in the mixed infected non treated subgroups (up to 7.9 and 7.6 $\log_{10}$ CFU/g, respectively). Compared with the infected non treated subgroups, the numbers of tracheal MG and ORT organisms presented a less obvious trend in subgroups treated with A, Z or both at 24 and 26 d of age (3 and 5 d after the beginning of the treatment). Of note, there were significant ($P < 0.05$) differences in the viable counts between all infected treated subgroups and positive controls at 26 d of age. At the same interval, both therapies displayed the lowest ORT and MG counts (up to 4.9 and 5 $\log_{10}$ CFU/g, respectively), followed by A (5.1 and 5.7 $\log_{10}$ CFU/g, respectively) and finally Z (5.5 and 6 $\log_{10}$ CFU/g, respectively) in single infected groups. Moreover, all treated subgroups did not show any viable bacterial

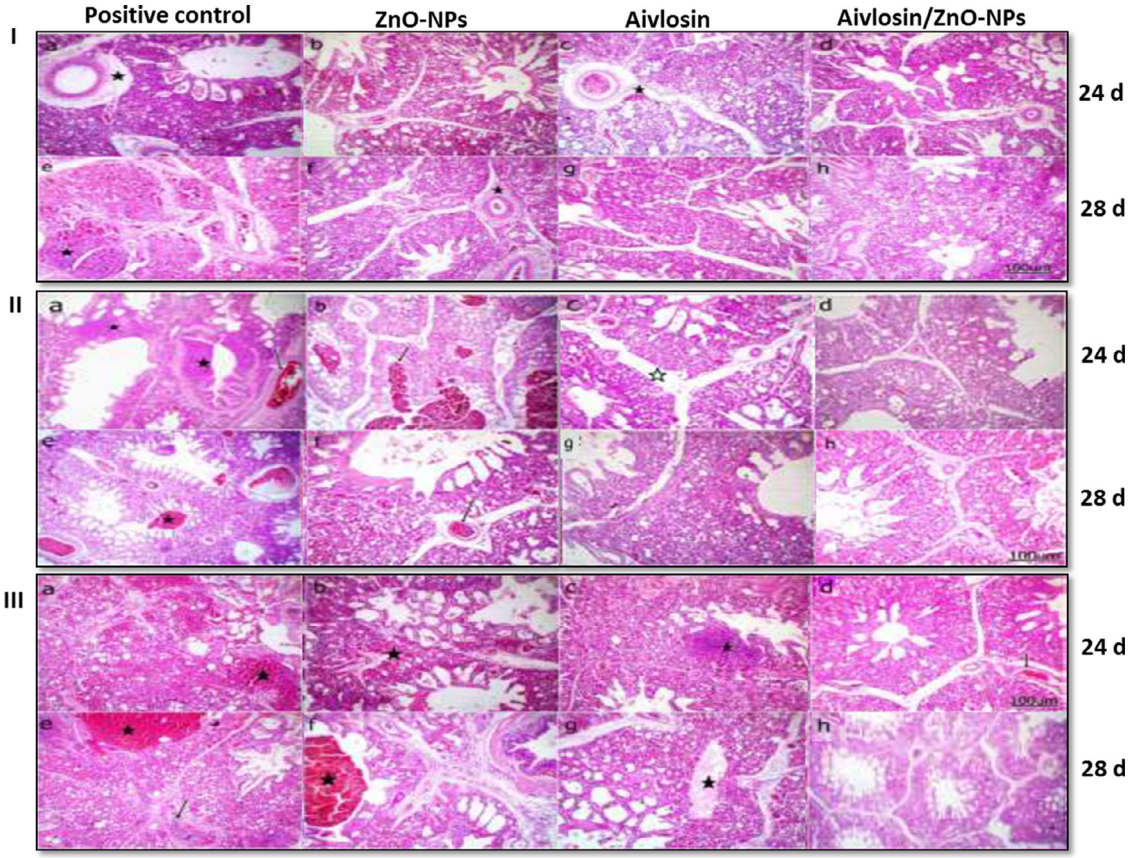

Figure 6. Histopathological sections of lung of *Mycoplasma gallisepticum* (I), *Ornithobacterium rhinotracheale* (II) and *Mycoplasma gallisepticum/ Ornithobacterium rhinotracheale* (III) experimentally infected chickens without treatments (a and e) and post treatment with ZnO-NPs (b and f), aivlosin (c and g) and aivlosin/ZnO-NPs (d and h) at 24 days (a, b, c, and d) and 28 d (e, f, g, and h) of age (H&E stain, magnification = X400, Scale bar = 100 μm).

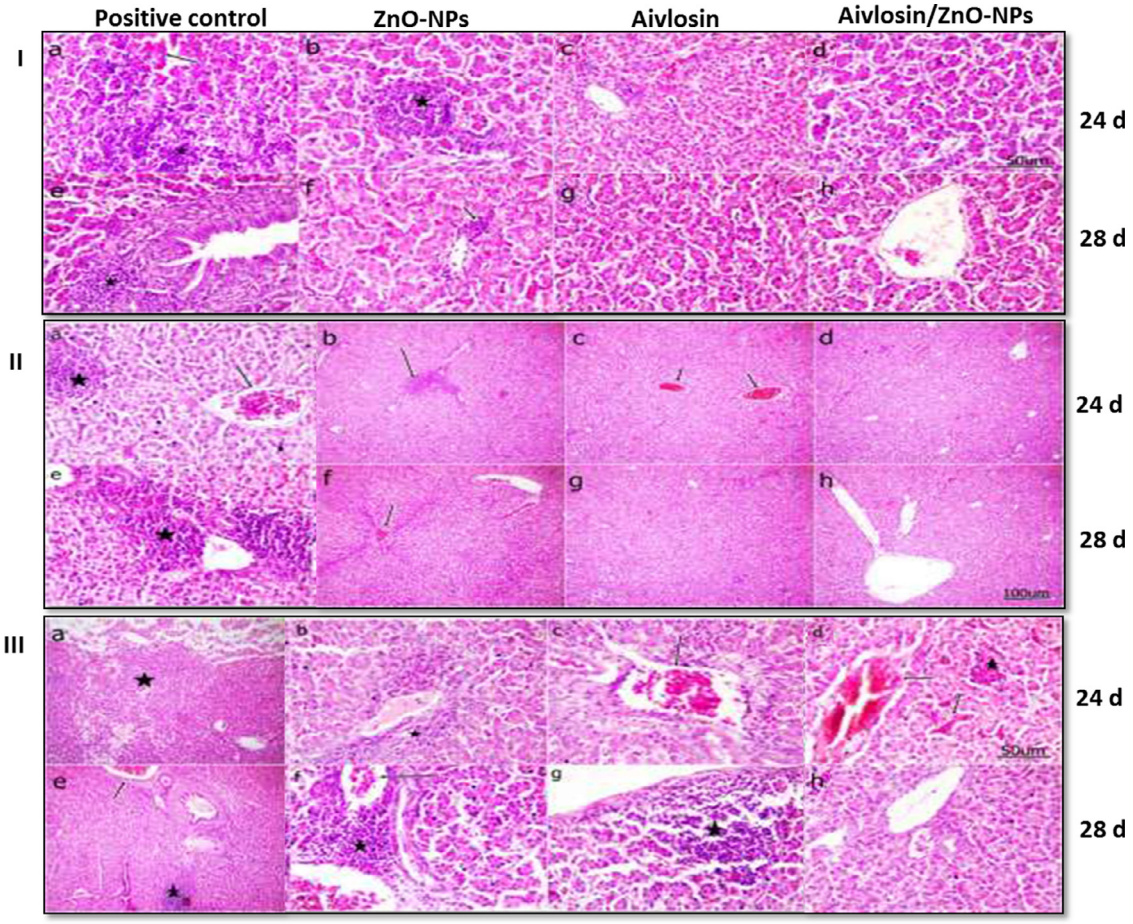

Figure 7. Histopathological sections of liver of *Mycoplasma gallisepticum* (I), *Ornithobacterium rhinotracheale* (II) and *Mycoplasma gallisepticum/ Ornithobacterium rhinotracheale* (III) experimentally infected chickens without treatments (a and e) and post treatment with ZnO-NPs (b and f), aivlosin (c and g) and aivlosin/ZnO-NPs (d and h) at 24 days (a, b, c, and d) and 28 days (e, f, g, and h) of age (H&E stain, magnification = X400, Scale bar = 50 μm).

counts in the trachea at the end of the treatment. Interestingly, treatment of MG and/or ORT infections using A/Z showed better reductions in viable bacterial counts than A or Z alone.

Validation of Analytical HPLC Technique and Occurrence of Aivlosin Residues in Tissue Samples using HPLC

The results showed that the R2 value was >0.99 indicating excellent linearity. The LOD and LOQ of aivlosin were 0.008 and 0.025 μg/g in all tissue samples, respectively. The intra- and inter-day precision and recovery rates of aivlosin are indicated in Table 1. The recovery averaged 98.06 ± 6.32 to 103.7 ± 7.01% with intra-day and inter-day RSD (%) values of < 2.00. The concentrations of aivlosin in liver, muscle, and skin of both healthy chickens in groups 4 and 5 and infected ones in subgroups treated with A and A/Z at 1, 3, 5, and 7 d after the end of the treatment are illustrated in Figure 9. The examined tissues exhibited lower values of aivlosin in infected and treated subgroups as compared with healthy groups at the 1st day after administering the last dose of treatment. Notably, the use of ZnO-NPs significantly ($P < 0.0001$) decreased aivlosin residues in all investigated tissues of both healthy and all infected chickens at all intervals. In all tested groups, high distribution of aivlosin was observed in liver (up to 7.781 ± 0.25 μg/g), followed by muscle (up to 5.741 ± 0.43 μg/g) tissues, while skin showed the lowest concentrations (up to 4.521 ± 0.23 μg/g). Interestingly, ZnO-NPs reduced the withdrawal time of aivlosin in the investigated tissues of both healthy and infected birds. Aivlosin was still detected in the liver and muscle tissues of healthy chickens in group 4 till the fifth day post treatment and in group 5 till the third d post treatment. On the other hand, it was detected only in liver tissues of all infected chickens treated with aivlosin and A/Z at first d after the last dose of the treatment, but it was undetected in muscle tissues of all infected birds treated with A/Z at all intervals. Regarding aivlosin residues in the skin of healthy birds in groups 4 and 5, it was still detected only till the third day post treatment. Meanwhile, it was detected only in the skin of all infected birds treated with aivlosin only at first day after the last dose of the treatment.

Tissue Distribution of ZnO-NPs

The liver, lung, spleen and kidney distribution pattern of ZnO-NPs was determined by measuring the total zinc

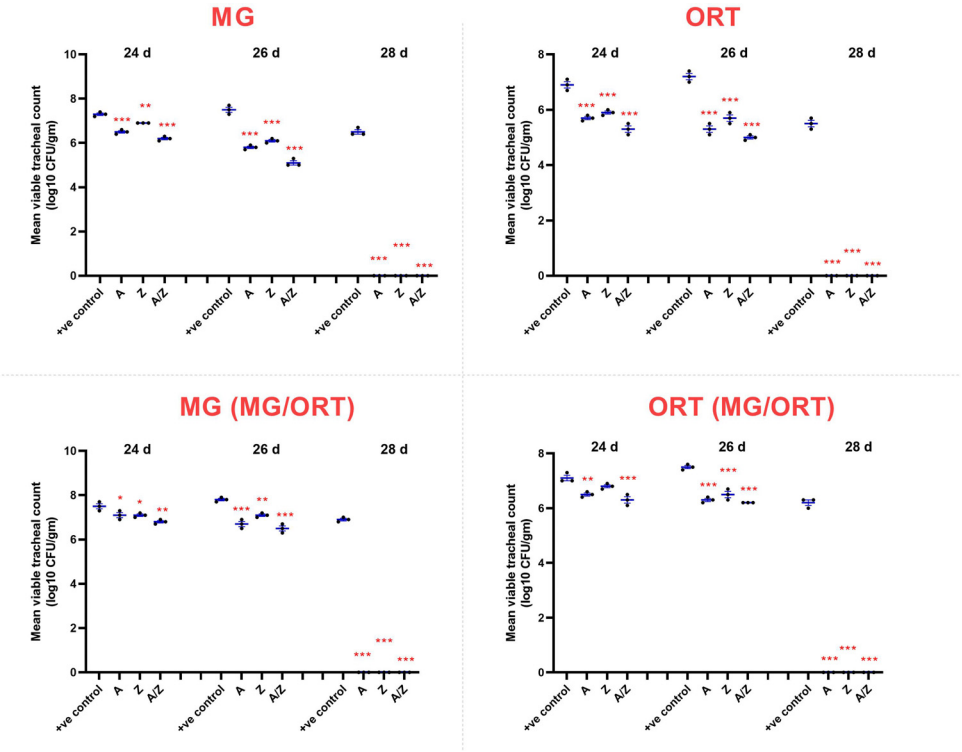

Figure 8. The effects of aivlosin (A), ZnO-NPs (Z), and A/Z (x-axis) treatments on the viable tracheal counts of *Mycoplasma gallisepticum* (MG) and *Ornithobacterium rhinotracheale* (ORT) (log10 CFU/gram) (y-axis) inoculated singly and in combination in experimental birds (n = 3/each subgroup) at 24, 26, and 28 d (d) of age. Each dot refers to the viable bacterial counts in one bird. Stars refer to statistical differences in the viable bacterial counts between infected treated subgroups and positive controls (+ve). Statistical significance was calculated using One-way ANOVA with Bonferroni's Multiple Comparison Test at a cutoff level of 0.05. Significance levels; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

levels with FAAS. Comparing the concentrations of total zinc levels between all infected subgroups treated with Z and A/Z and the negative control group, no statistically ($P > 0.05$) significant increases were noticed in the examined tissues at all time intervals.

DISCUSSION

Ornithobacterium rhinotracheale and *Mycoplasma gallisepticum* have been identified as primary respiratory bacterial pathogens causing respiratory diseases and severe economic losses in broilers. The severity of these diseases was exacerbated due to mixed infections with both pathogens (Ellakany et al., 2019a). Therefore, the current work demonstrated the effects of single and dual respiratory infections with MG and/or ORT in broilers and the efficacy of aivlosin and/or ZnO-NPs therapies for the control of these infections. Moreover, we designed to evaluate the effect of ZnO-NPs on aivlosin concentrations in poultry tissues.

Table 1. Validation parameters of analytical HPLC technique utilized for investigation of aivlosin residues in tissue samples.

Tissue	Average recovery (%)±SD	Intraday RSD (%)	Interday RSD (%)
Liver	103.7 ± 7.01	0.78	0.95
Muscle	99.28 ± 10.45	0.89	0.63
Skin	98.06 ± 6.32	1.04	0.62

SD, standard deviation; RSD, relative standard deviation.

Although no interaction was observed between ORT and MG under field conditions in broilers, a high synergism between both bacterial agents has been noticed under the experimental conditions carried out in the current study. This was evidenced by observable high mortality rates and severe respiratory signs in the mixed infection cases. The aggravation of this clinical disease was reflected by the clear outspoken necropsy findings observed in air sacs, trachea and lung of the dually infected birds. Moreover, extensive histopathological lesions and higher tracheal MG and ORT counts were observed in the birds infected with both agents in comparison to the singly infected ones. Exacerbation of the severity of the respiratory diseases as a result of mixed infections was reported earlier (Kleven, 1998; Marien, 2007). Recently, only few experimental studies demonstrated MG and ORT synergism in layers (Sivaseelan et al., 2015) and broilers (Ellakany et al., 2019a). This may be attributed to the minimal pathological effects of ORT infection alone on broilers, which were aggravated when there was a concurrent infection with other respiratory bacteria such as MG causing more severe respiratory disease (Sivaseelan et al., 2015).

The efficacy of aivlosin and/or ZnO-NPs treatments against the experimental MG and/or ORT infections in broilers was evaluated. All treatment schemes were able to improve the body weights, relive the clinical respiratory signs, reduce the mortality rates, macroscopic lesions in the examined respiratory organs and viable MG and ORT counts and ameliorate the trachea, lung

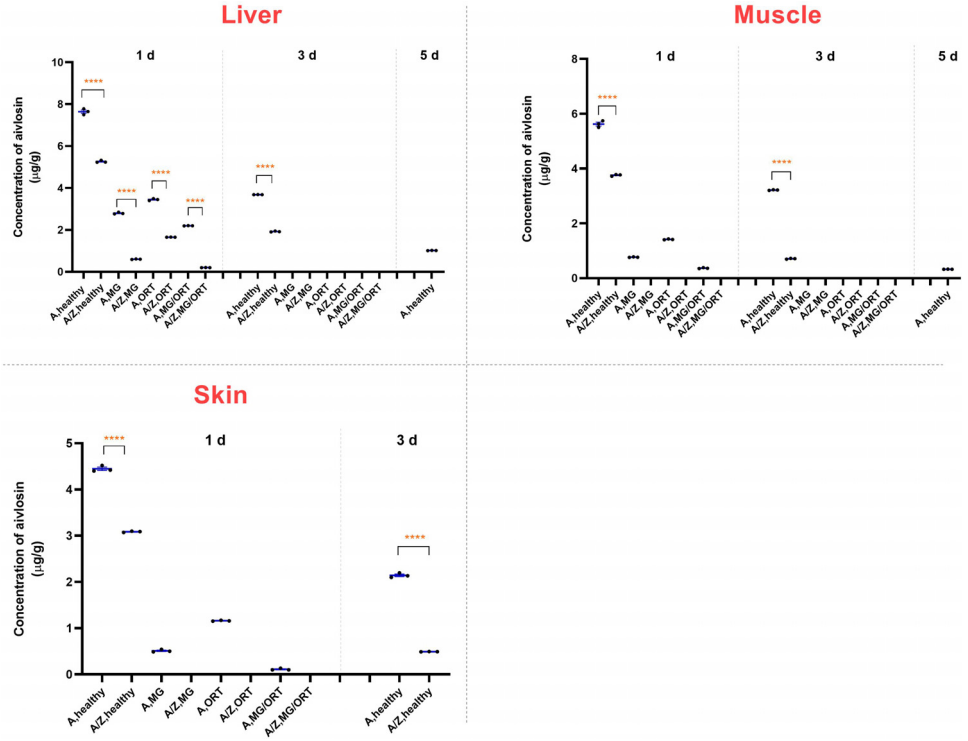

Figure 9. The effect of ZnO-NPs on the concentrations of aivlosin residues (μg/g) (y-axis), measured by HPLC analysis, in liver, muscle and skin of healthy and *Mycoplasma gallisepticum* (MG), *Ornithobacterium rhinotracheale* (ORT) and MG/ORT infected birds (n = 3/each treatment) those were treated with aivlosin alone (A) or in combination with ZnO-NPs (A/Z) (x-axis) at 1, 3, and 5 d (d) after the end of the treatment. Each dot refers to the aivlosin concentrations in one bird. Stars refer to statistical differences in the aivlosin tissues' concentrations between healthy and infected birds treated with aivlosin alone or in combination with ZnO-NPs. Statistical significance was calculated using student t-test at a cutoff level of 0.05. Significance levels; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and liver histopathological pictures compared to the infected non treated subgroups. These results assume that for clinical MG and/or ORT infections in broilers, treatment with aivlosin and/or ZnO-NPs is recommended.

Aivlosin is well known to be effective for the treatment of respiratory diseases in chickens (Giguère, 2013; Stipkovits and Mockett, 2007). Herein, we have illustrated that the field MG and ORT isolates previously recovered from broiler chickens suffering from respiratory manifestations were highly sensitive to aivlosin (MICs = 0.062 and 0.016 μg/mL, respectively) providing robust evidence for its *in vitro* antibacterial efficacy against MG and ORT. This was supported by its *in vivo* efficacy in ameliorating the respiratory diseases experimentally induced in our study. The aforementioned efficacy concurs with previous studies using aivlosin as a therapeutic regimen for the treatment of MG (Stipkovits and Mockett, 2007; Salman et al., 2016; Abd El-Hamid et al., 2019b) and ORT (Varga, 2002; Tasker, 2006; Cerdá et al., 2010) in broilers. The possible reason for these effects is the fact that aivlosin, in common with other macrolides, is absorbed from the gastrointestinal tract and enters and accumulates in the epithelial cells lining the respiratory tract leading to an inhibition in the synthesis of bacterial proteins (Stuart et al., 2007). Avian MG has recently been anticipated to be capable of invading chicken cells (Vogl et al., 2008). This penetration into epithelial cells combined with the high sensitivity of avian MG to aivlosin would maximize its antibacterial efficacy.

There is a continuous interest in the use of nanoparticles as an effective and safe alternative therapy against several bacterial agents without driving antimicrobial resistance. Today, many researchers explored new alternative antimicrobials such as metal nanoparticles. Among these metal nanoparticles, ZnO-NPs are highly concerned. Many previous studies have demonstrated the potential antibacterial effects of ZnO-NPs against a broad spectrum of microorganisms including MG (Jones et al., 2008; Petros and DeSimone, 2010; Raghupathi et al., 2011; Taha and Eissa, 2014). Consistent with these previous reports, ZnO-NPs exhibited robust antibacterial activities against MG and ORT isolates with MIC values of 0.5 and 0.125 μg/mL, respectively. Our *in vitro* potent inhibitory effects of ZnO-NPs concur with their optimistic role in the impedance of MG and ORT experimental infections in broiler chickens. Similarly, it has previously been reported that zinc nanoparticles were highly effective in controlling MG experimental infection in chickens (Taha and Eissa, 2014) suggesting its therapeutic use in the field. Reviewing the available previous literature, it seems that the *in vitro* and *in vivo* potential applications of ZnO-NPs as therapeutic agents against ORT are investigated for the first time in the current study. Although the definite antibacterial mechanism of action of ZnO-NPs is not fully explained, some researchers suggested

that the bacterial cell death is due to increased permeability leading to penetration of the cell membrane, damage of the bacterial cell wall and leakage of its cytoplasm contents (Siddiqi et al., 2018). Another possible mechanism is attributed to the electromagnetic attraction of ZnO-NPs resulting in oxidation and bacterial cell death (Arabi et al., 2012). It was also proposed that ZnO-NPs released ions that combine with the thiol groups of proteins leading to protein inactivation and bacterial cell death (Rajendra et al., 2010). More importantly, the concentration and surface area of nanoparticles are the key factors influencing their antibacterial activities; the smaller the nanoparticles size, the larger the surface area attainable for fundamental interaction with bacteria and consequently the powerful bactericidal effects (Arabi et al., 2012).

With the objective of developing new highly effective therapeutic combinations of nanoparticles and antibacterial agents, we applied a checkerboard method to evaluate the *in vitro* interaction between aivlosin and ZnO-NPs. The combination of aivlosin and ZnO-NPs showed synergistic interaction against both MG and ORT with FICI values of 0.3 and 0.1, respectively. It is worth noting that this combination also exhibited a remarkable correction in the clinical picture of MG and/or ORT experimental infections in chickens indicating that the use of aivlosin/ZnO-NPs treatment is successful in combating these clinical infections in the field. A recent study investigated the synergistic effects of ZnO-NPs and various antibiotics combinations against some Gram negative clinically bacterial isolates (Fadwa et al., 2021a, 2021b,). This success could be attributed to the combination of both therapies with their different modes of actions. Owing to the available limited data for the combination of ZnO-NPs with aivlosin, our data suggest that their synergistic effect would be possible due to the formation of complexes of Zn^{2+} ions, which were released from ZnO-NPs surface with aivlosin increasing its antibacterial activity. Additionally, ZnO-NPs could hinder the efflux transporters leading to an increase in the efficacy of aivlosin against the investigated pathogens as previously documented (Fadwa et al., 2021b). Future studies are warranted to investigate the pharmacokinetic and pharmacodynamic interactions of ZnO-NPs and aivlosin concurrently.

Notably, the use of ZnO-NPs decreased aivlosin residues in liver, muscle and skin tissues of healthy and infected chickens. Moreover, the examined tissues exhibited lower values of aivlosin in infected and treated subgroups as compared with the healthy groups. In this context, lower values of tylvalosin residues were demonstrated in the investigated tissues of *M. gallisepticum* infected and infected prebiotic pretreated broilers as compared with the healthy and healthy prebiotic pretreated ones (Salman, 2017). The reason to explain the agreement between our findings and the previous published article may be contributed to that tylvalosin was consumed by the infected bacteria in all infected subgroups indicating its higher clearance rate in all infected chickens. Remarkably, the withdrawal times of aivlosin were 5 to 7 and 3 d in healthy and infected birds, respectively. The obtained results go in parallel with another previous report, where the tylvalosin withdrawal times were 5 and 4 d after its oral administration for healthy and infected broiler chickens at the dose of 25 mg/kg (Elkomy et al., 2019). Moreover, the withdrawal times of tylvalosin were previously reported to be 5 to 7 and up to 3 d in healthy and infected broiler chickens, respectively (Salman, 2017). The prolonged aivlosin residues in healthy vs. infected birds is interesting, particularly considering the possible metaphylaxis and treatment of both healthy and infected birds in the field. Interestingly, ZnO-NPs reduced the withdrawal time of aivlosin in the investigated tissues of both healthy and infected birds suggesting the use of ZnO-NPs as an efficient tool to reduce the aivlosin withdrawal time alongside with boosting its efficacy. This is probably could be achieved through loading the aivlosin on ZnO-NPs leading to the delivery of aivlosin to the target sites at lower doses and subsequently reduce its residues and toxicity (Hemeg, 2017).

Regarding the liver, lung, spleen and kidney distribution patterns of ZnO-NPs, there were no statistically ($P > 0.05$) significant increases in the total zinc levels in all infected subgroups treated with Z and A/Z comparing with the negative control group at all time intervals. Similarly, Baek et al., revealed that significantly elevated zinc levels were observed in the rat kidney, lung, and liver 6 h after oral administration of 50 mg of ZnO-NPs/kg, followed by a decrease in the zinc levels reaching the normal levels (Baek et al., 2012). The previous authors also demonstrated that when 2,000 mg of ZnO-NPs/kg was administered, high accumulation of ZnO-NPs was evident within 2 and 3 d. This is probably due to the fact that the dose of ZnO-NPs is an important factor influencing its tissue distribution suggesting that the low dose of ZnO-NPs s is rapidly distributed to the tissues and does not accumulate in them. Concerning the toxic effect of ZnO-NPs on animals and/or consumers, our findings confirmed that ZnO-NPs at the used dose (30 mg/kg bodyweight) revealed no accumulation in the tissues and this low dose did not lead to any observed adverse effects on the histopathological pictures of the investigated tissues. Previous studies also confirmed that the toxicological adverse effects for ZnO-NPs were detected only at the higher doses; greater than 125 mg/kg (Kim et al., 2014), 2,000 mg/kg (Baek et al., 2012) and 300 and 2000 mg/kg (Srivastav et al., 2016). More thorough toxicity studies are needed for safety evaluations of ZnO-NPs.

It is noteworthy to highlight that this is the first report evaluating the synergistic antibacterial effect of aivlosin and ZnO-NPs in controlling the experimentally induced MG and/or ORT infections along with studying the effect of ZnO-NPs on aivlosin residues in chicken tissues and the tissues distribution patterns of ZnO-NPs in Egypt. Of note, we limited our replicates to 3/condition in order to find a compromise between comparing multiple conditions and attaining the required statistical power. In conclusion, aivlosin/ZnO-NPs treatment was

efficient enough to alleviate the negative effects derived from MG and/or ORT infections. Moreover, ZnO-NPs used in the current study played a vital role in decreasing the aivlosin residues in broiler tissues. These beneficial findings recommend the use of aivlosin in combination with ZnO-NPs as a good choice in the treatment of these bacterial infections in the field. To support the results of our experimental trials, additional field studies should be applied in the future.

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Authors' contributions: NFSA, YMH and MIAE designed the study. NFSA, MIAE and YMH carried out the molecular analyses and participated in the data analysis. NSE performed the HPLC analysis. EK, RIH, HHN, EMAA, MAN and STE conceived the study and participated in the design. NFSA, YMH, NSE and MIAE wrote the initial draft of the manuscript. All authors approved the final manuscript.

DISCLOSURES

The authors declare that they have no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101884.

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