Methylsulfonylmethane ameliorates inflammation via NF-κB and ERK/JNK-MAPK signaling pathway in chicken trachea and HD11 cells during *Mycoplasma gallisepticum* infection

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ABSTRACT *Mycoplasma gallisepticum* (**MG**) is an avian pathogen that commonly causes respiratory diseases in poultry. Methylsulfonylmethane (**MSM**) is a sulfur-containing natural compound that could alleviate inflammatory injury through its excellent anti-inflammatory and antioxidant properties. However, it is still unclear whether MSM prevents MG infection. The purpose of this study is to determine whether MSM has mitigative effects on MG-induced inflammatory injury in chicken and chicken like macrophages (**HD11 cells**). In this research, White Leghorn chickens and HD11 cells were used to build the MG-infection model. Besides, the protective effects of MSM against MG infection were evaluated by detecting MG colonization, histopathological changes, oxidative stress and inflammatory injury of trachea, and HD11 cells. The results revealed that MG infection induced inflammatory injury and oxidative stress in trachea and HD11 cells. However, MSM treatment significantly ameliorated oxidative stress, partially alleviated the abnormal morphological changes and reduced MG colonization under MG infection. Moreover, MSM reduced the mRNA expression of proinflammatory cytokines-related genes and decreased the number of death cells under MG infection. Importantly, the protective effects of MSM were associated with suppression of nuclear factor-kappa B (**NF-κB**) and extracellular signal-related kinases **(ERK)**/Jun amino terminal kinases **(JNK)**-mitogen-activated protein kinases (**MAPK**) pathway in trachea and HD11 cells. These results proved that MSM has protective effects on MG-induced inflammation in chicken, and supplied a better strategy for the protective intervention of this disease.

Key words: *Mycoplasma gallisepticum*, methylsulfonylmethane, inflammation, chicken, NF-κB and ERK/JNK-MAPK pathway

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INTRODUCTION

Mycoplasma gallisepticum (**MG**) is a wall less prokaryotic microorganism (Li et al., 2019). It is the main pathogen that causes chronic respiratory disease (**CRD**) in poultry, causing significant economic damage to the chicken industry (Wang et al., 2021b). MG lacks a cell wall and has the property of adhesion and attachment to host cells (Levisohn and Kleven, 2000). MG-derived lipid-associated membrane proteins (**LAMPs**) are thought to be one of the major factors in *mycoplasma* pathogenesis and are potent inducers of the host innate immune response (Yu et al., 2018). The lipoproteins in *Mycoplasma* membranes play a major role in eliciting immune responses, and are potent stimulators of macrophages (Mühlradt et al., 1998). These lipoproteins have the ability to activate toll like receptors (**TLRs**)-mediated signaling pathway and results in the release of various inflammatory cytokines (Muneta et al., 2003). At present, the main treatment methods of relieving symptoms during the acute phase of MG infection are still antibiotics. Although the threat posed by MG has been alleviated to a certain extent, the continued use of antibiotics could lead to the growing levels of bacterial resistance (Gautier-Bouchardon, 2018; Wang et al., 2021a). Therefore, it is still the focus of future research to seek more treatment programs for MG infection.

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Nuclear factor-kappa B (**NF-κB**) is a pivotal transcriptional factor that adjusts immunity reaction and acts as an intermediary of inflammatory reaction. Besides, TLRs is able to activate NF-κB and sensitize immune interrelated signaling pathways (Jo et al., 2020). Mitogen-activated protein kinases (**MAPK**) exists in all eukaryotic cells and is a serine-threonine protein kinase that mediates cell response to extracellular stimuli. The general MAPK pathway is enjoyed by four different cascades, which are nominated according to their MAPK layer composition; the extracellular signal-related kinases (**ERK1/2**), Jun amino terminal kinases (**JNK1/2/3**), p38-MAPK, and ERK5 (Sun et al., 2015). Generous studies have shown that under hyperglycemia, reactive oxygen species (**ROS**) are upregulated and ROS induced IL-1β through phosphorylation of ERK1/2 and p38 MAPK, resulting in NF-κB activation (Volpe et al., 2018) and bringing about the release of inflammatory cytokines (Olson et al., 2007). NF-κB and MAPK signaling pathways are very closely related to inflammation and oxidative stress. It has been previously demonstrated that MG infection activated NF-κB and MAPK signaling pathways, which induced inflammatory injury and oxidative stress in chickens (Lu, et al., 2017; Ishfaq, et al., 2019b). Thus, the inhibition of NF-κB and MAPK signaling pathway could be a novel treatment approach for the prevention of MG-induced oxidative stress and inflammation.

Methylsulfonylmethane (**MSM**), dimethyl sulfone, is the oxidation metabolite of dimethyl sulfoxide (**DMSO**). It is a sulfur compound widely found in fruits, vegetables, grains, animals, and humans (Miller, 2018). It is the main substance in the body to maintain the balance of biological sulfur. There is evidence that MSM is relevant to the catabolism of amino acids by the intestinal microbiota (He and Slupsky, 2014). MSM has diverse functions, such as anti-inflammatory, anticancer, antioxidant, antiallergy, and anti-immunosuppression activities, which make it a potential drug in the future (Joung et al., 2016). MSM is the main source of sulfur in the antioxidant glutathione, and it plays a very important role in scavenging oxygen free radicals in the body. Therefore, numerous countries use a nontoxic form of dietary sulfur to remedy many diseases, and it can be used as a joint health supplement. MSM has been shown to protect against obesity-induced metabolic disorders, constipation, hyperacidity, mucous-membrane inflammation, intestinal cystitis, colitis, and liver damages (Parcell, 2002; Amirshahrokhi et al., 2011; Sousa-Lima et al., 2016). Moreover, the addition of MSM in the diet has beneficial effects on growth performance, immune function, meat, and egg quality of chickens (Jiao et al., 2017). However, the protective effects of MSM on MG-induced inflammatory injury in chicken trachea are currently unclear. Therefore, the objective of our study was to evaluate the preventive effects of MSM against MG infection and its associated cell damage. In addition, the research will provide scientific foundation for the use of MSM to prevent MG-induced inflammatory injury in the chicken industry.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted under the approval of Laboratory Animal Ethics Committee of Northeast Agricultural University (Heilongjiang province, China) in accordance with Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892-2018, National Standards of the People's Republic of China) in the present study.

Chemicals and Experimental Cells

Methylsulfonylmethane (purity ≥99.0%) was bought from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Chicken like macrophages (**HD11 cells**) were provided from the lab of Veterinary Pathology of Northeast Agricultural University.

Mycoplasma Gallisepticum Strains Culture

The MG strain R_{low} was obtained from Harbin Institute of Veterinary Medicine, Chinese Academy of Agricultural Science. MG was grown on modified Hayflicks medium containing 20% fetal bovine serum, 10% freshly prepared yeast extract, 0.05% Penicillins, 0.05% thallium acetate, and 0.1% Nicotinamide adenine dinucleotide at 37°C (Cai et al., 2008).The frozen stock of MG R_{low} strain was cultivated in MG culture medium at 37° C. The liquid medium containing MG strain was diluted to different concentrations by 10-fold in test tubes. In the logarithmic growth phase of MG, the medium color change was observed from phenol red to orange. After 7 days of incubation at 37°C, the highest dilution was observed as the color of the medium changes from phenol red to orange and was considered as the color change unit (**CCU**). While the concentration of the bacterial solution to 1×10^9 CCU/mL was adjusted for the subsequent experiment as previously described (Calus et al., 2010; Garcia-Morante et al., 2018).

Chickens and Treatments

Healthy one-day-old White Leghorn chickens were purchased from a local commercial chicken farm (Xianfeng Guangda Poultry Co. Ltd., Harbin, China). Chickens were housed in aseptic glass box of constant temperature, and provided with sterile feed and water. The antibacterial-free chicken feed was provided from Lenong Feed Co., Ltd. (Harbin, Heilongjiang). Chickens were randomly divided into 4 experimental groups. There were 10 chickens in each group (Figure 1). All experimental groups including (A) Control group, (B) MG group, (C) MG group treated with MSM (MG +MSM group) and (D) MSM alone treated group (MSM group). Control group and MG group 1-day-old age chickens were intranasally treated with 50 μL of PBS from d 1 to d 7, twice in a day. MSM group and MG+MSM group 1-day-old age chickens were

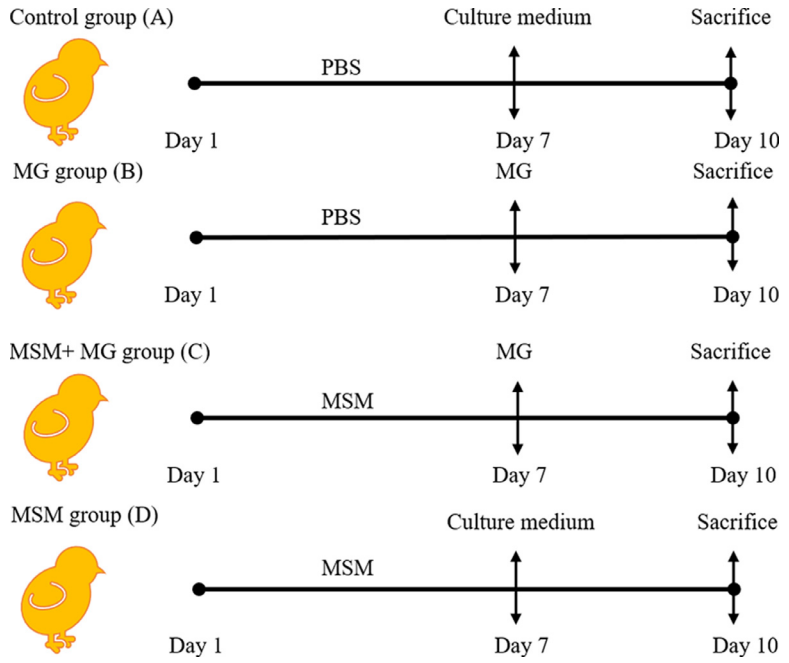

Figure 1. Schematic diagram of the experimental group in this study, details showed in experimental groups in Materials and Methods section.

intranasally treated with 50 μL of MSM from d 1 to d 7, twice in a day, MSM group (500 mg/kg) and MG+MSM group (1,000, 500, and 250 mg/kg). MG infected group and MG+MSM group chickens were inoculated with MG strain R_{low} (1×10^9 CCU/mL) in the left air sacs at d 7 (Ishfaq et al., 2020). After 3-d postinfection, the chickens were euthanized with intravenous barbiturates to minimize damage to the trachea and tracheal tissues were collected for the following experiment (Figure 1).

Mycoplasma Gallisepticum Quantification

In order to measure the extent of MG infection, a recombinant plasmid containing the cloned *mgc2* gene was used to detect the absolute abundance of MG by quantitative RT-PCR to establish a standard curve (Gao et al., 2017). In brief, tracheal tissues samples were weighed, dissolved in 9-fold volume of physiological saline solution, and homogenized at 4°C. The homogenized samples were then centrifuged at 1,000 × *g* for 10 min, and the supernatant was collected. The DNA of chicken tracheal tissues from each group was prepared by using E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Inc., Norcross, GA) following the manufacturer's guidelines. The method of DNA kit was DNA adsorption column purification. The abovementioned experimental operation was carried out in a sterile environment. *mgc2* gene was amplified by PCR as part of MG genome DNA and sequenced. The primers are shown in Table 1. PCR reaction conditions: 95°C-5 min, 95°C-10 s, 55°C-30 s, 72°C-60 s, 39 cycles.

Culture of HD11 Cells

HD11 cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37°C in 5% CO_2 (Garrido et al., 2017). The cells were cultured for 12 h in 96-well cell culture plate and 6-well cell culture plate prior to the start of the following experiment. After counting under a microscope, the cells were cultured in a 6-well cell culture plate with 1×10^6 cells/well and a 96-well cell culture plate with 1×10^5 cells/well for experiments. 1×10^6 cells/well in a 6-well cell culture plate and 1×10^5 cells/well in a 96-well cell culture plate were the average number of cells per well at the time of the experiment.

Cells Viability

Add *mycoplasma* with different multiplicity of infection (**MOI**) or different concentrations of MSM in 96-well cell culture plate. After incubating at different time points in a 5% CO_2 incubator (37°C), add the Cell Counting Kit-8 (**CCK-8**) solution and incubate for 2 h. Based on the results of CCK-8 test, the optimum concentration range of MG and MSM on HD11 cells was determined.

Table 1. List of primers used in qRT-PCR.

Genes	Primers (from 5′ to 3′)	Primers origin
TNF-α	F: CAGATGGGAAGGGAATGAAC R: AGAACAGCACTACGGGTTGC	JN942589.1
IL-1β	F: AGCAGCCTCAGCGAAGAGACC R: GTCCACTGTGGTGTGCTCAGAATC	NM_204524.1
IL-6	F: GAGGTTGGGCTGGAGGAGGAG R: TCTCGCACACGGTGAACTTCTTG	NM_204628.1
IL-8	F: TCAAGATGTGAAGCTGACGCCAAG R: GTCCAAGCACACCTCTCTTCCATC	DQ393272.2
GAPDH	F: GGTAGTGAAGGCTGCTGCTGATG R: AGTCCACAACACGGTTGCTGTATC	NM-204305
mgc2	F: TTGGGTTTAGGGATTGGGATT R: CCAAGGGATTCAACCATCTT	(Wang et al., 2020)

HD11 Cells Treatments

HD11 cells were divided into the following 4 experimental groups. (A) Control group: HD11 cells without MG infection and/or MSM; (B) MG-infection group: HD11 cells were infected with MG at 400 MOI and cultured for 6 h for different analyses; (C) MG-infection group treated with MSM: HD11 cells were treated with MSM (200 mmol/L) for 12 h. Then the medium was removed and fresh medium containing the MG at 400 MOI was added and incubated for 6 h before different analyses; (D) Methylsulfonylmethane-alone treated group: HD11 cells were treated with 200 mmol/L MSM dissolved in RPMI1640 cultured medium for 12 h.

Acridine Orange/Ethidium Bromide Staining

HD11 cells were inoculated in a 6-well plate and stained with Acridine orange/ethidium bromide (**AO/EB**; Leagene, China). Firstly, the cells were washed twice with PBS. After cell counting and resuspended in the desired volume of PBS. Then the 90 μL cell suspension were incubated with 5 μL AO and 5 μL EB at 37°C in the dark for 10 min (Chi et al., 2021). Lastly, the cells were photographed by a fluorescence microscope (OLYMPUS, Japan). Images were taken with an Olympus BX53 microscope containing the filters for long pass and band pass and an Olympus DP26 color camera. Green fluorescence emission wavelength was 530 nm and red fluorescence emission wavelength was 640 nm. Quantitative analysis of the fluorescence intensity was accomplished by using Image J software (V 1.42, National Institutes of Health, Bethesda, MD).

Histopathological Examination

Fresh tracheal tissues of each group were collected, washed with physiological saline to remove blood as much as possible, and then tracheal tissues were trimmed into little pieces of suitable size, and fixed in 10% neutral formalin at room temperature for more than 48 h (Ishfaq et al., 2021). Samples were processed in a series of ethanol, embedded in paraffin and cut into thin slices. Next, the slides were stained with eosin and hematoxylin dye and observed through a light microscope (Nikon ECLIPSE E100, Japan).

Ultrastructural Observations

Briefly, trachea and HD11 cells samples were fixed in 2.5% glutaraldehyde and rinsed twice in 0.2 M phosphate buffer (pH = 7.2) for 15 min (Zhang et al., 2020). Then the specimens were fixed in 1% osmium tetroxide for 1 h, dehydrated in a series of ethanol and embedded in epoxy resin. The samples were dried for 4 h and a metal film was plated on the surface of 1 mm^3 tracheal tissue with ion sputtering coating equipment. Lastly, samples were observed through transmission electron microscope (**TEM**, JEOL., Ltd. Japan) and scanning electron microscopy (**SEM**, SU8010, HITACHI., Ltd. Japan).

Determination of Antioxidant Activities

iNOS, H_2O_2, MDA, CAT, GSH-PX, and T-SOD enzyme activities and contents were measured in trachea tissues in the light of the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China). Nitric oxide synthase (iNOS, Cat no. A014-1), hydrogen peroxide (H_2O_2, Cat no. A064-1), malonaldehyde (MDA, Cat no. A003-1), catalase (CAT, Cat no. A007-1), glutathione peroxidase (GSH-Px, Cat no. A006), total superoxide dismutase (T-SOD, Cat no. A001-1). Samples were weighed, dissolved in 9-fold volume of physiological saline solution, and homogenized at 4°C. The homogenized samples were then centrifuged at 1,000 × g for 10 min, and the supernatant was collected and examined for the abovementioned oxidative stress-related parameters on a microplate spectrophotometer (Philes, Nanjing, China).

Measurement of Cytokines

Each group of HD11 cell samples were extracted and analyzed with enzyme-linked immunosorbent assay (**ELISA**) kit according to the production instructions (Beijing Cheng Lin Biological Technology Co., Ltd., China). Detection of inflammatory factor IL-1β, TNF-α, IL-6, and IL-8 (Wu et al., 2019).

Total RNA Extraction and qRT-PCR

Total RNA was isolated from chicken trachea and HD11 cells samples using Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions (Takara Biomedical Technology (Beijing) Co., Ltd., China) for reverse transcription of cDNA. LightCycler 96 (Roche, Basel, Switzerland) was used to perform quantitative RT-PCR analysis of gene expression (Wu et al., 2020). Primer sequences are shown in Table 1. Using GAPDH gene as an internal standard and the data were quantified by $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Western blotting was used to measure the related proteins. The total protein of tracheal tissue and HD11 cells was extracted by the whole-cell lysis method. Primary antibodies for TLR4 (bs-20379R, Bioss, Beijing, China), IκBα (10268-1-AP, Proteintech, Wuhan, China), p-IκBα (bs-2513R, Bioss), p65 (bs-0465R, Bioss), p-p65 (bs-0982R, Bioss), IL-1β (A16288, ABclonal, Wuhan, China), TNF-α (bsm-33207M, Bioss), JNK (bs-20760R, Bioss), p-JNK (bs-17591R, Bioss), ERK (bs-2637R, Bioss), p-ERK (bs-1645R, Bioss), and GAPDH (A5028, bimake, Houston, TX) (all at 1:1,000 dilution) protein were incubated for 12 h at 4°C. Secondary anti-rabbit IgG horseradish peroxidases (bs-0061R, Bioss) were incubated for 1.5 h.

Enhanced chemiluminescence (**ECL**) reagent (Beyotime, China) was used to visualize the bound immune complexes by automatic chemiluminescence image analysis system (Tanon, China) and the density of the protein bands was measured with Image J software (V 1.42, National Institutes of Health; Hu et al., 2021).

Statistical Analysis

The data were statistically analyzed by the Statistical Package for Social Sciences (**SPSS, Chicago, IL**) software (version 21.0) and GraphPad software (San Diego, CA) (version 6.01). For comparison among multiple groups, one-way ANOVA was used to determine the statistical significance at a value of $P \leq 0.05$ and $P \leq 0.01$, followed by least square difference (**LSD**) post hoc test. All the experiments were performed at least 3 times unless otherwise stated and the data were expressed as mean ± standard deviation (**mean ± SD**).

RESULTS

MSM Alleviated MG Colonization in Trachea Tissues

The amount of MG colonization in trachea increased significantly in MG group (Figure 2). Furthermore, the results showed that MSM (1,000 mg/kg and 500 mg/kg) treatment significantly reduced MG colonization.

Histological and Ultrastructural Observation

Pathological and ultrastructural observations are shown in Figure S1. Histopathological examination revealed that MG infection caused grievous tracheal tissue injury and a large number of cilia were shed (Figure S1B). SEM revealed that the cilia were broken, ruptured, and necrotic by MG-infection (Figure S1H). TEM showed that cilia were ruptured, inverted, cytoplasm swelling by MG-infection (Figure S1N). The morphology and appearance of trachea tissue micrographs in the Control group (Figure S1A, S1G, and S1M) and MSM alone group (Figure S1F, S1L and S1R) were normal. But after treatment with MSM (1,000 mg/kg and 500 mg/kg), the abnormal morphology and structural deterioration observed in the trachea of chickens partially disappeared (Figure S1C, S1D, S1I, S1J, S1O, and S1P). In MSM (1,000 mg/kg and 500 mg/kg) treatment group, a small number of the cilia were broken and exfoliated with less inflammatory cell infiltration compared to the MG group. MSM (250 mg/kg) treatment has slightly better effect on MG infection (Figure S1E, S1K, and S1Q). The severity of tracheal lesions was scored on a scale of 0−3 as described (Nunoya et al., 1987; Bao et al., 2020). The tracheal lesion scores of the MG group were significantly greater than the Control group, but the scores were significantly reduced in the MSM (1,000 mg/kg and 500 mg/kg) treatment group (Figure S2). The above results revealed that the optimal concentration of MSM treatment on MG infected chicken is 500 mg/kg for subsequent experiments.

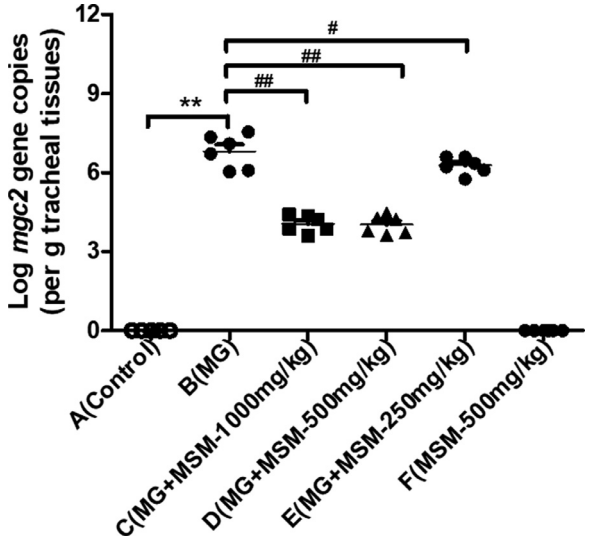

Figure 2. MG trachea burden 3 d post MG inoculation (n = 6). Each point represents a chicken, and the horizontal line represents the average value.

MSM Alleviated Oxidative Stress in Trachea Tissues

The oxidative stress parameters in tracheal tissue are shown in Figure 3. MG infection reduced antioxidant activities such as CAT, GSH-PX and T-SOD activity of trachea tissue. MG infection caused the level of iNOS activity, H_2O_2 and MDA contents were increased in the trachea of chickens (all $P < 0.01$). It has been noted that MSM treatment apparently recovered the normal level of these enzymes and alleviated trachea oxidative stress. Interestingly, compared with the Control group, MSM intervention alone had no notable influence on these enzymes.

Repression of Proinflammatory Cytokines and NF-κB and ERK/JNK-MAPK Signaling Pathway of Trachea by MSM

The mRNA expression of inflammatory cytokines (Figures 4A−4D) including TNF-α, IL-1β, IL-6, and IL-8 was markedly increased in tracheal tissues by MG infection. While MSM treatment obviously restrained the increase in the expression of these cytokines under MG infection. The western blotting results (Figure 5) showed that MG infection significantly increased NF-κB and ERK/JNK-MAPK signaling pathway related proteins expression of TLR4, p-IκBα/IκBα, p-p65/p65, IL-1β, TNF-α, p-JNK/JNK, and p-ERK/ERK. Meanwhile, compared to MG group, the protein expression level of NF-κB and ERK/JNK-MAPK signaling pathway were reduced ($P < 0.05$) with MSM treatment.

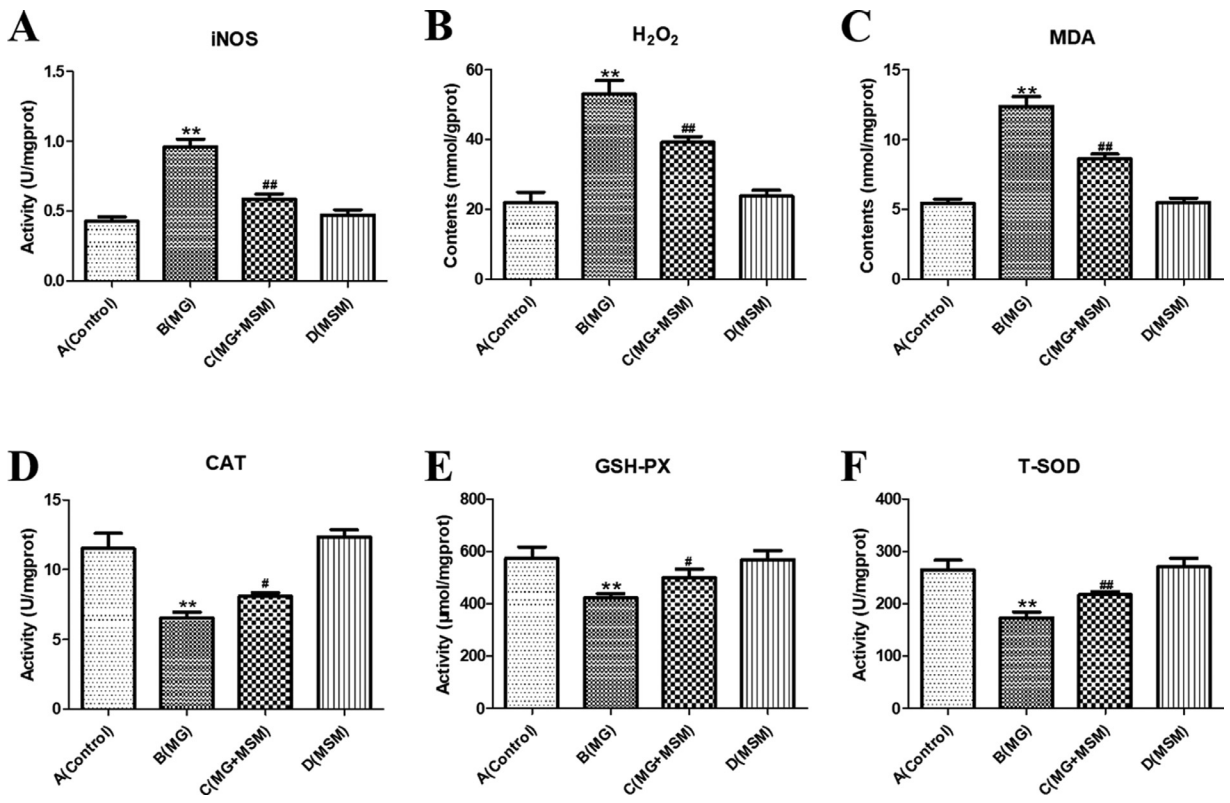

Figure 3. The effect of MSM was evaluated against MG infection on oxidative stress in chicken trachea. The parameters related to oxidative stress were (A) iNOS activity, (B) H_2O_2 contents, (C) MDA contents, (D) CAT activity and (E) GSH-PX activity, and (F) T-SOD activity and were measured on d 3 postinfection. All the bar graphs show mean results ± SD (n = 3). $0.01 < {}^{*}P < 0.05$ and ${}^{**}P < 0.01$ represent statistically significant difference compared to the Control group. $0.01 < {}^{\#}P < 0.05$ and ${}^{\#\#}P < 0.01$ represent statistically significant difference compared to the MG group.

Cells Viability Changes

CCK-8 assay was used to measure cells viability as shown in Figure 6. MG infection reduced the cells viability in a dose and time-dependent manner. Compared with the Control group, at 4, 6, 8, and 10 h, when the dose was greater than 400 MOI, and at 8 and 10 h, when the dose was greater than 200 MOI, notable cell death was observed (Figure 6A). Therefore, MG 400 MOI for 6 h was used as the best infection dose for the follow-up experiments. Within the range of 0 to 200 mM MSM concentration, there was no notable difference between 6 h and 12 h ($P > 0.01$). But the higher the MSM concentration, the survival rate of HD11 cells more obviously decreased ($P < 0.01$) in a time-dependent manner (Figure 6B). These results revealed that MSM with a concentration of less than 400 mm for 12 h has no apparent toxicity effect on the cellular activity of HD11 cells. Therefore, MSM concentrations of 200 mM for 12 h after infection were chosen for subsequent experiments.

MSM Alleviated MG Induced Ultrastructural Changes and Cell Death

It is worth to mention that nuclear membrane was disappeared, swollen or fractured mitochondria, and cellular damage by MG infection (Figure 7). However, MSM intervention effectively alleviated these abnormal ultrastructural pathologies under MG infection. As shown in Figure 8, green fluorescence intensity represented the amounts of live cells and red fluorescence intensity represented the amounts of dead cells. The AO/EB staining results signified that there were much more the amounts of live cells than dead cells in the Control group. Compared to the Control group, HD11 cells showed apparent death by MG infection, while MSM intervention obviously increased the amounts of live cells.

MSM Suppressed Proinflammatory Cytokines and NF-κB and ERK/JNK-MAPK Signaling Pathway in MG Induced HD11 Cells

MG infection caused an obvious increase in proinflammatory cytokines including IL-1β, TNF-α, IL-6, and IL-8 by ELISA. MSM intervention largely lessened IL-1β, TNF-α, and IL-6 activities under MG infection (Figure 9).

Proinflammatory cytokines and NF-κB and ERK/JNK-MAPK signaling pathway related protein expression were tested at mRNA and protein level. MG infection caused conspicuous increase in TNF-α, IL-1β, IL-6, and IL-8 mRNA expression compared to the Control group (Figure 10). Meanwhile, MSM caused reduction in the mRNA expression of cytokines compared to the MG infection group. Subsequently, the protein

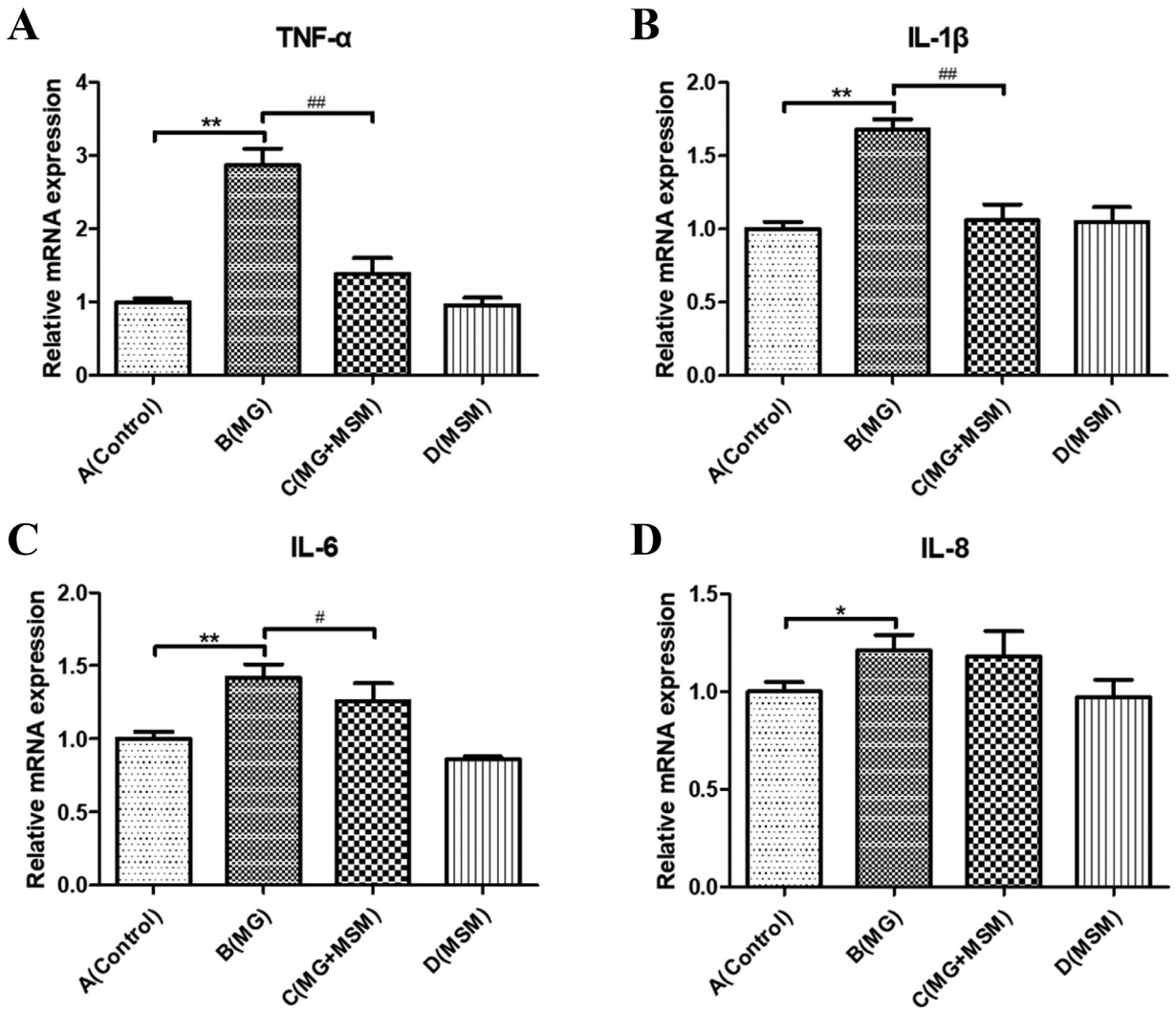

Figure 4. Effect of MSM and MG infection on inflammation-related genes mRNA measured in chicken trachea. A−D shows the mRNA expression of proinflammation-related genes including TNF-α, IL-1β, IL-6, and IL-8.

expressions of NF-κB and ERK/JNK-MAPK pathway-related genes were determined in HD11 cells (Figure 11). MG-infection apparently increased the protein expressions of TLR4, p-IκBα/IκBα, p-p65/p65, IL-1β, TNF-α, p-JNK/JNK, and p-ERK/ERK. Meanwhile, MSM abated increase the amount of these protein expressions by MG infection. A notable difference has been noted in the MSM treatment group compared to the MG infection group.

DISCUSSION

It is worthy to mention that MG infection caused chronic respiratory disease, and the main infection site of MG is the respiratory tract (Xiao et al., 2016). Studies demonstrated that MG infection caused severe inflammatory responses and oxidative stress lesion, and gives rise to considerable economic damage in the chicken industry (Ishfaq et al., 2019a). In the present study, MG induced abnormal morphological alterations in the trachea. Ultrastructural analysis demonstrated cilia were ruptured and inverted in chicken trachea and cellular damage in HD11 cells. Besides, oxidative stress-related parameters including MDA and H_2O_2 content, GSH-PX, T-SOD, iNOS, and CAT activities were subsequently altered, which revealed the increase of oxidative stress in MG infected chickens to a great extent. In the present research, MG infection produced inflammatory and oxidative stress which is the very probable reason of abnormal pathology and structural deterioration in chickens and HD11 cells. These findings were in consistence with previous studies that MG induced damage in the trachea and HD11 cells and caused oxidative stress (Lam, 2002, 2003, 2004; Vitula et al., 2011). Besides, the level of trachea inflammatory cytokines substantially increased in the MG group. Based on above results, it has been demonstrated that MG induced inflammation-injury of the respiratory tract in chickens.

Some studies revealed that *Mycoplasma* infection induced oxidative stress because of increased ROS generation (Ji et al., 2019). These results were further confirmed by the present experiments. We demonstrated that MG infection induced severe oxidative stress of trachea. There were a lot of significantly differentially expressed genes on MG infection and many of these

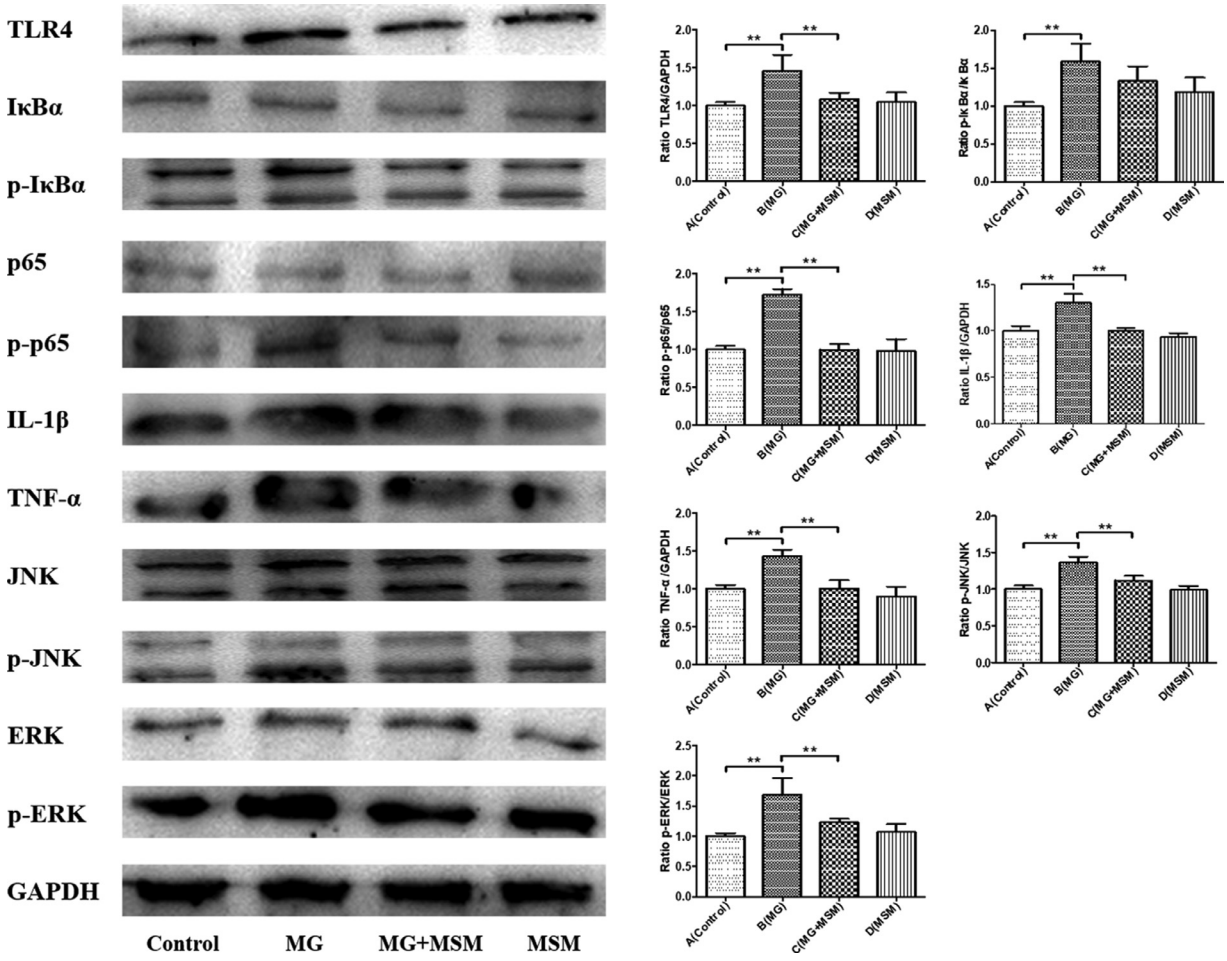

Figure 5. Effect of MSM and MG infection on NF-κB and ERK/JNK-MAPK signaling pathway correlative proteins expression measured on d 3 post-infection in the chicken trachea. Western blots revealed that the protein levels of TLR4, IκBα, p-IκBα, p65, p-p65, IL-1β, TNF-α, JNK, p-JNK, ERK, and p-ERK, GAPDH was used as internal control. The values with a star differ significantly ($0.01 < {}^{*}P < 0.05$; ${}^{**}P < 0.01$) between the one group and the other group.

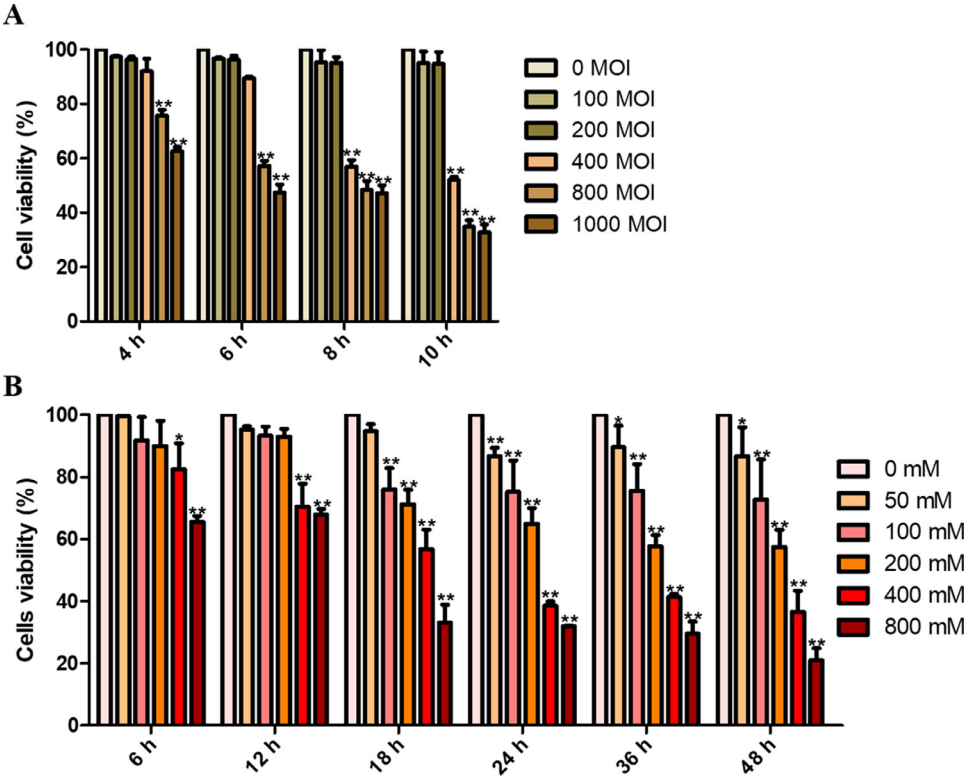

Figure 6. Cells viability determination. HD11 cells were incubated with different MOI MG (A) and treated with different concentration of MSM (B) for different time points.

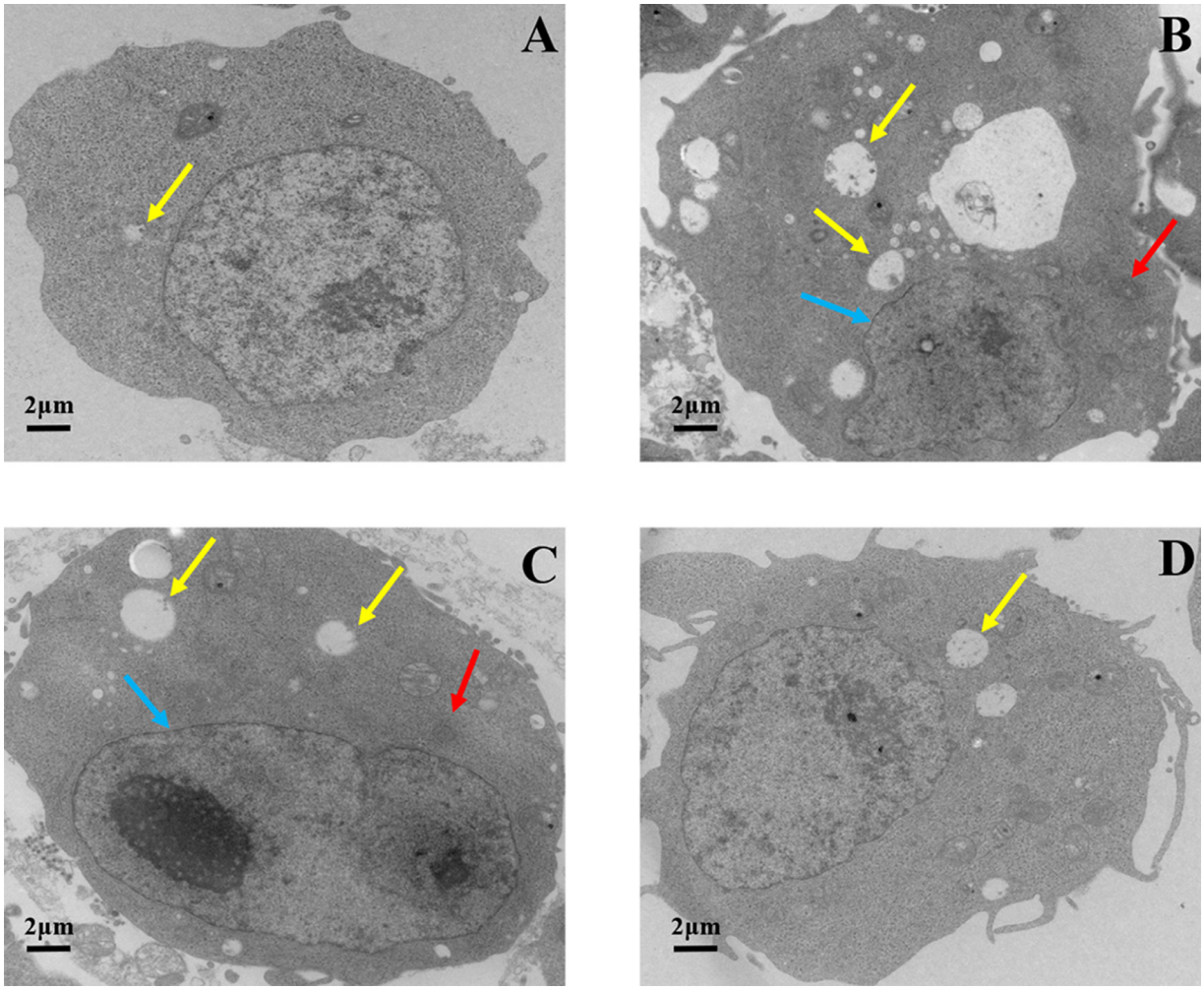

Figure 7. Ultrastructural examination of HD11 cells (A from Control group; B from MG group; C from MG+MSM group; D from MSM group). Red arrow means mitochondrial tumefaction, blue arrow signifies fragmented DNA and yellow arrow signifies vacuole.

target genes are bound up with immunological function involved in TLR and MAPK signaling pathway (Beaudet et al., 2017). Although MG does not match typical TLR4 ligand, MG can significantly increase the expression of TLR4. It is speculated that MG activates target genes of immune function and increases numbers of macrophage. (Smiley et al., 2001; Erridge, 2010; Ospelt and Gay, 2010; Kelsh and McKeown-Longo, 2013). It has been demonstrated that MG LAMPs mediated inflammatory reactions in poultry

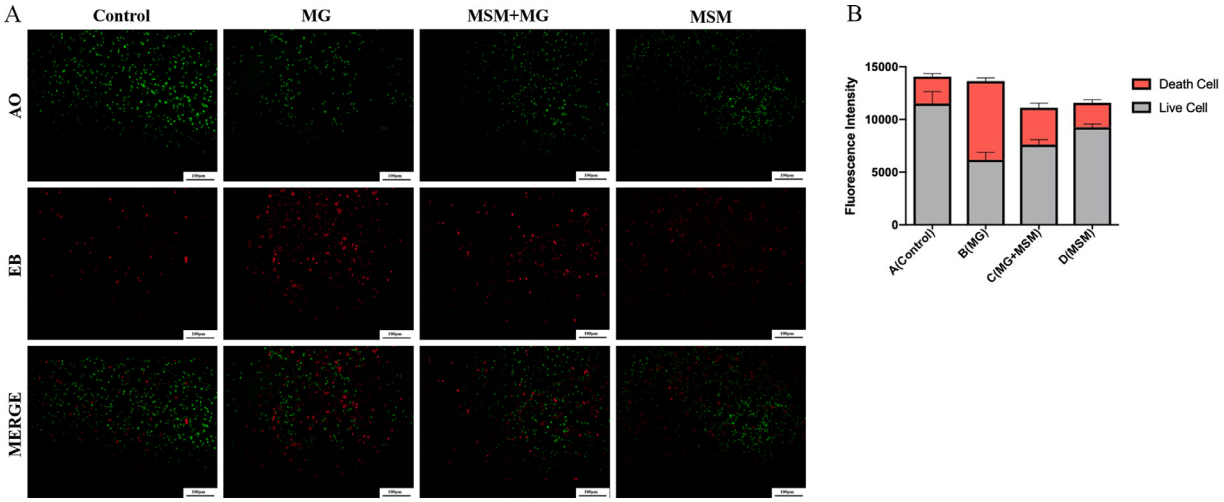

Figure 8. MG infections lead to cell death in HD11 cells. (A) AO (green) and EB (red) in HD11 cells under MG infection with or without MSM pretreatment through fluorescence microscope (100 μm). (B) Quantitative analysis for AO and EB fluorescence intensity of HD11 cells under MG infection with or without MSM pretreatment.

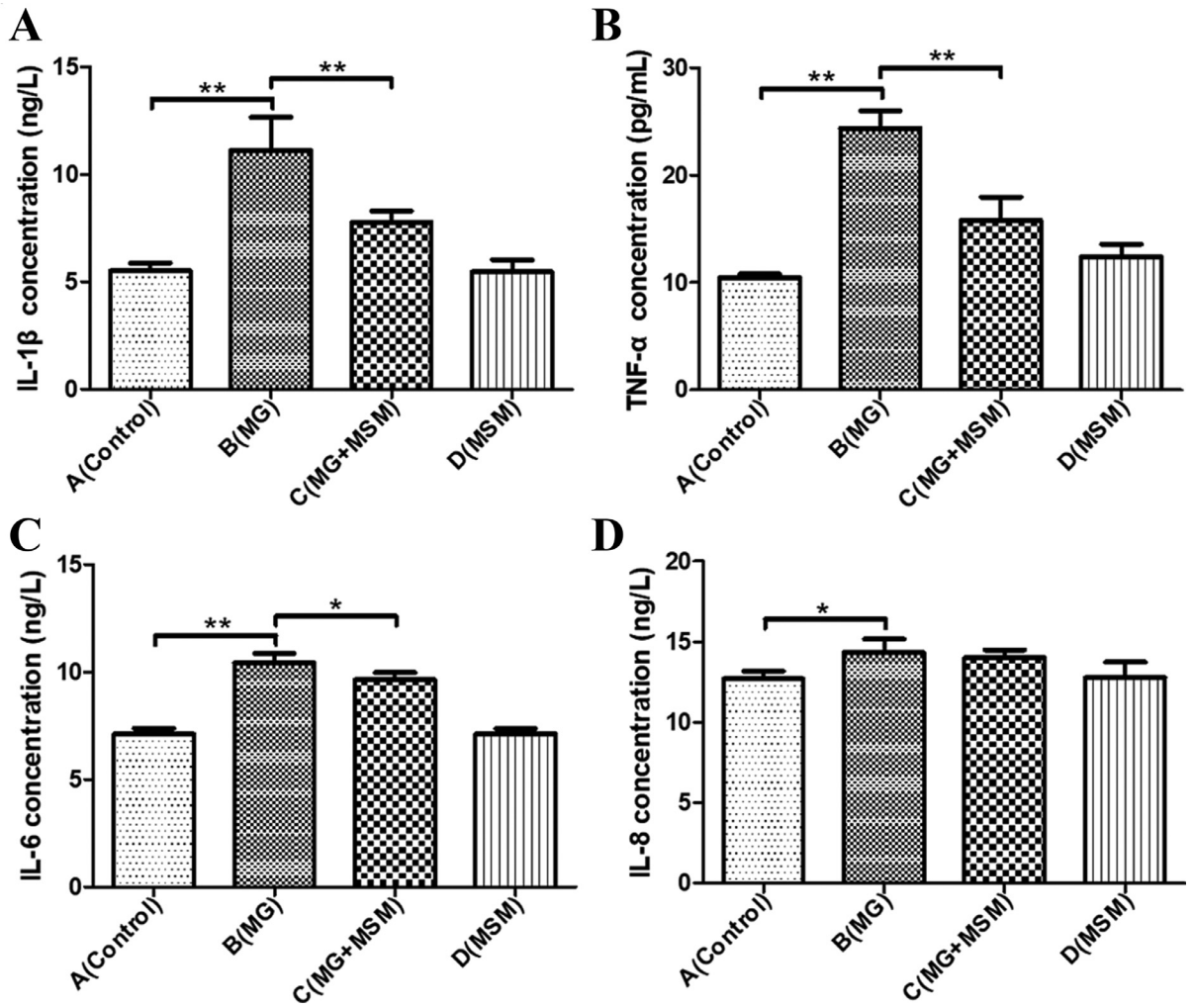

Figure 9. Effect of MSM and MG-infection on inflammatory mediators in HD11 cells. Inflammatory mediators including (A−D) IL-1β, TNF-α, IL-6 and IL-8 were detected by ELISA in HD11 cells.

respiratory epithelial cells via NF-κB signaling pathway, which activated extensive proinflammatory chemokines and cytokines (Majumder et al., 2014). Abundant studies made us think about whether the NF-κB and MAPK pathways play a momentous role in the in vivo and in vitro injury caused by MG infection. Therefore, we tested the expression level of NF-κB and ERK/JNK-MAPK pathway-related protein, and found that NF-κB and ERK/JNK-MAPK pathway was also involved in high expression at MG infection periods. The results suggested that TLR4 mediated the phosphorylation of IκBα, p65, JNK, and ERK led to the activation of IL-1β and TNF-α and activated the NF-κB and ERK/JNK-MAPK pathway of MG infection in vivo and vitro.

Abundant studies have shown that MSM has the potential protective effects on numerous diseases, including synovitis, osteoarthritis, stomach ulcer, allergic rhinitis, cancer, and pulmonary fibrosis (Kim et al., 2009). More importantly, oral MSM at either acute or subchronic concentrations did not cause any adverse effects on the growth or clinical outcomes in broilers and appeared to be absorbed and distributed throughout the body (Abdul Rasheed et al., 2019). Therefore, MSM could be a potential drug for the cure of MG-induced inflammatory injury. Chickens showed decreased MG colonization in trachea after MSM treatment. We demonstrated that MSM could inhibit MG directly and the minimum inhibitory concentration (**MIC**) of MSM against the MG strain was 221.61 mmol/L (Figure S3). Moreover, (Yan et al., 2020) reported the diet supplementation of MSM resulted in a positive effect on growth performance, immunity and antioxidant status in ducks. We speculated that MSM also could promote the chicken to reduce MG colonization indirectly by improving more efficient free radical−scavenging activity, increasing the levels of serum inflammatory cytokines and stimulating the proliferation of T lymphocytes. We also demonstrated that MSM significantly ameliorated MG-induced abnormal pathological damage and structural exacerbation in chickens and HD11 cells. In addition, we investigated the protective effects of MSM against MG-induced oxidative stress in the chicken trachea. MSM treatment significantly alleviated oxidative stress compared to the MG infection group. These findings were in consistence with previous studies that MSM prevented from oxidative stress (Rasheed et al., 2020a, 2020b). Moreover, MSM played an antioxidant role by providing a source of sulfur for

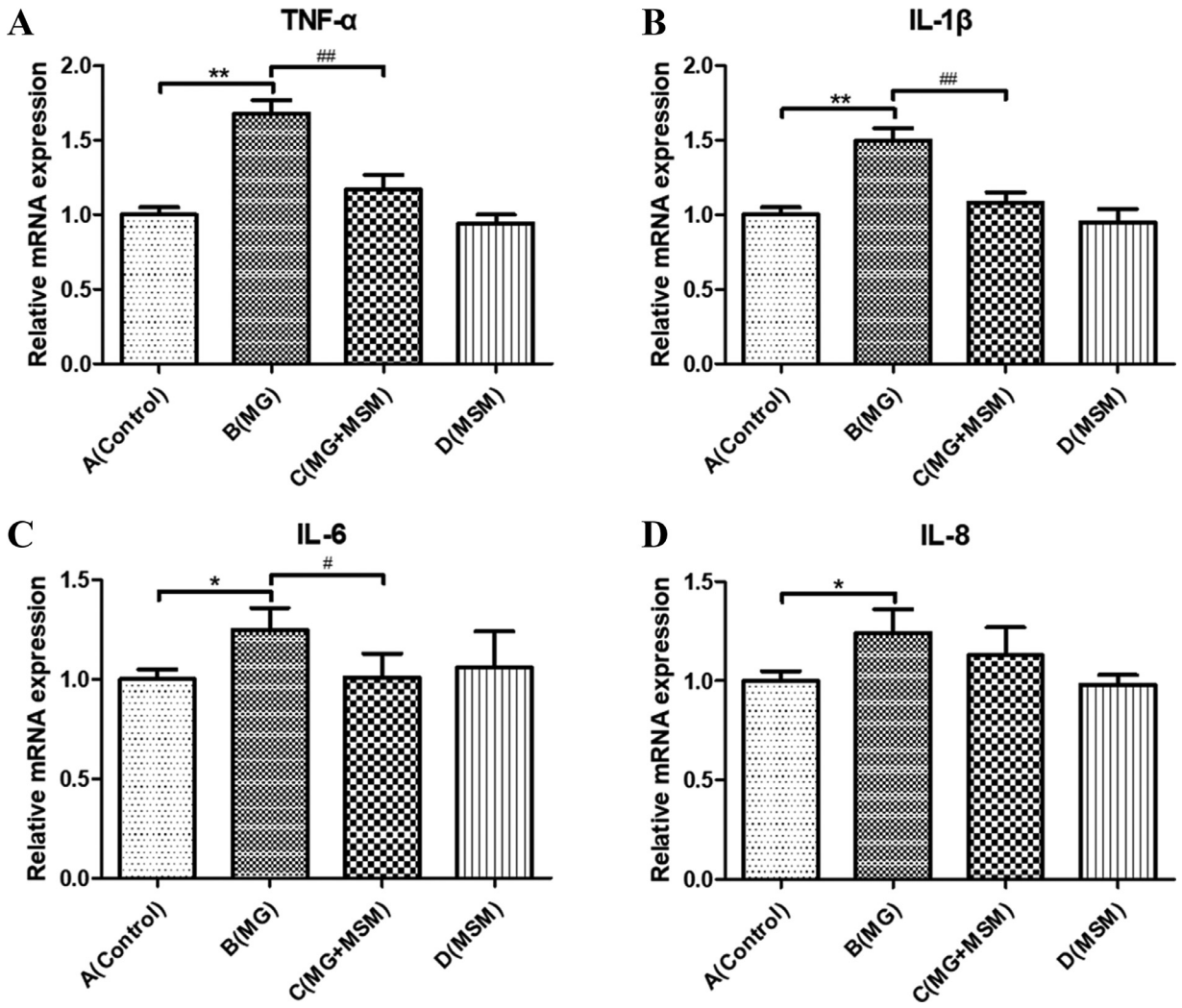

Figure 10. The effect of MSM and MG infection on inflammation-related genes mRNA measured in HD11 cells. Proinflammation-related genes mRNA expression levels including (A−D) TNF-α, IL-1β, IL-6, and IL-8.

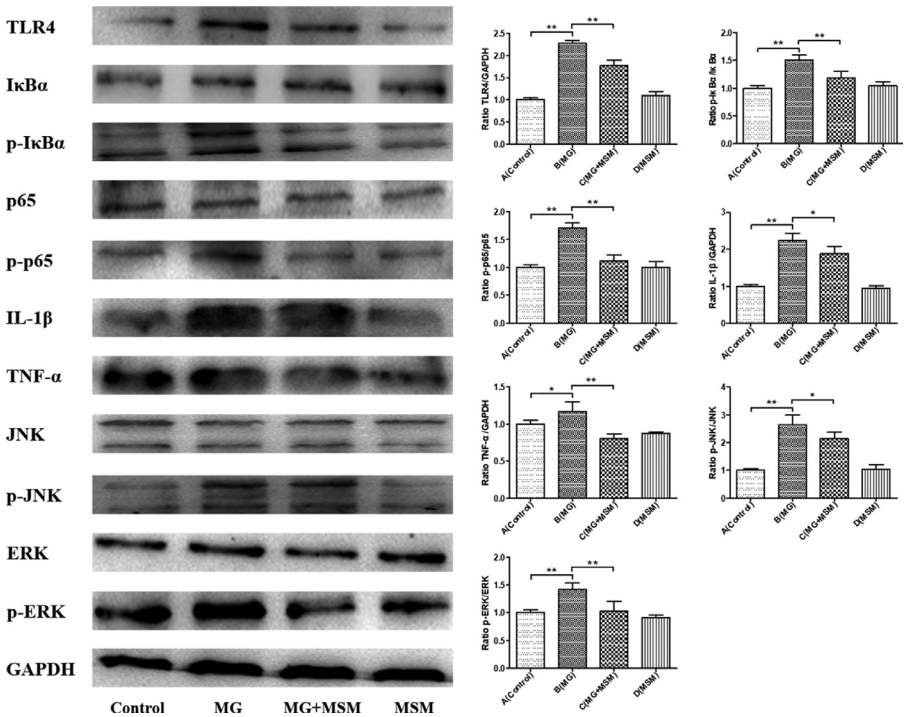

Figure 11. Effect of MSM and MG infection on NF-κB and ERK/JNK-MAPK signaling pathway correlative proteins expression measured in HD11 cells. Western blots demonstrated the protein levels of TLR4, IκBα, p-IκBα, p65, p-p65, IL-1β, TNF-α, JNK, p-JNK, ERK, and p-ERK. While GAPDH was used as an internal control in the experiments.

cysteine, taurine, and the strong antioxidant glutathione (Amirshahrokhi and Khalili, 2017). The previous study showed that MSM drastically downregulated these signaling molecules (LDH, iNOS, and PGE2) by reducing ROS production under inflammation, demonstrating their anti-inflammatory ability (Oliva et al., 2020). On the other hand, we also examined the protective effects of MSM against MG infection-mediated inflammation in the chicken trachea and HD11 cells. The results showed that MSM reduced the production of proinflammatory cytokines. More importantly, MSM strongly inhibited IL-6, IL-1β, and TNF-α mRNA expression and blocked the degradation of IκBα and nuclear translocation of p50/p65 in MG stimulated chicken and HD11 cells. These results suggested that MSM played a significant anti-inflammatory role by inhibiting NF-κB pathway. MSM also attenuated MG induced inflammation by decreasing the phosphorylation of ERK/JNK-MAPK pathway.

In summary, the research demonstrated that MG infection could induce severe inflammatory injury and oxidative stress in the trachea of chickens and HD11 cells. It is noted that MSM ameliorates inflammatory injury through the repression of NF-κB and ERK/JNK-MAPK signaling pathway, and suppressed proinflammatory cytokines in the trachea and HD11 cells. We also confirmed that MSM plays a significant role in the inflammatory response of MG infection both in vivo and vitro. The anti-inflammatory effect of MSM was a crucial factor, which may provide a solid theoretical foundation for the development of more potential drugs against MG infection. Nevertheless, further studies are required to investigate the specific molecular mechanism of MSM against MG infection.

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DISCLOSURES

The authors declared no potential conflicts of interests.

SUPPLEMENTARY MATERIALS

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