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Effects of Methylsulfonylmethane and Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor on Cell Proliferation, Cell Cycle and Anti-Inflammatory Effect in Chicken Intestinal Epithelial Cells with Lipopolysaccharide Challenge

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ABSTRACT

Background: Intestinal epithelial injury stress is one of the most common stresses in the contemporary poultry breeding. This study aims to evaluate the protective effects of Methylsulfonylmethane (MSM) and epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI, erlotinib) on injury stress induced by lipopolysaccharides (LPS) in chicken intestinal epithelial cells (IECs).

Methods: Cell Counting Kit-8 (CCK-8) and EdU assays were used to evaluate cell viability and proliferation. The cell cycle was analyzed by flow cytometry method. The mRNA transcript and protein expression levels of EGFR were detected by RT-qPCR and Western blotting (WB). The levels of pro-inflammatory cytokines were determined by using ELISA kits.

Result: The results have shown that MSM could promote the cell proliferation and regulate the cell cycle arrest when EGFR inhibited ($P<0.05$). In addition, both MSM and EGFR-TKI could decrease the expressions of IL-1β, IL-6 and TNFα ($P<0.05$). These results showed that the beneficial roles of MSM in chicken IECs induced by LPS may be related to promoting cell proliferation and inhibiting inflammation; meanwhile, our experimental results may provide valuable information for further study of the possible mechanism of MSM to improve the chicken intestinal epithelial function by regulating EGFR signaling pathway.

Key words: Anti-inflammatory, Cell proliferation and cycle, Chicken intestinal epithelial injury stress, Epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), Methylsulfonylmethane (MSM).

INTRODUCTION

Intestinal epithelial injury stress is one of the most common stresses in poultry feeding. Intestinal epithelial injury generally leads to the apoptosis of epithelium cell, the destruction of barrier function *etc*., which resulting in a series of reactions such as swelling, bleeding and necrosis of intestinal epithelial cells, causing the bird unable to digest the feed, resulting in significant economic losses to the breeding industry (Wu *et al*., 2019; Yang *et al*., 2021). The reasons of intestinal epithelial injury are often complex and diverse and the repair mechanism of intestinal epithelial injury has always been the focus and hotspot of research.

Methylsulfonylmethane (MSM), also known as dimethyl sulfone (DMOS), a sulfur-containing natural organic compound, which exists in food, animals and human. Previous studies focused that MSM had positive effects on daily gain, feed efficiency, average daily feed intake, nutrient digestibility in pigs, chickens, ducks and mice (Butawan *et al*., 2017). Our previous study also found that dietary supplementation of 0.20% MSM had positive effects on growth performance, meat quality, excreta microbiota and blood profiles in broilers ($P<0.05$) (Jiao *et al*., 2017). In recent years, studies have shown that MSM has significant anti-inflammatory and antioxidant effects on poultry (Rasheed Abdul *et al*., 2020; Yan *et al*., 2020; Miao *et al*., 2022). MSM has been proved to have the ability to inhibit the production of inflammatory response through nuclear factor kappa-B (NF-κB) signaling pathway; it plays an anticancer role in gastrointestinal cancer cells and colon cancer cells by inducing pathways related to cell cycle arrest and apoptosis (Butawan *et al*., 2017; Kim *et al*., 2020a; Miao *et al*., 2022). Previous studies have also shown that MSM can be used in combination with other drugs to inhibit tumor growth and regulate the expression of key genes (Sarkhani *et al*., 2017). However, there are few reports on the repair of intestinal epithelial injury in chicken by MSM.

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Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein belonging to erbB receptor tyrosine kinase family (TKs), which are related to cell growth, proliferation, differentiation, survival, adhesion, invasion and angiogenesis (Herbst, 2004; Upadhyay *et al*., 2022). Studies have shown that abnormal expression of EGFR is strongly associated with tumor cell proliferation, invasion, metastasis and apoptosis inhibition (Kersting *et al*., 2018; Wee and Wang, 2017). At the same time, EGFR and its downstream signaling pathways are involved in the process of intestinal epithelial injury and improve gut health by regulating intestinal epithelial function (Ayati *et al*., 2020; González-Mariscal *et al*., 2008). First, EGFR signaling pathway can protect intestinal epithelial junctions by inhibiting epithelial cell apoptosis. Studies have shown that EGFR can significantly inhibit cell apoptosis and promote cell survival. Secondly, increasing evidence actually suggests that the EGFR pathway has a major impact on the inflammatory/ immune reactions of the epithelial cells. Meanwhile, the latest researches reported that MSM has been proved to be involved in more and more signal pathways, including EGFR downstream signaling pathways MAPK and Akt (Miao *et al*., 2022; Butawan *et al*., 2017). However, in the intestinal epithelial injury of chicken, it is unclear whether MSM regulates the EGFR signaling pathway and what is the specific regulatory mechanism during intestinal epithelial repair. Therefore, in this study we decided to evaluate the combined effect of EGFR inhibition (EGFR-TKI) and MSM regulate chicken small intestinal epithelial cells.

Considering the characteristics of MSM and the beneficial effects of combination therapy in most clinical and animal studies, the purpose of this study is to explore whether MSM has a certain damage repair function on chicken intestinal epithelial cells and we hypothesize that the repair process may be related to the EGFR signaling pathway. The current study might provide a new idea for the treatment of intestinal epithelial injury stress in livestock and poultry.

MATERIALS AND METHODS

The experiment was conducted from September 2021 to February 2022 at Wuchang Shouyi University, Wuhan, China. And this study was approved by the Ethics Committee of Wuchang Shouyi University. All the experiments on animals used in the study were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Chemicals and reagents

The following chemicals were used in the experiments: LPS (Lipopolysaccharides) from *Escherichia coli* O55:B5, Methylsulfonylmethane (purity≥99.0%), erlotinib (EGFR-TKI), advanced dulbecco's modified eagle medium (DMEM) cell culture medium, fetal bovine serum (FBS), phosphate buffer (PBS), Hank's Balanced Salt Solution (HBSS) and horseradish peroxidase were bought from Wuhan Biofavor Biotech Services Co., Ltd. (Wuhan, China).

Isolation and culture of chicken small intestinal epithelial cells (IECs)

Take 3-5 white feather chicken embryos without specific pathogen (18-19 days old), take their small intestinal tissue blocks, remove adipose tissue and mesentery and wash them repeatedly with PBS to make the supernatant clear. Collagenase I was used, digested in 37°C water bath for 50 min, then the small intestinal tissue block was transferred to a 50 mL small beaker, cut it up with scissors, let it stand for 5 min, precipitate by itself and discard the supernatant. Continue to shear, add serum-free medium to suspend the tissue block, transfer it to a 15 mL centrifuge tube for cleaning, centrifuge at 1000 R/min for 4 min, repeat twice and discard the supernatant. Add DMEM/F12 complete medium containing 10% serum to suspend tissue blocks, collect crypt cell blocks through 100 mesh screen, purify and obtain small intestinal epithelial cells and culture them in 5% CO_2 incubator. After incubation for 24-48 hours, the solution was changed every two days. After stable passage, the cells were grouped for subsequent experiments.

Cell treatment

The stable cells were seeded in the 6-well dishes and cultivated at 37°C, 5% CO_2 atmosphere. Morphological features of the primary cultures were inspected daily using an inverted phase-contrast microscope. Later, cells were washed twice with PBS and then exposed to fresh media containing 0 or 10 Mmol/L LPS for an additional 24 h. IEC without any treatment were used as controls and IEC only treated with 10 µg/mL LPS were used as positive controls. After treatment, the cells were collected for further analyses. All experimental groups including control group (CON), 10 µmol/L LPS group (CON+LPS), LPS group treated with EGFR-TKI (20 umol/L erlotinib, 24 h) group (LPS+TKI), LPS group treated with 100 mM MSM group (LPS+MSM) and LPS group treated with EGFR-TKI and MSM combination (20 umol/L erlotinib+100 mM MSM) group (LPS+TKI+MSM).

Cell proliferation viability tested by CCK-8 assay

Cell proliferation viability was determined using a CCK-8 cell viability assay kit. All cells were pretreated with cultured methods as indicated for 24 h in a 96-well plate at a density of 2×10^5 cells/well. 10 uL of cell viability assay kit solution was added to each well of the plate. After incubation for 1 h at 37°C in the dark, absorbances were measured at 450 nm using a multi-well plate reader.

EdU assay

The cells were inoculated in 96-well plate with 2×10^5 cells/ well density and cultured for 24 h, 20 mM EdU solution was added and co-incubated for 2 h. Cells were collected into a suspension and fixed with 1 mL of 4% paraformaldehyde at room temperature for 15 min and then washed the cells 3 times per well. Incubated with 1 mL PBS containing 0.3% TritonX-100 for 15 min at room temperature and washed the cells 2 times per well. Prepare click reaction solution

(10 mL), add 0.5 mL click reaction solution to each well, gently shake the culture plate to ensure that the reaction mixture can cover the sample evenly and incubate in the dark for 30 min and then wash 3 times. Dilute Hoechst 33342 (1000×) with PBS in the ratio of 1:1000. After absorbing the washing solution, add 1 mL of 1× Hoechst 33342 solution to each well, incubate in the dark for 10 minutes, absorb 1× Hoechst 33342 solution, dry the liquid on the climbing sheet with absorbent paper, seal the sheet with sealing solution containing anti fluorescence quenching agent and then observe the collected image under fluorescence microscope.

Cell cycle analysis

Cells at logarithmic growth stage were selected and the cell density was adjusted to 2.5×10^5 cells/mL. The cells were connected to a six-well plate with 2mL cell suspension per well and cultured in an incubator at 37°C and 5% CO_2 for 24 h. After 24 h of incubation, cells were collected and harvested after trypsinization, washed with PBS, fixed with cold 70% ethanol and conserved at -20°C overnight and the cell nuclei were stained with cold propidium iodide (PI) solution (50 µg/mL) containing RNase A (50 µg/mL) in PBS (pH 7.4) for 30 min in the dark following the manufacturer's protocol. The stained cells were analyzed with a flow cytometer (Beckman coulter, cytoFLEX, USA). A 488 nm laser line was used to excite the dye and the 595 to 660 nm detector to capture the PI signal. The results are presented as the percentage of cells from a given population at certain stages of the cell cycle. Data are presented as mean±SD of 3 independent experiments.

ELISA assay

The levels of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in the cells tissue were determined by using chicken ELISA kits (Beijing Solarbio Science and Technology Co., Ltd., China) according to the manufacturer's instructions. The results were expressed as pg/mg protein. Data are presented as mean±SD of 3 independent experiments.

RT-qPCR

Total RNA in cells was extracted by Trizol method and reverse transcribed into cDNA as a template. Prepare 20 µL reaction system according to the instructions of PCR kit. The reaction procedure is: pre denaturation at 95°C for 2 min; denaturation at 95°C for 15 s, annealing at 60°C for 1 min, 40 cycles in total. After the reaction, calculate the CT value of each reaction, take GAPDH as the reference and use $2^{-\Delta\Delta Ct}$ method calculation results for comparison and analysis between groups. The forward and reverse primer of EGFR are shown as follows: EGFR (F) 5′-GCGCTACCTTGTCATTCAGG-3′, (R) 5′-TATCAATGCAAGCCACGGTG-3′.

Western blotting

Take logarithmic growth cells for experiment and 200 µL cell lysate containing protease inhibitor was added into the culture well. After cell centrifugation, BCA protein quantitative kit was used to determine the protein concentration, 50 µg/well was subjected to SDS-PAGE gel electrophoresis. Transfer membrane, after blocking with 5% nonfat milk for 1.5 h, the membrane was incubated with solutions of the appropriate primary antibodies overnight at 4°C. Then, incubation with the respective horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h. Add ECL reaction solution for 1 min and observe the results. The imaging system collects images and ImageJ software analyzes the gray value.

Statistical analysis

Data were expressed as mean±SD. Statistical differences among groups were tested by one-way analysis of variance (ANOVA). Post hoc comparisons were made by Tukey's test. GraphPad Prism 9.0 was used for statistical analyses. Values were considered significantly different when $P<0.05$.

RESULTS AND DISCUSSION

As shown in Fig 1A, CCK-8 assay was employed to investigate the effect of MSM and EGFR-TKI on the viability of chicken IECs. According to the results of the CCK-8 assay, it was shown that at 24 h after transfection, no significant difference was found in cell viability among the LPS+MSM, LPS+TKI and LPS+TKI+MSM groups ($P>0.05$). Similar study has found that in fetal horse liver cells, MSM reduced ROS generation without affecting cell viability (Kim *et al*., 2020b). The latest research showed that within the range of 0-200 mM MSM concentration, there was no notable difference between 6 and 12 h ($P>0.01$). But the higher the MSM concentration, the survival rate of chicken macrophages (HD11 cells) more obviously decreased ($P<0.01$) in a time-dependent manner (Miao *et al*., 2022). According to the results (Fig 1B), the EdU incorporation assay revealed that LPS+MSM group (38.81%) could markedly promote cell proliferation in chicken IECs ($P<0.05$) compared with the LPS+TKI group. Shah *et al*. (2019) modelled acquired resistance in EGFR-mutant lung adenocarcinoma cells and found that AKIs synergistically inhibited cancer cell growth in combination with EGFR inhibitors in mouse and patient-derived xenograft models. Meanwhile, Gong *et al*. (2020) found that a combination of erlotinib plus anifrolumab resulted in significant ($P<0.05$) suppression of tumor growth. These results indicated that MSM could promote the proliferation of chicken IECs at an appropriate concentration, while inhibition of EGFR affects the recovery of proliferation in chicken IECs induced by LPS.

We next investigated the cell cycle in chicken IECs by using a flow cytometry analysis. The current results showed that in the LPS+TKI group the percentage of chicken IECs in G0/G1 phase increased significantly ($P<0.01$), while S phase decreased significantly ($P<0.01$), indicating that EGFR-TKI can block the synthesis of essential proteins in cell cycle from G1 to S phase, leading to cell cycle arrest and apoptosis (Fig 2). Meanwhile, the percentage of chicken IECs in G2/M phase decreased significantly ($P<0.05$) in LPS+TKI+MSM group compared with the LPS+TKI and LPS+MSM groups, indicating that the combination of MSM

and EGFR-TKI can block the cell cycle arrest from S phase to G2/M phase (Fig 2). These results were similar to most experimental results, research on EGFR-TKI showed that the anti-EGFR monoclonal antibody C225 had effects on cell proliferation, cell cycle phase distribution and apoptosis in squamous cell carcinoma (SCC) cell lines. C225 could have inhibited SCC proliferation and induced accumulation of cells in G1, which is accompanied by a 2-3-fold decrease

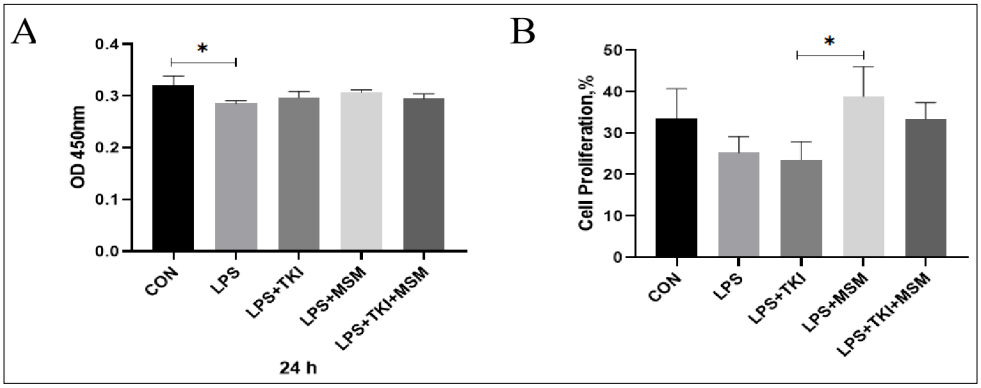

Fig 1: Effects of MSM and EGFR-TKI on cell viability and proliferation in LPS-treated chicken IECs. CCK-8 (A) and EdU assays (B) were used to evaluate chicken IECs' viability and proliferation. Data are expressed as the mean±SD (n=3). **P<0.01, *P<0.05, one-way ANOVA followed by Tukey post hoc tests.

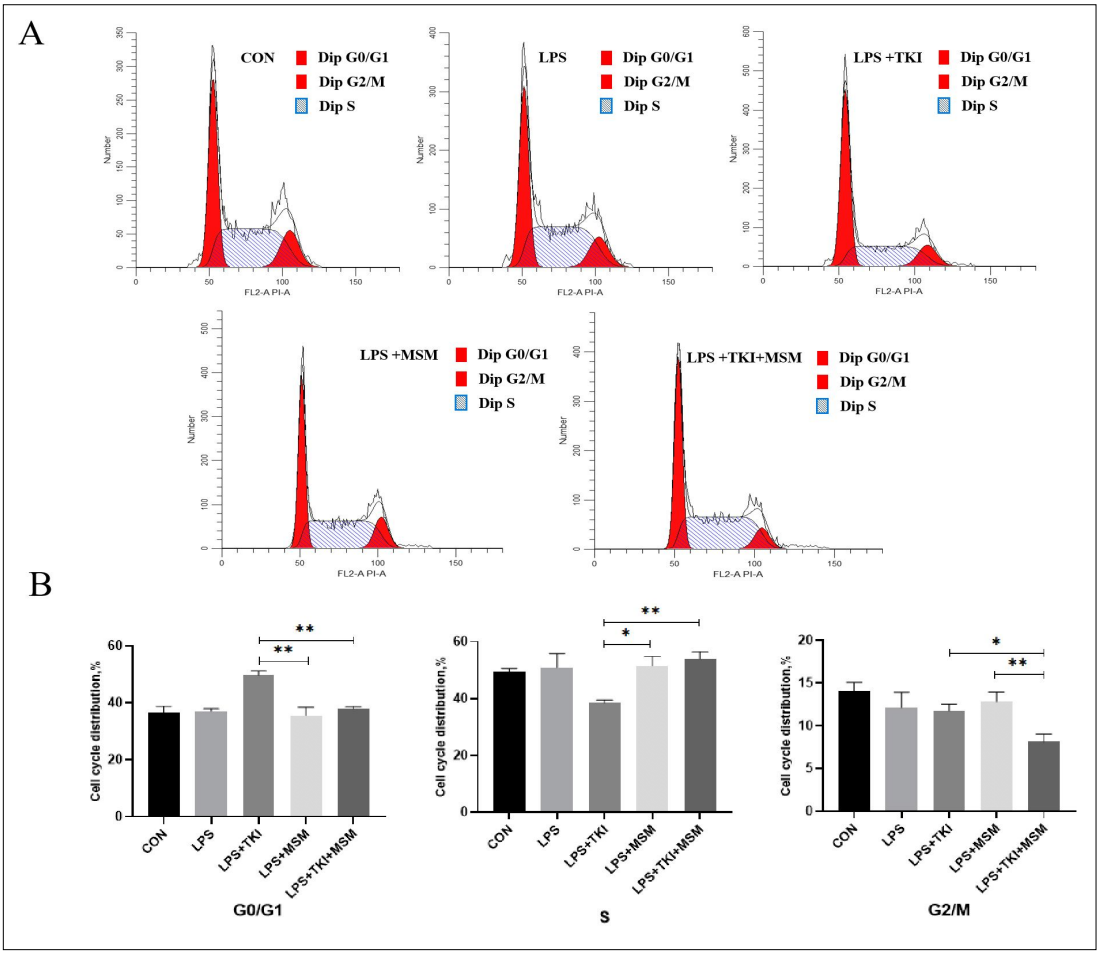

Fig 2: Effects of MSM and EGFR-TKI on cell cycle in LPS-treated chicken IECs. The percentage (%) of G0/G1, S and G2M phase of IECs affected by MSM and EGFR-TKI. Data are expressed as the mean±SD (n=3). **P<0.01, *P<0.05, one-way ANOVA followed by Tukey post hoc tests.

in the percentage of cells in S phase (Huang *et al*., 1999). Kersting *et al*. (2018) also found that the inhibition of EGFR causes cell cycle arrest in G1 prior to DNA synthesis. Ling *et al*. (2018) demonstrated that erlotinib-induced cell growth inhibition in EGFR high-expressing human H322 NSCLC cells was accompanied by G1/S phase arrest, which was largely caused by a decrease in expression of G1/S-related cyclins, suppression of activities of cyclin-dependent kinase (CDK) 2 and CDK4. Meanwhile, previous study also indicates that MSM can induces cell cycle, Sarkhani *et al*. (2017) found that the combination treatment (MSM+allicin) inhibited $CD4^-$ and $CD44^+$ cells in the G2/M and S phases of the cell cycle, respectively. In addition, MSM supplementation to human gastric, hepatocellular and esophageal carcinoma cell cultures did elicit cytotoxic effects by arresting the G2/M phase of the cell (Jafari *et al*., 2012). Therefore, according to the results of the current study, we found that both EGFR-TKI and MSM have effect on the proliferation and cell cycle of chicken IECs and the addition of MSM can promote the recovery of cell proliferation and cell cycle of S phase when EGFR inhibited. However, at present, the research on EGFR is mostly focused on human cancer and tumor cells, the relationship between EGFR and other signal pathways, the mechanism of interaction regulation, *etc*. are not yet totally clear. At the same time, there are few reports on the mechanism of EGFR in the regulation of anti-inflammatory drugs and MSM, as an anti-inflammatory drug, has a complex mechanism in regulating cell proliferation and apoptosis of various tumors and cancer cells. Furthermore, different cell types and different concentrations of MSM had different effects on cell proliferation and cell cycle arrest, more studies are needed to further explore.

As shown in Fig 3, ELISA assay was used to measure the concentrations of TNF-α, IL-1β and IL-6 in the chicken IECs. The levels of TNF-α, IL-1β and IL-6 in the LPS group were significantly higher than those in the other groups ($P<0.01$); and the levels of IL-1β and IL-6 in the LPS+TKI group were significantly lower than those in the LPS+TKI+MSM groups ($P<0.01$), these results indicating that both MSM and EGFR-TKI had an anti-inflammatory effect on chicken IECs induced by LPS. EGFR inhibitors are well-known as anticancer agents, recent studies tried to develop EGFR inhibitors as anti-inflammatory agents. Elkamhawy *et al*. (2019) proved that compound 4d (EGFR inhibitor) significantly and dose-dependently inhibits LPS-induced iNOS expression and IL-1β, IL-6 and TNF-β production *via* NF-κB inactivation in peritoneal macrophages. TNF-β, IL-1β and IL-6 are important inflammatory mediators, a number of *in vitro* studies suggest that MSM exerts an anti-inflammatory effect through the reduction in cytokine expression (Butawan *et al*., 2017). The main finding of previous studies was that the inhibitory effect of NF-κB results in the down regulation of mRNA for IL-1β, IL-6 and TNF-α *in vitro*. The latest study have shown that 500 mM MSM has a mitigating effect on mycoplasma (MG)-induced inflammatory damage in chicken and chicken HD11 cells and MSM can strongly inhibit IL-6, IL-1β and TNF-α mRNA expression by reducing phosphorylation of the ERK/JNK-MAPK pathway (Miao *et al*., 2022). ERK/JNK-MAPK is a classical downstream signal pathway of EGFR. Meanwhile, in the current study, we noticed that both EGFR-TKI and MSM had anti-inflammatory effect, the concentrations of TNF-α, IL-1β and IL-6 in LPS+TKI+MSM group had no significant difference compared with LPS +MSM group, indicating that when the expression of EGFR is inhibited, the anti-inflammatory effect of MSM is not weakened, which may be due to a variety of reasons, such as the dose of MSM added, the anti-inflammatory effect of EGFR-TKI mentioned earlier and the extremely complex regulation mechanism of EGFR signaling pathway. As shown in Fig 4, we preliminarily explored the regulation of MSM on the dynamic expression of EGFR. The results have shown that MSM could significantly up-regulate the mRNA expression of EGFR and the p-EGFR protein expression in LPS-induced chicken IECs ($P<0.01$), this also might be the reason that when the expression of EGFR is inhibited, the anti-inflammatory effect of MSM is not weakened. These results concluded that EGFR-TKI, MSM and EGFR-TKI plus MSM played a significant anti-inflammatory role in chicken IECs induced by LPS, this seems to provide new ideas for our

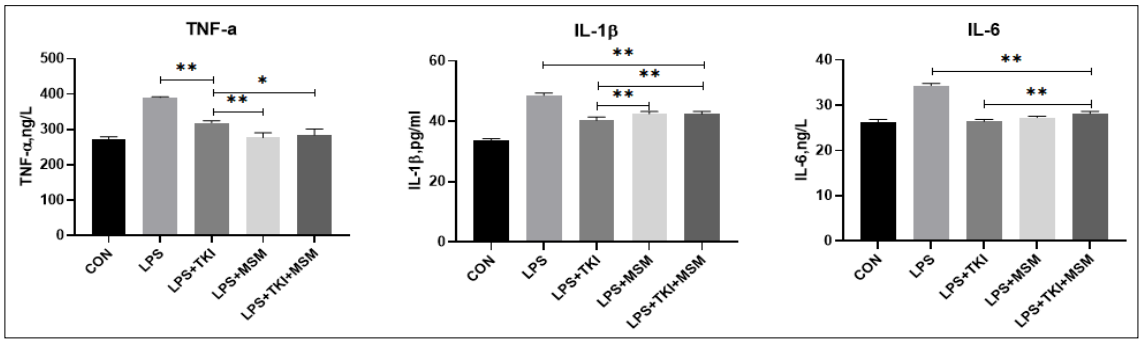

Fig 3: Effects of MSM and EGFR-TKI on inflammation reaction in LPS-treated chicken IECs. The levels of TNF-α, IL-1β and IL-6 were estimated in culture media by ELISA Kit. Data are expressed as the mean±SD (n=3). **P<0.01, *P<0.05, one-way ANOVA followed by Tukey post hoc tests.

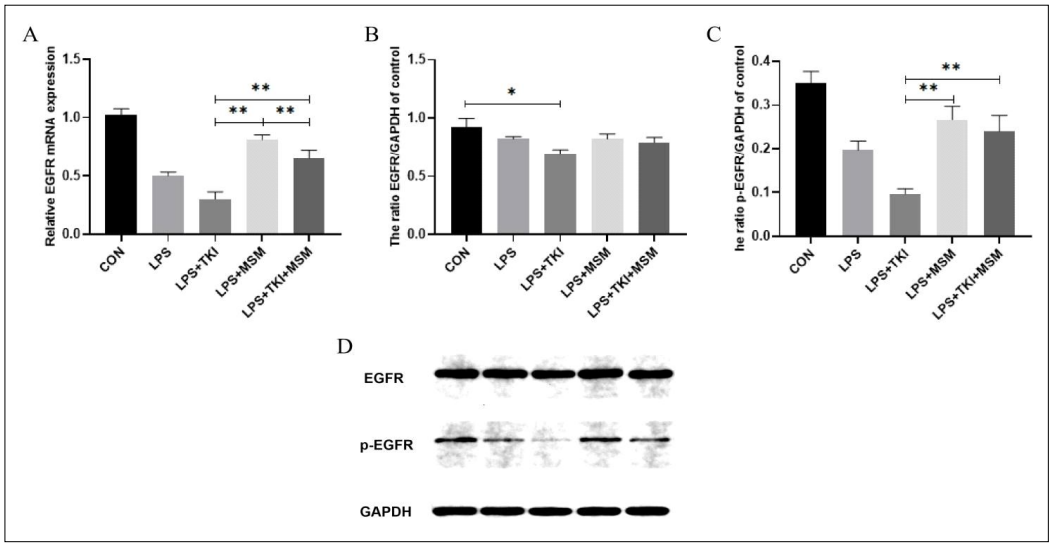

Fig 4: Effects of MSM and EGFR-TKI on the EGFR expression in LPS-treated chicken IECs. The mRNA expressions of EGFR was detected using real-time PCR (A). EGFR and p-EGFR protein expressions in IECs were determined via Western blotting (B-D). Data are expressed as the mean±SD (n=3). **P<0.01, *P<0.05, one-way ANOVA followed by Tukey post hoc tests.

follow-up research to explore a new ways of supplementation MSM in poultry.

CONCLUSION

In summary, the present study showed that MSM played beneficial roles in the improvement of chicken IECs injury induced by LPS. The protective effect of MSM may be related to regulating the expression of EGFR, inhibiting inflammation and regulating cell proliferation.

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Disclosures

The authors declare that there are no conflicts of interest.

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