

# Baicalin ameliorates oxidative stress and apoptosis by restoring mitochondrial dynamics in the spleen of chickens via the opposite modulation of NF- $\kappa$ B and Nrf2/HO-1 signaling pathway during *Mycoplasma gallisepticum* infection

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**ABSTRACT** *Mycoplasma gallisepticum* (MG) infection produces a profound inflammatory response in the respiratory tract and evade birds' immune recognition to establish a chronic infection. Previous reports documented that the flavonoid baicalin possess potent anti-inflammatory, and antioxidant activities. However, whether baicalin prevent immune dysfunction is largely unknown. In the present study, the preventive effects of baicalin were determined on oxidative stress generation and apoptosis in the spleen of chickens infected with MG. Histopathological examination showed abnormal morphological changes including cell hyperplasia, lymphocytes depletion, and the red and white pulp of spleen were not clearly visible in the model group. Oxidative stress-related parameters were significantly ( $P < 0.05$ ) increased in the model group. However, baicalin treatment significantly ( $P < 0.05$ ) ameliorated oxidative stress and partially alleviated

the abnormal morphological changes in the chicken spleen compared to model group. Terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling assay results, mRNA, and protein expression levels of mitochondrial apoptosis-related genes showed that baicalin significantly attenuated apoptosis. Moreover, baicalin restored the mRNA expression of mitochondrial dynamics-related genes and maintain the balance between mitochondrial inner and outer membranes. Intriguingly, the protective effects of baicalin were associated with the upregulation of nuclear factor erythroid 2-related factor 2 (Nrf2)/Heme oxygenase-1 (HO-1) pathway and suppression of nuclear factor-kappa B (NF- $\kappa$ B) pathway in the spleen of chicken. In summary, these findings indicated that baicalin promoted mitochondrial dynamics imbalance and effectively prevents oxidative stress and apoptosis in the splenocytes of chickens infected with MG.

**Key words:** *Mycoplasma gallisepticum*, spleen, baicalin, apoptosis, Nrf2/HO-1 pathway

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## INTRODUCTION

*Mycoplasma gallisepticum* (MG) causes severe inflammatory response in the birds respiratory tract results in sneezing, nasal discharge and coughing. The disease is known as chronic respiratory disease in chickens and infectious sinusitis in turkeys, which causes great economic losses in the poultry industry (Gaunson et al., 2000; Jacob et al., 2014; Roussan et al., 2015). Unlike other bacteria, MG establish a firm attachment to host

cells via cytoadhesion and colonizes, essential for its progression and development (Chen et al., 2001; Purswell et al., 2012). MG infects a variety of non-phagocytic cells such as fibroblasts, HeLa cells, and chicken red blood cells (RBCs). Additionally, this pathogen invade, multiply and survive in extra pulmonary tissues such as heart, blood, liver, brain, and spleen (Majumder et al., 2014). Immune system protects the body from invading pathogens and damages. Spleen is one of the immune organs which plays a key role in innate immune system (Li et al., 2017). Previous studies reported the effect of MG infection on lymphoid organs of broilers such as thymus, spleen, and bursa of fabricius, and explained that lymphocytes depletion has been noticed in these organs (Lockaby et al., 1998; Manafi et al., 2015). This means that MG infection modulated the immune response and development of immune organs.

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Accumulative evidences showed that excessive reactive oxygen species (**ROS**) generation and/or oxidative stress results in impaired immune responses in the host body and induced apoptosis in immune organs (Li et al., 2012; Gostner et al., 2015; Hu et al., 2018). However, the effect of MG infection-mediated oxidative stress and apoptosis in the spleen is still unknown.

Mitochondria are pivotal organelles for the cell survival by regulating apoptosis, providing energy and calcium buffering. Excessive generation of ROS, alteration in mitochondrial respiratory chain and imbalance in mitochondrial dynamic-related proteins cause mitochondrial dysfunction (Halliwell, 1992; Praharaaj et al., 2018), playing a critical role in the pathogenesis of different diseases. Studies demonstrated that increased expression of dynamin-related protein 1 (**Drp1**) caused mitochondrial fragmentation (Xu et al., 2013). Besides Drp1, mitochondrial fission factor (**Mff**) induced mitochondrial fission, and optic atrophy 1 (**Opa1**), mitofusin 1 (**Mfn1**) and mitofusin 2 (**Mfn2**) proteins are responsible for mitochondrial membrane fusion, which in turn, facilitated apoptosis (Alaimo et al., 2013). Cytochrome C is released upon mitochondrial damage followed by the activation of executioners of apoptosis such as caspase 9 and caspase 3 to initiate apoptosis (Caroppi et al., 2009). In addition, BCL2 Associated X (**Bax**) and B-Cell Lymphoma-2 (**Bcl2**) proteins are also involved in mitochondrial-dependent apoptosis. Bcl2 inhibits apoptosis by preventing the release of cytochrome C from mitochondria (Yip and Reed, 2008). It would be enthralling to investigate whether MG infection induce apoptosis and its underlying mechanism in the spleen of chickens that will help in exploring new drug targets for the prevention of MG infection.

Recently, plant-derived natural flavonoid compounds are becoming more and more popular due to their excellent pharmacological properties. Researchers reported that flavonoids possess anti-inflammatory, anti-tumor, anti-hepatotoxic, antioxidant, antimicrobial, anti-allergic and analgesic properties (Tian et al., 2019). Among them, one such flavonoid is baicalin extracted from the root of *Scutellariae radix*, has been proved to possess pharmacological effects against a variety of ailments including infection, inflammation, oxidant, and immune dysregulation (Hsu et al., 2016). The chemotherapeutic properties of baicalin may be attributed to its ability to modulate various transcription factors that are involved in various signaling pathways (Gong et al., 2017). Previous studies showed that baicalin suppressed the transcription factor nuclear factor-kappa B (**NF- $\kappa$ B**), a critical regulator of inflammation (Cheng et al., 2017). Importantly, **NF- $\kappa$ B** overexpression exacerbate the inflammation reaction through excessive production of pro-inflammatory mediators leading to immune dysregulation (Byun et al., 2013). Therefore, suppression of **NF- $\kappa$ B** pathway could prevent immune impairment in the spleen of chickens during MG infection. In addition, nuclear factor erythroid 2-related factor 2 (**Nrf2**) is a critical reg-

ulator of cellular redox balance to maintain homeostasis inside the cells (Itoh et al., 1999). In normal conditions, Nrf2 is found in the cytoplasm bound with Kelch ECH associating protein 1 (**Keap1**) (Zipper and Mulcahy, 2002). Increase in oxidative stress or exposure to electrophilic agents react with keap1 and causes the nuclear translocation of Nrf2. Nrf2 binds to the antioxidant response element (**ARE**) in the nucleus leading to the transcriptional activation of several antioxidant and detoxifying genes (Kensler et al., 2007). Numerous studies reported that baicalin exerts beneficial effects in part through the activation of Nrf2/HO-1 signaling pathway activation (Zhang et al., 2012). However, the effect of baicalin on **NF- $\kappa$ B** and Nrf2/HO-1 signaling pathway in the spleen of chicken is still unknown. Thus, the inhibition of **NF- $\kappa$ B** pathway and activation of Nrf2/HO-1 signaling pathway could be a novel pharmacological approach for the prevention of MG-induced oxidative stress and apoptosis. The present study was aimed to investigate the preventive effects of baicalin against oxidative stress and apoptosis induced by MG infection in the chicken spleen.

## MATERIALS AND METHODS

### Ethical Statement

All the experimental procedures and guidelines were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SYXK (Hei) 2012–2067) in the present study.

### Chemicals and Reagents

Baicalin (purity  $\geq 98.0\%$ ) was bought from Huifeng Animal Health Co., Ltd. (Heilongjiang, China). Methanol, Ethanol (99.0%), glycine, isopropanol and chloroform are the common reagents used in the study.

### Strain $r_{low}$ of mg and Culture Conditions

Strain  $R_{low}$  of MG was used in these experiments provided by Harbin Veterinary Research Institute (Chinese Academy of Agricultural Science, Heilongjiang, China). The culture conditions for growing MG were kept the same as mentioned in our previous study (Lu et al., 2017). Briefly, modified Hayflicks medium containing 0.05% Penicillins, 0.1% Nicotinamide adenine dinucleotide (**NAD**), 10% freshly prepared yeast extract, 20% fetal bovine serum and 0.05% thallium acetate. At mid-exponential phase of MG, a color change was observed from phenol red to orange. Chickens were challenged at a density of  $1 \times 10^9$  color change unit per milliliter (**CCU/ml**) for the pathogens.

### Chickens and Treatments

120 one-day-old white leghorn chickens were bought from Chia Chau chicken farm situated in Harbin

(China). Chickens were reared for one week to adapt to experimental conditions prior to experiments. Ad libitum feed and fresh drinking water were provided to chickens. After 1 wk, chickens were divided into 4 experimental groups in 3 replicates. Each group were randomly assigned 10 chickens. Experimental groups including (A) Control group (normal chickens) (B) Model group (MG infected group) (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). Chickens were challenged with MG strain R<sub>low</sub> ( $1 \times 10^9$  CCU/mL) in the bilateral air sacs in the thoracic region as reported previously (Xiao et al., 2014). After 3 d of post challenge, baicalin solution (0.5 ml) was given orally (Cheng et al., 2017), once in a day at a dose of 450 mg/kg. After 7 d of baicalin post treatment, chickens were humanely sacrificed to avoid pain and suffering of chickens and spleen were collected for further experimental analyses.

### **Measurement of Antioxidant Activities**

Spleen samples from all experimental groups were examined for malonaldehyde (MDA, Cat no. A003-1) and nitric oxide (NO, Cat no. A012) content, and catalase (CAT, Cat no. A007-1), inducible nitric oxide synthase (iNOS, Cat no. A014-1), superoxide dismutase (SOD, Cat no. A001-1), glutathione peroxidase (GSH-Px, Cat no. A006), gamma glutamyl transferase ( $\gamma$ -GT, Cat no. C017) activities were measured on a spectrophotometer (UV190PC, Shanghai Drawell Scientific Instrument Co., Ltd. China). Spleen samples were prepared at 4°C in nine-fold volume of physiological normal saline solution in a chilled homogenizer as mentioned previously (Li et al., 2017; Muhammad et al., 2018). After, the solution was centrifuged ( $1000 \times g$  for 10 min), supernatant was collected and assayed for the contents or enzyme activities. All the above mentioned kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### **Histopathological Examination of Chicken Spleen**

For histopathological examination, 10% buffered formalin were used for fixing spleen samples for 12 h. Following dehydration with graded ethanol, samples were processed for paraffin wax and cut into sections (5  $\mu$ m thickness). Then, the sections were mounted on slides, stained with hematoxylin and eosin dye and observed under a light microscope (Nikon E100, Japan, 40X magnification).

### **Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick Endlabeling Assay**

Terminal deoxynucleotidyl transferase–mediated dUTP nick endlabeling (TUNEL) assay was employed

to detect apoptotic cells in the chicken spleen. The specimens were first fixed in formalin, dehydrated in graded ethanol and embedded in paraffin wax. The sections were mounted on glass slides, and apoptotic cells were detected by an apoptosis cell detection kit (Beyotime biotechnology, Jiangsu, China) according to the instructions of the manufacturer. Hydrogen peroxide was used to inhibit endogenous peroxidase activity following treatment with proteinase K. The slides were incubated at 37°C for 1 h with terminal TdT/nucleotide mixture and rinsed in phosphate buffer solution (PBS). After nuclear labelling developed with diaminobenzidine and horseradish peroxidase, the slides were counterstained with hematoxylin and examined under a fluorescence microscope.

### **Determination of Cytokine Activities**

The splenic tissues were first washed in normal saline to get rid of excess debris and homogenized in normal saline solution. It was then centrifuged at  $1000 \times g$  for 10 min in PBS and the supernatant was collected in new ependorff tubes. Cytokines activities were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions. The samples were loaded in duplicate along with a blank control sample in 96 well plate and run on iMARKTM microplate reader (Bio-Rad Co., Ltd. Shanghai, China).

### **Protein Extraction and Western Blotting**

Protein was extracted from spleen samples using radioimmunoprecipitation assay and a protease inhibitor phenylmethyl sulfonyl fluoride as described earlier (Muhammad et al., 2018). Proteins were separated on SDS-PAGE (10 to 15%) after equal loading and transferred to membranes made of nitrocellulose. Membranes were blocked with 5% non-fat dry milk in TBST for 1 h and incubated with specific primary antibodies overnight. The membranes were again washed 3 times with TBST 10 min each time and incubated with secondary anti-mouse or anti-rabbit IgG peroxidases for 1 h at room temperature. After, bound immune-complexes were visualized with enhance chemiluminescence (ECL, Biosharp Life Sciences, China) reagent. Image J software (National Institute of Health, Bethesda, Maryland) was used to analyze the blots.

### **RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction**

Trizol reagent (catalog no. 15,596–018, Thermo Fisher Scientific, Carlsbad, California) was used to extract total RNA from spleen samples. RNA quality was evaluated by absorbance at 260/280 ratio (Wang et al., 2016). First strand cDNA was synthesized using

**Table 1.** Primers for target genes in RT-PCR.

Target gene	Primers (from 5' to 3')	Length
Bax	Forward 5'-ACTCTGCTGCTGCTCTCCTCTC-3' Reverse 5'-ATCCACGCAGTGCCAGATGTAATC-3'	174
Caspase-3	Forward 5'-TACCGGACTGTCTATCTCGTTCAGG-3' Reverse 5'-ACTGCTTCGCTTGCCTGTGATCTTC-3'	166
Caspase-8	Forward 5'-GGAAGCAGTGCCAGAACTCAGAAG-3' Reverse 5'-TTGTTGTGGTCCATGCACCCGATAG-3'	174
Caspase-9	Forward 5'-CCGAAGGAGCAAGCACGACAG-3' Reverse 5'-CATCTAGCATGTCAGCCAGGTCAC-3'	121
P53	Forward 5'-GGAGATGGAACCATTGCTGGAACC-3' Reverse 5'-GCTCCTGCCAGTTGCTGTGATC-3'	113
Bcl2	Forward 5'-GAGTTCGGCGGCGTGATGTG-3' Reverse 5'-TTCAGGTACTCGGTCATCCAGGTG-3'	92
Cytochrome C	Forward 5'-CCTAATCGCCGTGGCCTTCTTAAC-3' Reverse 5'-GGAGGAGGTAGATGGTCCGATTGG-3'	163
Nrf2	Forward 5'-GATGTCACCCTGCCCTTAGA-3' Reverse 5'-TCGTTCCATTTGTTCTCTG-3'	124
HO-1	Forward 5'-TCATTGGCAAGAAGCATCCAGAGC-3' Reverse 5'-GAACTTGGTGGCGTTGGAGACTC-3'	176
iNOS2	Forward 5'-GAAGTGGTATGCTCTGCCTGCTG-3' Reverse 5'-GTCTCGCACTCCAATCTCTGTTCC-3'	115
NF- $\kappa$ B	Forward 5'-CACATGGTGGTGACCCCAATAG-3' Reverse 5'-GTGCCATCGTATGTAGTGTGCTGTC-3'	194
TNF- $\alpha$	Forward 5'-TGATCGTGACACGTCTCTGC-3' Reverse 5'-CAACCAGCTATGCACCCCAG-3'	88
IL-6	Forward 5'-TTCACCGTGTGCGAGAACAGC-3' Reverse 5'-CAGCCGTCCTCCTCCGTCAC-3'	80
IL-1 $\beta$	Forward 5'-AGCAGCCTCAGCGAAGAGACC-3' Reverse 5'-GTCCACTGTGGTGTGCTCAGAATC-3'	90
Mff	Forward 5'-GGCTCCTCAGAATGCTGACCTTG-3' Reverse 5'-CACTACAATCCGCTCTGGAACCTG-3'	91
Mfn1	Forward 5'-CCTGCTGCAACTCCAGAGAACAC-3' Reverse 5'-TCACTCCGCCAACAACGATGATG-3'	115
Mfn2	Forward 5'-AGCTGGCTGCGTACATCAATGAG-3' Reverse 5'-GCCTTGCCAACACTTCACTAATGC-3'	150
Opa1	Forward 5'-TATGTGAAACGGGCCACTGT-3' Reverse 5'-ATGGTCCACACCAGCCAAAA-3'	85
NQO1	Forward 5'-TCGCCGAGCAGAAGAAGATTGAAG-3' Reverse 5'-GGTGGTGAAGTACAGCATGGC-3'	191
GSTA2	Forward 5'-GGAGTCAATCCGGTGGCTGTTAG-3' Reverse 5'-GGCTCTGCTCTGCACCATCTTC-3'	163
$\beta$ -actin	Forward 5'-CAACACAGTGTCTGTGGTGGTAC-3' Reverse 5'-CTCCTGCTTGTGATCCACATCTG-3'	199

Prime Script<sup>TM</sup> RT reagent Kit with gDNA Eraser kit purchased from Takara, Dalian, China. Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed with a kit obtained from Takara, Dalian, China (catalog no. RR820A) in a Roche LightCycler instrument (Shanghai, China) and mRNA expression of the target genes (primers shown in Table 1) were evaluated according to  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001), and  $\beta$ -actin was used as internal control gene for normalization.

### Data Analysis

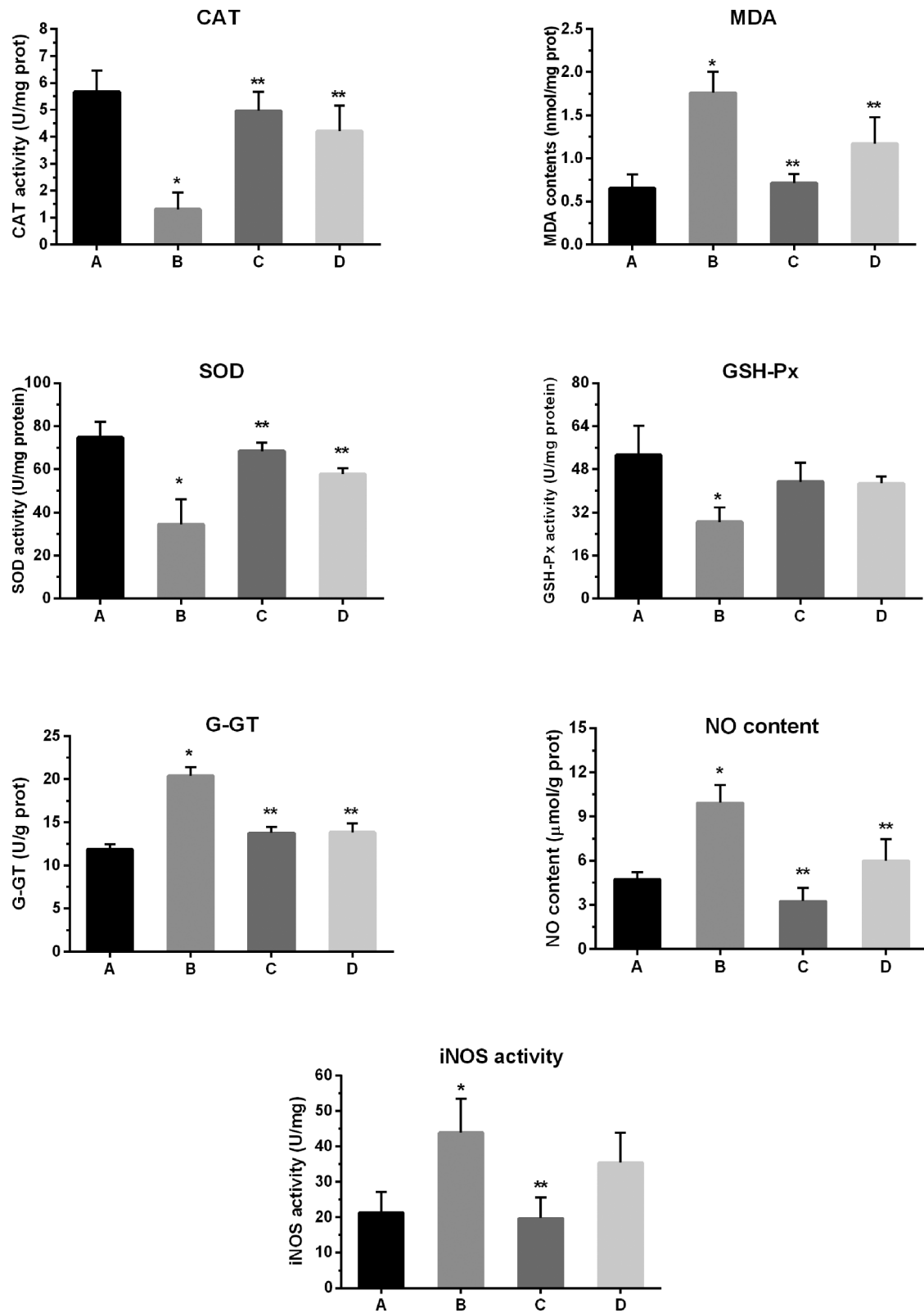
All the analysis of data was performed by statistical package for social sciences (SPSS window version 21.0, Chicago, Illinois) software and one-way analysis of variance was applied to determine the statistical sig-

nificance among data at a  $P$  value  $< 0.05$  followed by LSD test. The experiments were performed in triplicates ( $n = 3$ ) and the data were expressed as mean  $\pm$  SD. All the graphs were made by GraphPad prism (window version 6.01, San Diego, California).

## RESULTS

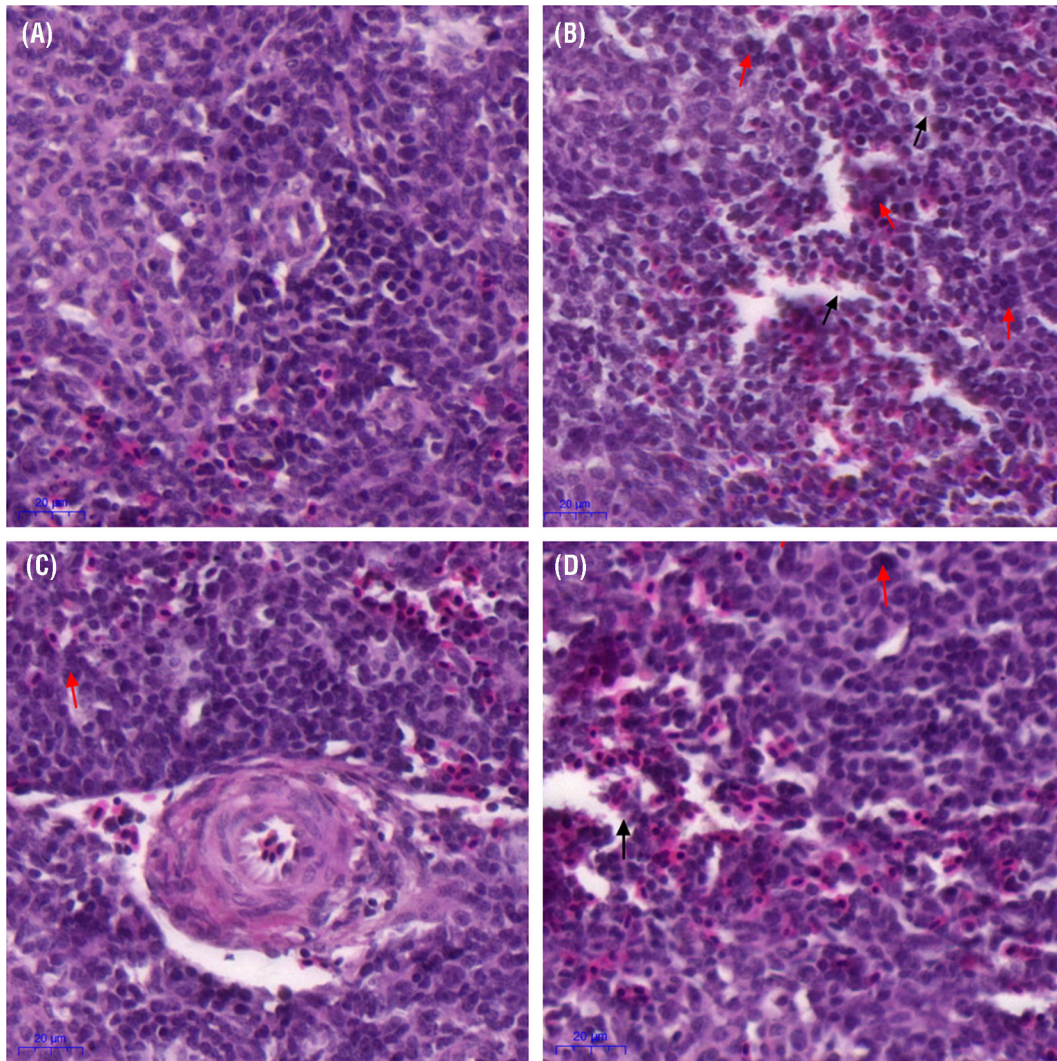
### Baicalin Alleviated Oxidative Stress in Spleen Tissues

Oxidative stress-related parameters (Figure 1) were observed in the chicken spleen infected with MG. SOD, GSH-Px and CAT activities were significantly ( $P < 0.05$ ) reduced in the model group, and iNOS and G-GT activities were significantly ( $P < 0.05$ ) enhanced in the model group compared to control and baicalin



**Figure 1.** Oxidative stress-related parameters were assessed in the spleen of chicken. Experimental groups are represented as (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). All the bar graphs represent mean results  $\pm$  SD (n = 3). Statistical significance were represented as \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. model group.





**Figure 2.** Spleen photomicrographs stained with hematoxylin and eosin (scale bar = 20  $\mu$ m) are shown in the Figure 2 ( $n = 3$ ). Black arrows represent lymphocytes shedding, and red arrows show mononuclear hyperplasia. Experimental groups including (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg).

alone group (Figure 1). Similarly, NO and MDA content were significantly ( $P < 0.05$ ) increased in the model group. However, compared to model group, baicalin treatment significantly restored the normal level of these enzymes and alleviated oxidative stress in the spleen of chickens. Intriguingly, it is worthy to mention that baicalin alone treatment has no significant effect on these enzymes compared to control group.

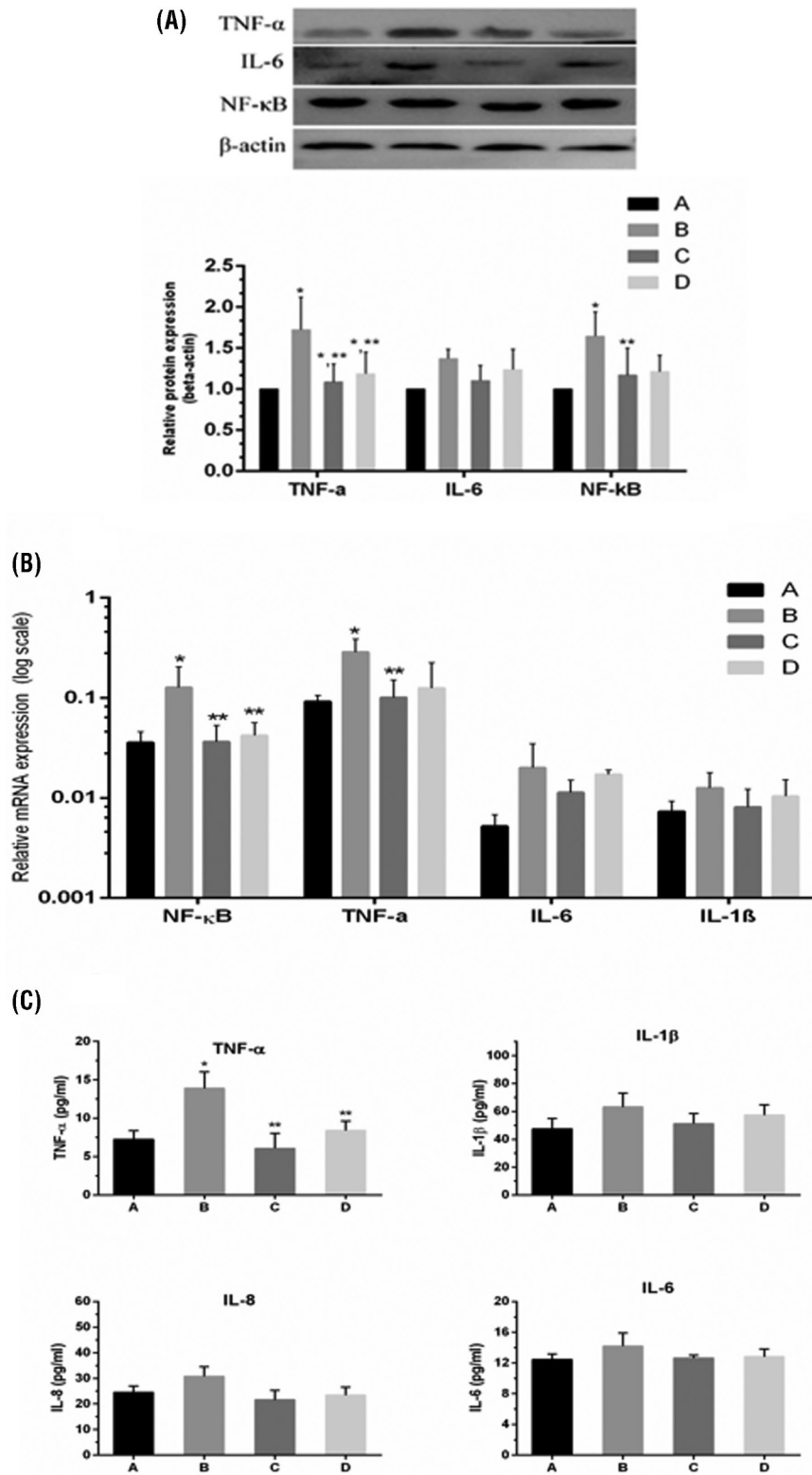
### Histopathological Assessment of Spleen Tissues

Histopathological observation (Figure 2) showed morphological changes in the tissue sections of spleen in the model group compared to control and baicalin alone group. Abnormal morphology including lymphocyte reduction, cells hyperplasia, and red, white pulp were not clearly visible in the model group (Figure 2B). Spleen tissue micrographs from control (Figure 2A)

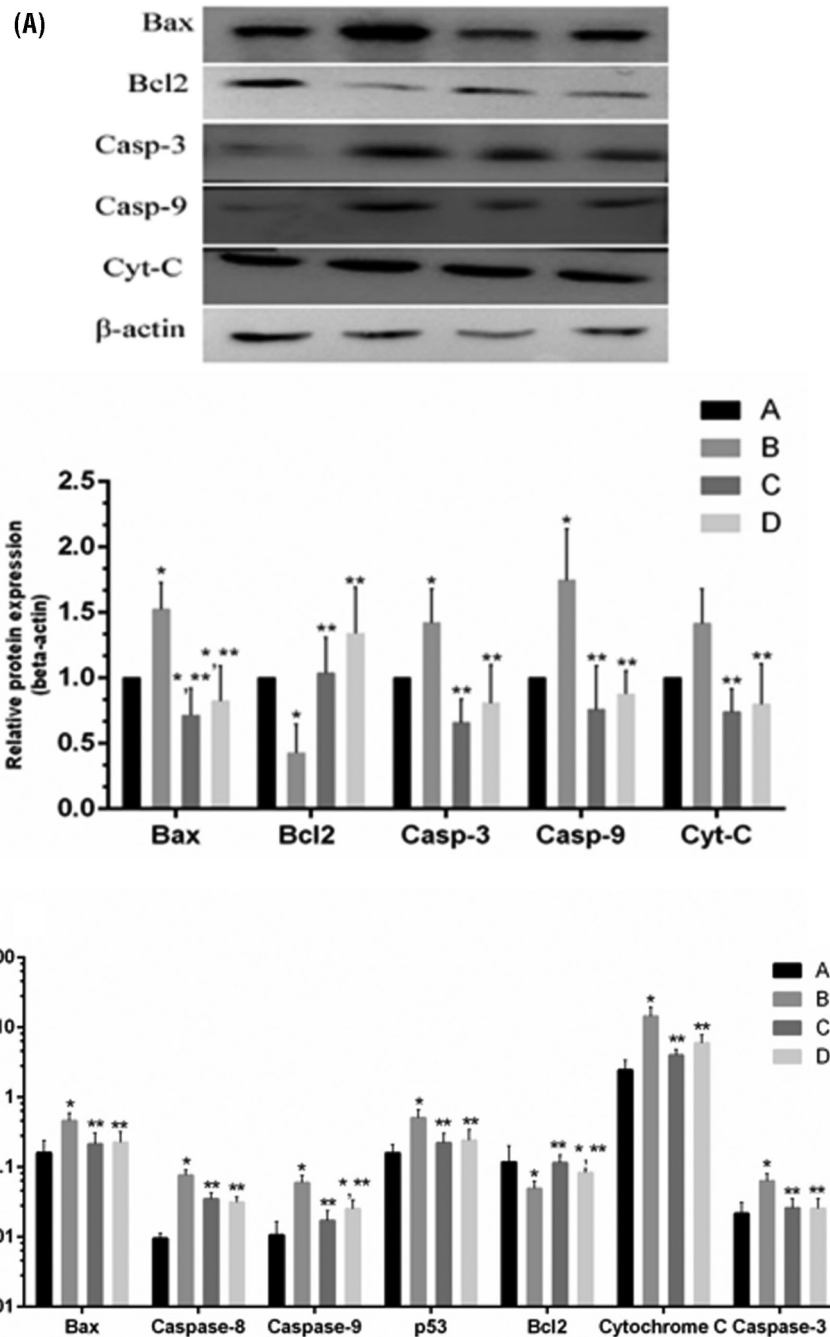
and baicalin alone group (Figure 2C) showed normal morphology and appearance. However, the observed abnormal morphological signs and structural deterioration partially disappear with baicalin treatment in the spleen of chickens infected with MG (Figure 2D).

### Suppression of Proinflammatory Cytokines and NF- $\kappa$ B by Baicalin

The mRNA and protein expression level of proinflammatory cytokines and NF- $\kappa$ B are shown in Figure 3 (A, B). NF- $\kappa$ B, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and IL-1 $\beta$  mRNA expression (Figure 3B) were significantly ( $P < 0.05$ ) enhanced in the model group compared to control and baicalin alone group. Protein expression results (Figure 3A) of NF- $\kappa$ B, TNF- $\alpha$ , and IL-6 showed the same trend as mRNA in the model group and significantly ( $P < 0.05$ ) increased



**Figure 3.** Panel (A) shows the protein expression of NF- $\kappa$ B, TNF- $\alpha$  and IL-6, panel (B) shows the mRNA expression of NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  and panel (C) shows enzyme activities of cytokines TNF- $\alpha$ , IL-6, IL-8, and IL-1 $\beta$  in the spleen tissues of chicken. Whereas, experimental groups represented as (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). Statistical significance were represented as \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. model group. All the values shows mean results  $\pm$  SD ( $n = 3$ ).



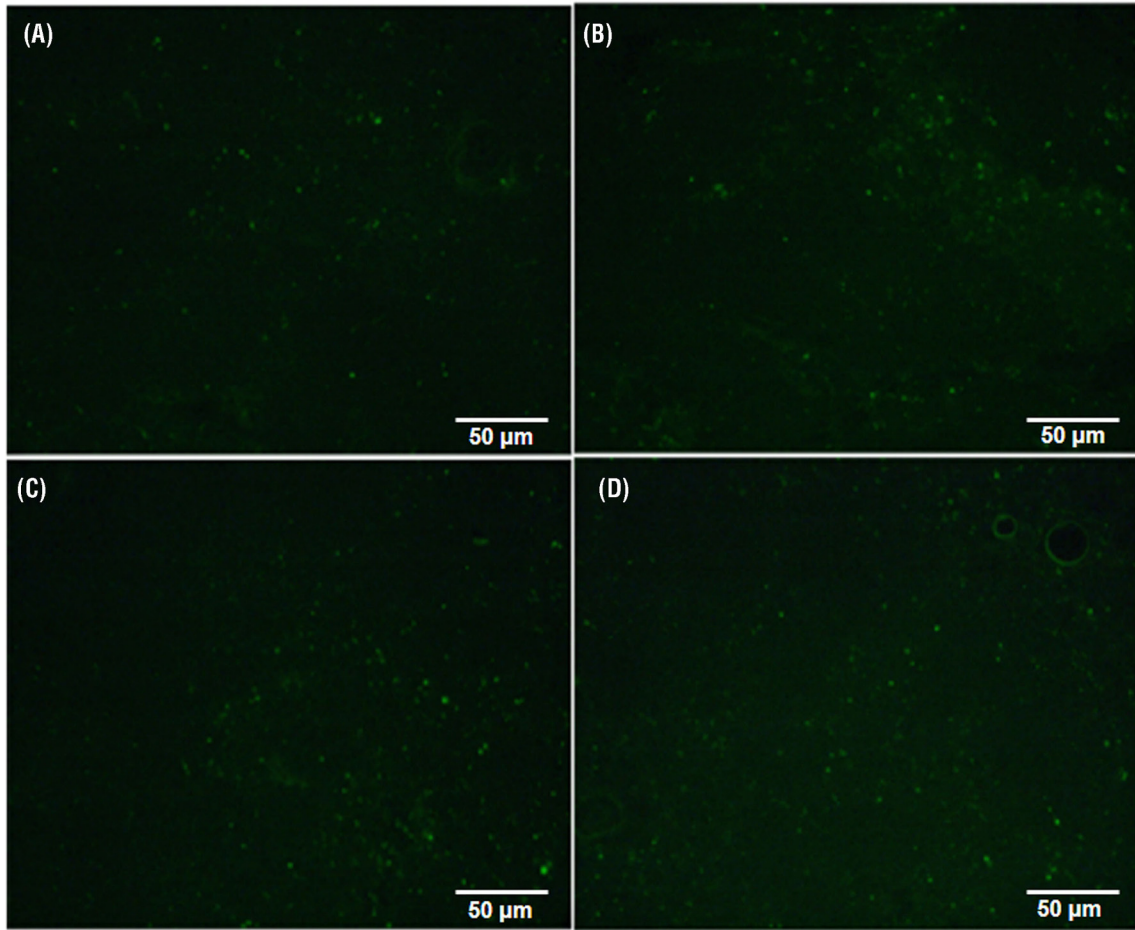
**Figure 4.** The mRNA and protein expression of mitochondrial-related apoptosis genes. Panel (A) shows protein expression level and panel (B) shows the mRNA expression of mitochondrial apoptosis related genes. Whereas, experimental groups consisting of (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). Statistical significance were represented as \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. model group. All the values shows mean results  $\pm$  SD ( $n = 3$ ).

in the model group compared to control and baicalin alone group. Meanwhile, the mRNA and protein expression level of NF- $\kappa$ B, and proinflammatory cytokines were reduced ( $P < 0.05$ ) with baicalin treatment in comparison to model group. Proinflammatory cytokine activities (Figure 3C) were increased in the model group compared to control and baicalin alone group. While, baicalin treatment significantly ( $P < 0.05$ ) alleviated the increased expression of these cytokines in the spleen.

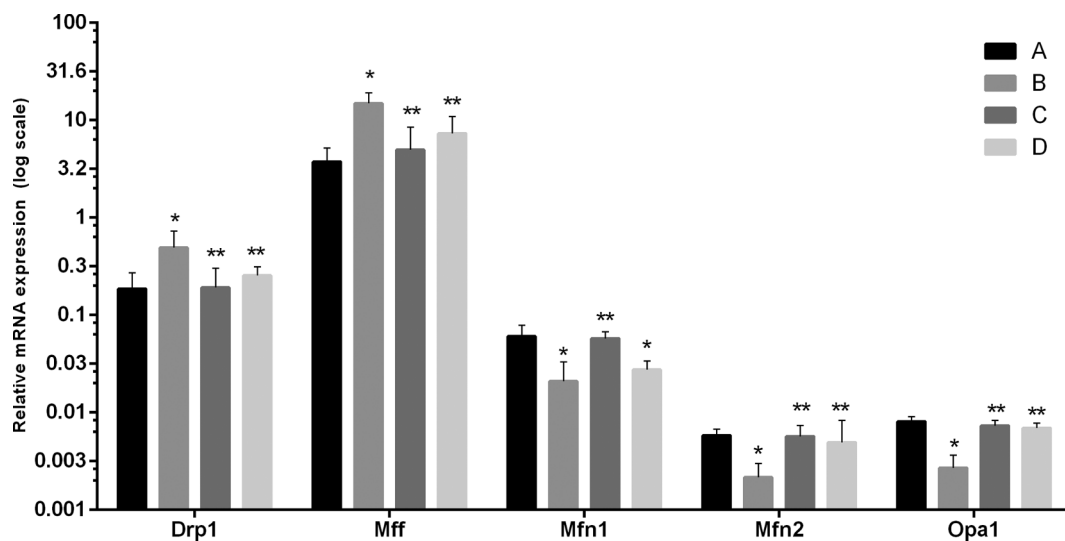
### **Baicalin Attenuated Apoptosis in the Spleen**

Level of apoptosis (Figures 4 and 5) was measured in the spleen of chickens to evaluate the preventive effects of baicalin against MG-induced immune impairment. The mRNA and protein expression level (Figure 4) of mitochondria-related apoptosis genes showed significant ( $P < 0.05$ ) upregulation in the model group compared to control and baicalin alone group with the exception of Bcl2. Bcl2 level significantly





**Figure 5.** Apoptosis were measured by TUNEL assay at day 7 post-treatment after infection. Positive stained nuclei (apoptotic cells) were observed in the spleen of chickens (scale = 50 µm). Whereas, experimental groups consisting of (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg).



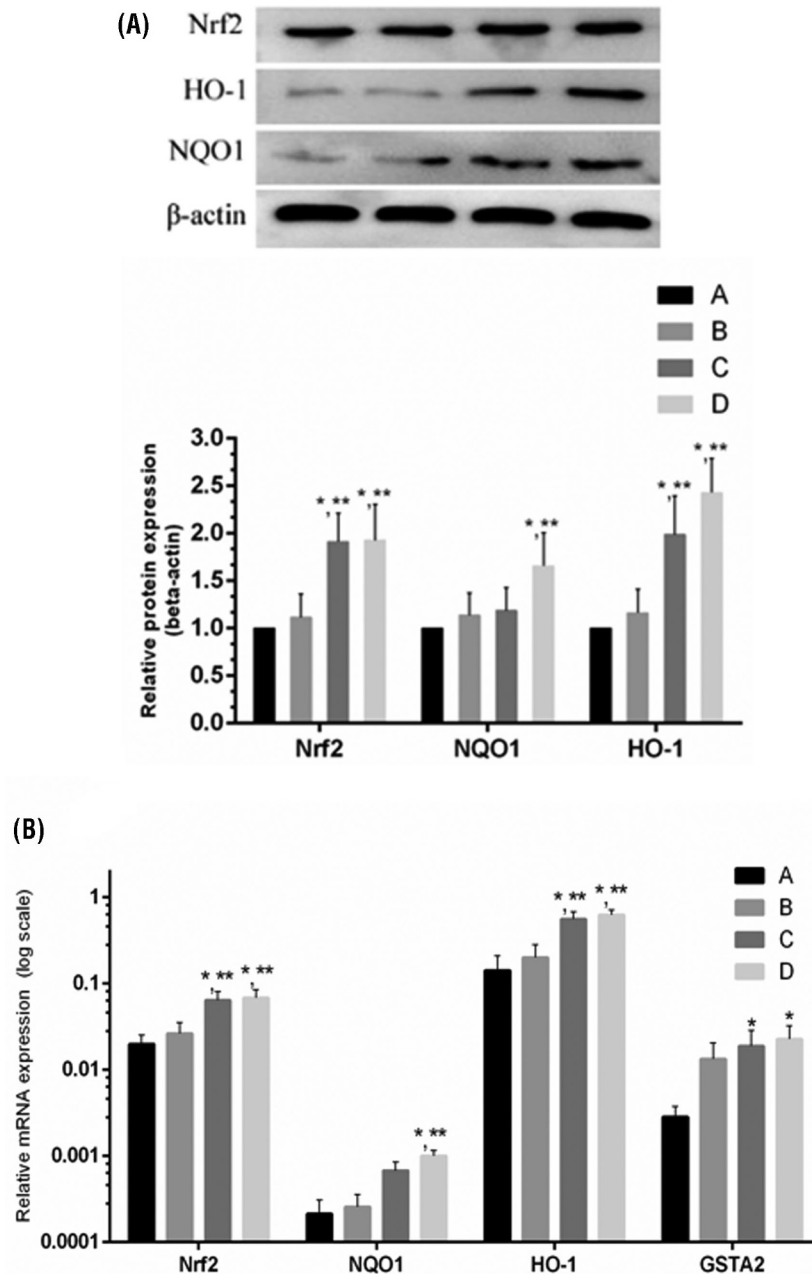
**Figure 6.** The effect of MG infection and/or baicalin on mitochondrial dynamics related genes mRNA expression were assessed in the spleen tissues. Whereas, experimental groups including (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). Statistical significance were represented as \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. model group. All the bar graphs shows mean results  $\pm$  SD (n = 3).

decreased in the model group both at mRNA and protein expression level. More importantly, significant ( $P < 0.05$ ) decrease has been noted in mRNA and protein expression level of these enzymes with baicalin treatment compared to model group. In addition, anti-apoptotic gene (Bcl2) mRNA and protein levels were significantly ( $P < 0.05$ ) enhanced with baicalin treatment in comparison with model group. In addition, TUNEL results (Figure 5) showed extensive positive-stained nuclei in the model group compared to control and baicalin alone treated group. Meanwhile, baicalin treatment signifi-

cantly reduced positive-stained nuclei in the spleen of chickens infected with MG as compared to model group.

### Effect of Baicalin and MG Infection on Mitochondrial Dynamics in the Chicken Spleen

Figure 6 represents the level of mRNA expression level of mitochondrial dynamics of 4 experimental groups. MG infection significantly altered the mRNA



**Figure 7.** The effect of MG infection and/or baicalin on transcription factor Nrf2 and its downstream genes (HO-1, NQO1, GSTA2). Panel (A) shows the protein expression levels of Nrf2, NQO1 and HO-1 and panel (B) shows the mRNA expression level of Nrf2, NQO1, HO-1, and GSTA2. Whereas, experimental groups consist of (A) Control group (B) Model group (C) Baicalin alone treated group (450 mg/kg) and (D) Model group treated with baicalin (450 mg/kg). Statistical significance were represented as \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. model group. All the bar graphs shows mean results  $\pm$  SD (n = 3).

expression level of these genes in comparison to control and baicalin alone group. The expression level of Drp1 and Mff were significantly enhanced in the model group. While, Mfn1, Mfn2, and Opa1 mRNA expression level were significantly ( $P < 0.05$ ) decreased in the model group. Meanwhile, baicalin treatment significantly prevented the altered mRNA expression level of these genes.

### Effect of Baicalin and MG Infection on the Expression of Nrf2/HO-1 Signaling Pathway

The mRNA and protein expression level of Nrf2 and its downstream genes are represented in Figure 7. Interestingly, Nrf2 mRNA and protein expression level increased ( $P > 0.05$ ) in the model group compared to control group. Similarly, NAD(P)H: quinone oxidoreductase 1 (NQO1), HO-1 and Glutathione S-transferase-A 2 (GSTA2) mRNA also increased in the model group compared to control group. However, the increase in mRNA expression level of these genes is not statistically significant compared to control group. Meanwhile, baicalin treatment significantly ( $P < 0.05$ ) enhanced Nrf2 and its downstream genes mRNA and protein expression level compared to control and model group.

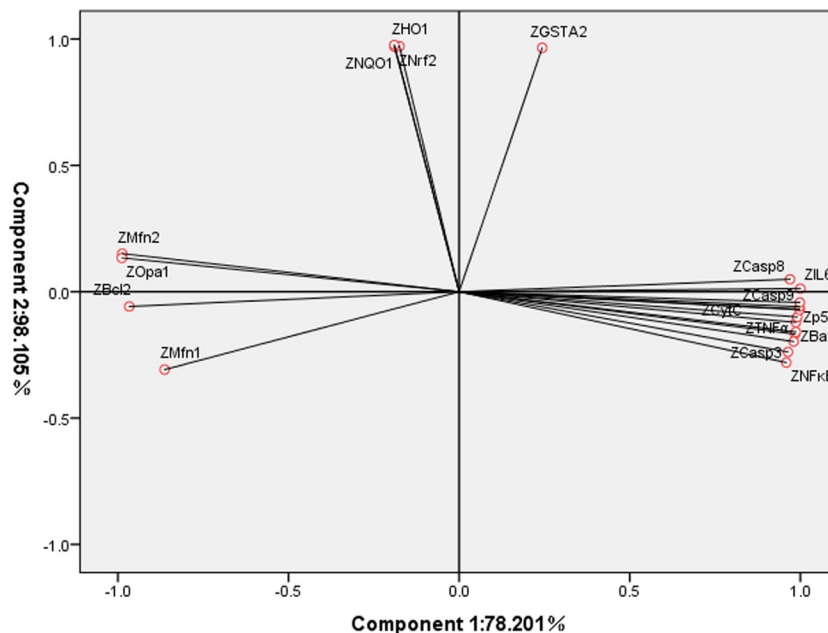
### Principal Component Analysis

Principal component analysis was carried out to determine the key factors involved in individual variations, which define the most important parameters with the advantage of compressing the data. Using Principal Component Analysis (Figure 8), the data is

divided into two principal components, the first principal component (78.201%) and second principal component (98.105%), respectively, except Bcl2. Mfn1, Mfn2, Opa1, Nrf2, NQO1, and HO-1 are positively correlated with principal component 1, but only Casp-8, Mfn2, Opa1, IL-6, Nrf2, NQO1, HO-1, and GSTA2 are positively correlated with principal component 2. The rotating component matrix obtained through principal component analysis is shown in Table 2.

## DISCUSSION

Baicalin has been used as a traditional medicine in East Asia for several decades (Ishimaru et al., 1995). A wide variety of pharmacological properties of baicalin such as anti-cancer, anti-pruritic and anti-inflammatory effects had been reported in literature (Lin and Shieh, 1996). Nowadays, natural products have been extensively used against bacterial diseases with the benefits of avoiding antibiotic resistance and harmful side effects (Jang et al., 2014). Baicalin is one of the natural flavonoids that showed potential therapeutic effects against bacterial diseases (Fujita et al., 2005). In addition, it shows synergistic effects with a variety of other antibiotics (Cai et al., 2016). A previous study demonstrated that baicalin prevents from mycoplasma pneumonia infection (Meng et al., 2013). (Garmyn et al., 2017) reported the efficacy of tiamulin alone or in combination with chlortetracycline against MG infection in chickens. However, the immunomodulatory effects of baicalin against MG infection in the chicken spleen is still not reported. In the present study, we investigated the preventive effects of baicalin against MG infection induced immune impairment involving oxidative stress and apoptosis in the chicken spleen. Histological and



**Figure 8.** The rotating components in space obtained from principal component analysis. The first and second principle components were 78.201 and 98.105%, respectively.

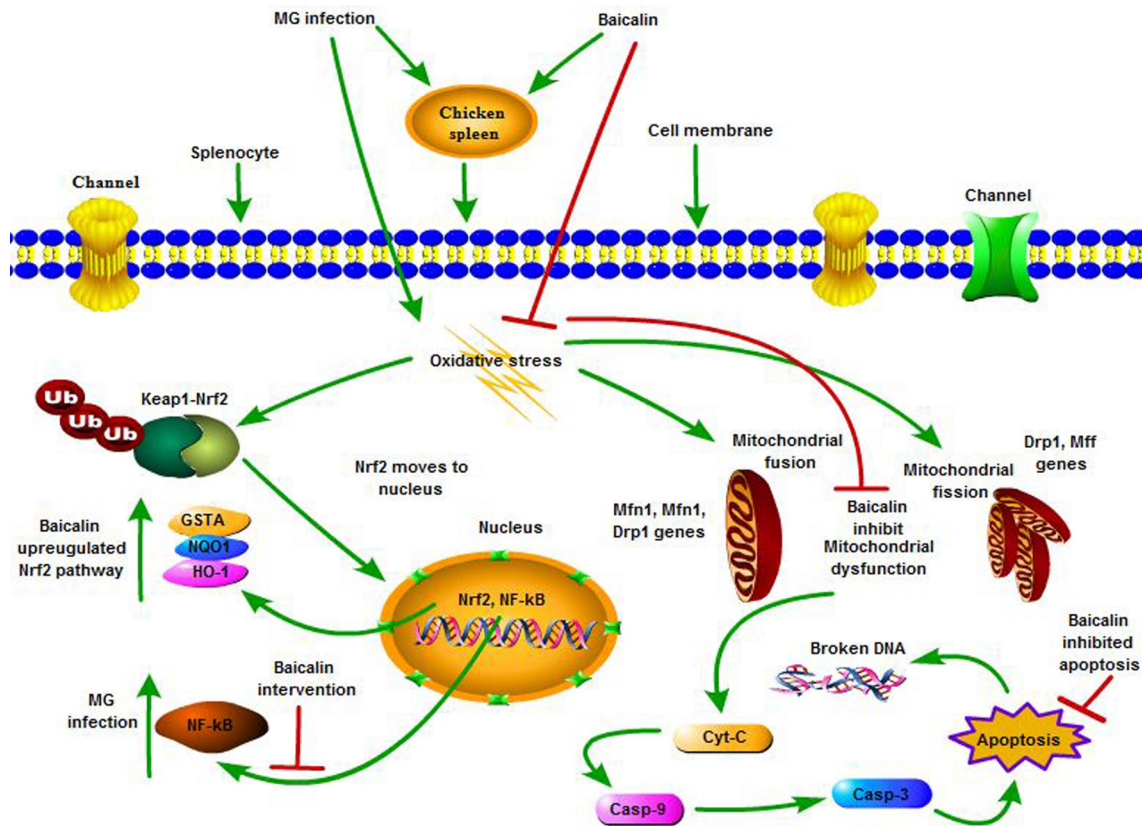
**Table 2.** Rotating component matrix.

Component	Bax	Casp-3	Casp-8	Casp-9	p53	Bcl2	Cyt-C	Drp1	Mff	Mfn2	Opa1	NF- $\kappa$ B	TNF- $\alpha$	IL-6	IL-1 $\beta$	Nrf2	NQO1	HO-1	GSTA2	iNOS2	Mfn1
1	0.987	0.964	0.970	0.998	0.991	-0.967	0.997	0.984	0.997	-0.987	-0.988	0.959	0.980	1.00	0.999	-0.190	-0.175	-0.190	0.243	0.987	-0.863
2	-0.121	-0.239	0.049	-0.069	-0.100	-0.058	-0.072	-0.168	-0.059	0.151	0.134	-0.280	-0.197	0.013	-0.042	0.970	0.972	0.977	0.977	-0.159	-0.308

ultrastructural observation showed that baicalin treatment partially ameliorated the pathological changes in the spleen. Intriguingly, MG infection produced oxidative stress which is the possible cause of these abnormal pathological changes and structural deterioration in the spleen of chickens. Oxidative stress-related enzyme activities were subsequently altered in the spleen tissues, confirmed an increase in oxidative stress in the chickens infected with MG. More importantly, baicalin treatment significantly alleviated oxidative stress compared to model group. These findings are in consistence with previous studies that baicalin prevented from oxidative stress (Wu et al., 2018). In addition, we noticed a profound level of apoptosis in the spleen of model group as compared to control and baicalin alone treated group. TUNEL assay results showed a number of positive stained nuclei in the model group. Subsequently, the mRNA and protein expression level of apoptosis-related genes were significantly changed. It has been suggested from these results that baicalin significantly attenuated apoptosis and could prevent immune impairment in the spleen of chickens infected with MG. Previous reports demonstrated that mitochondrial apoptosis is also associated with mitochondrial dynamics genes (Dabrowska et al., 2015). In the present study, we noted an increase in mRNA expression level of Drp1 and Mff genes in the model group, responsible for mitochondrial fragmentation and fission. While, mitochondrial membrane fusion-related genes such as Opa1, Mfn1 and Mfn2 mRNA expression level were significantly downregulated in the model group. These findings are in agreement with earlier studies (Halliwell 1992; Alaimo et al., 2013; Xu et al., 2013; Jin et al., 2017; Praharaaj et al., 2018), demonstrating that the alteration in mitochondrial dynamics results in mitochondrial dysfunction leading to apoptosis. In addition, our data showed that baicalin treatment significantly promoted the expression level of mitochondrial dynamics and prevents mitochondrial dysfunction during MG infection. However, further studies are needed to investigate the effect of baicalin on mitochondrial respiratory chain complex associated with energy metabolism in the spleen of chickens.

Previous studies demonstrated that upregulation of Nrf2 pathway protects the body from oxidative stress and injuries (Wang et al., 2018), and Nrf2 is a key therapeutic drug target in case of oxidative stress and various other diseases (Zhang and Gordon, 2004). Antioxidant enzymes including GST, HO-1, and NQO1 prevent cells from oxidative stress (Banerjee et al., 1999). Recently, the upregulation of these antioxidant enzymes by chemical or natural products is a common strategy to provide protection to cells in case of diseases as well as cancer (Hwang et al., 2011). In addition, iNOS plays a crucial role in inflammatory response often induced by oxidative stress via NF- $\kappa$ B pathway (Chung et al., 2007). Therefore, the molecular inhibition of iNOS through the modulation of NF- $\kappa$ B is considered to be a key target in the spleen of chickens during MG infection. Our





**Figure 9.** The schematic diagram showing the preventive effects of baicalin against MG infection-mediated oxidative stress and apoptosis in the spleen of chickens. Red arrows shows inhibition, while green arrows shows upregulation or increased expression.

results showed that baicalin significantly inhibited the expression of NF- $\kappa$ B pathway. It could be speculated that baicalin alleviated oxidative stress-mediated alteration in cytokines expression. In addition, baicalin significantly upregulated the transcription factor Nrf2 and its downstream genes such as GST, HO-1 and NQO1 both at mRNA and protein level. The data provided an evidence that the cytoprotective action of baicalin is partially attributed to its ability to induce cytoprotective genes during MG infection, and Nrf2 promoted defense system against MG infection-mediated immune impairment in the chicken spleen. These results are in line with previous findings (Kim et al., 2010; Shen et al., 2017) that baicalin inhibited NF- $\kappa$ B and upregulated Nrf2 signaling pathway to attenuate oxidative stress and confers cytoprotection. Moreover, further molecular and mechanistic studies are needed to scrutinize the underlying mechanism of baicalin behind the regulation of NF- $\kappa$ B and Nrf2 signaling pathway. In conclusion, baicalin efficiently prevented oxidative stress and apoptosis in the chicken spleen during MG infection (refer to schematic diagram, Figure 9). In addition, baicalin promoted the balance among mitochondrial dynamics and protect mitochondrial dysfunction. Overall, these findings suggested that baicalin protect immune dysfunction through the activation of Nrf2/HO-1 signaling pathway, and suppressed NF- $\kappa$ B and proinflammatory

cytokines in the spleen of chickens. Nevertheless, further studies are required to investigate the crosstalk between these pathways to better understand the preventive mechanism of baicalin against various infections.

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## CONFLICT OF INTEREST STATEMENT

All authors declared that there are no potential conflicts of interests.

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