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Original Article

The Expression of NF-κB Signaling Pathway Was Inhibited by Silencing TGF-β4 in Chicken IECs Infected with E. tenella

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ABSTRACT

Chicken coccidiosis is a parasitic disease caused by one or more species of *Eimeria* infection with severe consequences. The NF-κB signaling pathway and TGF-β4 play an important role in the inflammation and immune response caused by coccidiosis infection. This study was designed to investigate the changes of NF-κB signaling pathway and the effects of TGF-β4 silencing on the expression of NF-κB signaling pathway in chicken's intestinal epithelial cells (IECs) after infecting with *E. tenella* sporozoites. TGF-β4 small interfering RNA (TGF-β4 siRNA) sequences were transfected into chicken IECs for reducing TGF-β4 expression. The results showed that compared with uninfected control group (Con.), the proinflammatory factors of NF-κB, IL-6, IL-2, IL-1β increased significantly in the *E. tenella* infected group (ET) ($p<0.05$). In comparison with the IECs in the ET, the expression level of NF-κB, IL-6, IL-2, IL-1β dropped significantly in the group infected both by *E. tenella* and TGF-β4 siRNA vector (ET+siRNA) ($p<0.05$). The results of this experiment indicate that NF-κB signaling pathway is closely correlated with TGF-β4 in IECs infected with coccidia sporozoites. TGF-β4 plays an important role in regulating the immune function of coccidia-infected chicken epithelial cells through NF-κB signaling pathway and preventing immune-mediated pathological changes.

INTRODUCTION

Chicken coccidiosis is a common but highly dangerous and acute epidemic parasitic disease caused by one or more coccidia (Ahmad, 2016). The disease is characterized by intestinal damage and gastrointestinal infection is the only way to cause the disease (Ovington, 1995). As a result, chicks fall ill due to the ingestion of coccidia sporulated oocysts. The disease has strong pathogenicity and rapid spread, causing substantial economic losses (Dalloul & Lillehoj, 2006). At present, antibiotics and chemical drugs are used in farms to prevent and treat chicken coccidiosis. Because of this, chicks have developed resistance to a variety of anticoccidial drugs and the abuse of drugs has caused environmental pollution (Noack, 2019; Liu, 2020). It is therefore urgent to find safe and effective ways to prevent and treat coccidiosis. Since cytokines play an important role in the immune response and anti-infection process, their immuno-protective effects on coccidiosis infection are receiving more and more attention. Some of them were used as molecular adjuvant in live attenuated anticoccidial vaccines, which were the highly efficient strategies for controlling avian coccidiosis (Peek & Landman, 2011; Tang, 2018).

Widely distributed in the intestine, TGF-β directly regulates the immune function of the intestine and synergizes with nutrients to regulate the intestinal immune system (Howe, 2005; Rautava, 2012; Li,

2014). TGF-β plays an important role in maintaining the balance between the control and clearance of infectious microorganisms and immune-mediated pathologies (Omer, 2000). Jakowlew *et al*. (1997) reported that after infected with coccidia, the expression of TGF-β4 in intestinal epithelial lymphocytes of chicks increased by 5 to 8 fold while the expression of TGF-β2 and TGF-β3 remained unchanged. Song *et al*. (2010) found that the expression of TGF-β4 in the spleen and cecal tonsil of coccidia infected chicks increased three-fold. These studies indicate that TGF-β4 is the subtype that displays most prominent changes after chicks infected with coccidia.

Nuclear transcription factor (NF-κB) is a multi-directional transcriptional regulatory protein involved in regulation of various genes and is closely related to various physiological and pathological processes such as inflammation, immune response, and cell proliferation, transformation and apoptosis. NF-κB signaling pathway have certain effect in the immune process of chicken against coccidiosis and E.tenella infection can stimulate significantly increased secretion of NF-κB in chicken ceca (Jiao, 2018). Two or more cytokines may have antagonistic, increased or synergistic effects in animal immunization. However, it remains unclear as to whether the expression of TGF-β4 affects the NF-κB signaling pathway after chickens infected with coccidia. In this report, by constructing a TGF-β4 siRNA recombinant lentiviral vector, we used the in vitro chicken IECs coccidia infection model to study the relationship between TGF-β4 and NF-κB signaling pathways to provide experimental evidence for further study of the regulatory mechanism of the immune response in chickens infected with coccidia.

MATERIALS AND METHODS

Experimental materials

IECs (preserved in the laboratory), coccidial stock solution, fetal bovine serum (Hyclone), culture medium (Corning), trypsin (Gibco), pLVshRNA-EGFP (2A) Puro, Lipo2000 Transfection reagents (Invitrogen), SYBR Premix EX Taq (Perfect Real Time)(TaKaRa) PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa) TRIzol (Tiangen Biotech Co. Ltd, Beijing), Antibody (Abcam), horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Promega).

Cell culture

The frozen IECs were taken out of the liquid nitrogen tank, put in the frozen IECs in the flask containing the complete medium, placed in a 37°C incubator for static culture, which was then replaced with the fresh complete medium upon cell adherence on the next day. IECs was cultured continuously, observed for growth, and subcultured based on the cell growth. The medium was removed from the flask, which was then rinsed with PBS and added with 1mL trypsin. The cellular digestion was observed under an inverted microscope. When the cells were mostly rounded, the flask was quickly taken back to the console and the cells were resuspended with the complete medium. The cells were counted by cell counter and adjusted to the concentration of 2.5×10^5 /mL before 2 mL of each was inoculated into a 6-hole plate.

Sporozoites excystation

A 2.5% concentration of potassium dichromate solution was added to the rejuvenated *E. tenella* oocysts before it was placed in a constant temperature shaker for sporulation. When the sporulation rate reached 80%, the number was counted. After centrifugation, the potassium dichromate solution was discarded, and the precipitation was washed with PBS for 3 times. The precipitate obtained from centrifugation was resuspended in 30% sodium hypochlorite and allowed to stand at room temperature for 10 min before the upper spore-forming oocysts were washed with PBS and the sterilized glass beads were added for manually crush for 3 min. When the sporangia release reached over 80% after the microscopic examination, it was centrifugated and the sediment was collected. It was then resuspended in Hank's solution containing 0.25% trypsin and 0.75% deoxycholate before it was digested in a constant temperature shaker for 40 min and examined with microscope. The digestion was stopped when 80% sporozoites were released and then washed with PBS. The sporozoite sediment in PBS solution was resuspended and filtered through a funnel. The filtrate was collected, centrifuged, and the pellet was resuspended in PBS and counted, and stored at 4 °C. The above steps were all taken under aseptic conditions.

Construction and identification of chicken TGF-β4 gene lentivirus

Ambion software was used to design the interference sequence according to the sequence of chicken TGF-β4. The shRNA sequences (Table.1) were designed for subsequent viral packaging and was sent to the Sangon Biotech (Shanghai) for synthesis according to the designed sequence. It was then annealed to form a double strand: 5 uL endonuclease buffer 3; single-stranded primer (20 umol) for 5 uL

each; ddH2O was added to form a 50 uL system; it was then kept at 100°C for 1 min and naturally cooled down to room temperature. The lentiviral vector pLVshRNA-EGFP (2A) Puro was digested with BamHI and EcoRI endonucleases. The enzyme digestion system included: Buffer MIX: 5 uL; EcoRI: 1 uL; BamHI: 1 uL; DNA: 2 uL; ddH 20: 1 uL. The gene fragment was ligated to the viral vector. The recombinant vector was transformed into *E. coli* DH5a and placed on the plate at 37 °C overnight. On the next day, the monoclonal bacterium was picked and amplified, and the plasmid was extracted and verified by sequencing.

Table 1 – shRNA sequence.

shRNA	Oligonucletides sequence
TGF-β4 -1	5-AATTCGTGCAGAGCATTGCCAAGAACTCGAGTTCTTGGCAATGCTCTGCATTTTTG-3
	3-GCACGTCTCGTAACGGTTCTTGAGCTCAAGAACCGTTACGAGACGTAAAAACCTAG-5
TGF-β4 -2	5-AATTCGGTACCAGGGTTACGGCAATCTCGAGATTGCCGTAACCCTGGTACTTTTTG-3
	3-GCCATGGTCCCAATGCCGTTAGAGCTCTAACGGCATTGGGACCATGAAAAACCTAG-5
TGF-β4 -3	5-AATTCGTCGACACCGACTACTGCTTCTCGAGAAGCAGTAGTCGGTGTCGATTTTTG-3
	3-GCAGCTGTGGCTGATGACGAAGAGCTCTTCGTCATCAGCCACAGCTAAAAACCTAG-5
NC	5-AATTCGTCGATCCCTACGACAGAGTCTCGAGACTCTGTCGTAGGGATCGATTTTTG-3
	3-GCAGCTAGGGATGCTGTCTCAGAGCTCTGAGACAGCATCCCTAGCTAAAAACCTAG-5

Packaging and Amplification of Recombinant Lentivirus

The low-generation 293T cells were resuscitated and 293T cells were seeded at a density of 5 × 10^6 and cultured in 10 ml of complete medium at 37°C with 5% CO_2 for 24 hours. When the cell density reached 80%, four TGF-β4 shRNA lentiviral plasmids that had been constructed were transfected with the transfection reagent. They were then incubated at 37°C and changed with 10 ml of fresh medium after 4-6 hours. After 48 hours of culture, the effect of virus-infected cells was observed under a microscope. In addition, the cell supernatant was collected, and the virus was concentrated by ultracentrifugation to determine the virus titer, which was sub-packed and stored at -80 °C.

Measuring TGF-β4 Silencing Effect using RT-PCR

The isolated chicken ICEs were inoculated into the culture dish 24 hours prior to the virus infection. The virus solution was then dissolved and mixed well at room temperature. After that, the viral solution and polybrene were diluted with cell culture medium before they were transferred to the cell culture. After they were cultured for 24 hours, the virus infection solution was replaced by the new complete medium. Exogenous gene expression could be observed 48-72 hours after viral infection, and the cells can be used for fluorescence detection of cell lines. After the transfection effect was determined by fluorescence, the transfected cells were collected and subjected to real-time PCR to determine the transfection efficiency. The relative changes of gene expression were analyzed using $2^{-\Delta\Delta C(t)}$ method (Livak & Schmittgen, 2001).

Experimental design

A control group (Con.), a coccidial sporozoite infection group (ET), a TGF-β4 siRNA silencing group (siRNA), and a coccidial sporozoite infection + TGF-β4 siRNA group (ET+siRNA) were set up. Treatment methods for each group were described as follows. When the cells in the 6-hole plate grew to the point that the fusion state was 80% or more, for the *E.T* group: the medium was discarded, the cells were washed three times with PBS before the complete medium and the sporozoites were added at the doze of 3×10^5/mL, the cells were then collected after being cultured for 24 hours. For the siRNA group: The virus solution and polybrene were diluted with cell culture medium and added to the cell culture holes to get cells cultured for 24 hours. The virus infection solution was replaced with a new complete medium and the cells were further cultured for 48 hours before collection. For the ET+siRNA group: Cells infected with the recombinant lentivirus in the same as siRNA group before the culture medium was discarded and the cells were washed 3 times using PBS. After that, the complete cell culture medium was added and the coccidia sporozoites were added at a dose of 3×10^5/mL before the cells were collected 24 hours later. At the same time, control holes (Con.) were set and cultured in a CO_2 incubator at 37°C for 24 hours. There were three replicates in each group.

Detecting Gene Expression of NF-κB Signaling Pathway using RT-PCR

The cells were washed twice with cold PBS and the RNA was extracted by adding Trizol. A small amount extract was tested for OD value and the concentration and purity of total RNA was measured. The cDNA was

synthesized according to the instructions of the reverse transcription kit. The resulting cDNA was stored at -20°C. Fluorescence quantitative PCR reaction was carried out in a 20 μL reaction system, consisting of SYBR® Premix Ex TaqTM II (2×) 10 μL, PCR Forward Primer (10 μM) 1 μL, PCR Reverse Primer (10 μM) 1 μL, cDNA template 2 μL, ddH2O 6 uL with the total amount of 20uL. There are three double tubes in the sample tube and the inner tube respectively at the reaction conditions of 95°C 30 sec denaturation, 95°C 5 sec, 60°C 31 sec, and 40 cycles. The primer sequence (synthesized by Shanghai Sangon Biotech Co., Ltd.) was shown in Table 2.

Table 2 – Primer of Real-time PCR.

Gene	Synthesized sequence (5'-3')
TGF-β4	5-ATGCAGAGCATTGCCAAGAA-3
	5-CGAAGCAGTAGTCGGTGTCG-3
NF-κB	5-TACTGATTGCTGCTGGAGTTGATGTC-3
	5-TTGTGCCATCGTATGTAGTGCTGTC-3
IL-6	5-CTGCTGCCGCTGCTGCTG-3
	5-TCTCGCACACGGTGAACTTCTTG-3
IL-2	5-TTGGCTGTATTTCGGTAGCA-3
	5-CTCCTGGGTCTCAGTTGGTG-3
IL-Iβ	5-AGCAGCCTCAGCGAAGAGACC-3
	5-GTCCACTGTGGTGTGCTCAGAATC-3
GPADH	5-GACAGCCATTCCTCCACCTT-3
	5-TGCCATGTGGACCATCAAGT-3

TGF-β4, transforming growth factor β4; NF-κB, nuclear factor-κB; IL-6, Interleukin-6; IL-2, Interleukin-2; IL-1β: Interleukin-1β; GAPDH, Glyceraldehyde-3-phosphate-dehydrogenase

Detecting IL-6 Protein Expression using Western Blot

Total protein was extracted out of cells from each group to determine the protein concentration. The sample concentration was adjusted to 3 ug/uL with the loading quantity of 10 uL/hole. Separation gel and spacer gel were prepared. The cells were electrophoresized, transmembraned, and observed for the effect of transmembrane. After that, the membrane was completely immersed in 3% BSA-TBST and shaken at room temperature for 30 min. The primary antibody (Abcam) was diluted with 3% BSA-TBST at the dilution ratio of IL-6 at 1:1000, incubated at room temperature for 10 min, and kept for overnight at 4°C. On the next day, the samples were incubated for 30 min at room temperature and the membrane was washed 5 times with TBST for 3 min each time. In addition, the secondary antibody was diluted with 5% skim milk powder-TBST and goat anti-rabbit IgG (H+L) HRP 1:3000, and gently shaken at room temperature for 40 min. Finally, the membrane was washed with TBST and exposed for image using chemical illuminator.

Statistical Analysis

One-way ANOVA analysis, significance test, and correlation analysis were performed using SPSS 18.0 statistical software and the experimental data was expressed as means ± standard error.

RESULTS

Sequencing Identification of Positive Clones

Sequencing results indicated that the synthetic TGF-β4 shRNA nucleotide sequence was inserted correctly with no base deletions and substitutions. Plasmids were extracted from the correctly sequenced monoclonal colonies. After the extraction was completed, the recombinant lentiviral plasmid DNA was measured using a protein nucleic acid analyzer and the value of A260/A280 was between 1.8 and 1.9, indicating that the purity and integrity of the plasmid DNA met the requirements.

Effects of Three shRNA on TGF-β 4 Gene Expression in IECs

As can be seen from the Table 3, there was no significant difference in the expression of TGF-β 4 gene in the control group compared with the negative control group. Compared with the control group, TGF-β 4 expression in the siRNA 1-3 group was 0.685, 0.379 and 0.139 in the control group respectively with extremely significant difference. The siRNA-3 had the best silencing effect and was thus selected as a silencing plasmid.

Table 3 – Effects of three shRNA on the expression of TGF-β4.

TGF-β 4 siRNA	mean	SE	*p* value	inhibition rate (%)
Control	1.000^{A}	0.068	0.000	
siRNA-1	0.685^{B}	0.056		31.876
siRNA-2	0.397^{C}	0.068		60.466
siRNA-3	0.139^{D}	0.019		86.196
NC	0.941^{A}	0.067		6.300

Verifying the Silencing effect of siRNA-3 Plasmid on TGF-β 4 Expression

As can be seen from Table 4, the relative expression level of TGF-β 4 mRNA significantly decreased after the siRNA-3 plasmid infected the IECs, indicating that the constructed TGF-β 4-RNAi-3 successfully down-regulated the expression of TGF-β 4 gene in chicken intestinal cells. The expression level of TGF-β 4 increased after the chicken was infected with coccidiosis. However, compared with the siRNA group,

there was no difference in terms of the expression level of TGF-β 4 in the ET+ siRNA group, but such level was significantly lower than that in the coccidia infected group.

Table 4 – Effect of siRNA-3 plasmid on the expression of TGF-β 4.

Treatments	Mean	SE	*p* value
Con	1.011[b]	0.006	<0.05
ET	7.599[a]	0.477	
siRNA	0.220[c]	0.054	
ET+siRNA	0.475[c]	0.042	

Effect of TGF-β 4 Silencing on Gene Expression of NF-κB Signaling Pathway

The results in Figure 1 show that compared with the control group, the expression of NF-κB significantly increased after the IECs were infected with coccidia sporozoites, and the expression levels of IL-1β, IL-2 and IL-6 were also significantly higher than those of the control group (p<0.05). The expression levels of NF-κB, IL-1β, IL-2 and IL-6 in the TGF-β 4 siRNA group were significantly lower than those in the ET group but were not significantly different from the control group. The expression levels of NF-κB, IL-2 and IL-1β in cells in ET+ siRNA group were significantly higher than those of the control group and the TGF-β 4 siRNA group, but significantly lower than the coccidial infected group (p<0.05), indicating that coccidiosis infection increases the expression of NF-κB, IL-1β, IL-2 and IL-6 in chicken IECs. Silencing the TGF-β 4 gene can reduce the expression level of NF-κB signaling pathway in chicken's intestinal cells infected with *E. tenella*. Silencing the TGF-β 4 gene did not reduce the expression level of the NF-κB signaling pathway in the absence of coccidial stimulation.

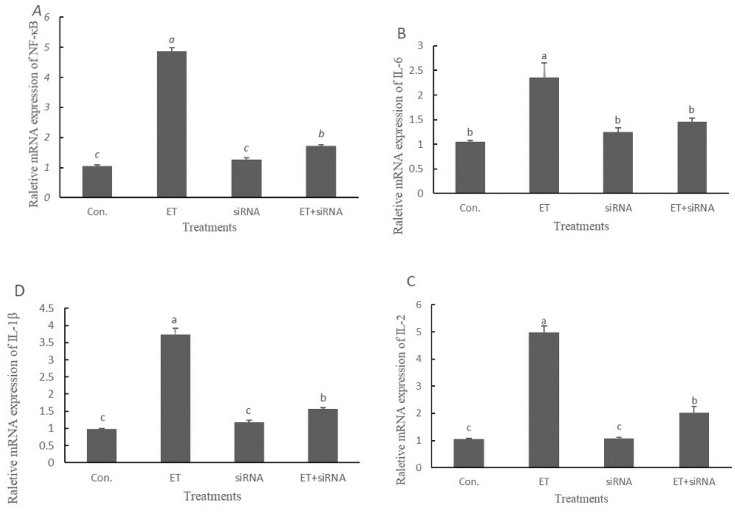

Figure1 – Effect of TGF-β 4 silencing on mRNA expression of NF-κB, IL-6, IL-2, IL-1β.

Effect of TGF-β 4 Silencing on the Expression of IL-6 Protein using WB

The WB results in Figure 2 show that the expression level of IL-6 protein in the coccidial infected group, TGF-β 4 silencing group, and ET+TGF-β 4 silencing group significantly increased compared with that in the control group. Compared with the coccidial infected group, the expression level of IL-6 in the TGF-β 4 silencing group and the ET+TGF-β 4 silencing group significantly decreased. These results indicated that coccidia infection increased the secretion of IL-6 protein in chicken's intestinal cells and silenced the TGF-β 4 gene, having the effect of reducing the secretion of IL-6 in chicken's intestinal cells.

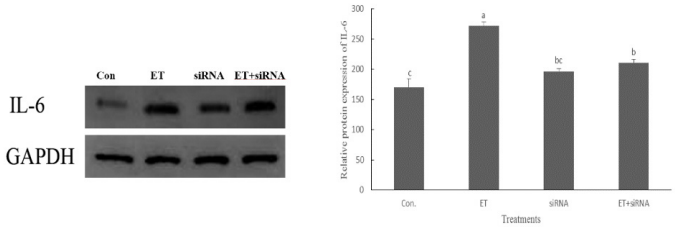

Figure 2 – Effect of TGF-β 4 Silencing on the Protein Expression of IL-6.

DISCUSSION

The global poultry production is growing rapidly out of the increasing demand for poultry and eggs. Intensive feeding in poultry farms is beneficial to the growth of chickens but also to the growth and reproduction of coccidia. Because of this, coccidiosis thus has become one of the most threatening diseases jeopardizing intensive farms. Chicken coccidiosis is characterized by intestinal damage and the proliferation of coccidia causes disintegration to the intestinal epithelial cells, damage to the intestinal mucosal barrier, excretion of blood, disordered digestive functions, lower production performance, and even death of chickens in severe cases (Laurent, 2001; Zhao, 2019). Chicken industry has suffered huge economic losses due to coccidiosis (Galli, 2019). Currently, antibiotics and environmental disinfection are the main methods employed to control chicken coccidiosis. However, the abuse of drugs triggers coccidia to have developed cross-over or multi-drug resistance to most anticoccidial drugs (Rothwell, 2004), causing clinical or subclinical coccidiosis to occur frequently in flocks. In addition, antibiotics go into the surrounding environment with the animal wastes or animal products, giving rise to the environmental pollution and the threat to the health of humans and other living creatures. It is increasingly urgent to find safe and effective ways to fight against coccidia. In

recent years, with the continuous advancement in genetic technology and the profound research on chicken cytokines, increasing attention has been given to the role of chicken cytokines in fighting coccidia (Rothwell, 2004; Kim, 2014).

The immune response caused by chicken coccidia infection is very complicated and the infected intestinal epithelial cells are the first line of defense against *Eimeria* (Laurent, 2001). When the chicken is infested with coccidia, the host cells respond to coccidial stimuli by selective expression of inflammatory factors. These inflammatory signals bind to the receptor and trigger a chain reaction of intracellular inflammatory signals, resulting in increased expression of NF-κB. After infected with coccidia, the expression of NF-κB mRNA in chicken cecal tissue significantly increased (Jiao, 2018). Similarly, the results of this experiment showed that the expression level of NF-κB in IECs infected with coccidia spores increased. NF-κB has the function of promoting the expression of inflammatory factors of IL-1, IL-6 and TNF-a. In turn, these inflammatory factors could activate the NF-κB signaling pathway and repeatedly promoted the inflammatory response, further aggravating intestinal cell damage. Therefore, NF-κB is a key factor in this signaling pathway, which acts to amplify the inflammatory signal of cytokines and regulate the persistence of inflammation throughout the process of inflammation. Overexpression of NF-κB led to inflammatory response and histopathological damage in the body (Song, 2013). The results of this experiment show that the expression level of NF-κB in the TGF-β 4 gene silencing group significantly decreased compared with the coccidial infected group, indicating that the inhibition of the expression of TGF-β 4 gene can effectively inhibit the expression level of NF-κB.

Inflammatory cytokines IL-1β,IL-2 and IL-6 were evidenced to participate in the avian Eimeira infection (Hong, 2006a; Jiao, 2018). IL-1β is a powerful pro-inflammatory cytokine secreted by macrophages and many other different cell types upon activation by stimuli (Hong, 2006b). IL-1β increased significantly seven days after *E. tenella* infection (Laurent, 2001). Chicken vaccinated in ovo with a cloned *Eimeria acervuline* gene (3-1E) plus IL-1 exhibited significantly greater serum IgG antibody levels and body weight gain following experimental infection with *E. acervuline* (Ding, 2004). IL-2 is a broad-spectrum biologically active cytokine that mainly stimulates the immune system to cause cellular immune responses, promotes the differentiation and proliferation of $CD4^+$ cells and $CD8^+$ cells, and plays an important role in the fight against viral, bacterial and parasitic infections. Miyamoto *et al* (2002) infected chicken with *E. tenella* and found that the level of IL-2 in serum was significantly higher than the control group 7 days after the first infection, and IL-2 caused the antibody to give anamnestic response to the reinfected cells. This experiment also demonstrated with in vitro experiment that the expression of IL-2 in chicken intestinal cells increased significantly after infected with coccidia sporozoites. In this experiment, an obvious increase of IL-1β, IL-6 and IL-2 were observed in the coccidia infected group, while, the expression of them down-regulated in the ET+ siRNA group, which indicating that the low expression of TGF-β inhibited the expression of IL-1β, IL-6 and IL-2. The reduction of NF-κB signaling pathway expression level can alleviate the intestinal immune response and is of great significance to the control and treatment of coccidiosis.

CONCLUSIONS

We found that the expression of NF-κB signaling pathway in chicken's intestinal cells infected with coccidia sporozoites increased. Silencing TGF-β 4 gene could effectively repress NF-κB signaling pathway. Changes in immune factors may be related to the body's mechanisms that regulate TGF-β4 activity or neutralize its effects.

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