

Immunologic Improvement of Vaccine for Newcastle Disease via Co-Inoculation of Cattle Transfer Factor in Broilers

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Abstract

Background: Transfer factors (TFs), a novel immunostimulatory reagent, have found use as auxiliary or primary therapy for many diseases. The aim of this study was to explore whether TFs are able to strengthen immune responses of Newcastle Disease (ND) vaccines in broilers.

Results: The serum antioxidant status was increased in TF-treatment broilers. TF subcutaneous injection could significantly increase ($P < 0.5$) the antibody titers at 14 and 21 days of the experiment. Moreover, TF treatment increased development of organs of the immune system, such as spleen, thymus, and bursa of Fabricius through inhibition of apoptosis and promotion of proliferation. Cellular immune responses were found to have higher levels in the groups with TF co-inoculation compared to those groups only treated with the ND vaccine, showing phenomena of higher expressions of interleukin (IL)-2, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ mRNA in the thymus, spleen, and bursa of Fabricius. Moreover, the immunostimulatory effect of TF subcutaneous injection treatment is better than oral and intramuscular injection.

Conclusion: our findings suggest that TF treatment can improve antioxidant status and strengthen immune responses to ND vaccine, including antibody production and cellular immunity (lymphocytes proliferation and cytokines production) of the broilers, and the subcutaneous injection of TF is the appropriate inoculation way. Thus, TFs are a potent adjuvant and can serve as a medicine for immunoregulation.

Background

Transfer factors (TFs) are low molecular weight peptides or immune messengers [1]. It has demonstrated that TFs can transfer immunity mediated by cells from immune donors to the non-immune recipient. Previous studies have found the mechanism underlying TF-upregulated immune responses [2], including promoting thymocyte differentiation and maturation in T lymphocytes, restoring function of malfunctioning peripheral lymphocytes, recovering humoral immunity via B lymphocyte differentiation, forming specific humoral antibodies, increasing rejection capacity of allogeneic graft, producing lymphokine and increasing activity of mononuclear phagocytic systems [3].

Due to the immuno-regulation function, TFs have found use as auxiliary or primary therapy to treat viral, fungal, parasitic, and some bacterial infections [4–7]. Many clinical reports have established that TFs could function as a tool for prevention allowing immunity transfer prior to infections [4, 8]. It has been indicated by several clinical experiments that TFs can modulate immune systems [9–11]. Pizza et al. [12] and Roda et al. [13] have confirmed that the effectiveness of TFs in the treatment of viral hepatitis. Willeford et al. [8] have reported that TF treatment can against the toxic of Salmonella in mice, including reduction of the mortality and increase in INF- γ production. Several studies have reported that TFs can act as an adjuvant prompting a heightened immune response to the vaccine [14]. Wang et al. [15] have

found that levels of cellular immune responses were significantly enhanced in TF co-inoculated groups, when compared with PPV oil emulsion vaccine alone.

Here, broilers were inoculated with Newcastle Disease (ND) vaccines and TFs to explore whether, or not, TFs are able to strengthen immune responses of ND vaccines. We studied effects of TFs combining with ND vaccines on antibody tiers of serum, the development of immune organs including apoptosis and proliferation of lymphocytes, and the cytokines production.

Meanwhile, in order to find out the appropriate TF treatment method, we compare the different of oral, intramuscular injection and subcutaneous injection. The work aimed to ascertain immune enhancing effects of TFs on ND vaccines and to provide theoretical supports for developing novel adjuvants for ND vaccines.

Materials And Methods

Animals

A total of 48 one-day-old healthy broilers were caged for 21 days and warmed with electrical heaters. Feed and water, in addition to experimental diets mentioned below, were offered *ad libitum*. Experiments relating to use of broilers and all procedures concerning animals were approved by the Animal Care and Use Committee, Sichuan Agricultural University. The diet used was a basal diet containing corn and soybean designed by the National Research Council [16].

All experiments with animals were carried out in accordance with guidelines recommended by the Animal Care and Use Committee, Sichuan Agricultural University

Chemicals

Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Bioengineering Institute of China (Nanjing, China). PE Annexin V Apoptosis Detection Kit (BD Biosciences, USA). Anti-proliferating cell nuclear antigen (PCNA) (1:100) antibody (Boster, Wuhan, China)

TF preparation

Preparation of TFs followed the method proposed by Wang et al. [17], with some modifications. Briefly speaking, frozen cattle spleens underwent mechanical crashing, centrifugation, filtration and ultrafiltration in succession, to then prepare non-specific TFs, followed by quality examination. All laboratory experiments conformed to China Biologicals Regulations. Concentration of polypeptides was detected as 2 mg/ml through conducting biuret reactions [19].

Experimental design

Four groups were formed by the 48 one-day-old healthy broilers (N = 12). Table 1 lists the treatment schedule and the treatment time is showed in Fig. 1. After 7 days of acclimatization, all the broilers were inoculated the ND vaccine. Group I is the control group, which didn't treat with TF. Some 0.2 ml TFs were added in groups II, III and IV by oral, intramuscular injection and subcutaneous injection, respectively. Samples were collected at days 14 and 21 during experiments.

Table 1
Treatment for the different groups

Design	Incubation	TF administration method	Dose per chicken
Group I	Vaccine		
Group II	Vaccine + TF	Oral administration	0.2 ml
Group III	Vaccine + TF	Intramuscular injection	0.2 ml
Group IV	Vaccine + TF	Subcutaneous injection	0.2 ml

Table 2
Primer sequences of genes selected for analysis by qRT-PCR

Target gene	Accession number	Primer	Primer sequence (5'-3')	Product size	T _m (°C)
IL-2	AF000631	Forward	TCTGGGACCACTGTATGCTCT	138 bp	60
		Reverse	ACACCAGTGGGAAACAGTATCA		
IL-6	AJ309540	Forward	AATCCCTCCTCGCCAATCTG	106 bp	60
		Reverse	GCCCTCACGGTCTTCTCCATA		
IL-8	HM179639	Forward	CTGGCCCTCCTCCTGGTT	105 bp	60
		Reverse	GCAGCTCATTCCCCATCTTTAC		
IL-10	AJ621614	Forward	CGGGAGCTGAGGTGAA	192 bp	55
		Reverse	GTGAAGAAGCGGTGACAGC		
INF- γ	Y07922	Forward	AGCTGACGGTGGACCTATTATT	157 bp	58
		Reverse	GGCTTTGCGCTGGATTC		
TNF- α	NM204267	Forward	CCCCTACCCTGTCCCACAA	100 bp	58
		Reverse	TGAGTACTGCGGAGGGTTCAT		
β -actin	L08165	Forward	TGCTGTGTTCCCATCTATCG	178 bp	62
		Reverse	TTGGTGACAATACCGTGTTCA		

Serum antioxidant parameters detection

At 14 and 21 days, the e blood samples were taken from retro-ocular artery. The serum was obtained by blood centrifugation ($3,500 \times g$, 15 min). The serum SOD, CAT, GSH-Px activity and GSH contents were detected by biochemical methods according to the instructions of the reagent kits.

Serum antibody assay

At days 14 and 21 during experiments, serum samples were collected from six broilers from each group. The ND antibody titer was determined using a hemagglutination inhibition test.

Apoptosis evaluation based on flow cytometry

At days 14 and 21 during the experiment, we selected six broilers from each group to determine apoptosis in the spleen, thymus, and bursa of Fabricius through use of flow cytometry. The method description can refer to Guo et al. [18]. A cell suspension was formed by grinding samples of the spleen, thymus, and bursa of Fabricius and then filtered using nylon screens (350 mesh). After being washing twice with cold phosphate buffer solution (PBS; pH 7.2–7.4), the cells were then allowed to suspend in PBS to reach a final concentration of 1×10^6 cells/mL. After being transferred to 5 mL culture tubes, cell suspension (100 μ L) was stained by 7-aminoactinomycin (7-AAD) and PE Annexin V. The mixture experienced gentle vibration and incubation for 15 min in dark place. Thereafter, each tube was added with $1 \times$ binding buffer (400 μ L). Finally, the BD FACS Calibur flow cytometer was used to analyze rates of hepatic apoptosis.

Evaluation of proliferation by PCNA immunohistochemistry

In each group, six broilers were sacrificed humanely to perform gross examination at days 14 and 21 during the experiment. After being taken and fixed in 10% neutral buffered formalin, the spleen, thymus, and bursa of Fabricius were treated and trimmed, followed by paraffin embedding.

Description of the method can refer to Guo et al. [18]. Briefly, slices underwent xylene dewaxing, rehydration by a graded series of ethanol, washing with distilled water and PBS, and blocking for endogenous peroxidases through 15 min of incubation using 3% H_2O_2 in methanol. Antigen retrieval procedure was then undertaken on the slices via microwave processing in the sodium citrate buffer (0.01 M, pH 6.0). Before being incubated again at 37 °C in 10% normal goat serum for 30 min, the slices were washed again with PBS. Incubation of the slices lasted overnight at 4 °C with the anti-PCNA (1:100) antibody (Boster, Wuhan, China). The slices washed with PBS were allowed to expose to 1% biotinylated secondary antibody goat anti-mouse IgG (Boster, Wuhan, China) at 37 °C for 1 h, followed by incubation

with strept avidin-biotin complex (SABC) (Boster, Wuhan, China) at 37 °C for 30 min. For visualizing immunoreaction, diaminobenzidine hydrochloride (DAB) (Boster, Wuhan, China) was used to immerse the slices. Immediately after a brown color staining was visualized, the slices were observed under a microscope and stopped through immersion in distilled water. The slices were then subjected to light counterstaining by hematoxylin, dehydration by ethanol, xylene clearing and then mounted.

A computer-supported imaging system interfaced with an Olympus AX70 light microscope was used to count the PCNA protein expression. Quantification of integrate optical density (IOD) of each protein was conducted through use of the Image-pro Plus 5.1 (USA) as mentioned above. Five slices were observed in each group, with each slice observed with five visions and results averaged.

Quantitative real-time PCR

At days 14 and 21 during the experiment, six broilers were taken from each group to detect cytokines mRNA expression levels in the spleen, thymus, and bursa of Fabricius by using flow cytometry. Samples of the spleen, thymus, and bursa of Fabricius underwent homogenization and then were stored in liquid nitrogen for RNA extraction. The RNAiso Plus (9109; Takara, China) was used for extracting total RNA of the liver. A Prim-Script™ RT reagent Kit (RR047A; Takara, China) was utilized to synthesize the cDNA following the manufacturer's instructions. The qRT-PCR analysis was conducted with cDNA as the template. Primer sequences were attained from Genbank and NCBI. Primer 5 was used to design the primers, and primer synthesis was realized at Sangon Biotech (Shanghai, China). The SYBR® Premix Ex Taq™ system (DRR820A, Takara, Japan) was used for qRT-PCR on a Thermal Cycler (C1000, BIO RAD, USA). The $2^{-\Delta\Delta CT}$ method was used for analyzing relative expression of target genes [19].

Results

Changes in body weight and development of immune organs

In this study, we found that there are no changes in body weight and development of immune organs at day 14 during experiments. At day 21, body weight only significantly increased in the group IV (TF subcutaneous injection) ($p < 0.05$) compared to those in the control (Fig. 2a). The spleen, thymus, and bursa of Fabricius showed significant increments ($p < 0.05$) in relative weight relative to the control (Fig. 2a, b, c) at 1 days of experiment, and the group IV is the highest group, it seems that subcutaneous injection of TFs is the best way.

Histological changes in the immune organs

Thymus, spleen, and bursa of Fabricius are the main immune organs in broilers. Thymus, a specialized primary lymphoid organ, is the place for maturation of T cells. The histological results showed that the

lymphocytes numbers of cortex and medulla were increased in the TF treatment groups (III, IV) when compared with that in the non-TF treatment group at 14 and 21 days of experiment (Fig. 3a). Spleen is a peripheral lymphoid organ and substantially associated with humoral immunity and cellular immunity through B and T lymphocytes. There are no significant changes among TF-treatment and non-TF-treatment groups (Fig. 3b). As a unique lymphoid organ in birds, bursa of Fabricius is a primary site for differentiation, development and maturation of B lymphocytes, and antibody production. The histological results showed that TF treatment (III, IV) promote the numbers of lymphocytes in bursa of Fabricius at 21 days of experiment (Fig. 3c).

The changes of serum antioxidant capacity

As shown in Fig. 4, the serum antioxidant enzymes SOD, CAT and GSH-Px activity of TF-treatment groups were significant ($p < 0.05$) increase in comparison with those in the control group. SOD, CAT and GSH-Px are portion of free radical-scavenging enzymes and can ameliorate the damage effects of ROS by converting them into oxygen and then into water. Meanwhile, the GSH contents were significant ($p < 0.05$) higher increase in TF-treatment groups than those in the control group (Fig. 4d) at 14 and 21 days of experiment.

Changes in ND antibody titers

To examine the effect of the TF on the new castle disease vaccines responses, the new castle disease HI antibody titers were detected. The antibody titer was found to be highest in the group IV, which was higher ($P < 0.05$) than groups I, II, and III at days 14 and 21 of the experiment. The groups II and III displayed higher antibody titers relative to the group (I) only receiving vaccine on days 14 and 21 of the experiment, with no significant difference ($P > 0.05$).

Changes in apoptosis in the immune organs of broilers

Results in the current study indicated that apoptosis involved in the effect of TFs on immune function. From Fig. 4, no significant difference was found in thymus and spleen across four groups at day 14 of the experiment. Apoptotic percentages in bursa of Fabricius in the group III and IV were significant lower ($P < 0.05$) compared with groups I and II at day 14 during experiments. At day 21, the percentage of apoptosis in the spleen, thymus, and bursa of Fabricius was significant decreased ($P < 0.05$) in TF-treated groups (II, III, and IV) in comparison with that in the group (I) not treated with TFs. The TF subcutaneous injection (group IV) treatment method is better than oral (groups II) and intramuscular injection (group III).

The effect of TFs on the cell proliferation of immune organs of broilers

Above-mentioned results showed that TF treatment can inhibit the apoptosis of the spleen, thymus, and bursa of Fabricius. Here, we detected whether TFs influence the cell proliferation of the immune organs. PCNA is a proliferation marker and plays a critical part in many cellular processes. As shown in Fig. 5, the PCNA protein expression levels were significant increase ($P < 0.05$) in TF treatment groups (II, III, and IV) relative to that in the group (I) not treated with TFs in the spleen, thymus, and bursa of Fabricius. And, the TF subcutaneous injection (groups IV) treatment method is better than oral (groups II) and intramuscular injection (groups III).

The effect of TFs on the mRNA expression of cytokines

Cytokines are key components in immune functions. The research found that TF-treatment also can increase the cytokines production. As shown in Fig. 6, the IL-2, IL-6, IL-8, IL-10, TNF- α , and IFN- γ mRNA exhibited significant higher expressions ($P < 0.05$) in groups (II, III, IV) treated with TFs relative to those in the non-TF treatment group (I) in the thymus, spleen, and bursa of Fabricius at days 14 and 21 during the experiment. And, the TF subcutaneous injection (group IV) treatment method is better than oral (groups II) and intramuscular injection (group III).

Discussion

It has been several decades since the both clinical and basic science researches in the immunostimulatory function and application of TFs [20]. To date, it has been evaluated and confirmed in several animal researches that TFs can enhance the ability to challenge infectious disease and cancer [21].

In the present experiment, we investigated TFs as an adjuvant for enhancing immunogenicity of ND vaccines in broiler models. Furthermore, we also compare the different of TF treatment (oral, intramuscular injection and subcutaneous injection) on the vaccine response, then find out the appropriate mode of administration. Firstly, the enhancement of serum antioxidant levels including antioxidant enzymes (SOD, CAT and GSH-Px) and GSH contents were observed in TF-co-treatment groups. These results demonstrated that TF can improve the ability to resist disease through stimulation of antioxidant activity. Next, the results suggested significant enhancement of immune responses by virtue of co-administration with TFs, especially in the TF subcutaneous injection group. All of the results made it clear that TFs imposed great adjuvant effects in strengthening efficacy of ND vaccines. In agreement with our results, Mohymen et al. [22] have also reported that the birds that treated with TFs could resist virus infection and have a high level of anti-adenovirus antibodies.

TFs were tested in this study as an adjuvant agent, we found that TF treatment can increase growth performance and the immune organs index. The important immune organ in the broilers was the thymus, spleen, and bursa of Fabricius, controlling production and maturation of lymphocytes. Next, we examined the reason of TF-promoted immune organs index. Because of the importance of the balance between cell apoptosis and proliferation for normal development of a diverse types of tissues, the ratio of proliferation

to apoptosis was detected in thymus, spleen, and bursa of Fabricius were detected. Apoptosis (programmed cell death) acts as a key part in terms of homeostasis and development of all multicellular organisms. The result suggested that the apoptotic percentages were significant decreased in the TF-treatment groups at 21 days of experiment, however, there are no changes at 21 days of experiment. Meanwhile, we detected the proliferation marker protein PCNA, the PCNA-positive cells were significant increased at 14 and 21 days of experiment. Consist with our findings, Holeva et al. [23] and Vetvicka et al. [24] also report that TFs can activated the proliferation of splenocytes. Our above results indicate that apoptosis inhibition and proliferation promotion are the basic mechanism of TF-induced immune organs development.

Antibody titers of serum are an indicator for measuring humoral immunity. It can be seen from the results that TF treated groups (II and III) did not differ significantly from the vaccine alone group (I), and ND antibody titer was significantly higher in TF groups (II, III, and IV) relative to the group (I) only receiving vaccines alone, however, only the increase of TF subcutaneous injection group (IV) was significant ($P < 0.5$). These indicated that TFs could significantly affect immune functions mediated by B cells in the broilers. In contrast with our results, Wang et al. [15] and Lawrence [25] reported that TFs do not show significant influences upon immune functions mediated by B cells.

Cellular immunity is regarded as an important factor for clearance of chronic virus infections. It has indicated that TFs can strengthen cellular immunity [15, 26, 27]. Previous study has demonstrated that CD3+, CD3 + CD4 + and CD3 + CD8 + T lymphocyte percentages in peripheral blood of broilers treated with TFs [28]. It is also worth noting that the T lymphocyte has functions in both antibody generation and cellular immunity. Generally, T lymphocytes fall in two subsets: Th1 and Th2 cells [29]. The former has contributions to differentiation of cytotoxic T cells and can mediate immune responses of cells. Th1 cytokines comprise IL-2, IFN- γ , and TNF. The latter has a wide range of secrete, such as IL-4, IL-5, IL-6, IL-10 and IL-13, and facilitate the proliferation and differentiation of B cells, as well as the up-regulation of antibody production. In this study, TF treatment can increase mRNA expressions of cytokines of Th1, including IL-2, IFN- γ , and TNF- α , and those of Th2, such as IL-6, IL-8 and IL-10, in the spleen, thymus, and bursa of Fabricius of broilers. Moreover, the change in IFN- γ is more sensitive than others. These results made it clear that TFs could enhance immune responses of ND vaccines in cells, implying that responses mediated by T cells have direct influences on immune systems against ND. The result conforms to several studies that TFs as an immunoenhancement can rise IFN- γ production [15, 30, 31].

Conclusion

In conclusion, above-mentioned results suggest that TF treatment improves antioxidant activity and immune responses to ND vaccines, including antibody production and cellular immunity (lymphocytes proliferation and cytokines production) of the broilers, and the subcutaneous injection of TFs is an appropriate inoculation way. Thus, TFs are a potent adjuvant and can serve as an agent for immunoregulation.

Abbreviations

TFs: Transfer factors; ND: Newcastle Disease; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; GSH-Px: glutathione peroxidase; PCNA: proliferating cell nuclear antigen

Declarations

Ethics approval and consent to participate

All experimental procedures involving the use of broilers were approved by the Animal Care and Use Committee of Sichuan Agricultural University. As suggested by the animal welfare protocol, all efforts were made to reduce animal suffering and to use only the number of animals required to produce dependable scientific data.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared no conflict of interest.

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Author Contributions

H. Guo, W. Lai and K. Wang designed and performed experiments, collected and analyzed data, and wrote the paper. X. Li, X. Li, X. Jiang, Y. Geng, P. Ouyang, Y. Xie, G. Yang, X. Gu, R. He, and H. Tang performed experiments, collected and analyzed data. All authors contributed discussions and interpretations.

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Figures

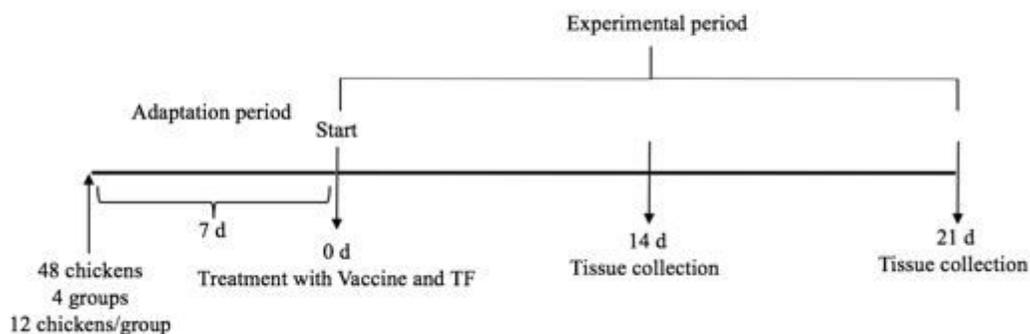


Figure 1

Experimental design of the trial. 48 one-day-old healthy broilers were divided into four groups, after 7 days of acclimatization, all the broilers were inoculated the ND vaccine. Group I is the control group, which didn't treat with TF. Group II, III and IV were added 0.2 ml TF by Oral, intramuscular injection and subcutaneous injection respectively. Samples were collected at the 14 and 21 days of the experiment.

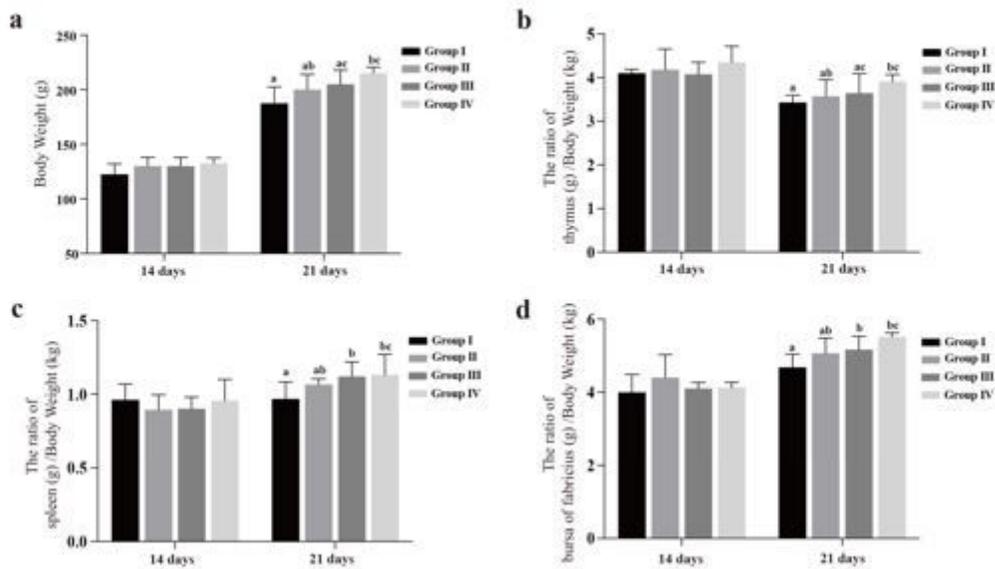


Figure 2

The changes of body weight and relative weight of immune organs. (a) The changes of body weight. The relative weight of thymus (b), spleen (c), and bursa of fabricius (d) of broilers. Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.

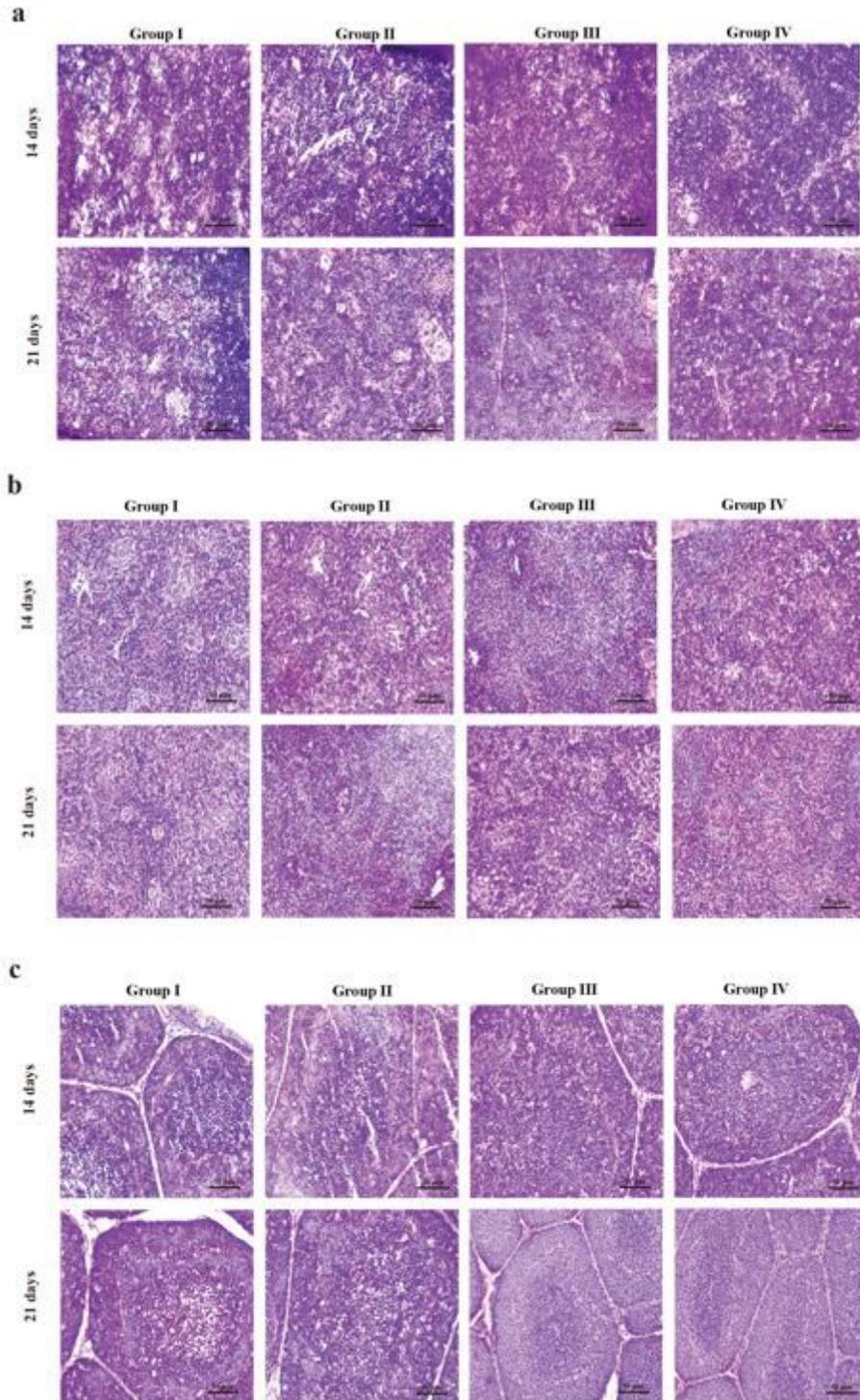


Figure 3

Histological changes in the immune organs of broilers. The histopathological changes in thymus (a), spleen (b), and bursa of fabricius (c) of broilers.

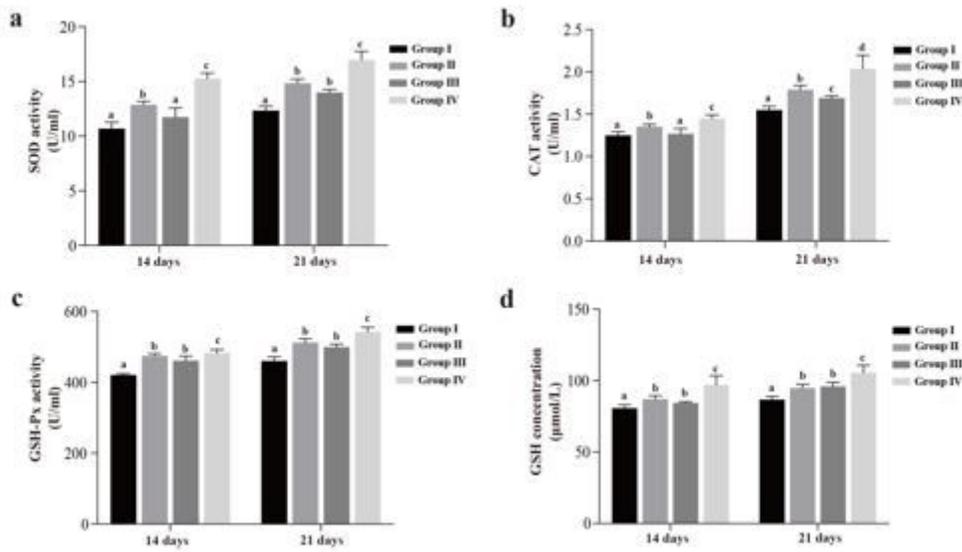


Figure 4

The changes of antioxidant capacity. The changes of serum SOD activity (a), CAT activity (b), GSH-Px activity (c) and GSH concentration. Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.

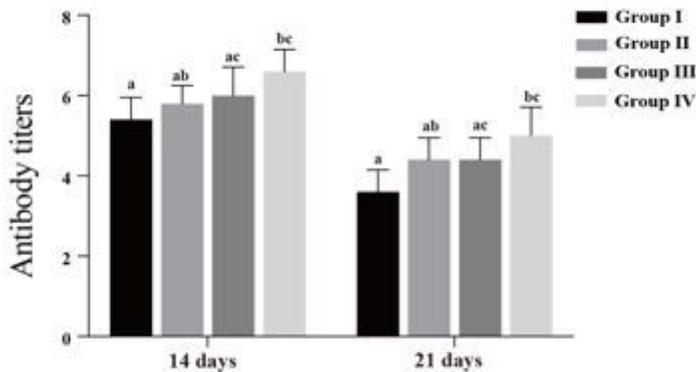


Figure 5

The changes of ND antibody titers. Broilers were immunized with ND vaccine with or without TF. Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.

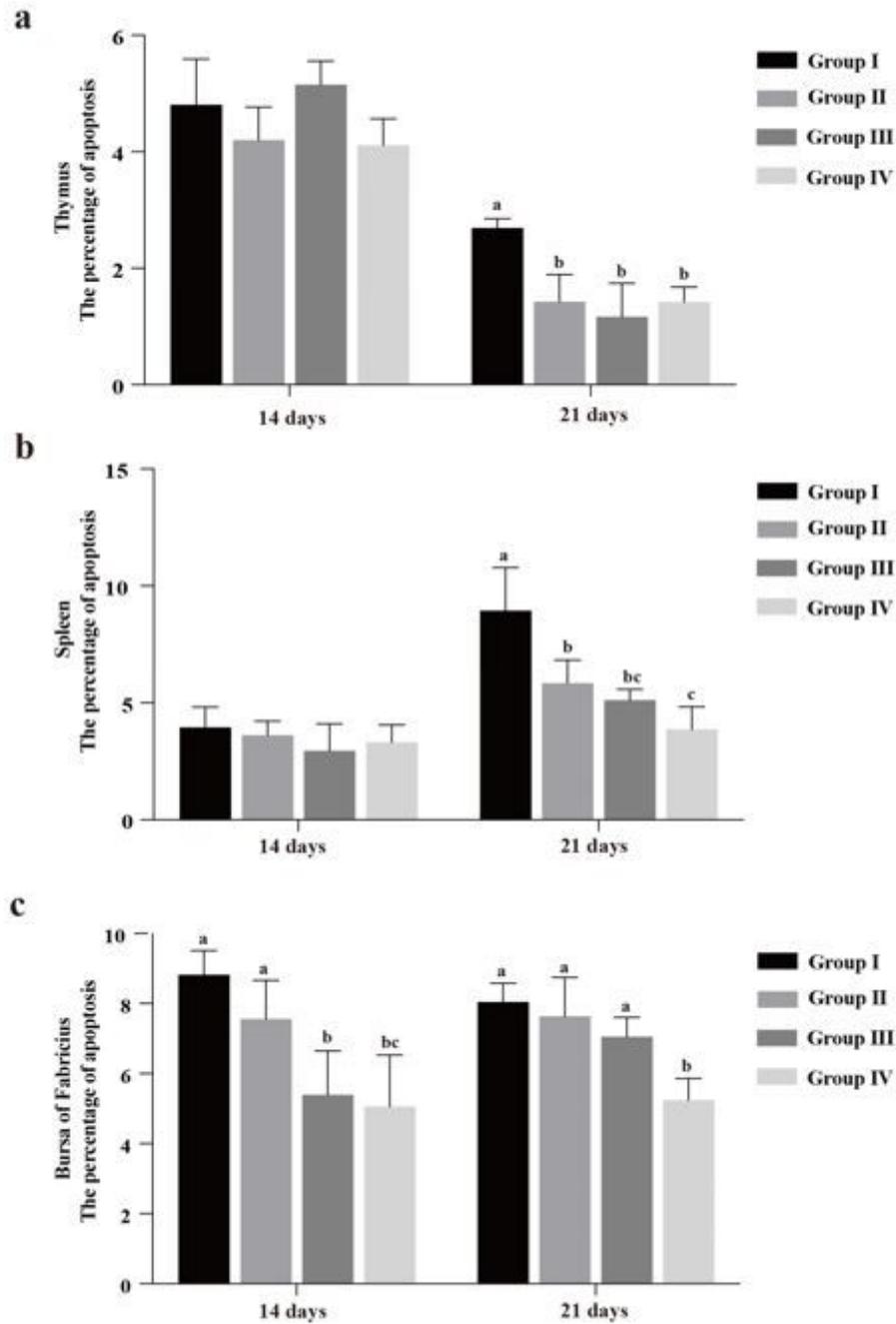


Figure 6

The changes of apoptosis in the immune organs of broilers. The changes of apoptosis in thymus (a), spleen (b), and bursa of fabricius (c) of broilers Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.

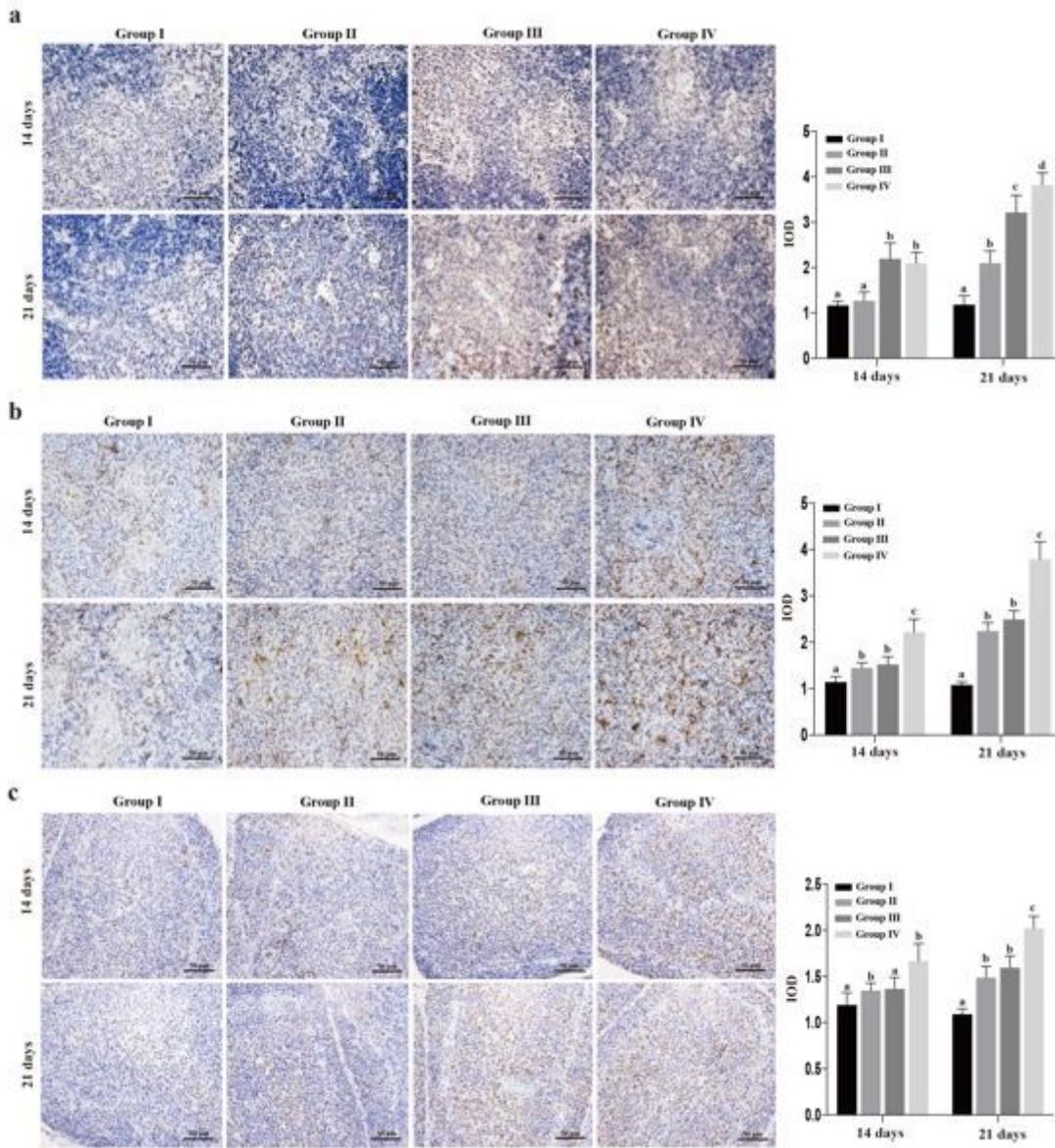


Figure 7

Immunohistochemical analysis of PCNA in the immune organs of broilers. The result of PCNA staining and quantification in thymus (a), spleen (b), and bursa of fabricius (c) of broilers. Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.

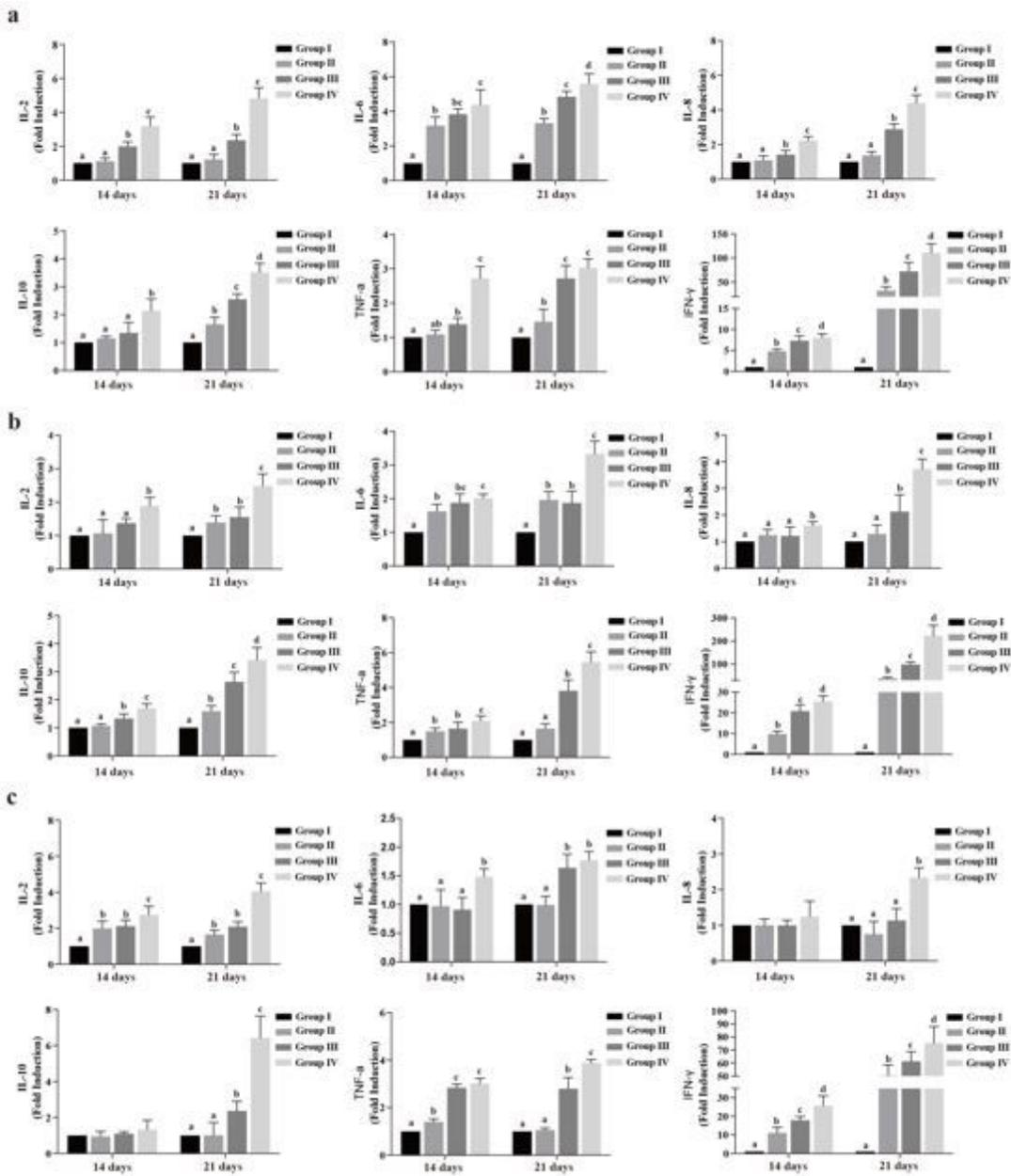


Figure 8

The changes of cytokines mRNA expression in the immune organs of broilers. The result of IL-2, IL-6, IL-8, IL-10, TNF- α , IFN- γ mRNA expression in the thymus (a), spleen (b), and bursa of fabricius (c) of broilers. Data are presented with the means \pm standard deviation (n=6). The letters a, b, c, and d represent a difference ($p < 0.05$) among the four groups.