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Research paper

Different doses of tannin reflect a double-edged impact on broiler chicken immunity

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ABSTRACT

Tannin is one of the most common phytochemical secondary phenolic metabolites, which is widely distributed in various plant production systems. Dietary intake of tannin can exert different actions on the immune system. The aim of this study was to evaluate the influence of different concentrations on broiler chicken immunity. Three groups (n = 10 in each group) were evaluated: control group given a normal basal diet, high tannic acid (HT) group given high-dose tannic acid (30 g/kg diet) and low tannic acid (LT) group given low-dose tannic acid (0.5 g/kg diet) for 35 days. Feed intake and body weight were measured weekly. Cells were isolated from thymus, spleen and caecal tonsils at the end of the study. Lymphocyte subsets, monocytes phagocytosis and cytokine mRNA expression in spleen were evaluated. The results showed that HT group chickens had decreased daily gain, final body weight, daily feed intake and relative weights of lymphoid organs compared with other groups. In thymus, spleen and caecal tonsils, relative $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ cell populations in the HT group were significant decreased in comparison with those of other groups. The relative B cell population in the HT group was also significant decreased. Cytokine mRNA expression in spleen cells of the HT group was also significantly lower than that in other groups. Conversely, $CD4^+CD8^+$ subpopulations in spleen and caecal tonsils, $\gamma\delta^+$ cells in spleen and B cells in caecal tonsils of the LT group were significantly higher than those of the control group. In addition, IFN-γ mRNA expression in the LT group was significantly higher than that of the control group. These results demonstrated that dietary intake of tannin had positive and negative effects on broiler chicken immunity in a dose-dependent manner.

1. Introduction

Tannins are important plant secondary metabolites that are widely distributed in various plants utilized for feed and food such as cereals, fruits and vegetables, and comprise a group of chemicals called polyphenols (Abo-Donia et al., 2017; Huang et al., 2018; Zhang et al., 2018; Valenti et al., 2019). Tannins are categorized as hydrolysable or condensed tannins (Lee et al., 2010; Parisi et al., 2018) and largely impact animal health in a dose-dependent manner (Makkar, 2003; Barszcz et al., 2018). Feeding farm animals with excess amounts of tannin impairs animal performance and feed intake (Barszcz et al., 2011; Amesa and Asfaw, 2018).

Previous studies have shown that livestock fed diets containing excess amounts of tannin isolated from feedstuffs or with commercial tannins have decreased growth rate and feed intake, enhanced loss of essential vitamins and minerals, impaired immune response and increased liver and protein destruction (Lee et al., 2010). Furthermore, rats fed 10–25 g tannic acid (TA) /kg diets developed iron deficiency anemia (Afsana et al., 2004). TA effects on farm animals can vary from positive to toxic. Higher TA doses induce a stress condition in chickens, which might be involved in immune system impairment (Kaleem et al., 2014; Huang et al., 2018).

Several recent studies have revealed dietary intake of low amounts of tannins from several sources improved health status, nutrition and animal performance (Brus et al., 2013; Huang et al., 2018). Interestingly, chickens fed a diet supplemented with tannin obtained from chestnuts had enhanced feed efficiency (Schiavone et al., 2008; Starcevic et al., 2015). The reasons for differences in results could be different sources of tannin, animals species, health status and tannin dietary concentration (Clauss et al., 2007; Jamroz et al., 2009).

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Immunosuppressive effects of chicken fed 25 or 30 g TA/kg diet included extremely decreased numbers of white blood cells (WBCs), and serum IgM and IgY levels were decreased in a dose-dependent manner (Marzo et al., 1990). Conversely, exposure of chickens to a low concentration of condensed tannin by oral administration increased proliferation of peripheral blood mononuclear cells (PBMCs), splenocytes and thymocytes. In addition, chicken administered low doses of tannin orally for 5 weeks had increased $CD4^+$ and Bu-1^+ cell populations in the spleen (Park et al., 2013). Another study showed that *Emblica officinalis* containing low concentrations of tannin had an immunomodulatory effect by stimulating T cells and macrophages and potentiated effects on humoral immune responses in chickens (Madhuri et al., 2011; Kaleem et al., 2014).

However, effects of high- and low-dose TA orally administered on cell-mediated immunity and cytokine mRNA expression have not yet been studied. Therefore, the purpose of this study was to evaluate the effects of high and low TA concentrations orally administered on broiler chicken immune status. We described here that low-dose TA partly improves the immune response, however, high-dose TA decreased cell-mediated immunity and immune response in broiler chickens.

2. Material and methods

2.1. Animals, diets and experimental design

Thirty newly hatched broiler chickens (male chunky strain) were obtained from a local hatchery (Miyazaki, Japan). Chickens had a body weight of 40–50 g after 7 days acclimatization with free access to diet and drink. Chickens were randomized into three groups: control group (n = 10), fed a basal diet; high tannic acid (HT) group (n = 10), basal diet supplemented with 30 g TA/kg; and low tannic acid (LT) group (n = 10), basal diet supplemented with 0.5 g TA/kg diet for 35 days. TA was purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Practical grade, Osaka, Japan). All groups were fed mashed diets that were formulated to meet nutrient requirements of broiler chickens. The basal diet contained crude protein (> 17 %), crude fat (> 3 %), crude fiber (< 6 %), crude ash (> 13 %), calcium (> 3.1 %), phosphorus (> 0.45 %) and metabolizable energy (> 2,850 Cal/kg).

At 21 days of experiment, all chickens were intravenously immunized with 200 μg of keyhole limpet hemocyanin (KLH; Fujifilm Wako Pure Chemical Co., Ltd.). After 7 days, blood was collected to measure primary immune responses, and all chickens received a secondary immunization of 200 μg of KLH. At the end of the experiment, a blood sample was collected from each chicken to evaluate secondary immune responses.

The Animal Care and Use Committee of the Feed Research Institute of the University of Miyazaki approved all experimental procedures (No. 2018-039-1). Feed intake and body weight were recorded weekly for all chickens.

2.2. Sample collection

Thymus, spleen and caecal tonsil (CT) were removed and weighed after dissection.

2.3. Preparation of cells

Small pieces of each lymphoid organ were removed and cut into pieces. A cell suspension was prepared by mincing tissue in ice-cold Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) and removing residual tissue by a cell-strainer to obtain a single-cell suspension. The cells were purified by density gradient centrifugation with Ficoll-Paque (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, U.K.). The cells were then washed with phosphate-buffered saline (PBS) and erythrocytes were lysed with NH_4Cl lysis buffer. Cells were suspended in RPMI 1640 medium (Fujifilm Wako Pure Chemical Co., Ltd.) supplemented with 10 % fetal calf serum and antibiotics. The cell number was adjusted to 3.0×10^6 cells/ml.

2.4. Lymphocyte subsets

For immunofluorescence assays, cells were suspended in PBS supplemented with 0.5 % bovine serum albumin and 0.05 % sodium azide (BSA-PBS). Viable cells ranging from 1×10^5 to 1×10^6 were incubated with fluorescence-labeled monoclonal antibodies (mAb) as described below at 4 °C for 1 h. Stained cells were washed three times with BSA-PBS and re-suspended in BSA-PBS containing propidium iodide (10 μg/ml, Sigma-Aldrich). Relative immunofluorescence intensities were determined by flow cytometry using the FACS Canto™ II system (Becton Dickinson, Franklin Lakes, NJ, USA). Anti-CD4 (400× dilution, CT4, Southern Biotech, Birmingham, AL, USA), anti-CD8 (400× dilution, CT8, Southern Biotech), anti-γδ (400× dilution, TCR1, Southern Biotech), anti-MHC class II (100× dilution, 2G11, Southern Biotech) and anti-Bu-1b (200× dilution, 5-11G2, Southern Biotech) mAb were used. For fluorescence labeling of mAb, FITC labeling kit-NH_2, HiLyte™ Fluor 555 labeling kit-NH_2 and HiLyte™ Fluor 647 labeling (F647) kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan) were used according to the manufacturer's instructions.

2.5. Phagocytosis assay

Spleen cells were suspended in RPMI 1640 medium supplemented with 10 % fetal calf serum and antibiotic then incubated with 1 μl of 2.5 % FITC-labeled latex bead suspension (1-μm diameter, L1030, Sigma-Aldrich). Cells were then incubated at 37 °C for 1 h in 5 % CO_2 humidified atmosphere. After incubation, cell-free beads were washed out with EDTA-PBS, and then cells were stained with F647 labeled anti-MHC class II (100× dilution, 2G11, Southern Biotech) mAb and analyzed by flow cytometry using the FACS Canto™ II system. Macrophage was gated according to their relative size (forward scatter) and complexity (side scatter). Phagocytic index of MHC class II^+ macrophage was reported as the ratio of MHC class II^+ $FITC^+$ (phagocytizing latex beads) cells to all MHC class II^+ cells in the fraction of macrophage.

2.6. Analysis of IL-1β, IL-2, IFN−γ, IL-4 and IL-10 mRNA expression in spleen cells using real-time PCR

Total RNA was extracted from spleen cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed using a one-step TB Green PrimeScript PLUS RT-PCR Kit (Takara Bio., Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR primers pairs were designed by using Oligo7 software (Molecular Biology Insights, Colorado Springs, CO, USA) and are described in Table 1. Real-time PCR conditions consisted of reverse transcription at 42 °C for 5 min and initial PCR activation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 57 °C for 30 s and 70 °C for 30 s, and a dissociation curve was added to the protocol. A real-time RT-PCR assay was performed using QuantStudio™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The mRNA expression level of each target gene was normalized against that of GAPDH. GAPDH expression levels in each sample were not different, therefore, we determined that GAPDH could be used as the reference gene for real-time PCR. Data were analyzed by the QuantStudio™ software (Applied Biosystems).

2.7. Enzyme-linked immunosorbent assay for anti-KLH antibody titers

Each well of a 96-well microplate (Nunc, Roskilde, Denmark) was coated with 60 μl of KLH (10 μg/ml) and incubated at 37 °C for 2 h. After washing with 0.05 % Tween 20-PBS, 1 % BSA-PBS was added to each well for blocking and incubated overnight at 4 °C. One hundred-fold diluted chicken sera were added to antigen-coated wells and

Table 1
Primer sequences used for real-time PCR.

Gene	Primer	Sequences (5′–3′)	Product size	Accession number
GAPDH	F	AAGCGTGTTATCATCTCAGCTC	162	NM204305.1
	R	AATGCCAAAGTTGTATGGAT		
IL-2	F	AATTAAAGAAGAATTGTAACTGC	145	AF000631.1
	R	GGTCATTCATGGAAAATCAG		
IL-1β	F	CTACACCCGCTCACAGTC	126	NM204524.1
	R	TTGAGCCTCACTTTCTGG		
IFN-γ	F	CATGATTTATTATGGACATACTGC	178	NM205149.1
	R	GCTCAGTATGATCCTTTTCTC		
IL-4	F	TGTGCCCACGCTGTGCTT	169	GU119892.1
	R	AACAATTGTGGAGGCTTTGCATA		
IL-10	F	CCACCTGCCTGCACTTCTCT	149	NM001004414.2
	R	CCCCTTAAACTCATCCAGCAGT		

incubated at 37 °C for 1 h. The wells were then washed, and 60 μl of goat anti-chicken IgM (1000×, Bethyl Lab. Inc., Montgomery, TX, USA) or goat anti-chicken IgY-Fc fragment (1000×, Bethyl Lab. Inc.) were added. After incubation at 37 °C for 1 h, the wells were washed, supplemented with 60 μl of peroxidase-labeled rabbit anti-goat IgG-Fc fragment (40000×, Bethyl Lab. Inc.) and then incubated at 37 °C for 1 h. The wells were washed, supplemented with 60 μl of substrate buffer of ELISA POD substrate ABTS (Nacalai Tesque, Inc., Kyoto, Japan) and then incubated for 10–30 min at room temperature. Optical density was measured at 405 nm using a microplate reader (Benchmark Plus- Bio-Rad Lab, Inc, USA).

2.8. Statistical analysis

Data obtained were subjected to analysis variance. Data were analyzed using statistical R software version 3.5.1. Comparison between normally distributed continuous experimental group was carried out by one-way analysis of variance (ANOVA), followed by Tukey's HSD for comparison of individual means. A p value < 0.05 was considered significant. Data are expressed as mean ± SEM.

3. Results

3.1. Effects of TA on body weight, daily gain, daily food intake and organ weight

Effects of TA on body weight, daily gain, daily food intake and organ weight are shown in Table 2. Initial body weight was not significantly different among the three groups (data not shown). The final body weight (42 days of age) of chickens in the HT group was significantly lower than that of chickens in control and LT groups ($p < 0.01$). Daily gain during the last week of experiment (35–42 days of age) was affected by dietary intake of tannin. Chickens in the HT group had a significant decrease in, daily weight gain compared with chickens in control and LT groups ($p < 0.01$). Furthermore, daily feed intake of chickens (35–42 days of age) in the HT group was significantly lower than that of chickens in control ($p < 0.01$) and LT groups ($p < 0.05$). There was a marked reduction in relative weights of bursa of Fabricius and CT ($p < 0.05$) in the HT group compared with those of the control group. Moreover, there was a significant decrease in relative spleen weight in the HT group compared with those of control ($p < 0.01$) and LT groups ($p < 0.05$).

Table 2
Effect of graded doses of tannic acid on body performance, feed consumed and weight of lymphoid organs.

Groups	Control	LT	HT
FBW (g)	2528 ± 9.9	2204 ± 7.6	419.4 ± 4.7[a]
ADG (g)	354.3 ± 9.9	229.5 ± 7.6	48.2 ± 4.7[a]
ADFI (g)	209.3 ± 3.9	206.8 ± 4.1	118.7 ± 4.1[a, b]
Organ weight (g)			
Bursa	3.20 ± 0.01	2.21 ± 0.02	1.72 ± 0.03[a]
Spleen	2.40 ± 0.01	2.30 ± 0.02	0.12 ± 0.01[a, b]
Cecal Tonsils	1.03 ± 0.01	0.50 ± 0.01	0.30 ± 0.01[a]

FBW: Final Body Weight on 42 day of age, ADG: Average Daily Gain, ADFI: Average Daily Feed Intake during last week of experiment (35–42 day of age) and the organ weight show relative weight of lymphoid organs of three groups were expressed as gram per 100 g of body weight. Data are reported as mean ± SEM. Uppercase letters (a: $P < 0.05$ vs control, b: $P < 0.01$ vs LT) indicated significantly different.

3.2. Effects of TA on lymphocyte subsets and phagocytosis

Relative $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ cell populations in the thymus are shown in Fig. 1. $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ subpopulations of chickens in the HT group were significantly lower than those of the control group ($p < 0.01$). There was no significant difference between control and LT groups, while chickens fed HT showed significant decreases in $CD4^+$ cell populations compared with control and LT groups ($p < 0.05$).

Relative $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ cell populations in spleen are shown in Fig. 2. $CD4^+$ cell populations of chickens in the HT group were significantly lower than those in the control group ($p < 0.01$). There was no significant difference in $CD4^+$ cells between control and LT groups. $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ subpopulations in the spleen of the HT group were significantly lower than those of control and LT groups ($p < 0.05$). Meanwhile, chickens in the LT group showed a significant increase in $CD4^+CD8^+$ and $\gamma\delta^+$ cell populations compared with those of the control group ($p < 0.01$).

Relative $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and $\gamma\delta^+$ cell populations in CT are shown in Fig. 3. Relative $CD4^+$ and $CD8^+$ cell populations in CT of the HT group were significantly decreased ($p < 0.01$ and $p < 0.05$,

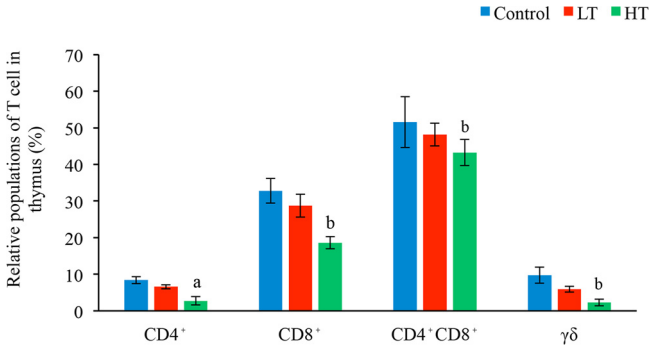

Fig. 1. Effect of tannic acid (TA) on relative T cell subsets in the thymus. Control group was fed a normal basal diet, LT group was fed a 0.5 g TA/kg diet and HT group was fed a 30 g TA/kg diet. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control) indicated significantly different.

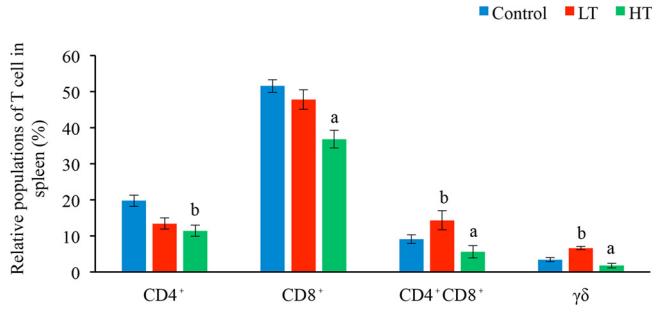

Fig. 2. Effect of tannic acid (TA) on relative T cell subsets in the spleen. Control group was fed a normal basal diet, LT group was fed a 0.5 g TA/kg diet and HT group was fed a 30 g TA/kg diet. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control) indicated significantly different.

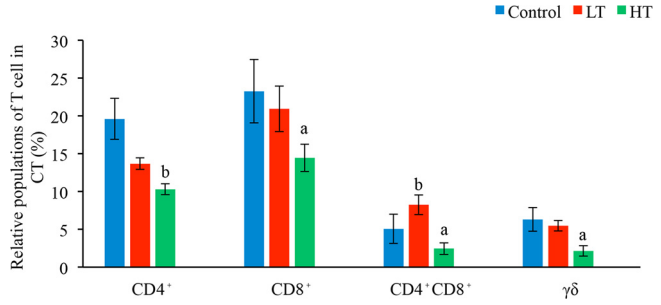

Fig. 3. Effect of tannic acid (TA) on relative T cell subsets isolated from caecal tonsil (CT). Control group was fed a normal basal diet, LT group was fed a 0.5 g TA/kg diet and HT group was fed a 30 g TA/kg diet. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control) indicated significantly different.

respectively) compared with that of the control group. The $CD4^+CD8^+$ population of CT in the HT group was significantly lower than those of other groups ($p < 0.05$). In addition, the $\gamma\delta^+$ cell population in CT of the HT group was significantly decreased compared with that of the control group ($p < 0.05$). Conversely, the $CD4^+CD8^+$ population in CT of the LT group was significantly higher than that of the control group ($p < 0.05$).

As shown in Fig. 4, the MHC class $II^+Bu1\text{-}b^+$ (B cell) population in the spleen of the HT group was significantly decreased compared with that of the control group ($p < 0.05$). In addition, the number of B cells in CT of the HT group was significantly lower than that of control and LT groups ($p < 0.01$). However, the B cell population in the LT group was significantly higher than that of the control group ($p < 0.05$). A significant difference in the percentage of macrophage phagocytosis among groups was not observed as shown in supplement data.

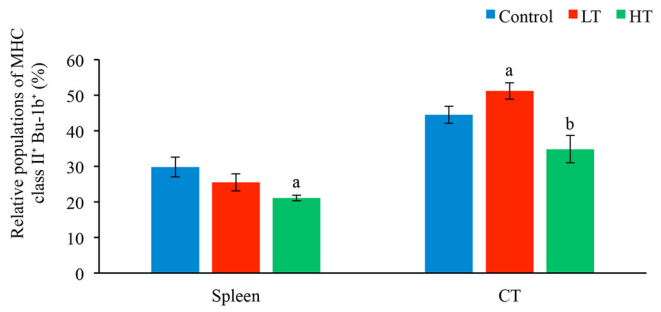

Fig. 4. Effect of tannic acid (TA) on relative B cell populations in spleen and caecal tonsil (CT). Control group was fed a normal basal diet, LT group was fed a 0.5 g TA/kg diet and HT group was fed a 30 g TA/kg diet. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control) indicated significantly different.

3.3. Effects of TA on IL-1β, IL-2, IFN-γ,IL-4 and IL-10 mRNA expression levels in spleen

As shown in Fig. 5, IL-1β mRNA expression levels in the HT group were remarkably decreased compared with that of control ($p < 0.01$) and LT groups ($p < 0.05$). Additionally, IL-2 mRNA expression levels were significantly decreased in the HT group compared with those of the control group ($p < 0.01$). The IFN-γ mRNA expression level in the LT group was significantly higher than that of the control group ($p < 0.01$). However, IFN-γ mRNA expression in the HT group was significantly decreased compared with that of control and LT groups ($p < 0.05$). IL-4 mRNA expression in the HT group was lower than that of other groups ($p < 0.01$). In addition, IL-10 mRNA expression level was not significant difference among group.

3.4. Effects of TA on primary and secondary immune responses

As shown in the upper figure of Fig. 6a, a significant difference in anti-KLH IgM in primary immune response between groups was not observed. However, the secondary immune response of anti-KLH IgM indicated significant suppression in the HT group compared with that of the control group ($p < 0.05$). As shown in the bottom figure of Fig. 6b, primary and secondary immune responses of anti-KLH IgY in the HT group were significantly decreased compared with those of control and LT groups ($p < 0.01$).

4. Discussion

Tannin is an anti-nutrient that has a complex and varied chemical structure and belongs to secondary metabolites, which are commonly distributed in the plant kingdom (Torregrossa and Denise Dearing, 2009; Lee et al., 2010; Parisi et al., 2018). Tannins have varied effects on animal health according to sources and concentrations of dietary tannin, animal species and health status (Clauss et al., 2007; Jamroz et al., 2009).

In the present study, final body weight and daily gain in the HT group was significantly lower than those in control and LT groups. Tannin may reduce body weight gain by its ability to form an insoluble protein complex with many hydroxyls groups, causing nutrients to be less digestible (Afsana et al., 2004; He et al., 2007; Jamroz et al., 2009). Moreover, reduction of both duodenal villus height and crypt depth induce mucosal necrosis in animals fed high-dose TA (Nyamambi, 2007; Torres et al., 2013). It is possible that decreased feed intake in the HT group could be attributed to tannins with a stringent taste, which reduces palatability and feed consumption (Mueller-harvey, 2006; Huang et al., 2018).

This study also demonstrated that high-dose TA supplementation in the diet can reduce relative weight of the bursa of Fabricius, spleen and CT, indicating that TA might affect the development of primary and secondary lymphoid organs. Chickens receiving the highest dose of TA have reduced liver weight (Marzo et al., 1990, 2002; Barszcz et al., 2018). High-dose TA caused serious systemically decreased growth and affected the development of primary and secondary lymphoid organs. However, further detailed analysis of the effect of high-dose TA on decreased growth of chicken by organ histology and biochemical examination of blood is needed.

This study also investigated the effect of tannin on relative T and B cell subsets and revealed these cell populations were reduced in the HT group compared with those of the other groups. The marked reduction of T and B cell subsets could be associated with the suppression of development of primary and secondary lymphoid organs. We speculate that TA forms an insoluble toxic complex with protein in the intestine, which causes a deficiency in protein utilization, leading to impaired immune function (Marzo et al., 1990). In addition, TA decreases the intestinal absorption of essential amino acids, minerals and vitamins. Therefore, protein synthesis in lymphoid organs might be diminished

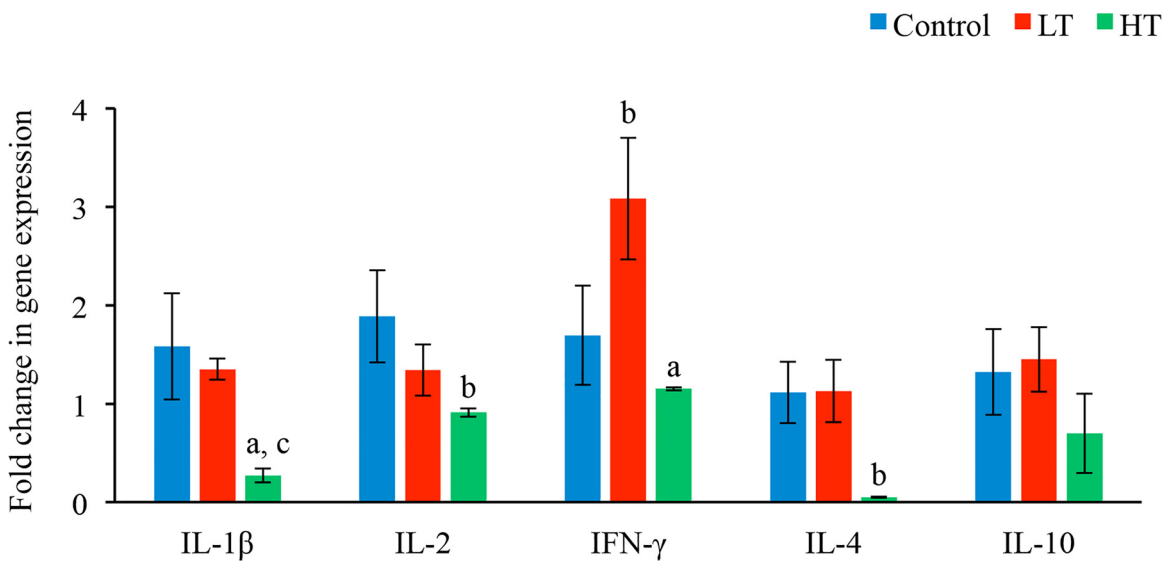

Fig. 5. IL-1β, IL-2, IFN-γ, IL-4 and IL-10 mRNA expression levels in spleen. Total RNA was obtained from spleen tissue of chickens in control, LH and HT groups. Expression levels were measured by real-time PCR. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control, c: $p < 0.01$ vs LT) indicated significantly different.

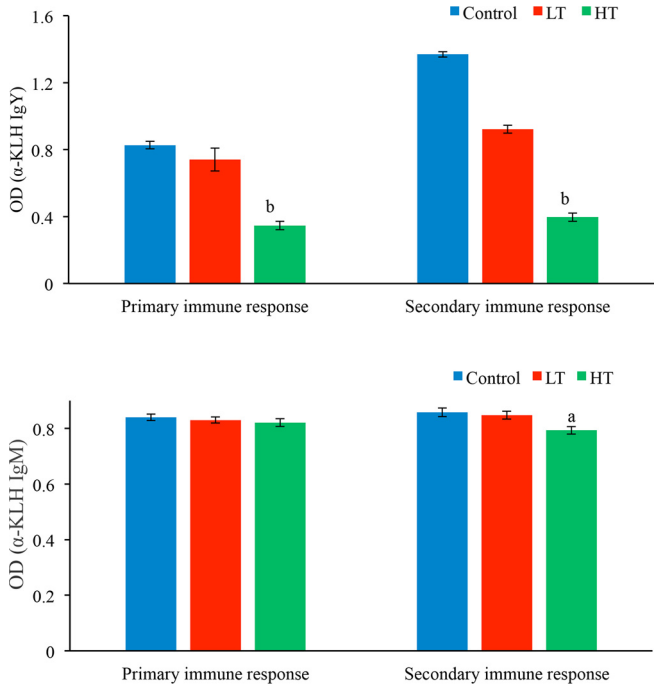

Fig. 6. Anti-KLH specific IgM and IgY immune responses. The upper figure shows primary and secondary IgM responses to KLH in chickens. The bottom figure shows primary and secondary IgY responses to KLH in chickens. Control group was fed a normal basal diet, LT group was fed a 0.5 g TA/kg diet and HT group was fed a 30 g TA/kg diet. Chickens were injected with 200 μg of KLH at 21 days of experiment. After 7 days, sera for primary response evaluation were collected and chickens were again injected with 200 μg of KLH. After 7 days, sera for secondary response evaluation were collected. Data represent mean ± SEM. Letters (a: $p < 0.05$ vs control, b: $p < 0.01$ vs control) indicated significantly different.

(Maggini et al., 2007). Chickens are stressed by high-dose tannin, which elevates corticosterone levels in plasma that might be involved in the impairment of the immune system (Marzo et al., 1990; Chung et al., 1998; Franchimont et al., 2000; Huang et al., 2018).

Our results also showed that low-dose TA increased some lymphocyte subsets such as $CD4^+CD8^+$ cells and γδ T cells in spleen and $CD4^+CD8^+$ cells and B cells in CT compared with those of the control group. In chicken CT, microbiota regulate the population of $CD4^+CD8^-CD25^+$ and $CD4^+CD8^+CD25^+$ T cells and have important role for gut immunity (Lee et al., 2018). However it needs further detail study about relationship among kinetics of these subsets, microbiota and tannin. Peripheral blood and secondary lymphoid tissues of swine, chickens and monkey have a substantial number of $CD4^+CD8^+$ cells (Zuckermann, 1999). Moreover, a large $CD4^+CD8^+$ cell population was found in the peripheral blood (20 ± 40 %), spleen (10 ± 20 %) and intestinal epithelium (5 ± 10 %) of chickens. These double positive lymphocytes have been shown to proliferate in response to mitogen stimulation and signals delivered through their T cell receptor (Luhtala et al., 1997). In chicken spleen, the proportion of $CD4^+CD8^+$ cells increased after hatching, and these cells are defined as mature antigen specific effector memory cells that have the ability to secrete IL-2 and IFN-γ (Zuckermann, 1999; Nascimbeni et al., 2004).

In the present study, a marked decline of IL-1β, IL-2, IFN-γ and IL-4 mRNA expression in the spleen was found in the HT group. Cytokines act as important modulators of immune function and have a potential role in the control of infectious diseases in poultry (Wigley and Kaiser, 2003). IL-1β is a pro-inflammatory cytokine, which induces activation, proliferation and differentiation of T cells into effector cells (Madrigal-Estebas et al., 2002; Wigley and Kaiser, 2003). Chicken IL-2 is referred to as T cell growth factor and produced by activated T lymphocytes (Choi and Lillehoj, 2000; Girish and Smith, 2008). IFN-γ, produced mainly by T and NK cells, is an organizer of various immunological functions such as immunomodulation and leukocyte trafficking (Schroder et al., 2004). IL-4 plays a crucial role in immunoglobulin class switching (Tangye et al., 2002; Blanchard et al., 2004). The reduction mRNA expression of cytokines in the HT group indicated impairment of the immune function of splenocytes in broilers chicken. One of the mechanisms may have been due to the decreased proportion of T cell subsets. In contrast, chickens in the LT group had increased IFN-γ mRNA expression level compared with that of the control group. This result might reflect the increasing γδ T cell population in the spleen.

Noticeable differences were observed in both primary and secondary immune responses, as measured by anti-KLH IgM and anti-KLH IgY antibody titers, and were reduced in chickens of the HT group. These findings are consistent with previous studies that showed high-dose TA fed chickens had impaired humoral immunity and decreased

numbers of total WBCs and lymphocyte populations (Marzo et al., 1990). This impaired humoral immunity might be attributed to the fact that TA impairs intestinal utilization of essential elements such as sugars, amino acids, trace elements and vitamins, thereby forming insoluble tannin complexes (Marzo et al., 2002; Makkar, 2003). Therefore, lack of these essential nutrients probably inhibits antibody production (Kaminogawa and Nanno, 2004; Maggini et al., 2018). T cell suppression results in decreased cytokine production related to B cell differentiation and IgG class switching (Gore, 2006; Imamura et al., 2009; Kawai et al., 2013). Especially, IL-4 is a very important cytokine for antibody production (Amoils, 2006). In the present study, reduced IgY titer was confirmed along with decreased IL-4 mRNA expression.

In conclusion, dietary TA positively and negatively affects immune status of broiler chickens in a dose-dependent manner. Thus, low-dose tannin partly improves the immune response, whereas high-dose tannin decreases T and B cell subsets and suppresses humoral immunity and cytokine mRNA expression in the spleen. Overall, high tannin concentration suppresses immune response in broiler chickens.

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Appendix A. Supplementary data

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