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Immune responses to genestein in male broiler chicks

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This study was conducted to evaluate the effect of dietary genistein (Gn) on broiler immune system. A corn-soybean meal basal diet was supplemented with five genistein levels (10, 20, 40, 80 and 160 mg of Gn/kg) in a completely randomised design with 5 replicates of 10 birds. Dietary control treatments were included the negative control diet with no feed additive supplementation and two positive control groups supplemented with either virginiamycin or zinc bacitracin. Three hundred and fifty day-old male broiler chicks were fed with the experimental diets from 1 to 42 days of age. On days 13 and 23, chicks were vaccinated against Newcastle disease virus (NDV) and infectious bronchitis virus disease (IBV), respectively. The 10 chicks from each dietary treatment (two per pen replicate) were bled on day 6 and 12 after vaccinations. Sera samples were use in hemagglutination inhibition test for NDV and ELISA test for IBV. Dietary supplementation with 20 mg/kg Gn caused increases ($p < 0.01$) in antibody titres against Newcastle and infectious bronchitis diseases viruses at day 12 after vaccine administration. However, diet modifications had no significant effect on blood leukocyte sub-populations and heterophil to lymphocyte ratio. The present results suggest that dietary supplementation with genistein especially at the levels of 20 and 40 mg/kg can improve immunological responses of broiler chicks.

Keywords: genistein; immune responses; broilers; zinc bacitracin; virginiamycin

Introduction

Genistein (4′,5,7-trihydroxyisoflavone) is a natural isoflavone phytoestrogen present in soybean seeds and flour, and the structurally similar coumestrol is found in alfalfa (Franke et al. 1995). Genistein has been recognised as an inhibitor of tyrosine kinases (Setchell and Cassidy 1999). In vitro studies demonstrated that high levels of genistein could reduce macrophage and natural killer cell numbers and phagocytosis rates by inhibiting tyrosine kinases (Steele and Brahmi 1988), and decrease T and B lymphocyte production by inhibiting topoisomerase II (Chang et al. 1995). Low levels of genistein, however, could elicit natural killer cell activity (Zhang et al. 1999) and antiviral replication (Yura et al. 1993). In pigs challenged with porcine reproductive and respiratory syndrome (PRRS), Greiner et al. (2001a) demonstrated that soy genistein could enhance serum PRRS virus elimination, decrease interferon activity in the serum and increase α1-acylglycoprotein (AGP). The pig growth performance also improved. The authors concluded from these results that soy genistein at 200–400 mg/kg can be an orally active immune modulator.

Micro-array analysis of the effects of estradiol and genistein on neonatal thymus indicated that estradiol affected genes involved in transcription, apoptosis, cell cycle and thymic development and function; genistein had similar effects on many estradiol target genes but also had unique actions not replicated by estradiol. Despite extensive work showing inhibitory effects of genistein on immunity, other rodent studies reported that genistein or other phytoestrogens stimulate various aspects of immune function. Although the present data strongly indicate that genistein can regulate immune function, possibly at physiologic concentrations, further work is required to definitively establish overall thymic and immune effects of genistein and soy, which may vary with age, species and specific end point (Paul et al. 2006). To our knowledge, there has been limited research conducted with genistein in poultry. Therefore, the objectives of this research were to determine the effects of genistein on immune responses of male broiler chicks.

Materials and methods

Birds and experimental diets

This study was carried out in experimental farm of Ferdowsi University of Mashhad, Mashhad, Iran. Three hundred and fifty day-old male broiler chicks were randomly allocated to 7 treatments each of which had 5 replicate pens of 10 chicks per pen. The temperature was maintained at $34 \pm 1^{\circ}$C up to 7 days

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of age and then gradually decreased to $26 \pm 1^{\circ}C$ by 21 days of age. Lighting was continuous and water and feed were provided ad libitum all over the experimental period, which lasted for 42 days of age. The chicks received the same basal starter diets based on corn-soybean meal, and genistein (Beijing, china) was added to the basal diet at 20, 40, 80 and 160 mg/kg, respectively. Dietary control treatments included the negative control group with no feed additive and two positive controls supplemented with either virginiamycin antibiotic premix 10%, or zinc bacitracin (each 60 g sachet contains 240,000 IU Basitracin MD). Diets were offered for 0–3 weeks and 3–6 weeks. Nutrition levels of the diets (Table 1) were based on the NRC (1994). The ingredients were analysed for crude protein, ether extract, crude fibre and ash content according to the methods of AOAC (1995).

Analysis of antibody production against infectious bronchitis virus disease (IBV) by ELISA protocol

The chicks were vaccinated with infectious bronchitis virus vaccine at day 23 via eye dropping of two chicks per pen. Blood samples were collected from the wing vein at day 6 and 12 after vaccine administration. Serums were isolated and stored at $-20^{\circ}C$ until further analysis. Serum samples were defrosted at room temperature and diluted 500-folds (1:500) in diluents. Diluted serums were added (100 μL) to 96-well plates coated with infectious bronchitis antigens. The plates were incubated for 30 min at room temperature, washed with 350 μL of sterile distilled water and incubated again for additional 30 min at room temperature with 100 μL of a conjugate. Substrate was then dispensed (100 μL) in the wells to facilitate a colour reaction, as plates were allowed to incubate at room temperature for an additional 15 min. A stop solution was then added to end the enzymatic process. Plates were read on a Microplate Reader at 650 nm to measure the infectious bronchitis antibody titres (Kidd et al. 2001).

Hemagglutination inhibition test for Newcastle antibody titre

The antibody response to Newcastle disease virus (NDV) was quantified to examine the humoural immune response. The 10 chicks from each dietary treatment (two per pen replicate) were inoculated by eye drop of B1 strain of NDV at 13 days of age. Serum samples were collected from the same birds (wing-banded) by brachial venipuncture at day 6 and 12 after vaccine inoculation. The sera samples were assessed by hemagglutination inhibition test as previously described by Marquardt et al. (1984).

Heterophil to lymphocyte (H/L) ratio

At day 42, blood samples were obtained via wing vein of two birds per pen and collected into the tubes containing EDTA as anticoagulant. Two drops of blood were placed on the slide, and blood smear was prepared using Giemsa staining method (Lucas and Jamroz 1961). All slides were coded, and then heterophils and lymphocytes were counted to a total number of 100 cells per slide.

Table 1. Ingredients and nutrient composition of basal diets during starter and grower periods.

Ingredients	Starter (1–21 day)	Grower (21–42 day)
Corn, yellow	54.68	62.25
Soybean meal	38.19	31.30
Sunflower oil	3.00	2.14
Dicalcium phosphate	1.85	1.36
Limestone	1.23	1.30
Common salt	0.40	0.30
Mineral premix[a]	0.25	0.25
Vitamin premix[b]	0.25	0.25
DL-methionine	0.15	0.05
Nutrient composition		
ME (kcal kg^{-1})	3000	3030
Crude protein (%)	21.57	18.94
Methionine (%)	0.49	0.36
Lysine (%)	1.18	1.01
Calcium (%)	0.94	0.85
Non-phytate P (%)	0.43	0.34

[a]Supplied per kilogram of diet: vitamin A (retinal acetate), 15,000 IU; vitamin D3, 5,000 IU; vitamin E (dl-α-tocopheryl acetate), 80 mg; vitamin K, 5 mg; thiamin, 3 mg; riboflavin, 10 mg; pyridoxine, 5 mg; vitamin B12, 0.02 mg; niacin, 70 mg; folic acid, 2 mg; biotin, 0.4 mg; pantothenic acid, 20 mg.
[b]Supplied per kilogram of diet: manganese, 80 mg; zinc, 75 mg; iron, 70 mg; copper, 10 mg; iodine, 2 mg; selenium, 0.3 mg.

Table 2. Effect of dietary genistein supplementation on antibody response to Newcastle (log2) and infectious bronchitis (log10) disease viruses.

		Infectious bronchitis		Newcastle	
Treatments	Supplemental level (mg/kg of diet)	6 dpi	12 dpi	6 dpi	12 dpi
Control	0	3.043	3.508[b]	2.93	3.73[abc]
Zinc bacitracin	200	3.033	3.499[b]	3.10	3.52[bc]
Virginiamycin	200	3.058	3.514[b]	3.37	3.06[d]
Genistein	20	3.025	3.543[a]	3.78	4.08[a]
	40	3.040	3.544[a]	3.20	3.84[ab]
	80	3.057	3.501[b]	3.29	3.81[ab]
	160	3.067	3.495[b]	2.93	3.37[dc]
Probability					
P value		0.8597	0.0001	0.0780	0.0001
SEM		0.0231	0.0066	0.2011	0.1235
Contrasts					
Negative control vs. Gn		0.8525	0.0890	0.1111	0.7471
Positive controls vs. Gn		0.9192	0.0229	0.7118	0.0001

Note: [abcde] means with no common superscript within each column are significantly ($p < 0.05$) different. dpi, days post-inoculation.

Statistical analysis

Data from experiments were subjected to ANOVA according to the GLM procedure. All statistical procedures were done with an SAS statistical software package (SAS Institute 2002). A completely randomised design was considered for analysis of variance and pen was the experimental unit for all measurements. All treatment means were compared by Duncan's multiple range tests (Duncan 1955) at $p < 0.05$ statistical level. The single degree of freedom contrasts were made among the treatment means to compare the negative control versus genistein and two positive controls versus genistein treatments.

Results and discussion

The influences of dietary treatments on antibody productions against Newcastle and infectious bronchitis diseases viruses are shown in Table 2. At day 6 post-inoculation, different experimental diets had no significant effect on antibody titres. However, on day 12 after vaccine administration, dietary supplementation with 20 mg Gn/kg increased ($p < 0.01$) antibody titres against both Newcastle and infectious bronchitis diseases viruses. Furthermore, dietary supplementation with 20, 40 and 80 mg of Gn/kg resulted in a significant ($p < 0.01$) increase in antibody production titre against NDV compared with either zinc bacitracin or virginiamycin antibiotic diets. Genistein

Table 3. Effect of dietary genistein concentration on blood leukocyte sub-population.

Treatments	Supplemental level (mg/kg of diet)	Heterophil (%)	Lymphocyte (%)	H/L[a]
Control	0	24.7	69.25	0.356
Zinc bacitracin	200	25.0	70.0	0.356
Virginiamycin	200	25.7	70.25	0.364
Genistein	20	24.7	70.5	0.349
	40	24.5	70.75	0.345
	80	25.0	70.5	0.354
	160	24.0	71.25	0.336
Probability				
P value		0.6727	0.6996	0.5483
SEM		0.6551	0.8600	0.0098
Contrasts				
Negative control vs. Gn		0.8392	0.1154	0.3729
Positive controls vs. Gn		0.1571	0.6539	0.1100

Gn, Genistein.
[a]Heterophil to lymphocyte ratio.

treatments had no significant ($p > 0.05$) effect on Newcastle antibody titres in comparison with the negative control (not-supplemented diet). This effect may be explained by the activation of macrophages. It should be noted that genistein has been found to be an inhibitor of protein tyrosine kinases at high concentrations, while acting as an estrogenic compound at low concentrations (Makela et al. 1995; Stahl et al. 1998). Trevillyan et al. (1990) showed that genistein, a selective protein tyrosine kinase inhibitor (Akiyama et al. 1987), blocked the activity of p56lck in a concentration-dependent manner. Inhibition of enzyme activity could be associated with reduced IL-2 secretion. Studies with the protein tyrosine kinase inhibitors support the contention that tyrosine phosphorylation is an obligatory event in IL-2 secretion (Stanley et al. 1990). Our findings also showed that dietary supplementation with 20 and 40 mg Gn/kg had an increasing effect ($p < 0.01$) on antibody response against infectious bronchitis diseases virus compared with other treatments. With increasing dietary genistein supplementation, we observed that high concentration of genistein (160 mg/kg) failed to additionally boost the antibody response to IBV vaccination. In human dendritic cells, excess antioxidant can down-regulate the immune response (Verhasselt et al. 1999). Because genistein likely acts as an antioxidant (Jiang et al. 2007a, b), it is possible that at high doses it could prevent from the initial increase in reactive oxidant species necessary to stimulate lymphocytes.

The pattern change in peripheral blood leukocyte sub-populations is observed in Table 3. As noted, dietary treatments had no significant effect on proportion of heterophils and lymphocytes, as well as on heterophil to lymphocyte (H/L) ratio. A low H/L ratio indicates low levels of stress, but it remains unaffected in our study. In conclusion, genistein promotes humoural immune responses in broiler chicks. Moreover, dietary supplementation of genistein at the levels of 20 and 40 mg/kg in replacement with antibiotics can improve overall immune functions in birds. However, further researches are needed to quantify and/or specify the effects of these herbal components on cellular and humoural immune functions of broiler chicks at molecular levels.

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