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Effect of Dietary Oil Extract of Propolis on Immune Response and Broiler Performance

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Abstract: This investigation was conducted to determine the effect of different levels of Oil Extract of Propolis (OEP) on immune system of broiler chickens from 1 to 47 days of age. Chicks were fed soybean meal-corn diets supplemented with levels of 0, 40, 70, 100, 400, 700 and 1000 mg kg^{-1} of OEP from hatching. On days 20 and 32, they were immunized with Newcastle Disease Virus (NDV) and examined 10 days later. In general, the immune system can be stimulated by addition of OEP, without affecting the performance. At 30 days of age after immunization at 20 days, antibody response to NDV was significantly increased in chickens fed with 70 and 100 mg kg^{-1} of OEP in the diet ($p<0.05$). No effect of dietary OEP was observed on the ratio of gamma globulins to total serum proteins. Heterophil to lymphocyte ratio was significantly decreased in chickens fed with 40 and 70 mg kg^{-1} of OEP in the diet ($p<0.05$). Investigation of histological sections revealed that the number of proliferating cells of bursa of fabricius was increased in chickens fed with 1000 mg kg^{-1} of OEP in the diet. When broilers received the high levels (400, 700 and 1000 mg kg^{-1}) of OEP in the diet the number of leukocytes in the lamina propria of intestine was higher compared with other levels of dietary OEP. The number of lymphoid cells in the preportal area of the liver increased at the highest dose (1000 mg kg^{-1}) of OEP and the thickness of blood vessels wall increased at control treatment. The results indicate that both humoral and cellular immune responses were modulated by different levels of OEP in the diet and suggest that low levels (40 and 70 mg kg^{-1}) of dietary OEP developed the immune response, whereas the chickens fed highly enriched OEP diet, showed lower immune response.

Key words: Propolis, immunity, broiler, NDV, OEP

INTRODUCTION

Interest in using immunomodulators to improve cellular and humoral immune functions and resistance against infections in chickens and other domestic animals has increased in the last decade. Among the natural products that have been found to modulate immune functions, propolis (bee glue) and its ingredients have been considered. Propolis is a natural resinous product of honey bees, which is rich in biochemical constituents, including mostly a mixture of polyphenols, flavonoids (major ingredient), phenolic acid and their esters, caffeic acid and their esters, phenolic aldehydes and ketones, respectively[1,2].

Clear evidence exists from other studies in poultry that administration of propolis extracts affects the immune system. Antibody production[3-5], T lymphocytes stimulation[3], peripheral lymphocytes proliferation[5] and phagocytosis percentage have been reported to increase after propolis extracts treatment[4].

Interaction between different cells involved in the immune response and production of cytokines by activated T cells has been found to play an important role in whether immune responses develop into primarily cell-mediated or primarily antibody-mediated response pathways[6]. However, there is evidence that administration of propolis (extracts or ingredients) affect both specific and nonspecific arms of the immune system in mouse and humans, including an increase of interleukin-1 (IL-1)[7-10], IL-2[9,11], IL-4[11], antibody response[11,12], T lymphocytes[13], T lymphocytes blastogenesis[11,14], CD^{+}_{4}/CD^{+}_{8} ratio[11,13], plaque forming cells[11,12] and macrophages[15].

However, the effect of a wide spectrum of dietary Oil Extracts of Propolis (OEP) intake on immune response has not been examined in broilers and this constituted the objective of the present study. To evaluate these responses, we measured antibody response to Newcastle Disease Virus (NDV), heterophil to lymphocyte (H/L) ratio, electrophoresis of serum proteins and investigated

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histological sections of bursa of fabricius, liver and small intestine (jejunum and duodenum).

MATERIALS AND METHODS

In a completely random design, 672 1-day-old (Ross 308) broiler chickens were divided to seven treatments with four replications per treatment (in 28 cages) and 24 chickens in each replication (or cage). Chicks had free access to water and the experimental diets. The basal diet used in the study was a typical corn-soybean diet and formulated to meet nutrient requirements[16], which were supplemented with 0, 40, 70, 100, 400, 700 and 1000 mg kg^{-1} of OEP equivalents (termed treatments 1 to 7). Body weight gain, feed intake and feed conversion ratio were determined over three age periods (1-21, 21-47 and 1-47 day old). Chickens were weighed on days 1, 21 and 47 on cage basis to determine weight gain and feed consumption per replicate was measured every week. Propolis was collected from Ziaran, Qazvin province, Iran. Extraction was carried out with moderate shaking for 15 min at 85°C in Sunflower oil. This extract was filtered and the final concentration was calculated, obtaining the solution (OEP). This solution was added to the diets to obtain the appropriate treatments. Chicks were routinely vaccinated on day 1 against Infectious Bronchitis (IB) (Nobilis H120, Intervet) and on day 14 against Infectious Bursal Disease (IBD) (Nobilis D78, Intervet). On days 20 and 32, each bird received one dose of commercially available NDV vaccine (by eyedrop and oral method, respectively). On day 40, all chickens of each group were boosted with IB (Nobilis H52, Intervet) vaccine via the water. On days 30 and 42, two chickens were sampled randomly from each cage (eight birds per treatment) for determination of serum antibody titer of the blood. Antibody responses to NDV were measured by ELISA using the Newcastle Disease Antibody Test Kit from IDEXX Laboratories Inc (Westbrook, ME 04092). Titers were obtained and calculated as described by IDEXX. On day 47, two chickens were sampled randomly from each cage (eight birds per treatment) for determination of gamma globulins to total serum proteins ratio by electrophoresis. In brief, cellulose acetate was wetted with barbital buffer (pH 7.4). Then, protein fractions were separated on cellulose acetate strips at 200 V for 25 min. Albumin and globulins were stained with ponceu S and lipoproteins with Oil Red O. Slides were dyed in an acetic acid solution and then put in a lightening solution[17,18]. All strips were scanned and then edited with related software. Two chickens were sampled randomly from each cage (eight birds per treatment) for determination of heterophil to lymphocyte (H/L) ratio by differential count of White Blood Cells (WBC). On day 47 blood was taken from eight chickens of each treatment placed in EDTA-coated tubes and examined for differential count of WBC. Differential counts were made by screening Giemsa-Stained slides[19]. The different types of WBC were counted and the H/L ratio was calculated. In addition, two chickens from each cage after blood collection were killed; liver, bursa of fabricius and small intestine (duodenum and jejunum) were sampled for investigation of their histological changes. Tissues were collected from each bird in treatments that had 0, 70, 400 and 1000 mg kg^{-1} of OEP in the diet (n=8 for each tissue). Tissues were immediately fixed at 10% buffered formalin. These formalin-fixed tissues were embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin for histological examination. The carcass percentage to live weight measured on day 47 was determined.

Statistical analysis: Statistical analysis of variance was performed on data using the General Linear Models procedure of the SAS Institute[20]. Means were compared by Duncan's Multiple Range Test at $p<0.05$.

RESULTS

Performance: The results of the experiment (Table 1) showed that the body weight, average daily weight gain, daily feed intake, feed conversion ratio and carcass percentage to live weight were not influenced by supplemental OEP. Although, all levels of supplemental OEP tended to increase body weight and average daily weight gain.

Antibody responses to NDV: The antibody response to NDV was significantly affected ($P<0.05$) by the levels of OEP in the diet (Table 2) at 30 days of age. The maximum values belonged to levels of 70 and 100 mg kg^{-1} of OEP in the diet. At levels of 400, 700 and 1000 mg kg^{-1}, antibody titers decreased and were similar to control. At 42 days old, antibody titer of NDV was not significantly different ($p<0.05$) among treatments, however the average antibody titer increased at low levels (40, 70, 100 and 400 mg kg^{-1}) of OEP in the diet compared with control. Also, similar to data at 30 day old, at high levels (700 and 1000 mg kg^{-1}) of OEP in the diet, antibody titer decreased.

Gamma globulins to total serum proteins ratio: Results obtained from electrophoresis of serum proteins showed that gamma globulins were not significantly different,

Table 1: Effect of diets containing different levels of OEP on bird performance

	Diet (mg of OEP kg^{-1})						
	0	40	70	100	400	700	1000
BW (g) 1 d	42.57	42.56	42.69	42.7	42.67	42.5	42.5
BW (g) 21 d	536.26	562.94	566.63	556.36	535.9	535.55	550.82
BW (g) 47 d	1912.08	1928.49	1916.64	1929.44	1919.88	1917.1	1935.67
Day 1 to 21							
ADG (g d^{-1})	23.5	24.78	24.94	24.46	23.48	23.47	24.2
ADFI (g d^{-1})	43.45	42.7	45.33	44.69	37.48	43.1	44.05
FCR (g g^{-1})	1.84	1.72	1.8	1.82	1.77	1.84	1.8
Day 21 to 47							
ADG (g d^{-1})	52.91	52.52	51.86	52.81	53.19	53.13	53.24
ADFI (g d^{-1})	126.58	126.3	123.98	117.55	129.46	131.75	126.87
FCR (g g^{-1})	2.38	2.4	2.39	2.22	2.43	2.48	2.37
Day 1 to 47							
ADG (g d^{-1})	39.77	40.12	39.9	40.14	39.96	39.88	40.31
ADFI (g d^{-1})	89.44	88.95	88.84	85.00	90.31	92.14	89.87
FCR (g g^{-1})	2.24	2.21	2.22	2.11	2.25	2.31	2.22
CP/LW (%)	74.04	73.84	74.93	75.23	74.44	73.61	74.61

BW = Body Weight; ADG = Average Daily Weight Gain; ADFI = Average Daily Feed Intake; FCR = Feed Conversion Ratio; CP/LW = Carcass Percentage to Live Weight

Table 2: Antibody titer to NDV (Ab to NDV) and gamma globulins to total serum proteins (G/TP) ratio in chickens fed containing different levels of OEP[1]

	Diet (mg of OEP kg^{-1})						
Variables	0	40	70	100	400	700	1000
Ab to NDV[2] (30 d old)	648.5^{c}	1028.8^{a-c}	1537.9^{ab}	1589.1^{a}	758.3^{bc}	814.9^{a-c}	760.6^{bc}
Ab to NDV[3] (42 d old)	2194.4	3382.9	3057.3	2984.9	3292.4	2163.1	1718.5
G/TP (47 d old)	16.11	22.25	20.36	14.15	15.75	9.5	9.5

$^{a-c}$Means without common superscript differ significantly ($p<0.05$). [1]Data are presented as means of eight birds, [2-3]Antibody titer was measured by ELISA

however average of gamma globulins to total serum proteins ratio had a similar trend to antibody titer at low levels of OEP and decreased in a manner similar to that of antibody titers obtained at high levels of OEP (Table 2).

Differential leukocyte count: No effect of dietary OEP was observed for the percentage of eosinophils and monocytes. With dietary OEP supplementation, the percentage of circulating heterophils decreased significantly, whereas the lymphocyte percentage in the peripheral blood increased at 47 days old. In absolute terms, this change reflects a statistically significant decrease in heterophils in peripheral blood, which led to a reduced H/L in blood with dietary OEP supplementation (Table 3). H/L decreased at levels of 40 and 70 mg kg^{-1} of OEP in the diet, they were significantly different ($p<0.05$) among treatments (Table 3). Using high levels of OEP induced slight increase in H/L.

Histological changes

Bursa of fabricius: The number of proliferating cells in the bursa of fabricius increased at the highest level (1000 mg kg^{-1}) of OEP in the diet. The germinal center

Table 3: Effect of diets containing different levels of OEP on percentage eosinophil, monocyte, heterophil, lymphocyte and heterophil to lymphocyte (H/L) ratio in chickens[1]

	Diet (mg of OEP kg^{-1})						
Variables	0	40	70	100	400	700	1000
Eosinophil	1.33	0.62	0.62	1.75	1.12	1.33	1.12
Monocyte	4.11	5.12	4.37	5.25	5	4.33	3.37
Heterophil	27.55^{a}	19.87^{c}	21.25^{bc}	23.12^{a-c}	23.87^{a-c}	26.44^{ab}	21.75^{a-c}
Lymphocyte	67.0^{c}	74.62^{a}	73.75^{ab}	69.87^{a-c}	70.0^{a-c}	67.88^{bc}	73.75^{a-c}
H/L	0.41^{a}	0.26^{c}	0.28^{bc}	0.33^{a-c}	0.34^{a-c}	0.38^{ab}	0.29^{a-c}

$^{a-c}$ Means without common superscript differ significantly ($p<0.05$)

[1]Data are presented as means of eight birds

Table 4: Histological changes in the bursa of fabricius, liver and small intestine on 47 day old chickens fed diet containing different levels of OEP[1]

		Diet (mg of OEP kg^{-1})			
Organ		0	70	400	1000
Bursa of fabricius	Germinal center	++[2]	++	++	+++
Liver	Thickness of blood Vessels wall	++	+	+	+
	Number of lymphoid cells	++	++	++	+++
Small intestine (duodenum)	Number of leukocytes	+	+	+++	+++
Small intestine (jejunum)	Number of leukocytes	+	+	+++	+++

[1]Mean of histological sections of two chickens of each group

[2]Number of +'s indicates severity of the histological changes

increased noticeably compared with control at this level (Table 4).

Liver: Investigation of histological sections showed an increase in the thickness of blood vessels walls at control level and the number of lymphoid cells in preportal area of liver at the highest level (1000 mg kg^{-1}) of OEP in the diet (Table 4).

Small intestine: When broilers received the high levels (400 and 1000 mg kg^{-1}) of OEP in the diet the number of

leukocytes in the lamina propria of duodenum and jejunum was higher compared with other levels of dietary OEP (Table 4).

DISCUSSION

The results showed that dietary OEP did not affect body weight gain or growth performance. An effect may be been predicted as the hydroxyl group of the flavonoid's aglycone are positioned similar to those of an estrogen[21]. Since estrogens have anabolic effects[22], one might suspect that flavonoids might be able to act as growth hormones in animals as well. No effect of dietary OEP was observed for Feed Conversion Ratio (FCR). Although, flavonoids can influence the cholesterol synthesis[21], present results showed numerically an increase of carcass percentage to live weight at level of 100 mg kg^{-1} of OEP in the diet.

At 30 days of age, antibody response to NDV was affected by dietary OEP. Flavonoids (one ingredient of propolis) stimulate the production of antibodies in a yet poorly known fashion[23]. Other researchers have observed that administration of propolis (extracts or ingredients) in mice cause an augmentation of IL-1, IL-2, IL-4 and CD^{+}_{4}/CD^{+}_{8}[7-10,11,13], that can increase antibody production. Also antioxidant[24] and anti-inflammatory effects[14] of propolis can influence immune system. In the present study low levels of OEP in the diet (70 and 100 mg kg^{-1}) increased antibody titer. Our results showed that antibody titer decreased at high levels (400, 700 and 1000 mg kg^{-1}) of OEP in the diet, thereby exhibiting a bell-shaped dose-response relation. At 42 days old, antibody titer to NDV was not affected by dietary OEP. However, antibody titer increased at low levels and decreased at high levels of OEP in the diet that was in a manner similar to that of pattern of antibody titer at 30 days old. The major reason for the differences between the antibody response at 30 and 42 days old may be due to method of vaccination and residual antibody from primary immunization; at 20 days old vaccination was carried out by eyedrop, whereas the oral method was used for the 32 day old birds. Although, gamma globulins to total serum proteins ratio had a similar pattern to antibody titer of NDV, these results were not affected by dietary OEP. Results also showed that heterophil to lymphocyte ratio were affected by OEP levels in the diet. Lymphocyte percentage increased at levels of 40 and 70 mg kg^{-1} of OEP in the diet compared with control and decreased at high levels of dietary OEP. These results had a similar pattern to antibody response and gamma globulins to total serum proteins ratio. The relation between cellular and humoral immunity should be considered because lymphocytes increase in viral infections before the antibody production[25,26]. For instance, Merz *et al.* [27] reported that after vaccination of chickens with NDV, cell-mediated immunity comes first; then humoral immunity follows. In the present study, chickens also received NDV which was viral. On the other hand, the increase of percentage lymphocytes should be due to T lymphocytes because the number of T lymphocytes in circulating blood is 80 to 90% among total lymphocytes[25,26] and secretion of IL-2 and IL-4 from T_4 lymphocytes and diffusion to B lymphocyte is necessary for antibody production. These cytokines cause B lymphocytes to convert into plasma cells which can synthesize antibody[25,26]. The increase of T lymphocyte proliferation in broiler chickens was reported by Kong *et al.*[5] and an increase of T lymphocytes blastogenesis has been observed in mice[11,14]. Ivanovska *et al.*[9] have described the immunostimulant effect of cinnamic acid, a phenolic acid present in propolis, on IL-2 and IL-4 production and lymphocyte proliferation. Thus, in our study a possible explanation to this immunostimulant activity could be attributed to presence of the cinnamic acid or to a synergic effect between the components.

The present study showed that OEP can increase antibody production and lymphocytes at levels of 40 to 70 mg kg^{-1} of OEP in the diet, whereas results obtained by others[3,4] indicated an increase at levels of 20 to 40 mg kg^{-1} of propolis extract in the diet. Differences between our results and those reported by others could be related to a variety of aspects in the experimental design, such as the type of propolis and its extracts in the diets and duration of feeding with experimental diets, the age and strains of chickens and type of vaccination used.

On the other hand, a possible explanation to this immunosuppressive activity on antibody or gamma globulin production and lymphocyte proliferation at high levels of OEP in the diet could be attributed to a synergism between the components of OEP. Reduction of plaque forming cells in mice is reported by Scheller *et al.*[12]. They observed that further increase in the propolis dose or in the frequency of its administration, have an inhibitory effect on the formation of the plaques in immunized mouse spleen cells and antibody production. Also previous studies have shown that synergism of flavonoids has an immunosuppressor effect on the lymphoproliferative response, especially at high doses[28-31], due to nitric oxide production from macrophages that is responsible for the inhibition of DNA synthesis in several cells[32].

Histological sections of bursa of fabricius only showed a little increase at germinal center at the highest level (1000 mg kg^{-1}) of OEP. Although, bursa of fabricius is a major organ in antibody production in chickens, researchers have reported that other organs can produce

immunoglobulins too[33-36]. On the other hand, propolis has affected the spleen and antibody production in mice[11,12]. Thickness of blood vessels wall decreased in liver by dietary OEP. This indicates that OEP has anti-inflammatory effects. Anti-inflammatory effects of propolis were reported by other studies[14,37]. High levels of OEP could increase the number of leukocytes in the duodenum and jejunum compared with other levels of dietary OEP and lymphoid cells in preportal area of liver at level of 1000 mg kg^{-1} of OEP in the diet. Since ingredients of propolis have stimulator effect on Antigen Presenting Cells (APC)[21], one might suspect that OEP might be able to act as immunostimulant factor in animals, because the small intestine and liver have antigen presenting cells.

In summary, both cellular and humoral immune response of broiler chickens was shown to be modulated by levels of OEP in the diet. Results indicated that birds maintained on low OEP diet developed strong antibody response to NDV, whereas birds maintained on the diet containing 700 and 1000 mg kg^{-1} of OEP showed reduced antibody responses to NDV. The ratio of gamma globulins to total serum proteins and the percentage of lymphocytes also had a similar pattern to antibody titer. Further investigation with fractioned part of propolis seems interesting.

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