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Regulation of Inflammatory Gene Expression by Echinacea purpurea in Avian Models

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***Abstract*—**This study was conducted to evaluate the effect of *Echinacea purpurea* on the expression of cyclooxygenase-2 (COX2), interleukin-17F (IL-17F) in seven-day-old broiler chickens. Four groups were fed with concentration of 0 g/kg, 5 g/kg, 10 g/kg and 20 g/kg from the root of *E. purpurea* in the basal diet and two other groups were only fed with the basal diet for 21 days. At the 28th day, lipopolysaccharide (LPS, 2 mg/kg diet) was injected in four groups and the basal diet group was injected by saline as control. The chickens' spleen RNA expression was measured for the COX-2 and IL-17F genes by Real-Time PCR. The results have shown that chickens which were fed *E. purpurea* had a lower COX-2 and IL-17F mRNA expression. The chickens who have received LPS only, lymphocyte was lower than other treatments. Vital organ weights were not significantly different, but body weight loss was recovered by dietary herbs inclusion. The results of this study have shown the positive effect of an anti-inflammatory herb to prevent the undesirable effect of inflammation.

I. Introduction

POULTRY are constantly exposed to infectious agents. In addition to systemic infections, microbes and their LPS that are presented in the hen houses air could challenge the lungs of chickens. LPS of microbes also penetrate from the intestine to the blood circulation [1]. These factors stimulate an immune response and create inflammation. Systemic inflammation causes different metabolic responses and symptoms including change in behavior, fever, physical weakness, loss of appetite, weight loss and interference in energy balance which are called "disease syndrome" [2].

In response to extracellular stimulating like LPS, the intercellular messaging pathways express the inflammatory cytokine genes via activation of the transcription factor NF-kB (Nuclear Factor Kappa B) [3].

The intermediate pathway in the inflammatory cytokine synthesis chain is IL23-IL17 pathway [4]. During activation of T cells, IL-17 is synthesized and stimulates the epithelial cells, fibroblasts [5]. Interleukin 17 (IL-17) runs many inflammatory responses, such as synthesis IL-1, IL-6, TNF-α, COX-2that they can again stimulate inflammation [6]. Prostaglandins (PGs) are an important group of these physiological and pathological intermediates of inflammation which are synthesized by the biosynthetic waterfall of arachidonic acid; Phospholipids are catalyzed into arachidonic acid due to release of certain stimuli which is then converted into PG H2 by the cyclooxygenase (COX) enzyme [7]. COX is a key enzyme in the first two steps of PG synthesis with its COX2 isoform being induced mainly during inflammatory response [8]. COX-2 is a key enzyme in the PG synthesis that PG could inhibit the brain control of feeding [9]. They also have inhibitory or stimulatory effect on the regulating feed intake hormones such as Ghrelin and Cholecystokinin, respectively. These effects of COX-2 on neurons, endocrines and behavior of chickens reduce feed consumption, energy cost and body weight [10].

Using the immune system regulators in chicken diet can be effective in reducing inflammatory cytokines [11] and [12]. There is convincing evidence that plant secondary metabolites especially polyphenols have anti-inflammatory activity which is found in many plant products including fruits, vegetables and herbs [13]. Data of some studies have shown that *Echinacea purpurea* could prevent inflammation response with several biological activities which down regulate COX gene [14]. Therefore, the purpose of this study was to evaluate effect of *E. purpurea* induced on expression of IL-17F and COX-2 in broiler chickens.

II. Materials and Methods

Eighty one-day-old Ross 380 broiler chickens were fed by pre-starter diet without any treatments. At the end of the first week, the chickens were weighed and 40 of them were allocated into the five equal experimental groups. The hens were treated as following, three groups were fed with three different concentration of *E. purpurea* (5 g/kg, 10 g/kg and 20 g/kg, in basal diet), and two group were fed by basal diet. On day 28, the entire three herb fed groups, plus one group from the basal diet, were injected with 2 mg/kg LPS (E-coli 055:B5, Sigma-Aldrich, USA) into their abdominal cavity. In addition, 1 ml of normal saline was injected to the control group [15].

After 24 hours, the spleens were immediately frozen in liquid nitrogen. *E. purpurea* roots were provided by Zardband Co. (Karaj, Iran). The roots were fine milled and added to the basal diet. After 24 hours, LPS injections and its change was calculated. Then, 2 ml blood of each chicken

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was drained from vein in a bottle containing ethylenediaminetetraacetic acid (EDTA), as anticoagulant [16]. To measure the expression level of mRNA of IL-17F and COX-2 in spleen, total RNA was extracted by GeneJET RNA purification kit (Thermo Scientific Co., USA) and cDNA synthesized with BioRT cDNA synthesis Kit (Bioer, China). Table I has shown Specific primers for COX-2, IL-17F and β-actin (as an internal control).

For Real-Time PCR amplification condition, 15 min at 95°C for initial denaturation, then 40 cycles were followed as 15 s denaturation at 95°C, annealing temperature of 30 s at 62°C for COX-2, 30 s at 64°C for IL-17F, 30 s at 62°C for βactin, and 30 s at 72°C for amplification step. The final extension step was run 5 min at 72°C. Comparative gene expression estimations of COX-2 and IL-17F were carried out using $2^{-\Delta CT}$ formula ($\Delta C_T = C_T$ gene COX-2 or IL-17F $-C_T$ βactin). To compare fold change gene expression (normalized gene expression) in the groups in comparison with the control, $2^{-\Delta CT}$ of each group was divided per $2^{-\Delta CT}$ of the control [17].

III. Statistical Analysis

Data were analyzed by the general linear models (GLM) procedure and Duncan's new multiple range test was used to determine the differences by SAS 9.1 software (SAS Institute Inc., Cary, NC).

IV. Results

Results of this experiment in Table II have shown that feeding the chickens with 5 g/kg, 10 g/kg and 20 g/kg *E. purpurea* from 7 days of age for 21 days, was significantly (P<0.001) decreased COX-2 gene expression for about 1.88, 2.37 and 2.72 times, respectively, in comparing with when LPS was injected (3.41, 2.7 and 2.35 *E. purpurea* groups vs. 6.40 LPS group). However, there were no differences between varying levels of herb (P>0.05). Otherwise, the negative control group was the lowest COX-2 expression (0.040) among groups (P<0.001).

Data of the IL-17F gene expression showed that feeding chickens with 5 g/kg and 20 g/kg of *E. purpurea* statistical significantly (P=0.046) decrease (2.04 and 1.78) in contrast to LPS group (2.73). There was no difference between the groups which supplemented with herbs (P>0.05) but control group was lower in IL-17F expression (0.067) than other groups (P=0.003). White blood cell differential count is presented in Table III which shows that challenging the immune system with LPS caused a significant (P=0.047) reduction in the lymphocyte percent (61.17%) compared with the control (67.37%). The use of LPS with *E. purpurea* increased the percent of lymphocytes although, only E20+LPS group showed a significant difference compared with the LPS group (P=0.047). No significant differences were found between the groups in percentage of monocytes, eosinophils, heterophil and heterophil/lymphocyte ratio (P>0.05).

TABLE II

Effect of Supplementation *Echinacea Purpurea* on IL-17 and COX-2 Gene Expression in Broiler Chickens

Treatments	IL-17 gene expression		COX-2 gene expression	
	Comparative C_T	Normalized C_T	comparative C_T	Normalized C_T
NC (LPS-)	0.067^{c}		0.040^{b}	
PC (LPS+)	0.185^{a}	2.73^{a}	0.256^{a}	6.41^{a}
E5+ LPS	0.138^{ab}	2.04^{b}	0.136^{ab}	3.41^{b}
E10+ LPS	0.146^{ab}	2.16^{ab}	0.108^{b}	2.70^{b}
E20+ LPS	0.121^{bc}	1.78^{b}	0.094^{b}	2.35^{b}
SEM	0.018	0.156	0.013	0.866
P-value	0.003	0.046	0.036	0.001

Negative Control: (normal saline injection without feed additive), Positive Control: (LPS injection without feed additive), C_T: Threshold cycle; EP: *Echinacea purpurea*. a-b; Different labels for each group compared with others shows significantly (p<0.5) statistical difference.

TABLE III

Effect of Dietary Feed Additives on Differential Leukocytes Count

Treatments	L	H	M	E	H/L
NC (LPS-)	67.37 ^{a}	23.37	9.50	2.17	0.35
PC (LPS+)	61.17 ^{b}	27.00	6.50	2.50	0.44
E5+ LPS	64.25 ^{ab}	24.87	7.75	2.12	0.39
E10+ LPS	65.00 ^{ab}	25.12	7.00	2.00	0.39
E20+ LPS	67.17^{a}	23.83	6.83	2.33	0.36
SEM	1.260	1.000	0.854	0.397	0.023
P-value	0.0472	0.3409	0.4244	0.4553	0.1723

Negative Control: (normal saline injection without feed additive), Positive Control: (LPS injection without feed additive) EC5+LPS (5 g *E. purpurea* with LPS injection), EC10+LPS (10 g *E. purpurea* with LPS injection), SEM; Standard Error of the Mean, H/L: Heterophil/ Lymphocyte, M: Monocyte, L: Lymphocyte, E: Eosinophil, $^{a-b}$; Different labels for each group compared with others shows significantly (p<0.5) statistical difference.

TABLE IV

Effect of Dietary Feed Additives on Percentage of Body Weight Loss and Mass of Spleen, Bursa, Liver and Heart (g/100g Body Weight)

Treatments	BWL[1]	Spleen	Bursa	Liver	Heart
NC (LPS-)	5.10 ^{a}	0.086	0.24	2.30	0.58
PC (LPS+)	-5.14 ^{b}	0.080	0.21	2.81	0.64
E5+ LPS	-2.66 ^{b}	0.096	0.19	2.55	0.59
E10+ LPS	-2.18 ^{b}	0.094	0.18	2.45	0.63

E20+ LPS	-2.35 [b]	0.091	0.22	2.53	0.58
SEM	1.593	0.008	0.018	0.100	0.036
P-value	0.0306	0.9107	0.2998	0.1348	0.9476

Negative Control: (normal saline injection without feed additive), Positive Control: (LPS injection without feed additive) EC5+LPS (5 g *E. purpurea* with LPS injection), EC10+LPS (10 g *E. purpurea* with LPS injection), SEM; Standard Error of the Mean, BWL: Body weight loss,

[1] Percentage of body weight difference before and 24 h after LPS injection in LPS group and the normal, [a-b]; Different labels for each group compared with others shows significantly ($p<0.5$) statistical difference.

Results in Table IV shows that all the chickens which received LPS had weight loss, with the highest reduction in the LPS group in comparison to the negative control ($p=0.031$). By supplementation of *E. purpurea* to the basal diet, the percentage of weight loss decreased as compared to the LPS group, although no statistically significant difference was observed between these groups ($p>0.05$). The relative weights of vital organs were not different between the groups ($p>0.05$).

V. Discussion

Results have shown that LPS caused to higher express of COX-2 and IL-17F genes in chickens comparing to those were not received LPS. Infection of broiler chicken with *Salmonella enterica* increased IL-17 expression in cecum on day 4 after infection [18]. Also, it was reported that LPS injection increased the expression of other inflammatory cytokine such as Interleukin 1 beta (IL-1β) and Interleukin 6 (IL-6) [19]. It was stated that the effect of inflammation on the growth rate and metabolic process could be mediated by COX-2 expression and PGs release [20]. Since PGs activate special neuron pathways related to disease syndrome in the brain and suppress appetite centers and stimulated digestive hormones to anorexia [10].

Although some documents supported the increase of IL-17F expression by LPS injection in mice or rat [21] but it seems that there were scanty reports on the effect of LPS on IL-17F production in poultry. IL-17F has a major role in the inflammatory response and induces the production of other proinflammatory cytokines [22]. Infection of broiler chicken with Salmonella enterica increased IL-17F expression in cecum on day 4 after infection [23].

LPS leads to the increase in COX-2 gene expression which is consistent with the results of [24] and [19]. It was stated that inflammatory response on growth rate and metabolic process due to the immune system response in poultry are the result of proinflammatory cytokines released and COX-2 expression which affects PG-induced changes [25]. According to the results, COX-2 and IL-17F gene expression down regulated by *E. purpurea* supplementation in basal diet. This result is similar to the studies have shown that Echinacea has antiinflammatory and its bioactivity compounds including polysaccharides, caffeic acid and especially alkamides can reduce the ratio of inflammatory to anti-inflammatory cytokines and significantly prevent COX [25] and [26]. The results of in vivo and in vitro experiments have shown that administering Echinacea with stimulating immune systems prevented the production of inflammatory cytokines such as TNF-α and IL-1β and increased anti-inflammatory cytokines IL-4 and IL-10 [27].

The results of this study have noted that use of *E. purpurea* can affect the white blood cell count in chickens which have been implemented with inflammatory stress and reduce the ratio of heterophil in these chickens. It has been reported that changes in the amount of non-lymphoid and lymphoid leukocytes can be used to evaluate the immune system's condition [28]. The lymphocyte counts reduced and heterophil counts or heterophil to lymphocyte ratio increased 3 h and 24 hours after immune stress [29]. For heterophil, the ratio of heterophil to lymphocyte and eosinophile increased by 24 h and 12 h after injection of LPS in chickens, respectively [30] and [31].

Chicken body weight of LPS group was decreased 24 hours after the LPS challenge in present study [32]. Excessive immune response such as inflammation in poultry suppress growth performance which is marked by reduced feed consumption, muscle protein mass or accretion and wasted energy and protein for acute protein synthesis [33]. Compared with the saline injection, LPS injection decreased the body weight gain by 9.36% and feed intake by 14.87% in the LPSchallenged chickens [34]. In pair-feeding (the same amount of feed is fed) experiment with chickens, LPS injection significantly reduces weight gain and feed efficiency [35]. Also, body weight growth was lower in LPS treated chickens compared to negative control, also bursa weight reduction after 48 h, but no changes were observed for spleen and heart weights [30]. The non-existent weight change in vital organs in this study can be for the reason that the decrease in organ weight was relative to reduction in body weight.

VI. Conclusion

In general, results of the current study have stated that the use of *E. purpurea* can interfere in the IL-17F gene expression as inflammatory cytokine and down regulate the key enzyme COX. The changes in white blood cells minimizes by using this herb. In addition, Lymphocyte response was higher by 20% of *Echinacea Purpurea* herb.

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