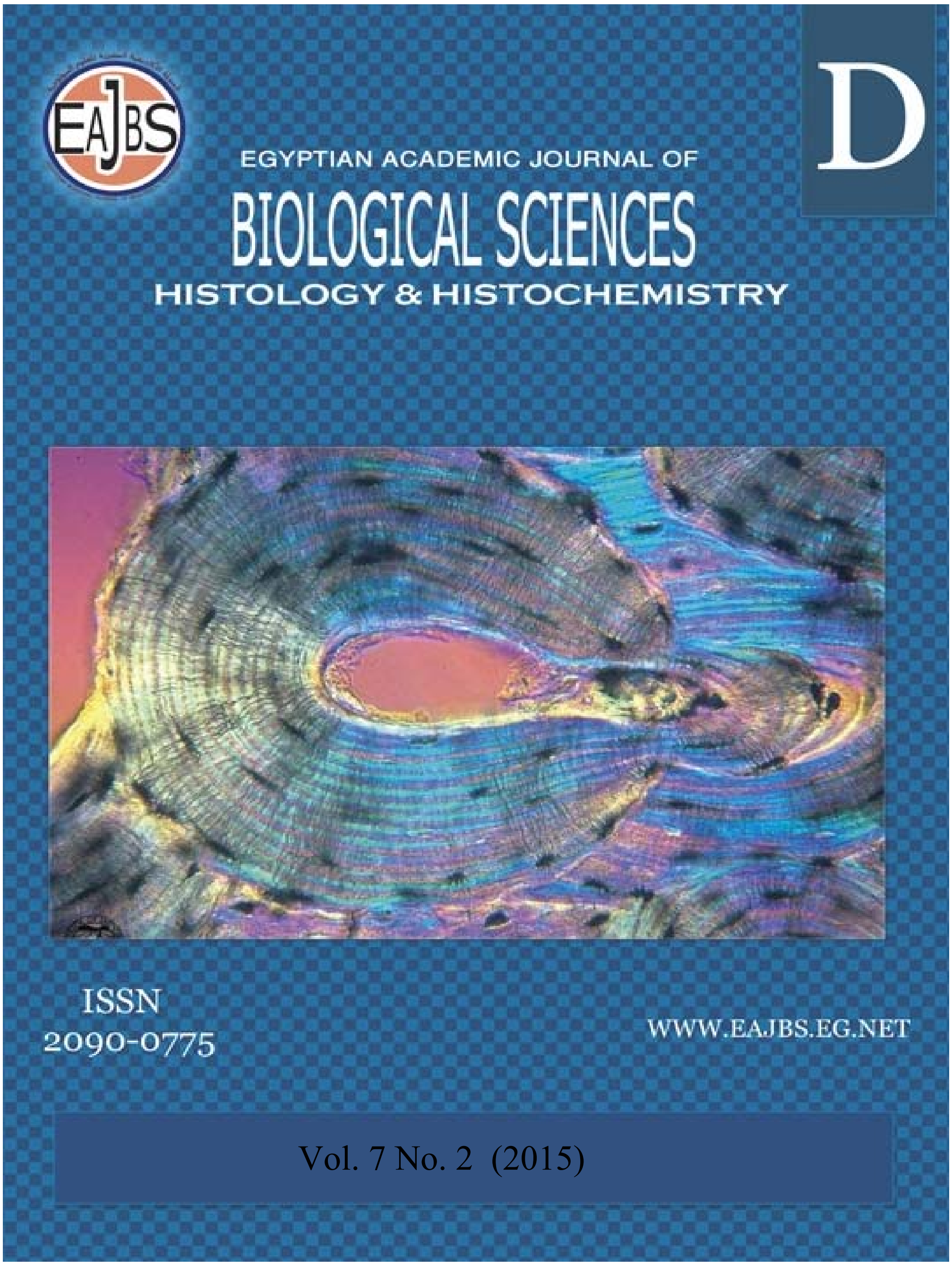

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Effect of Dietary Inclusion of Black Cumin (*Nigella sativa*) Seeds on Histological Parameters of Caecal Tonsils and Bursa of Fabricius in Broiler Chickens

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ABSTRACT

This study aimed to evaluate the effect of long term administration of *Nigella sativa* seed as an herbal immunomodulatory agent on histological features of caecal tonsils and bursa of Fabricius in broiler chickens. Fifty, one-day old chick were randomly divided into five equal groups and fed with diets containing 0, 0.5, 1, 1.5, and 2% of *N. sativa* seeds for 45 days. Then birds were slaughtered and 6µm-thick cross sections were made from caecal tonsils and bursa of Fabricius and stained with hematoxylin and eosin. Villus height and villus basal width, nodular unit width and height, follicular width and muscular layer width in caecal tonsils as well as height of plicae, follicular width, thickness of follicular cortex and medulla in bursa were determined. In addition, number of follicles in each plica in bursa of Fabricius and number of follicles per nodular unit in caecal tonsils were determined under light microscope. The results showed a change in all histomorphometric parameters of caecal tonsils due to *N. sativa* administration which followed a dose-dependent pattern in most cases. Villus height, nodular unit width and muscular layer width were decreased while other parameters increased as compared to control. Although all parameters in bursa increased due to *N. sativa* seed administration, plica height was the only parameter which showed a dose-dependent change. In conclusion, dietary inclusion of *N .sativa* especially at 2% dose, can improve immune structures of both bursa of Fabricius and caecal tonsils of broilers.

INTRODUCTION

Stressful conditions in intensive poultry farming can change immune responses and increase the health risks including the chance of development and spreading of microbial diseases.

Vaccination and antibiotics are used routinely as main strategies for protection and /or treatment of industrial chickens. However development of resistant pathogenic strains, vaccination stress or incomplete protection after vaccination as well as presence of drug residues in edible tissues adversely affect efficiency and decrease the benefits of using these approaches.

A strong immune status is a key factor to fight against infectious diseases and can result in better protective responses after vaccination.

Nutritional immunomodulators for example vitamin D3 and yeast cell wall products (Huff *et al.* 2009) as well as different immunostimulant agents with herbal origins are investigated for their possible immunomodulatory properties in chickens. Botanical agents are more accessible at least in some areas and are usually well known for their high safety in both humans and animals. Today extensive investigations for finding alternatives especially for antibiotic use are in progress and of course the use of medicinal plants has gained a good niche in this regard.

Nigella sativa Linn. (Ranunculaceae) is an annual flowering plant native to south and southwest Asia. The seeds of *N. sativa* are commonly known as black seed or black cumin, are used as an spice as well as in folk medicine as diuretic and antihypertensive, digestive and appetite stimulant, antidiarrheal, analgesic, anthelmintic and antibacterial agent (Al-Gaby 1988; Zaoui *et al.* 2000; Gilani *et al.* 2004; Gilani *et al.* 2001; Khan *et al.* 1999; Agarwal *et al.* 1979; Chowdhury *et al.* 1998; Ferdous *et al.* 1992; El-Kamali *et al.* 1998). Moreover antidiabetic, anticancer, anti-inflammatory, spasmolytic and bronchodilatory, hepatoprotective, renoprotective and antioxidant properties of black cumin seeds (BCS) are discovered (Meral *et al.* 2001; Abuharfeil *et al.* 2001; Farah *et al.* 2003; Al-Ghamdi 2001; Janbaz *et al.* 2003; Badary *et al.* 2000; Mansour *et al.* 2002).

One of the interesting aspects of BCS is its immunomodulatory effects which have been addressed by different investigators. Immune functions such as helper T cells to suppressor T cells ratio as well as natural killer cell activity have been improved in human volunteers (El-Tahir *et al.* 1993) and serum proteins and total immunoglobulin levels were increased in rainbow trout fish (Dorucu *et al.*. 2009) due to BCS administration.

Immunostimulatory effects of BCS have also been demonstrated in broiler chickens (AL-Beitawi *et al.* 2009; Al-Mufarrej 2014), as described by higher antibody titers of birds received the seeds compared to control without any adverse effects on performance. This motivated us to evaluate the effect of dietary inclusion of different doses of *N. sativa* on histological parameters of caecal tonsils and bursa of Fabricius as basic lymphoid organs in broiler chickens, which can help in better description of its immunomodulatory effects in poultry.

MATERIALS AND METHODS

Plant, animals and experimental design

N. sativa seeds were purchased from a local medicinal herb shop in Tehran Province, Iran and authenticated by specialists in the Institute of Medical Plants, Tehran, Iran. Fifty, one-day old chickens (Ross 308) were housed in individual cages in a temperature-controlled room (33±1°C during the first week and then the temperature decreased 2°C per week and kept at 24±2°C from day 22 until the end of the experiment) under 16:8 h light/dark cycle. Birds were randomly divided into five equal groups and fed with diets contained 0.5, 1, 1.5, and 2% of BCS (experimental groups) or basal diet with the nutrient specification as control group for 45 consecutive days. Birds had free access to feed and tap water during the experimental period.

Institutional (Karaj branch of Islamic Azad University) ethical guidelines were strictly followed during the whole experimental period and also in the case of euthanasia and sample collection.

Sample preparation and histological evaluation

At the end of experiment, all birds were slaughtered by decapitation and bursa of Fabricius as well as proximal part of caeca or caecal tonsils were removed immediately. After fixation in 10% buffered formalin (1.33 Molar) and routine histological laboratory methods, 6µm-thick transverse sections were made. From each caecal tonsil or bursa of each bird a total number of 10 slides were prepared and stained with hematoxylin and eosin. Criteria for evaluation were as follows: villus height and villus basal width at the crypt-villus junction, nodular unit width and height, follicular width and muscular layer width in caecal tonsils as well as height of plicae, follicular width, thickness of follicular cortex and medulla in bursa that were measured by a linear graticule. Moreover, number of follicles in each plica in bursa of Fabricius and number of follicles per nodular unit in caecal tonsils were also counted under light microscope. Arithmetic mean of 15 measurements of each parameter per section was calculated.

Statistical analysis

Data were presented as mean±SD. Normality test was passed and one-way ANOVA method followed by Tukey's multiple comparison test was used for data comparisons with $p<0.05$ as the significant level (IBM SPSS statistics software 22). Analysis of trend for determining linear, quadratic and cubic polynomial contrasts for evaluation of the effects of different levels of BCS was also performed (IBM SPSS statistics software 22).

RESULTS

The results showed a change in all histomorphometric parameters of caecal tonsils due to BCS administration which followed a dose-dependent pattern in most cases (Table 1). Villus height, nodular unit width and muscular layer width were decreased while other parameters increased as a result of BCS consumption.

Table 1: Histomorphometric parameters of caecal tonsils of birds in different groups at the end of experiment

Parameters / Groups	Villus height (mm)	Villus width (mm)	Nodular unit width (mm)	Nodular unit height (mm)	Follicular width (mm)	Follicle number per nodular unit	Muscular layer width (mm)
control	$0.17±0.03^{a}$	$0.06±0.01^{b}$	$0.37±0.03^{a}$	$0.59±0.07^{b}$	$0.12±0.03^{b}$	$6.00±1.00^{c}$	$0.09±0.002^{a}$
*N.sativa*0.5%	$0.15±0.02^{a}$	$0.07±0.01^{a}$	$0.28±0.01^{b}$	$0.76±0.04^{a}$	$0.12±0.01^{b}$	$10.80±0.53^{b}$	$0.06±0.001^{b}$
N.sativa 1%	$0.13±0.03^{ab}$	$0.07±0.01^{a}$	$0.28±0.02^{b,c}$	$0.77±0.04^{a}$	$0.15±0.02^{a}$	$11.00±0.24^{b}$	$0.06±0.001^{b}$
N.sativa1.5%	$0.10±0.01^{b}$	$0.07±0.01^{a,b}$	$0.24±0.02^{c}$	$0.84±0.06^{a}$	$0.17±0.02^{a}$	$15.40±0.09^{b,a}$	$0.06±0.003^{b}$
N.sativa 2%	$0.10±0.01^{b}$	$0.09±0.01^{a}$	$0.28±0.02^{b,c}$	$0.82±0.03^{a}$	$0.17±0.02^{a}$	$16.60±1.07^{a}$	$0.05±0.01^{b}$
Contrast	Linear term p=0.000 Quadratic term P=0.568 cubic term P=0.249	Linear term p=0.000 Quadratic term P=0.420 cubic term P=0.342	Linear term p=0.000 Quadratic term P=0.000 cubic term P=0.624	Linear term p=0.000 Quadratic term P=0.002 cubic term P=0.286	Linear term p=0.000 Quadratic term P=0.468 cubic term P=0.549	Linear term p=0.000 Quadratic term P=0.179 cubic term P=0.451	Linear term p=0.000 Quadratic term P=0.000 cubic term P=0.001

Different superscript letters demonstrate significant difference in a column ($p<0.05$).

The most notable change was in follicle number per nodular unit of birds that received 2% BCS which was 2.8 times higher than that of control group (Fig. 1). As shown in Table 2, although all parameters in bursa increased due to BCS administration, pelica height was the only parameter which showed a dose-dependent change. Inclusion of BCS at the dose of 1% had the highest effect on number of follicles per plica although it was only slightly higher than that of 1.5% and 2% groups.

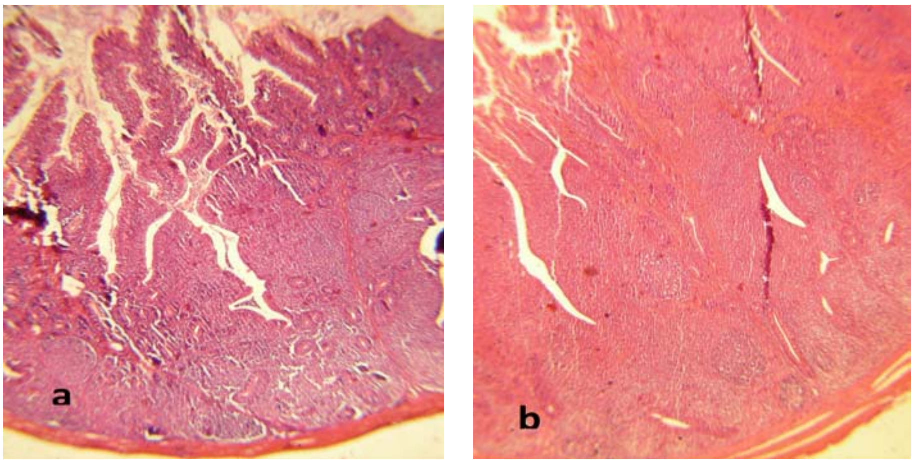

Fig. 1: Representative photomicrographs of caecal tonsil of birds in control group (a) treated with *N. sativa 2%* (b), (H and E, 40 x)(bar=40µm). Villi look shorter while the follicle number per nodular unit has been increased in *N. sativa* treated birds.

Doses of 0.5% and 2% had higher effect on follicular width, cortical and medullar thickness than other treated groups (Fig. 2). A linear trend in incremented doses for all measured parameters was observed both in caecal tonsils and bursa of Fabricius (Tables 1 and 2).

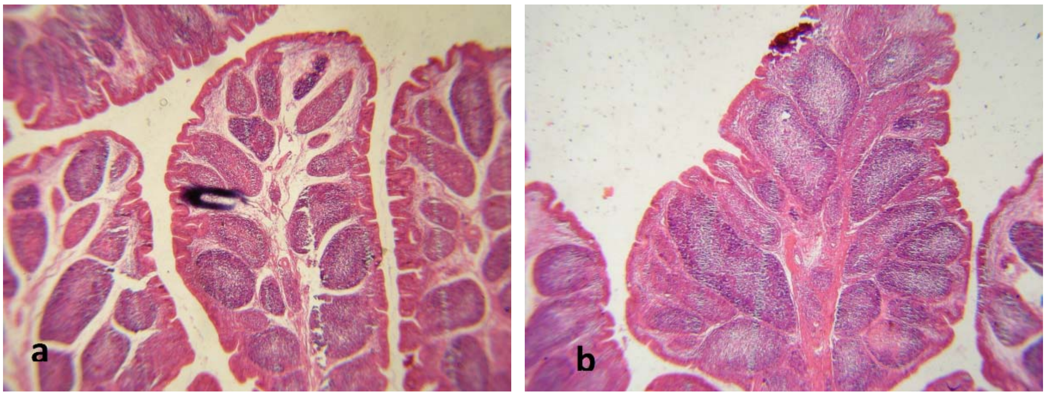

Fig. 2: Representative photomicrographs of bursa of Fabricius of birds in control group (a) and treated with *N. sativa 2%* (b), showing relatively higher number of wider follicles per plica in treated birds, (H and E, 40 x)(bar=40µm).

Table 2: Histomorphometric parameters of bursa of Fabricius of birds in different groups at the end of experiment.

Parameter / groups	Pelica height (mm)	Follicular width (mm)	Follicular cortex thickness(mm)	Follicular medulla thickness(mm)	Number of follicles per pelica
control	1.83 ± 0.14^{a}	0.24 ± 0.04^{a}	0.02 ± 0.01^{a}	0.19 ± 0.03^{a}	27.27 ± 5.11^{a}
N.sativa0.5%	2.34 ± 0.09^{b}	0.39 ± 0.05^{b}	0.07 ± 0.01^{b}	0.25 ± 0.03^{c}	31.20 ± 7.15^{a}
N.sativa 1%	2.72 ± 0.01^{c}	0.29 ± 0.04^{c}	0.04 ± 0.01^{c}	$0.21\pm0.03^{b,a}$	44.20 ± 3.73^{b}
N.sativa1.5%	2.90 ± 0.11^{d}	0.36 ± 0.03^{b}	0.06 ± 0.01^{b}	$0.23\pm0.02^{b,c}$	39.60 ± 8.22^{b}
N.sativa 2%	3.30 ± 0.14^{e}	0.46 ± 0.03^{d}	0.07 ± 0.01^{b}	0.32 ± 0.02^{d}	42.93 ± 10.10^{b}
Contrast:	Linear term p=0.000 Quadratic term P=0.000 cubic term P=0.000	Linear term p=0.000 Quadratic term P=0.102 cubic term P=0.000	Linear term p=0.000 Quadratic term P=0.010 cubic term P=0.000	Linear term p=0.000 Quadratic term P=0.001 cubic term P=0.000	Linear term p=0.000 Quadratic term P=0.009 cubic term P=0.848

Different superscript letters demonstrate significant difference in a column ($p<0.05$).

DISCUSSION

Regarding the remarkable role of caecal tonsil and bursa of Fabricius as primary and basic lymphoid organs in poultry, this study aimed to evaluate the effect of long term administration (from day one post hatch to slaughter at day 46) of *N. sativa* seed as an herbal immunomodulatory agent on histological features of these organs in broiler chickens.

Due to the back flowing of urine from the urodeum of the cloaca through the colon, caecum is constantly invaded by bacteria or non bacterial organisms of extra caecal origin (Kitagawa *et al.* 1998). Caecal tonsils are defined lymphoid tissues that are formed at the anti mesenteric side of the caeca and are considered as an important part of gut-associated lymphoid tissues (GALT) in chickens. In fact caecal tonsils are the largest lymphoid organ of the avian GALT that contain both T and B cells in germinal centers (Lillehoj and Trout 1996) therefore they may be an alternative location for B cell differentiation and antibody production as well as cell-mediated immune functions.

About 46% of lymph nodules are gathered in caecal tonsils of 6-months old white Leghorn chickens (Kitagawa *et al.* 1998) and caecal tonsils have appreciable role in immune responses against important infectious diseases of poultry such as New castle disease, influenza *etc.*

Acceleration of immune potential of this lymphoid organ may potentiate the ability of chickens to encounter infectious diseases and augment post vaccinational immunity.

The filtering capacity of interdigitating meshwork of villi at the caecal entrance blocks large particles and allows only fluid and fine particles to enter into the caeca by colonic anti peristalsis. Our data clearly demonstrate that consumption of BCS is dose dependently able to induce changes in histomorphometric parameters of caecal tonsils. In the present study BCS consumption resulted in shortening and flattening of the villi that increases the exposure of immune structures of caecal tonsils to potential antigens in the gut. On the other hand, thinning of muscular layer in BCS-treated groups lowers the strength for peristaltic movements and helps in longer presence of microorganisms and other antigens around the immune structures in caecal tonsils. The prominent increase in follicular width as well as number of follicles per nodular unit especially in 1.5 and 2% BCS-treated group may be due to the higher stimulation of immune structures as stated above.

Bursa of Fabricius is an epithelial and lymphoid organ which belongs to GALT in chickens. It is a dorsal diverticulum of proctodeal region of the cloaca. The luminal surface of the bursa is plicated. These plicae contain bursal follicles with follicle-associated epithelial cells, lymphocytes, macrophages, and plasma cells (Ciriaco *et al.,* 2003). A thick, smooth muscle layer surrounds bursa.

Development of the antibody-producing B-lymphocyte lineage in birds mostly takes place in bursa (Mustonen *et al.* 2010). Our data clearly demonstrate that *N. sativa* seed is able to induce changes in histomorphometric parameters of bursa of Fabricius where bursal follicles of birds treated with *N. Sativa* especially at the highest dose were larger and more frequent as compared to control group.

A great amount cell division occurs in the follicular cortex after hatch and most of the peripheral B cells are originated from the follicular cortex (Paramithiotis and Ratcliffe 1994). We observed an obvious increase in follicular cortex width in our study, which may be due to higher cell proliferation and/or lower cell migration from cortex to periphery. Each day only about 5% of the generated bursal cells immigrate to the periphery (Lassila 1989) and migration of cells from the cortex to the medulla is not considerable (Paramithiotis and Ratcliffe 1994); this strengthen the probability that higher cell proliferation is more responsible for the increase in follicular cortex as observed in our study, especially with regard to higher follicular width and thicker follicular medulla. Inhibition of apoptosis of B-lymphocytes may be another influencing factor for this observation. The exact distinction between these parameters and strict determination of their roles needs further investigations.

In conclusion, dietary inclusion of *N. sativa* especially at 2% dose during the rearing period of broilers, can improve immune structures of both bursa of Fabricius and caecal tonsils of broilers which may positively affect the ability these organs to encounter offending agents.

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