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IMMUNOMODULATION AND STRENGTHENING OF SERUM MINERAL PROFILE BY DIETARY SUPPLEMENTATION OF PROTEIN, PROBIOTICS AND VITAMINS (C AND E) IN MOLTED LAYER BREEDERS

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This study was executed to test the effect of low crude protein (12% CP), probiotics (Protexin®), vitamins (C and E) and combination of these treatments on serum mineral profile, cell mediated and humoral immunity of molted White Leghorn breeder males. Birds of 59 weeks age (n=270) were maintained at animal house of Faculty of Veterinary Science, University of Agriculture, Faisalabad, for this study. Normal breeder diet containing crude protein (CP) 16% (140 g/bird/day) was provided with fresh drinking water (*ad libitum*) and daily lighting schedule of 16 hours was fixed. After one week acclimatization, all the birds were forced molted by dietary ZnO (3g/Kg diet) until desired body weight reduction. These birds were divided into six groups (45 birds/group), post molting. Group A served as a control (normal diet). Other Groups were supplemented with diet containing vitamin C (500 mg/Kg), vitamin E (90 mg/Kg), probiotics (0.05 g/Kg), 12% CP and a combination of the aforementioned supplementations, respectively. Serum samples were collected for five consecutive weeks at one week interval after the start of semen production. Significant ($P \leq 0.05$) increase in serum concentrations of sodium, magnesium and calcium were found in vitamin E supplemented group. Macrophage engulfment percentage was also higher in both opsonized and unopsonized conditions (60 and 90 min) in vitamin E supplemented group. Moreover, serum antibody titers against ND (heamagglutination inhibition) and IB (indirect ELISA) were improved. In conclusion, vitamin E may be the best option for improving health and immunity against these diseases, during post molt period.

Keywords: Probiotics, cell mediated immunity, humoral immunity, macrophage engulfment percentage, opsonized, unopsonized.

INTRODUCTION

Molting is a natural phenomenon in birds during which periodic replacement and shedding of feathers occur. However, retired birds are commonly molted through forced molting. This technique is an economical and important management tool, used extensively in poultry industry to rejuvenate internal organs of birds for second and third cycle of production with an intention to improve their production efficiency. Molting is done by various methods *i.e.* feed withdrawal, photoperiod reduction, low aluminum, iodine diet (Khan *et al*., 2011), low energy, protein, calcium, sodium feed and high dietary zinc, (Wu *et al*., 2000). Among forced molting, Zn induced molting is considered to be superior (Khan *et al*., 2011), because of strengthening of acquired immune response in hen during second cycle of production (Sandhu *et al*., 2006) and more IgG and IgM production (Sandhu *et al*., 2007).

Besides molting, there are other factors such as restriction of dietary protein, supplementation of probiotics (Li *et al*., 2014) and vitamins, which are also known to affect the subsequent performance of molted birds (Khan *et al*., 2014). Crude protein (CP) is important for growth, reproductive performance and development of gastrointestinal tract and villus area. However, lower protein level improves beneficial intestinal microflora. Probiotics ('for life') are live microbial poultry feed supplements which improve microbial balance in intestine. Dietary inclusion of probiotics has also shown to improve immune response in chicks (Khan *et al*., 2013). A diet supplemented with probiotic or symbiotic or rich in protein enhances immunity of the body in molted hens (Anwar *et al*., 2015). Vitamins (C and E) play important role in improving health status of molted White Leghorn breeder males (Iftikhar *et al*., 2015). Vitamin E showed increased immunity in chickens against various diseases like infectious bursal disease (IBD), *E.coli* infection, coccidiosis and Newcastle disease (Akbari *et al*., 2008). It also plays important role in modulating serum mineral profile, as Mg level increases by the supplementation of vitamin E (Khan *et al*., 2013). Vitamin C plays an important role in the metabolism of minerals and amino acids. Vitamin C supplementation has been reported to improve IBD induced

immunosuppression, along with cellular and humoral immunity (Wu *et al*., 2000). Serum concentration of Ca, P, K, Fe and Zn was found to increase in quails with supplementation of ascorbic acid in their diet (Sahin *et al*., 2001).

Practices like protein restriction, provision of probiotics, vit. C and E have been used in broilers, layers and broiler breeders for determination of cell mediated immunity, humoral immunity and serum mineral profile, however, information regarding the use of these supplements in male layer breeders is scanty. This study was therefore, planned to investigate the impact of protein, probiotics vit. C and E on immunological (cell mediated immunity, humoral immunity) and health profile of retired male layer breeders after molting.

MATERIALS AND METHODS

***Experimental design*:** Two hundred seventy, White Leghorn breeder males (age=59 weeks) were procured from a commercial poultry breeder farm. The birds were acclimatized for one week and fed layer breeder diet (140 g/bird/day) having 16% CP. Fresh drinking water was available *ad libitum* and daily lighting schedule of 16 hours was fixed. Birds were immunized at 60th week against ND and IB (MSD-Intervet®) through drinking water. At the start of 2nd week, all the birds were subjected to forced molting process through feeding zinc oxide (3g/kg feed) and gradually decreasing day light from 16 to 12 hours. Dietary addition of ZnO was continued till the body weight of the birds was reduced by 20-25%. After completion of molting, the birds were randomly divided into six groups having 45 birds in each group. First group served as control and was kept on normal layer breeder diet. Whereas, the birds in remaining groups were fed diet supplemented with vitamin C (500 mg/Kg), vitamin E (90 mg/Kg), probiotics (Protexin®; @ 0.05 g/Kg of feed), protein (12%) and a combination of the aforementioned supplementations, respectively.

***Sample collection and determination of mineral profile*:** Serum samples from birds were collected after one week of first semen ejaculate and continued for the next five weeks. Collected serum samples from 6 birds/group were analysed for the determination of macro and micro minerals concentration. Serum samples (1 ml each) were subjected to wet digestion as described by Rahman and Akhtar (1993). The concentration of calcium (λ=422.7 nm), magnesium (λ=285.2 nm), copper (λ=324.8 nm), zinc (λ=213.9 nm), manganese (λ=279.6 nm) and iron (λ=248.3 nm) was determined by atomic absorption spectrometer (Hitachi Polarized Zeeman, Japan). Concentrations of Na and K were determined by flame photometer by subjecting all the samples to wet digestion.

Macrophage engulfment assay

***Harvesting abdominal macrophage cells*:** The method described by Qureshi *et al*. (1986) was used to collect the abdominal exudate macrophages. Briefly, 3% pre swollen Sephadex G-50® (Sigma) suspension was injected I/P @ 10 mL/kg of the body weight of the birds. Forty two (42) hours post injection, the birds were euthanized, and abdominal macrophage cells were collected for determination of macrophage engulfment percentage.

***Delayed type of hypersensitivity reaction (DNCB assay)*:** One percent solution of 2,4 di-nitro-chloro-benzene (DNCB) was prepared in acetone. Five birds from each group at each sampling stage were selected for this assay. One ml of DNCB was dripped drop by drop with an insulin syringe on to the skin of thigh region as a primary inoculation in one inch marked circle. The skin sensitization was done by the method of Tiwary and Goel (1985). The skin thickness before, then after 24 and 72 hours of the DNCB application were measured using a vernier caliper.

***Antibody titer against NDV and IBV*:** Antibody titres for ND and IB virus were measured in the serum samples using mesogenic strain of ND virus and pathogenic variant of IB virus obtained from the Institute of Microbiology. Micro titration haemagglutination inhibition (HI) test was applied for the determination of antibody titer against ND virus. Indirect ELISA test (IDEXX Kit, USA) was performed to evaluate antibody response to IB virus (Boulianne *et al*., 2013).

***Statistical analysis*:** Data obtained was subjected to two-way analysis of variance and the means were compared by using Duncan's Multiple Range test (Duncan, 1955). A two-way analysis of variance was conducted to compare the data statistically among different groups at various production stages. The data was analyzed by using CoStat 6.4® and GraphPad Prism 5.04® statistical softwares.

RESULTS

***Serum minerals*:** Serum macro mineral (Na, K, Ca, Mg) concentrations are shown in Table 1. Overall serum sodium concentration was significantly (P≤0.05) high in vitamin E and protein supplemented groups. Highest serum potassium concentration was detected in the birds of control group, followed by those of groups fed diets supplemented with vitamin C and combination diet. The highest serum calcium and magnesium concentrations were observed in the birds fed diet supplemented with vitamin E. Serum micro mineral (Zn, Fe, Cu, Mn) concentration showed that serum iron, copper and manganese concentrations did not vary among groups. Highest value of zinc was observed in birds fed diet supplemented with vitamin C and probiotics. These results advocate strengthening of serum mineral profile by vitamins E (Na, Mg, Ca), vitamin C (Mg, Zn) and probiotics (Zn) supplementation.

***Unopsonized and opsonized macrophage engulfment percentage (60 and 90 min)*:** Statistical analysis of the data showed a high percentage of macrophage engulfment at 60

Table 1. Mean serum sodium, potassium, calcium, magnesium iron, zinc, copper and mangnese (mg/L±SE) concentrations of molted control and treated Whit Leghorn breeder males.

	Sodium (Na; mg/L±SE)	**Potassium** (K; mg/L±SE)	**Calcium** (Ca; mg/L±SE)	**Magnesium** (Mg; mg/L±SE)	**Iron** (Fe; mg/L±SE)	**Zinc** (Zn; mg/L±SE)	**Copper** (Cu; mg/L±SE)	**Mangnese** (Mn; mg/L±SE)
Control	2341 ±146.4b	163.79 ±14.76a	191.9 ±11.35c	28.22 ±1.459c	32.70 ±2.613	7.360 ±0.323e	3.33 ±0.186	0.97 ±0.015
Vitamin C	2432 ±104.4ab	156.80 ±13.69ab	212.8 ±11.96c	33.24 ±1.659a	32.11 ±2.488	11.74 ±0.779a	3.55 ±0.126	1.00 ±0.016
Vitamin E	2521 ±75.30a	147.86 ±12.20bc	248.5 ±11.72a	34.52 ±1.351a	32.25 ±1.490	11.02 ±1.050b	3.40 ±0.125	0.98 ±0.013
Probiotics	2146 ±141.3c	143.57 ±8.103c	201.8 ±12.73bc	27.05 ±0.790c	30.07 ±1.279	11.60 ±1.283a	3.44 ±0.144	0.99 ±0.018
12% CP	2516 ±84.86a	150.22 ±14.97bc	229.2 ±10.96ab	30.70 ±1.519b	30.98 ±2.013	10.30 ±1.124c	3.27 ±0.219	0.99 ±0.014
Combination	2367 ±42.99b	156.73 ±6.787ab	225.2 ±18.40ab	28.62 ±2.027c	32.09 ±2.544	9.160 ±1.414d	3.47 ±0.307	0.98 ±0.009

A-E Mean values within a column, having different alphabets do differ significantly (P<0.05).

and 90 minutes under unopsonized (not sensitized with antibody) condition, in vitamin E supplemented group followed by vitamin C and combination groups. Similarly, macrophage engulfment percentage at 60 and 90 minutes under opsonized (sensitized with antibody) condition, was high in birds supplemented with vitamin E, followed by birds of vitamin C supplemented group (Table 2).

Table 2. Mean macrophage engulfment percentage (%±SE) at 60 & 90 minutes in opsonized & unopsonized condition of molted control and treated White Leghorn breeder males.

	Unopsonized		**Opsonized**	
	60 min. (%)	**90 min.** (%)	**60 min.** (%)	**90 min.** (%)
Control	10.77 ±0.192d	11.66 ±0.720d	42.16 ±1.170d	52.92 ±0.782e
Vitamin C	13.06 ±0.514b	14.67 ±0.652b	48.61 ±1.468b	60.73 ±1.003b
Vitamin E	15.52 ±0.797a	16.59 ±0.858a	52.76 ±0.532a	71.53 ±3.303a
Probiotics	11.61 ±0.253c	13.57 ±0.724c	45.96 ±1.326c	56.99 ±1.033d
12% CP	11.87 ±0.371c	13.46 ±0.758c	45.67 ±0.384c	56.21 ±1.319d
Combination	12.77 ±0.725b	14.87 ±0.425b	45.28 ±0.268c	59.12 ±1.591c

A-E Mean values within a column, having different alphabets do differ significantly (P<0.05).

***DNCB assay*:** Results of delayed type of hypersensitivity, after 24 and 72 hours of DNCB application showed significantly higher (P≤0.05) values of skin thickness in birds of vitamin E supplemented group when compared to control, followed by vitamin C and probiotics group (Table 3).

Table 3. Overall mean, increase in skin thickness (mm ± SE) of molted control and treated White Leghorn breeder males groups at 24 & 72 hrs after the inoculation of DNCB.

	24 hrs	**72 hrs**
Control	0.93±0.018d	1.01±0.058d
Vitamin C	2.10±0.117b	1.80±0.040b
Vitamin E	3.05±0.332a	3.14±0.206a
Probiotics	2.16±0.068b	1.73±0.104b
12% CP	1.18±0.067c	1.81±0.020b
Combination	1.29±0.032c	1.28±0.016c

A-D Mean values within a column, having different alphabets do differ significantly (P<0.05).

***Serum antibody titer against ND and IB*:** The results of geometric mean titer for ND and IB virus (Table 4) showed that in vitamin E supplemented group, GMT for ND virus was highest followed by vitamin C and probiotics supplemented groups. Similarly, GMT for IB virus was also highest in vitamin E supplemented group followed by probiotics and vitamin C supplemented groups.

Table 4. Serum geometric mean antibody titer (GMT) of molted control and treated White Leghorn breeder males against ND (New Castle Disease) & IB (Infectious Bronchitis) at different weeks of semen production.

	ND (GMT)	**IB** (GMT)
Control	168	5338.52
Vitamin C	337	5931.60
Vitamin E	445	7698.72
Probiotics	256	6219.72
12% CP	168	5825.54
Combination	222	5253.16

DISCUSSION

Avian immune system has two types; humoral and cell mediated immunity, which provides protection and prevention against pathogens. Cell mediated immunity destroys the cells which are infected with foreign agents like bacteria (Aderem *et al*., 2014). During this process, macrophage engulfs foreign particles and thus provides immunity to poultry birds. The results showed that birds fed diet supplemented with vitamin E exhibited the highest percentage of macrophage engulfment of sheep RBCs in opsonised and unopsonised (at 60 and 90 minutes) conditions. Meydani *et al*. (1990) have reviewed that membrane phospholipids of macrophages contain high concentration of arachidonic acid. Which upon stimulation release up to 50% arachidonic acid to form reactive oxygen species (ROS) like: prostaglandin, hydroxeicosatetraenoic acid and leukotriene. Being an antioxidant, vitamin E has been observed to decrease production of ROS in immune cells and enhance cell mediated immunity (macrophage engulfment). This highest percentage of macrophage engulfment is compatible with those observed by Gore and Qureshi (1997) who found that addition of 10 IU of vitamin E markedly increased the percentage of macrophage engulfment in chickens, when those were injected (*in ovo*) during embryonic stage of life. Increase in macrophage engulfment percentage has also been observed by Niu *et al*. (2009) due to dietary addition of vitamin E (200 mg/Kg diet) in stressed broilers. As suggested by Qureshi *et al*. (2000) the process of macrophage engulfment is a membrane mediated phenomenon and can be sustained by making available high levels of vitamin E required for the process of phagocytosis. Increased macrophage engulfment response in molted White Leghorn breeder males, may also be related to the decrease in cortisol level in the birds fed diet containing vitamin E when compared to those of control group, as has been observed by Anwar and Rahman (2011) in layers, probably due to the production of nitric oxide. Chicken macrophages have been known to produce nitric oxide (Hussain and Qureshi, 1997) under various immunological stimuli.

Higher dose of vitamin E maintains integrity of macrophage membrane, which is important for phagocytosis, being antioxidant it prevents the oxidation of the arachidonic acid, important for biosynthesis pathway of prostaglandin having immunosuppressive role at an increased level. A significant increase in mean nitric oxide has been observed by supplementation of vitamin E in the diet of male broiler breeders (Khan *et al*., 2012). Immune-modulation potential has been validated in a study through utilization of dietary vit. E (Sandhu *et al*., 2013). The immunomodulatory role of vitamin E has been found to be linked with its antioxidant potential (Khan *et al*., 2012). Role of vitamin E may be immuonomodulatory itself or its immunomodulatory effect may correspond to the level which is required for lipid peroxidation inhibition and protection of microsomes and mitochondria of liver from oxidative stress (Leshchinsky and Klasing, 2001). Being primary antioxidant of cell membrane, it is for the prevention of fatty acid peroxidation. Both *in vitro* and *in vivo* investigation showed that vitamin E inhibits peroxidation of lipid by breaking chain propagation (Niki, 2014).

Response regarding skin thickness of the birds was found to be more stable both after 24 and 72 hours of DNCB sensitization, depicting a significantly higher immune potentiating role of all the treatments. However, the effect due to the dietary inclusion of vitamin E was more pronounced as compared to all other treatment groups. The effect due to probiotics supplementation after 24 hours was also higher when compared to its counter parts but was still lower than vitamin E group. The delayed skin inflammation resolution may be attributed to body's strengthened immune responses.

Dietary supplementation of probiotics is known to have a significant immuno-stimulatory role in molted poultry birds (Anwar *et al*., 2015). Probiotics are powerful strategy for manipulation of host immune response and microbial composition. Direct interaction between gut microbiota and immune system has been reviewed that allows host to tolerate various antigens present in gut (Vieira *et al*., 2013). Growing number of studies showed beneficial effects of probiotics on host health (Mountzouris *et al*., 2010). A marked increase in cutaneous basophilic hypersensitivity response (CBH) in skin thickness of 64 weeks old White Leghorn layers after inoculating with phyto-haemagglutinin-P (PHP) has also been observed by Panda *et al*. (2003) due to the dietary addition of probiotics. They also reported that increased migration and accumulation of T-cells at the site of inoculation of DNCB was responsible for the increased hypersensitivity response of skin in the birds fed probiotics supplemented diets.

Serum antibody titers against ND and IB vaccine, in molted White Leghorn breeder males were found to be higher for all the treatments. In case of IB, the effect due to the dietary inclusion of vitamin E (GMT=7698.72) was more pronounced followed by probiotics (GMT=6219.72) and vitamin C (GMT=5931.6) supplemented groups. While serum antibody titers against ND vaccine were more pronounced vitamin E (GMT=445) followed by vitamin C (GMT=337) and probiotics (GMT=256) treated groups. Against ND, immunopotentiating effect is produced by Zn induced molting (Sandhu *et al*., 2007). Similar to our findings, ascorbic acid supplementation @ 800mg/Kg increased antibody titer in heat stressed broilers, against ND vaccine (Aengwanich *et al*., 2003). Similarly, birds supplemented with vitamin E used @ 20mg/Kg increased antibody titers against IB vaccine in cockerels (Lin, 2005). Dose dependant increase in production of antibody was found by Leshchinsky and Klasing (2001) in response to attenuated IB virus between 0 and 25 IU/Kg of supplementation of vitamin E.

***Conclusion*:** Vitamin E and vitamin C are useful in improving immunity (cell mediated and humoral), and strengthening of serum mineral profile during post molt period, in White Leghorn breeder males. Probiotics supplementation also have role in improving immune status of molted birds. However, vitamin E is found to be the best option for improving health and immune response against Newcastle and infectious bronchitis disease. Hence dietary supplementation of vitamin E is recommended in molted birds.

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