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Original study

Supplementation of vitamins, probiotics and proteins on oxidative stress, enzymes and hormones in post-moult male broiler breeders

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Abstract

This study was planned to investigate the comparative effect of vitamin E and C, probiotics, protein level and the combination of these treatments on body weight, serum total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), aspartate aminotransferase (AST), alanine aminotransferase (ALT), triiodothyronine (T_3), thyroxine (T_4), cortisol and some minerals in Zn-induced male broiler breeders. A total of 180 Hubbard male broiler breeders (65 weeks of age) were induced to moult. After moulting, the birds were randomly distributed into six groups. One group was fed vitamin E (100 IU/kg feed), second group was fed vitamin C (500 IU/kg feed), third group was fed probiotics (50 mg/L), fourth group was fed lower protein level (14 % crude protein) and the fifth group was fed the combination of the mentioned treatments. The sixth group served as control. The results revealed that serum TAC significantly increased ($P<0.05$) while TOS and OSI decreased ($P<0.05$) in the vitamin E fed group compared to the other treated groups. The analyses of the samples indicated that AST and ALT decreased significantly in the vitamin E supplemented group while T_3 and T_4 increased significantly as a result of this treatment. Cortisol concentration decreased significantly in the vitamin E fed group. Serum Mg increased significantly in the vitamin E supplemented group. The results indicated that the physiological biochemistry disrupted in male breeders as a consequence of moulting could be compensated by feeding vitamin E.

Keywords: Moulting, male broiler breeders, vitamins, probiotics, oxidative stress

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Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; CP: crude protein; OSI: oxidative stress index; T3: triiodothyronine; T4: thyroxine; TAC: total antioxidant capacity; TOS: total oxidant status

Introduction

Force moulting is an economical practice which enhances the productive and reproductive life span of the birds. This process has been associated with sudden change in physiological biochemistry (Berry 2003, Khan *et al*. 2011) which requires restoration before coming into production. Forced moulting has been conducted by a variety of techniques including fasting, photoperiod reduction or combination of both, hormone administration, feeding dietary salt of zinc, aluminium and iodine (Berry 2003, Park *et al*. 2004, Khan *et al*. 2011). The most important advantage of forced moulting is the rejuvenation of the reproductive system which originates from increased tissue efficiency, gonadal development, loss of adipose tissue, redevelopment of organs and tissue and hence better post-moult performance is achieved (Berry 2003, Park *et al*. 2004).

The level of crude protein (CP) is very important to control the body weight of broilers and male broiler breeders for optimum semen production (Zhang *et al*. 1999, Laudadio *et al*. 2012). Further, it has been commonly accepted that fertility of male broiler breeders could be maintained or can be raised by a lower CP level than those obtained with the level of 15-18 % CP (Wilson *et al*. 1971). The reported adverse effect of high CP has been explained by the increased production of uric acid due to more excretion of nitrogen from the excess protein catabolism (Wilson *et al*. 1987).

Probiotics are live microbial cultures which leave beneficial effect on the host body by balancing the intestinal micro flora (Fuller 1989, Houndonougbo *et al*. 2011, Dibaji *et al*. 2012). They may be bacteria, fungi or yeas, and they are isolated from the gut of the same organism to which the probiotics are administered (Fox 1988, Yoruk *et al*. 2004, O'Dea *et al*. 2006, Ayed & Ghaoui 2011). Probiotics and prebiotics have been successfully used to improve the health of the poultry to achieve better production (O'Dea *et al*. 2006, Li *et al*. 2008, Perić *et al*. 2010).

Vitamin C, also commonly known as ascorbic acid, is another important antioxidant. Although birds synthesise vitamin C in their body, however, its supplementation in the poultry diet has been recommended during stressful conditions (Khan *et al*. 2012a). Vitamin E is also a very important antioxidant which protects the body against the oxidative stress. Previously, we reported that reproductive efficiency of male broiler was improved as a result of vitamin E supplementation (Khan *et al*. 2012b). This vitamin is not synthesised by the birds and therefore, its inclusion in the feed cannot be avoided. We hypothesise that vitamin E may also have some role in the improved physiological biochemistry.

Therefore, the purpose of this study was to find the effectiveness of vitamins, probiotics and lower protein level on the amelioration of some physiological parameters associated with stress in moulted cockerels.

Material and methods

Experimental design and feeding of birds

One hundred and eighty Hubbard male broiler breeders at the age of 65 weeks were induced to moult after acclimatisation for one week, during which they were fed a ration having 16 % CP and lighting schedule was maintained at 16 hours per day. At the beginning of the second week, birds were subjected to moult with ZnO at the rate of 3 000 mg/kg of feed with a moderate decrease in lighting schedule from 16 to 12 h and they were offered 50 g/bird feed on the daily basis (Khan *et al*. 2012b). The phase of moulting continued for five weeks until 20 % reduction of their body weight was achieved. After completion of moulting birds were randomly assigned to six groups (five replicates per group) in a completely randomised design. Birds were reared on floor pens (3.96×3.96 m) in a window sided house with controlled temperature, ventilation and illumination. One group was kept as control (CP-16 %), second group was fed vitamin E (CP-16 %+100 IU/kg of feed DL- α-tocopherol), third group vitamin C (CP-16 %+500 IU/L of water L-ascorbic acid), fourth group probiotics (CP-16 %+50 mg/L of water probiotics; Protexin, HiltonPharma, The Netherlands), fifth group lower protein level (CP-14 %) and the last group was fed the combination of vitamin E, C, protein and probiotics (CP-14 % +100 IU DL- α-tocopherol/kg feed+500 IU L-ascorbic acid/L water+50 mg probiotics/L water) for five weeks. The detailed diet composition is reported in Table 1. Each bird was offered 140 g/day feed. The experiment lasted ten weeks after initiation of moulting. All animal procedures of this experiment were conducted in compliance with protocols approved by the Use and Care Committee, University of Agriculture, Faisalabad, Pakistan.

Blood collection

For determination of serum TAC, TOS, enzymes, hormones and minerals, about five ml blood samples from six birds per group were randomly taken by cervical dislocation. The blood samples were collected in sterile test tubes by decapitating the birds. Serum was separated after centrifugation the blood at 800×g for 15 min. Serum was collected in the small clean appendix for each sample and stored at –20 °C until further analyses. All samples were analysed in duplicate.

Determination of serum TAC, TOS and OSI

Total antioxidant capacity was measured in serum samples by using a novel automated method developed by Erel (2004), using *o*-dianisidine dihydrochloride (Sigma Chemical Co., London, UK) as a substrate. Total oxidant status of serum samples was determined by a new calorimetric measurement method described by Erel (2005). This assay is based on the oxidation of the ferrous into the ferric ions in the presence of various oxidant species, which are measured spectrophotometrically. The OSI, an indicator of the degree of oxidative stress, was calculated (TOS/TAS)×100 as described by Verit *et al*. (2006).

Table 1
Ingredient and chemical composition of basal diet fed to broiler breeders

Ingredients, g/kg	CP-16 %	CP-14 %
Yellow corn	430.0	450.0
Canola meal	170.0	180.0
Rice polishing	140.0	150.0
Rice tips	120.0	120.0
Soybean meal	60.0	20.0
Maize gluten	50.0	50.0
Dicalcium phosphate	10.0	10.0
Lime stone	10.0	10.0
Vitamin minerals premix*	5.0	5.0
Methionine	2.5	2.5
Lysine	2.4	2.4
Salt	0.1	0.1
Calculated composition		
Crude protein, g/kg	160.2	140.2
Crude fibre, g/kg	50.5	50.5
Ash, g/kg	70.0	50.0
ME, MJ/kg	12.24	12.22

*Provided per kg of diet: Mn 80 mg; Zn 60 mg; Fe 60 mg; Cu 5 mg; Co 0.2 mg; I 1 mg; Se 0.15 mg; choline chloride 200 mg; vitamin A (retinol) 12 000 IU; vitamin D3 (cholecalciferol) 2 400 IU; vitamin E (DL-α-tocopherol) 50 IU; vitamin K (menadione) 4 mg; vitamin B1 (thiamine) 3 mg; vitamin B2 (riboflavin) 6 mg; vitamin B5 (pantothenic acid) 25 mg; vitamin B6 (pyridoxine) 5 mg; vitamin B12 (cyanocobalamin) 0.03 mg; folic acid 1 mg

Determination of serum enzymes and hormones

Serum AST and ALT were measured spectrophotometrically (Biosystem, BTS-330, S.A. Costa Brava, Barcelona, Spain) using a commercially available kit (BT29 4 QY, Randox Laboratories Ltd., UK). Serum T_3 was measured by JD Biotech (Roseto degli Abruzzi, Italy) *in vitro* diagnostic T_3 EIA kit. The quantitative concentration of T_4 was determined by JD Biotech, Roseto degli Abruzzi, Italy) *in vitro* diagnostic T_4 EIA kit. The cortisol concentration in the samples was determined by using DRG (DRG Instruments GmbH, Marburg, Germany) ELISA kit. These hormones were measured using Stat Fax 303, ELISA reader at 450/620 nm.

Mineral determination

The preparation of serum samples for mineral analysis was carried out as described by Khan *et al*. (2012b). Briefly, the serum sample of 1 ml was taken into a digestion flask and added 10 ml concentrated nitric acid. The mixture was placed on a hot plate until all the fumes were evaporated. The flask was removed and cooled for 5 min. After cooling, 5 ml perchloric acid was added into the flask and heated the mixture again on a hot plate. When the volume was reduced to 1-2 ml, the flask was removed and the content was cooled again. The contents were diluted with 50 ml deionize water, filtered and kept in a clean bottle until analysis.

First standard solutions were run followed by samples. The concentration of minerals in the samples was obtained from the absorbance of standards and their corresponding concentrations (Khan *et al*. 2012b). Concentration of Na and K was measured using a flame photometer, while Ca and Mg concentrations were measured with the help of atomic absorption spectrophotometer.

Statistical analysis

Data was statistically analysed with the help of a statistical software SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to test the significance of treatment (six dietary treatments) on the studied traits (Steel *et al*. 1997). Means of the significantly affected traits were separated by Duncan Multiple Range Test (Duncan 1955). *P*-value less than 0.05 was considered to be statistically significant.

Results

In the present study, there was no significant difference between control and treated groups after moulting (Figure 1). The mean values of TAC, TOS and OSI have been presented in Table 2. Mean TAC was significantly higher in the vitamin E group compared to the rest of the groups at the end of the experiment. On the contrary, mean TOS was the highest ($P<0.05$) in the control group and lowest ($P<0.05$) in the vitamin E group. The vitamin E supplemented group also registered significantly higher ($P<0.05$) OSI compared to the other groups.

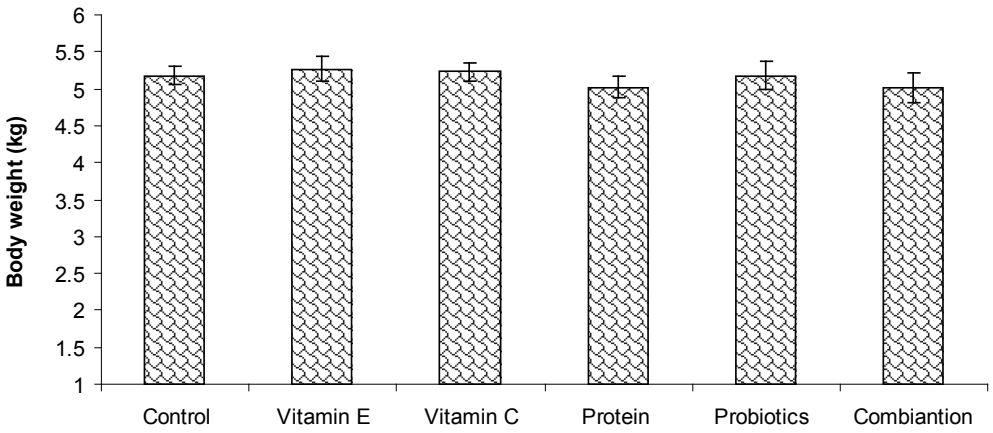

Figure1
Mean body weight of control and treated groups ($P>0.05$)

As shown in Table 3, mean AST and ALT decreased significantly ($P<0.05$) in the vitamin C supplemented group while T_3 and T_4 concentration increased significantly ($P<0.05$) as a result of this treatment. The cortisol concentration decreased significantly ($P<0.05$) in the vitamin E fed group. No significant change was observed in the mineral concentration except Mg which increased significantly ($P<0.05$) in the vitamin E supplemented group (Table 4).

Table 2
Mean TAC, TOS and OSI of post moult control and experimental male broiler breeders

Groups	TAC (mmol Trolox equiv./L)	TOS (μmol H_2O_2 equiv. /L)	OSI
Control	1.03 ± 0.09^{B}	1.40 ± 0.05^{A}	143 ± 9.87^{A}
Vitamin E	1.59 ± 0.13^{A}	0.97 ± 0.04^{D}	58.75 ± 9.54^{E}
Vitamin C	1.26 ± 0.11^{B}	1.11 ± 0.11^{CD}	80.45 ± 3.23^{D}
Protein	1.26 ± 0.19^{B}	1.16 ± 0.05^{BC}	83.45 ± 5.32^{D}
Probiotics	1.11 ± 0.05^{B}	1.32 ± 0.06^{AB}	100.34 ± 9.87^{D}
Combination	1.20 ± 0.08^{B}	1.25 ± 0.14^{BC}	95.47 ± 12.34^{C}

Control: 16 % CP; Vitamin E: 16 % CP + 100 IU/kg DL-α-tocopherol acetate; Vitamin C: 16 % CP + 500 IU/kg L-ascorbic acid; Protein: 14 % CP; Probiotics: 16 % CP + 50 mg probiotics (*Lactobacillus plantarum, L. bulgaris, L. acidophilus, L. rhamnosus, Bifadobacterum binfadum, Streptococus thermophilus, Enterococus faecium, Aspergillus oryzae, Candida pintolopesi*); Combination: 14 % CP+ 100 mg vitamin E+ 500 mg vitamin C+ 50 mg probiotics, $^{A-D}$Mean values within a column, not bearing a common superscript differ significantly ($P \leq 0.05$)

Table 3
Mean AST, ALT, T3, T4 and cortisol of post moult control and experimental male broiler breeders

Groups*	AST, U/L	ALT, U/L	T_3, ng/mL	T_4, μg/dL	Cortisol, ng/mL
Control	40.28 ± 1.87^{AB}	11.95 ± 0.43^{A}	3.34 ± 0.10^{C}	5.90 ± 0.10^{E}	42.19 ± 1.71^{A}
Vitamin E	36.86 ± 1.69^{BC}	7.29 ± 0.30^{B}	3.77 ± 0.12^{AB}	6.46 ± 0.20^{CD}	37.46 ± 1.33^{B}
Vitamin C	30.44 ± 1.17^{D}	5.49 ± 0.22^{C}	4.02 ± 0.12^{A}	8.28 ± 0.18^{A}	40.69 ± 1.62^{AB}
Protein	35.82 ± 1.19^{C}	7.22 ± 0.33^{B}	3.39 ± 0.22^{C}	6.22 ± 0.38^{DE}	41.90 ± 1.46^{AB}
Probiotics	41.81 ± 1.02^{A}	11.16 ± 0.53^{A}	3.63 ± 0.18^{BC}	6.87 ± 0.36^{BC}	42.68 ± 1.65^{A}
Combination	35.94 ± 1.25^{C}	6.24 ± 0.31^{BC}	3.86 ± 0.21^{AB}	6.98 ± 0.27^{B}	38.46 ± 1.45^{AB}

*Dietary treatments: see Table 2, ^{A,B}Mean values within a column, not bearing a common superscript differ significantly ($P \leq 0.05$)

Table 4
Mean Na, K, Ca and Mg of post moult control and experimental male broiler breeders

Groups*	Na (mg/L)	K (mg/L)	Ca (mg/L)	Mg (mg/L)
Control	$2\,083.05 \pm 137.70$	150.99 ± 9.23	168.41 ± 12.98	31.19 ± 1.76^{BC}
Vitamin E	$2\,267.90 \pm 90.20$	139.69 ± 11.22	225.23 ± 20.17	37.03 ± 1.54^{A}
Vitamin C	$2\,143.40 \pm 57.05$	148.25 ± 11.51	189.36 ± 15.12	36.03 ± 1.14^{AB}
Protein	$2\,255.00 \pm 99.10$	148.34 ± 8.48	206.11 ± 19.54	$35.06 \pm 1.74^{A-C}$
Probiotics	$1\,894.55 \pm 96.55$	134.98 ± 8.39	178.38 ± 12.13	29.62 ± 0.79^{C}
Combination	$2\,036.55 \pm 93.70$	148.15 ± 7.93	202.14 ± 22.61	31.15 ± 1.36^{BC}

*Dietary treatments: see Table 2, $^{A-C}$Mean values within a column, not bearing a common superscript differ significantly ($P \leq 0.05$).

Discussion

In the present study, the body weight did not change as a result of different supplements; however, TAC was significantly high in the vitamin E supplemented group. Previously, we

reported that an exogenous supplementation of antioxidants can ameliorate the injury of free radicals in the body (Khan 2011). Total antioxidant capacity is a summary variable of the different factors that measures the ability of serum to inhibit oxidative stress (Erel 2004, Khan 2011). Vitamin E is a potent antioxidant which directly scavenges the free radicals and regulates the activities of the antioxidant system (Khan *et al*. 2011). Sahin *et al*. (2004) reported that supplementation of melatonin, which is considered as a potent antioxidant, improved the antioxidant status in the plasma of Japanese quails, exposed to heat stress. The present study confirmed the previous general findings that supplementation of vitamin E suppresses oxidative stress (Khan *et al*. 2011). The combined effect of vitamin E and C on TAC was not much pronounced in this study which is according to the previous reports (Chen 1981, Aburto & Britton 1998). The discrepancy might be due to the dose of antioxidant vitamins or their interaction at the time of absorption and cellular level.

The TOS was significantly lower in the vitamin E and C groups compared to the other treatments. Oxidant molecules are produced endogenously by living organisms and these oxidants are neutralised via enzymatic and non-enzymatic antioxidants (Erel 2005, Khan *et al*. 2012). Oxidative stress develops as a consequence of the production of oxidants (Khan *et al*. 2011). The combined effect of oxidants can be measured collectively, which is quick and requires less labour (Erel 2005). In this study, we observed that the level of TOS was significantly higher in the absence of antioxidants (vitamin E and C). The lower TOS either in the vitamin E and C groups or higher TOS value in the control group are in agreement with detailed reports on TOS and TAC found by Erel (2005), Verit *et al*. (2006) and Verit *et al*. (2009). Upon supplementation of antioxidant vitamins, the production of corticosterone is depressed (Kucuck *et al*. 2003). In our study, the level of corticosterone was significantly decreased and we may speculate that the supplementation of vitamins at the present levels could have increased blood antioxidants and decreased corticosterone concentrations (Kucuck *et al*. 2003). By decreasing the synthesis of corticosteroids, antioxidant vitamins might have worked to alleviate the negative effects of stress, which might have resulted in less production of oxidants (Kucuck *et al*. 2003, Sahin *et al*. 2004). In the present study, the OSI was significantly low in the vitamin E supplemented group. Information on the OSI status in poultry is not available in the literature. However, reports on human studies indicate that the level of OSI is elevated in the condition of oxidative stress (Verit *et al*. 2006, 2009). The oxidative stress index gives an accurate measurement of oxidative stress and higher level of OSI indicates high oxidative stress (Verit *et al*. 2009). Moreover, this index is a reliable indicator and minimises the variability of individual parameters of oxidative stress (Verit *et al*. 2009).

To the best of our knowledge, it has not been reported on the serum biochemistry in moulted birds. In this study, serum AST and ALT decreased significantly in the vitamin E fed group. Lower AST and ALT levels are the indicator of better health in animals. According to Perić *et al*. (2009) the determination of AST and ALT is an indicator of oxidative damage in liver tissue and the elevated level of these enzymes is usually associated with liver diseases. In a study, Gursu *et al*. (2004) reported that serum ALT and AST decreased in response to vitamin C (250 mg/kg) in heat stressed Japanese quail. Jayasree *et al*. (2003) reported that the supplementation of vitamin E at the rate of 300 mg/kg reduced the AST and ALT concentrations which were increased in response to deltamethrin toxicity in broiler chicks. Perić *et al*. (2009) found a significant reduction in ALT and AST enzymes activities

in broiler chicks fed 0.3 ppm selenium and suggested that this reduction was due to the antioxidative effect of selenium. Sahin *et al*. (2001) reported that 250 mg/kg α-tocopherol acetate decreased serum AST and ALT in broilers reared under heat stress (32 °C). Franchini *et al*. (1995) also noted that the AST level increased with vitamin E supplementation in young turkey; however, it decreased in old turkey (140 days old) which is quite consistent to our study. In this study, the reduction of serum AST and ALT seemed to be due to the antioxidant effect of vitamin E supplementation.

In this study, the serum thyroid hormone concentration significantly increased in the vitamin E supplemented group. Thyroid hormones are well known to regulate energy metabolism, accelerate basal and oxidative metabolism rate by increasing the respiratory rate, mitochondrial mass and mitochondrial cytochrome contents of the cell (Lin *et al*. 2001). The plasma level of thyroid hormones changes with age, fasting, temperature, feeding and pathophysiology (Lin *et al*. 2001). It has been suggested that a decreased thyroid hormone level results in failure to provide adequate oxygen delivery to the tissues that may lead to hypoxia, heart failure and ascites (Hassanzadeh 2009). Nockles & Carnevale (1988) concluded that T_3 and T_4 values were not influenced by the amount of feed consumed, body weight or blood fluid volume in fasted male White Leghorn cockerels. Sahin *et al*. (2002) observed a linear increase in serum T_3 and T_4 concentrations in response to graded level of vitamin E in Cobb male broilers reared under heat stress.

In the current study, the serum concentration of cortisol was significantly low in the vitamin E fed group. Increased circulating level of corticosterone has been associated with stress response in poultry birds (Onbasilar & Erol 2007). The high level of corticosterone has been linked with deleterious effects on bird's performance. Upon the exposure to stress, hypothalamic-pituitary adrenal system is activated, which stimulates the hypothalamus to cause the pituitary gland to release adrenocarticotropic hormone (ACTH). The ACTH in the blood activates adrenal to secrete corticosterone (Virden *et al*. 2007). Satterlee *et al*. (1989) concluded that ascorbic acid reduced plasma corticosterone in broiler chickens prior to slaughter. Taniguchi *et al*. (2007) reported on chickens that vitamin E at the level of 500 and 5 000 mg/kg markedly decreased serum corticosterone concentration as well as the level of adrenal free cholesterol and concluded that vitamin E suppresses serum corticosterone by inhibiting the conversion of cholesterol esters to free cholesterol in the adrenal gland.

In this study, no significant difference was found in serum mineral concentration except Mg, where, it increased significantly in the vitamin E supplemented group. The Mg is a cofactor of several enzyme systems, where, thymine pyrophosphate is an essential component. Magnesium is necessary for the proper functioning of oxidative phosphorylation. Magnesium deficient diet leads to poor growth, decreased muscle tone, incoordination and ataxia (Merck 1986). Moreover, it is involved in the synthesis and degradation of DNA and plays a key-role in the immune system and neurotransmission (Sahin *et al*. 2005). It is well documented that Mg plays a significant role in attenuation of free radicals either by preventing the production of free radicals or by facilitating the scavenging of free radicals (Garcia *et al*. 1998). Magnesium deficiency elevates lipid peroxidation of hepatic tissue, although the mechanism of action is not clearly understood (Sahin *et al*. 2005). Guo *et al*. (2003) reported that Mg elevates significantly the activity of hepatic catalase and improves the antioxidant capacity of broilers. The role of vitamin E in elevation of Mg is still to be elucidated.

From the findings of the present study, it can be concluded that for an early recovery from the moulting stress, vitamin E could be supplemented in the diet of moulted broiler breeders.

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