

Egg production, egg quality, and lipid peroxidation status in laying hens maintained at a low ambient temperature (6°C) and fed a vitamin C and vitamin E-supplemented diet

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ABSTRACT: This experiment was conducted to evaluate the effects of vitamin C (L-ascorbic acid) and vitamin E (α -tocopherol acetate) on egg production, egg quality, lipid peroxidation status (measured as MDA), and some serum metabolites in laying hens (Hy-Line) maintained at a low ambient temperature (6°C). One hundred and twenty laying hens (18-wk-old) were divided into four groups, 30 hens per group. The laying hens were fed either a basal diet or the basal diet supplemented with either 250 mg of L-ascorbic acid/kg of diet, 250 mg of α -tocopherol acetate/kg of diet or 250 mg of L-ascorbic acid plus 250 mg of α -tocopherol acetate/kg of diet. Although feed consumption of the hens was similar ($P > 0.05$) among treatments, supplemental vitamin C and E significantly increased final body weight, egg production, and improved feed efficiency ($P < 0.05$). Egg weights were also greater ($P < 0.05$) in hens supplemented with the combination of vitamin C and E than that of hens supplemented either vitamin or no vitamin (control). Haugh unit did not change upon each vitamin supplementation, but the combination of the vitamin supplement yielded a higher Haugh unit ($P < 0.05$). Each dietary supplement of vitamin C and vitamin E improved the egg quality ($P < 0.05$) resulting in a greater specific gravity, thicker egg shell, and heavier egg shell weight. Separately or as a combination, supplemental vitamin C and E decreased MDA, glucose, cholesterol, and triglyceride concentrations ($P < 0.05$). Results of the present study showed that supplementing vitamin C and vitamin E, particularly as a combination, improved the performance of cold-stressed laying hens, offering a potential protective management practice in preventing cold stress-related losses in performance of laying hens. Results of the present study also indicated that the effects of vitamin C and vitamin E are additive.

Keywords: cold stress; vitamin C; vitamin E; laying hen; egg production; lipid peroxidation

Birds are naturally well adapted to cold due mainly to their highly efficient insulation provided by feathers (Ensminger *et al.*, 1990). However, production efficiency of poultry goes down at low ambient temperatures. Low ambient temperatures cause an increase in feed intake, but also result in decreased egg production and feed efficiency in laying hens (Sagher, 1975; Arad and Marder, 1982; Ensminger *et al.*, 1990; Spinu and Degen, 1993). Such cold conditions also cause decreases in serum concentrations of some vitamins, minerals, and insulin, and increases in serum corticosterone in poultry as well as humans (Datta and Gangwar,

1981; McDowell, 1989; Ensminger *et al.*, 1990; Siegel, 1995). Animals stressed due to environmental temperature are found to have reduced ascorbic acid, α -tocopherol, and retinol concentrations in plasma and blood cells (McDowell, 1989), whereas MDA levels were found high in plasma and tissues due to increased production of free radicals (Halliwell and Gutteridge, 1989; Klasing, 1998). Moreover, ambient temperature impairs absorption of vitamins A, E and C, and increases the dietary requirement of these vitamins (Freeman, 1967; Klasing, 1998).

Supplementing antioxidant vitamins is an effective way of alleviating the adverse effects of stress

on poultry production. Vitamin C and vitamin E are used in the poultry diet because of their anti-stress effects and also due to the fact that their synthesis is reduced during stress (Sykes, 1978; Hornig *et al.*, 1984; Cheng *et al.*, 1990; Kutlu and Forbes, 1993; Bollengier-Lee *et al.*, 1998; Sahin and Kucuk, 2001; Sahin *et al.*, 2001, 2002). As avian species are able to synthesize vitamin C, they do not require dietary source of vitamin C. It has been, however, reported that ascorbic acid synthesis and utilization are inadequate under stress conditions such as low or high environmental temperatures, humidity, high productive rate, and parasite infestation (Freeman, 1967; Sykes, 1978; Hornig *et al.*, 1984; Pardue and Thaxton, 1984; McDowell, 1989; Cheng *et al.*, 1990). Several researches have documented beneficial effects of ascorbic acid supplementation on growth rate, egg production, egg shell strength, and thickness in stressed-poultry (Thornton, 1962; McDowell, 1989; Bains, 1996). At temperatures above or below thermoneutral zone, corticosteroid secretion increases as a response to stress (Brown and Nestor, 1973). By decreasing synthesis and secretion of corticosteroids, vitamin C alleviates the negative effects of stress such as cold stress-related depression in poultry performance (McDowell, 1989; Kutlu and Forbes, 1993). Several studies have also indicated some benefits of dietary vitamin E supplementation to laying hens during environmental stress (Feenster, 1985; Whitehead *et al.*, 1998; Bollengier-Lee *et al.*, 1998; Bollengier-Lee *et al.*, 1999; Kirunda *et al.*, 2001; Puthongsiriporn *et al.*, 2001). Due mainly to its anti-stress properties, vitamin E alleviates negative effects of environmental temperature (stress).

Vitamin C and vitamin E are primary antioxidants in biological systems and break the chain of lipid peroxidation in cell membranes. Vitamin E is mainly found in the hydrocarbon part of membrane lipid bilayer towards the membrane interface and in close proximity to oxidizing enzymes which initiate the production of free radicals (Halliwell and Gutteridge, 1989; McDowell, 1989; Linder, 1991). Vitamin E protects cells and tissues from oxidative damage induced by free radicals (McDowell, 1989). Vitamin C is involved in several biochemical processes and its function is also related to its reversible oxidation and reduction characteristics in the endogenous of cells such as mixed function oxidation involving in incorporation of oxygen in the substrate (McDowell, 1989; Frei *et al.*, 1991; Linder, 1991). The objective of this study was to evaluate the effects of vitamin C and vitamin E supplementa-

tion on feed efficiency, egg production, egg quality, serum MDA, glucose, cholesterol, triglyceride, Ca, and P concentrations in laying hens reared under a low ambient temperature (6°C).

MATERIAL AND METHODS

Animals, dietary treatments and experimental design

One hundred and twenty, 18-wk-old Hy-Line laying hens were obtained from a commercial company (Umut, Elazig-Turkey). Hens were divided according to their body weights which were equal among treatments into four groups, 30 hens each. The birds were fed a basal diet or the basal diet supplemented with either 250 mg of L-ascorbic acid/kg of diet, 250 mg of α -tocopherol acetate/kg of diet or 250 mg of L-ascorbic acid plus 250 mg of α -tocopherol acetate/kg of diet. Vitamin C (ROVIMIX® STAY-C® 35; specifically produced for use as a stabilized source of vitamin C in feed) and vitamin E (ROVIMIX® E-50 SD; fairly stable source of vitamin E in feed) were provided by a commercial company (Roche, Levent-Istanbul, Turkey). Ingredients and chemical composition of the basal diet are shown in Table 1. The basal diet was a typical layer diet containing 11.62 MJ/kg metabolizable energy (ME) and 17.65% crude protein (CP), and was calculated to meet or slightly exceed the nutrient requirements recommended by the National Research Council (1994). Three hens were kept in each subcage with dimensions of 30 × 45 × 35 cm. Water and the diets were offered *ad libitum*. The hen house was lit for 17 h per day. During the experiment, hen house's temperature and humidity were measured four times a day (06.00, 12.00, 18.00, and 24.00). Average ambient relative humidity inside the hen house was 68.3 ± 5.1%. The mean value of daily temperature in the hen house was 6.5 ± 2.3°C. The length of the experiment was 100 days.

Performance and egg quality

Body weights were recorded at the beginning and at end of the study to determine body weight changes. Feed consumption was measured weekly. The number of eggs and egg weight were recorded daily throughout the experiment. Egg quality measurements were conducted monthly using all eggs of

Table 1. Ingredients and chemical composition of the basal diet fed to laying hens

Ingredients	%
Ground corn	63.05
Soybean meal	22.75
Wheat bran	1.68
Animal fat	1.50
Limestone	8.54
Dicalcium phosphate	1.40
Vitamin premix ^a	0.25
Mineral premix ^b	0.20
DL-methionine	0.20
Sodium chloride	0.40
Dry matter	91.10
Chemical analyses (DM basis)	
Metabolizable energy (MJ/kg)	11.62
Crude protein	17.65
Calcium ^c	4.15
Phosphorus ^c	0.78

^amix supplied per kg of diet: vitamin A – 7.750 IU; cholecalciferol – 1.250 IU; vitamin E – 7.5 IU; menadione – 1 mg; thiamin – 2.5 mg; riboflavin – 3.5 mg; d-pantothenic acid – 4 mg; pyridoxine – 1 mg; vitamin B₁₂ – 0.075 mg; folic acid – 750 mg; niacin – 15 mg

^bmix supplied per kg of diet: Mn – 40 mg; Fe – 30 mg; Zn – 25 mg; Cu – 3.5 mg; Iodine – 1.5 mg; Se – 0.75 mg; choline chloride – 200 mg

^ccalculated from tabular values (NRC, 1994).

one day from all treatments. Parameters included for egg quality measurement were specific gravity, egg shell thickness, egg shell weight, and Haugh unit. Specific gravity of eggs was determined by using the saline flotation method of Hempe *et al.* (1988). Salt solutions were made in incremental concentrations of 0.005 in the range from 1.065 to 1.120. Haugh units were calculated using the HU formula (Eisen *et al.*, 1962) based on the height of albumen determined by a micrometer and egg weight (Saginomiya, TLM-N1010, Japan). Shell thickness was determined measuring the thickness mean values taken at three locations on the egg (air cell, equator, and sharp end) by using a dial pipe gauge (Mitutoyo, 0.01–20 mm, Japan).

Laboratory analyses

Crude protein analysis of the basal diet was run in triplicates using international procedures of AOAC (1990). At the end of the experiment, blood samples were collected from vena brachialis from 10 birds randomly chosen from each treatment, and sera was prepared and stored at –20°C for determination of serum metabolite concentrations. Serum lipid peroxidation, as thiobarbituric acid reactive substances (TBARS), was determined by method of Placer *et al.* (1966) as modified by Matkovics *et al.* (1989). The values of TBARS material were expressed in terms of malondialdehyde (MDA) (nmol/ml serum). Serum glucose, cholesterol, triglyceride, Ca, and P concentrations were measured using a biochemical analyzer (Olympus AU-660, USA).

Statistical Analyses

The data were analyzed using the GLM procedure of SAS (SAS Institute, 1996). Significant differences at 5% among treatment means were determined using Duncan's new multiple range test.

RESULTS

The effects of supplemental dietary vitamin C and E during cold stress on performance of laying hens are shown in Table 2. A combination of vitamin C and E, rather than each separately, provided a greater performance. Although feed consumption of the hens was similar ($P > 0.05$) among treatments, supplemental vitamin C and E significantly increased final body weight, egg production, and improved feed efficiency ($P < 0.05$). Egg quality of laying hens is shown in Table 3. Egg weights were greater ($P < 0.05$) with hens supplemented with the combination of vitamin C and E than that of hens supplemented either vitamin or no vitamin (control). Generally, each dietary supplement of vitamin C and vitamin E improved the egg quality, but a combination of supplemental vitamin C and vitamin E resulted in a greatest specific gravity, thickest egg shell, and heaviest egg shell weight ($P < 0.05$). Haugh unit did not change upon each vitamin supplementation, but the combination of the vitamin supplement resulted in a higher Haugh unit ($P < 0.05$). The effects of vitamin C and E supplementation on serum values are presented in Table 4. Separately or as a

Table 2. The effects of supplemental vitamin C and vitamin E on the performance of laying hens reared under a low ambient temperature (6°C) ($n = 30$)

Parameters	Treatments*				SEM
	C	Vit C	Vit E	Vit C + E	
Final body weight (kg/hen)	1.33 ^c	1.52 ^b	1.49 ^b	1.67 ^a	0.06
Feed consumption (g/d)	115.63	114.08	114.83	113.46	2.93
Feed conversion**	1.72 ^a	1.68 ^b	1.67 ^b	1.60 ^c	0.01
Hen-day egg production (%)	84.40 ^c	87.33 ^b	87.40 ^b	89.83 ^a	0.20

^{a,b,c}mean values within a row with no common superscript differ significantly ($P < 0.05$)

*C = control (basal) diet; Vit C: control diet + 250 mg of L-ascorbic acid/kg; Vit E: control diet + 250 mg of α -tocopherol/kg of diet; Vit C + E: control diet + 250 mg of L-ascorbic acid/kg + 250 mg of α -tocopherol/kg

**kg feed consumed/kg egg produced

Table 3. The effects of supplemental vitamin C and vitamin E on the egg quality of laying hens reared under a low ambient temperature (6°C)

Parameters	Treatments*				SEM
	C	Vit C	Vit E	Vit C + E	
Egg weight (g)	56.3 ^c	57.8 ^b	57.4 ^b	58.9 ^a	0.1
Specific gravity	1.0826 ^c	1.0863 ^b	1.0864 ^b	1.0868 ^a	0.002
Egg shell thickness (μ m)	320.0 ^d	343.1 ^c	337.3 ^b	358.8 ^a	0.32
Egg shell weight (g)	5.01 ^d	5.28 ^c	5.26 ^b	5.60 ^a	0.02
Internal egg quality, HU**	81.4 ^b	82.3 ^b	82.1 ^b	83.6 ^a	0.2

^{a,b,c,d}mean values within a row with no common superscript differ significantly ($P < 0.05$)

*C = control (basal) diet; Vit C: control diet + 250 mg of L-ascorbic acid/kg; Vit E: control diet + 250 mg of α -tocopherol/kg of diet; Vit C + E: control diet + 250 mg of L-ascorbic acid/kg + 250 mg of α -tocopherol/kg

**HU = Haugh Unit

Table 4. The effects of supplemental vitamin C and vitamin E on some serum metabolites in laying hens reared at a low ambient temperature (6°C) ($n = 10$)

Parameters	Treatments*				SEM
	C	Vit C	Vit E	Vit C + E	
MDA (nmol/ml)	1.92 ^a	1.07 ^b	1.03 ^b	0.89 ^c	0.08
Glucose (mg/dl)	246 ^a	223 ^b	230 ^b	215 ^c	4.0
Cholesterol (mg/dl)	176 ^a	158 ^b	155 ^b	139 ^c	8.0
Triglyceride (mg/dl)	301 ^a	265 ^b	275 ^b	243 ^c	6.0
Ca (mg/dl)	9.2 ^a	12.3 ^b	11.9 ^b	13.7 ^c	0.5
P (mg/dl)	7.5 ^a	8.9 ^b	8.4 ^b	9.7 ^c	0.3

^{a,b,c}mean values within a row with no common superscript differ significantly ($P < 0.05$)

*C = control (basal) diet; Vit C: control diet + 250 mg of L-ascorbic acid/kg; Vit E: control diet + 250 mg of α -tocopherol/kg of diet; Vit C + E: control diet + 250 mg of L-ascorbic acid/kg + 250 mg of α -tocopherol/kg

combination, supplemental vitamin C and E decreased MDA, glucose, cholesterol, and triglyceride concentrations ($P < 0.05$). Serum concentrations of Ca and P both increased upon supplementation of vitamin C or E, particularly with the combination of the two vitamins ($P < 0.05$).

DISCUSSION

Vitamin C and E supplementation improved the performance in terms of live weight, feed efficiency, egg production as well as egg quality in laying hens reared under a low ambient temperature. With respect to dietary vitamin C supplementation, results of the present study are in agreement with the findings of other studies (Kafri and Cherry, 1984; Njoku, 1986; Kutlu and Forbes, 1993; Orban *et al.*, 1993). Several researches have documented a beneficial effect of ascorbic acid supplementation on egg production, egg shell strength, shell thickness, and interior egg quality in poultry kept under environmental temperature stress (El-Boushy *et al.*, 1988; Orban *et al.*, 1993; Bains, 1996). It is well known that growth rate and egg production decrease when ambient temperature goes below thermo-neutral zone (Arad and Marder, 1982; Ensminger *et al.*, 1990; Sarı, 1993). At temperatures above or below thermoneutral zone, corticosteroid secretion increases as a response to stress (Brown and Nestor, 1973). Kutlu and Forbes (1993) reported that ascorbic acid supplement reduces the synthesis of corticosteroid hormones in birds under stress conditions. By decreasing synthesis and secretion of corticosteroids, vitamin C alleviates the negative effects of stress such as cold stress-related depression in poultry performance (McDowell, 1989). It has been also postulated that the improved performance of stressed-poultry fed a vitamin C-supplemented diet results from a decrease in protein-derived gluconeogenesis (Pardue *et al.*, 1985). Ascorbic acid in birds also stimulates 1.25 dihydrox-cholecalciferol and increases calcium mobilization from bone, suggesting that vitamin C has an important role in egg shell formation thus egg quality (Dorr and Balloun, 1976; Demir *et al.*, 1995).

Similar to results of the present study, Bollengier-Lee *et al.* (1998) have shown that dietary supplementation with vitamin E can alleviate the negative effects of chronic temperature stress (heat) in laying hens. A supplement of 500 mg vitamin E/kg diet increased egg production by an average of 7% in

stressed hens compared to birds fed 10 mg vitamin E/kg diet (Bollengier-Lee *et al.*, 1998). Bollengier-Lee *et al.* (1999) also reported that dietary supplement of 250 mg vitamin E/kg provided before, during, and after stress partially alleviated the adverse effects of chronic stress in laying hens. Environmental stress decreases serum vitamin E concentration and also depresses ascorbic acid synthesis (McDowell, 1989; Sykes, 1978; Hornig *et al.*, 1984; Pardue and Thaxton, 1986), thus may exacerbate a marginal vitamin C and vitamin E deficiency or an increased vitamin C and E requirement, implying that both vitamin C and E should be supplemented as shown in the present study.

Serum MDA concentrations decreased when vitamin C and E were supplemented, indicating antioxidant effects of the two vitamins. Antioxidant effects of the vitamins were even more when supplemented as a combination. Similar to results of the present study, Morrissey *et al.* (1997) reported that dietary supplementation of α -tocopherol in chicken diets increased tissues α -tocopherol concentrations, while markedly decreased MDA concentration. It is well known that environmental stress causes an increased production of MDA in serum and liver and thus resulting in production of free radicals (Halliwell and Gutteridge, 1989; Bollengier-Lee *et al.*, 1999). Environmental stress also reduces the serum and liver concentrations of vitamin C and E, implying that stress must have some prooxidative effects in birds (Feenster, 1985; Klasing, 1998). Vitamin E, a nutritional antioxidant in biological systems, functions as free radical scavenger and inhibits lipid peroxidation within membranes (McDowell, 1989; Halliwell and Gutteridge, 1989). On the other hand, vitamin C can either exhibit antioxidative or prooxidative effects. At high concentrations of vitamin C, in the presence of relatively low concentrations of free or activated metal ions, the antioxidative properties usually predominate and vitamin C acts as a free-radical chain terminator (McDowell, 1989). Vitamin C is involved in several biochemical processes and its function is also related to its reversible oxidation and reduction characteristics in the endogenous of cells such as mixed function oxidation involving in corporation of oxygen in the substrate (McDowell, 1989; Frei *et al.*, 1991). In addition, vitamin C itself plays an important role not only in reacting with all aggressive oxygen species under formation of a practically inert radical but also in transferring radical equivalents from lipid phases to aqueous

compartments (Halliwell and Gutteridge, 1989; McDowell, 1989; Macpherson, 1994; Gey, 1998).

Supplemental vitamin C and vitamin E at the present study decreased serum concentrations of glucose, cholesterol, and triglyceride. These results might have been due to decreased corticosterone (catabolic) and increased insulin (anabolic) concentrations upon vitamin supplementations. Scarcity of corticosterone and abundance of insulin would yield less catabolic end products such as triglycerides. Low environmental temperature has been reported to cause decreases in insulin, ascorbic acid, α -tocopherol, and retinol concentrations in plasma and blood cells, and also increases in serum corticosterone as a response to stress in poultry as well as humans (Datta and Gangwar, 1981; McDowell, 1989; Ensminger *et al.*, 1990; Siegel, 1995; Teeter and Belay, 1996). Supplementing vitamins at the present study could have increased the blood vitamin concentrations thus decreased corticosterone concentrations. By decreasing synthesis and secretion of corticosteroids, vitamin C has been reported to alleviate the negative effects of stress such as cold stress-related depression in poultry performance (McDowell, 1989; Kutlu and Forbes, 1993).

Results from most of the parameters measured at the present study indicated that the effects of vitamin C and vitamin E are additive. Overall antioxidant potential has been reported to be more efficient and crucial than single antioxidant nutrients (Gallo-Torres, 1980). Many studies also suggest that vitamin C and vitamin E act synergistically (Tappel, 1968; Gey, 1998). Vitamin E acts as the primary antioxidant by quenching lipid peroxyl radicals. The resulting vitamin E-radical is regenerated by vitamin C (Halliwell and Gutteridge, 1989). To put another way, vitamin C and vitamin E work together such that vitamin E is the major chain-breaking antioxidant in lipid phases such as cellular membrane or low density lipoproteins, and the oxidizing free radical chain reactions are terminated in aqueous compartments, with vitamin C as terminal reductant (Tappel, 1968; Gey, 1998). In addition, not only is vitamin C a primary antioxidant in plasma and within cells, but it can also interact with the plasma membrane by donating electrons to the α -tocopheroxyl radical, a transplasma membrane oxidoreductase activity (McDowell, 1989).

Results of the present study showed that supplementing vitamin C (250 mg of L-ascorbic acid/kg of diet) and vitamin E (250 mg of α -tocopherol acetate/kg of diet), particularly as a combination, improved

the performance of cold-stressed laying hens. Such a combination of supplement can offer a potential protective management practice in preventing cold stress-related losses in performance of laying hens. Results of the present study also indicated that the effects of vitamin C and vitamin E are additive.

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