Effect of dietary supplemental vitamin C and zinc sulfate on productive performance, egg quality traits and blood parameters of laying hens reared under cold stress condition

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ABSTRACT
This study was conducted to investigate the effects of dietary supplemental vitamin C and zinc sulfate on performance, egg quality characteristics and blood parameters of laying hens reared under cold stress condition. A total of 144 Lohmann-LSL Lite laying hens (65 weeks old) were allotted to 24 groups. Based on a 2 × 2 factorial arrangement, four corn-soybean-based diets (2750 kcal/kg ME and 14.69% CP) including two levels of vitamin C (0, 240 mg/kg) and two levels of zinc sulfate (0, 40 mg/kg) were assigned to hens with six replicate cages (n = 6) during 3-month experimental period. The data were analysed in a completely randomized design using GLM procedure of SAS. Increased egg weight (EW), egg mass (EM) and albumin weight egg and heterophil to lymphocyte ratio, and reduced FCR were observed in hens fed the zinc-included diet (P < 0.05). Supplementing the diet of laying hens, with zinc sulfate increased egg mass and improved FCR. Significant interaction between supplemental vitamin C and zinc sulfate was detected on egg Haugh unit and shell thickness, so that the best results were observed when the diet of laying hens was supplemented by 240 mg/kg vitamin C.

Introduction

Animals are usually faced with various environmental stresses. Temperatures below 16°C have a significant impact on poultry performance. Cold stress stimulates physiological responses that are of higher priority and demanding energy according to homeotherms (Hangalapura et al. 2004b). In laying hens, cold stress causes some harmful effects including decreased nutrient digestibility, egg production, feed efficiency and increased feed intake (Sagher 1975; Arad and Marder 1982; Ensminger et al. 1990b; Spinu and Degen 1993). Additionally, cold stress increases energy requirements and energy consumption (Blahová et al. 2007). Environmental stress has been shown to decrease serum vitamins and increase mineral excretion (Anderson 1994; Siegel 1995). Mineral supplements, organics (Gajula et al. 2011) or vitamins (Rama Rao et al. 2011) reduce the side effects of stress in the birds. Therefore, the use of the suitable additives (vitamins, minerals) in the diet to respond to specific needs of organisms under cold stress conditions is a cost-effective to protect of bird welfare during cold periods (Lin et al. 2006).

Vitamin C or ascorbic acid is a water-soluble vitamin, which is required for a range of metabolic reactions in animals, such as inhibition of active oxygen species, collagen synthesis, adrenaline and bile acids (Murray et al. 1990). Poultry does not need any source of vitamin C rations because they have the ability to synthesize vitamin C. Pardue and Paul Thaxton (1986) reported that specific environmental stressors could change vitamin C utilization or synthesis in poultry. It has also been reported that under stress situation such as low or high environmental temperatures, high productive rate, humidity and parasite infestation, vitamin C is inadequate (Sykes 1978; Hornig et al. 1984; McDowell 1989a; Cheng et al. 1990). Several researchers documented useful effects of ascorbic acid supplementation on egg production, growth rate, thickness and eggshell strength in stressed bird (Thornton 1962; McDowell 1989b; Bains 1996). At temperatures upper or down the thermally neutral region (18–22°C), corticosteroid secretion increases as a response to stress (Brown and Nestor 1973). With decreasing synthesis and secretion of corticosteroids, ascorbic acid reduces the detrimental effects of stress such as cold stress related depression in poultry performance (McDowell 1989a; Kutlu and Forbes 1993).

Zinc is a mineral substance that is important in many metabolic processes (Bettger and O’Dell 1981). In addition, eggshell is one of the most important problems in the poultry industry and it influences the economic benefits of egg production. Rare elements such as zinc have affected mechanical properties of the eggshell by affecting the formation of crystal crystals and changes in the structure of the crystallography of eggshells (Mabe et al. 2003). One of the most important actions of Zn is the participation in the antioxidant defense system. Oxidative damage to the cell membrane has occurred through free radicals during zinc deficiency (Salgueiro et al. 2000; Prasad and Kucuk 2002). Trying to find the probable synergistic interactions between organic and inorganic substances added to the diet showed that the combination of vitamin C and chromium improved the performance of hens under cold stress conditions.
(Sahin et al. 2002a). They reported that chromium and zinc supplements increased live weight, egg production, feed efficiency, egg weight, shell thickness, egg density and Haugh unit in laying hens at low temperatures (Sahin et al. 2002b). Since, mineral or vitamins supplements have been reported to reduce the side effects of stress in birds, therefore the objective of this study was to evaluate the impact of dietary supplemental vitamin C and Zn, individually or in a combined form (to find out any probable synergistic effect), on productive performance, egg quality traits, and blood biochemical parameters of laying hens reared at a low ambient temperature.

Material and methods

Animals and diets

All experimental protocols were approved by the guidelines of the Animal Ethics Committee of Razi University (Kermanshah, Iran). One hundred and forty-four, 65-week-old Lohmann LSL-Lite laying hens, with mean body weight of (1528 ± 0.11 g), were allocated to 24 cages with 6 birds. Body weights were recorded at the onset, and at the end of the study to determine body weight changes. Hens in each six cages were randomly assigned to one of the four dietary treatments. A course of 14 days was used for bird adaptation to the experimental diets. The experimental period, lasted 12 weeks, started with the 67-week-old birds. The study was conducted in environmentally controlled house, and the birds were kept under 16 h of light/8 h dark cycle. This study was carried out between January 4 to April 4, and the temperature was kept as close as to a constant (13–15°C). Average ambient relative humidity inside the rearing house was 40%. Feed was offered on the basis of catalog (120 g/hen/day), and water was supplied ad libitum. A corn-soybean meal basal diet was formulated to contain adequate levels of all nutrients as recommended by the Lohmann LSL-Lite catalog (Lohmann LSL-Classic International 2011), except for zinc, which its analysed value for the basal diet was 36.8 mg/kg. Supplemental Zn (0 and 40 mg/kg diet) and vitamin C (0 and 240 mg/kg diet) were added to the basal diet (metabolizable energy (ME) = 2750 kcal/kg and Crude protein (CP) = 14.69 g/100 g diet) to create the four following experimental diets (basal diet with no additive as the control diet, treatments 1–3 included 40 mg Zn/kg diet, 240 mg vitamin C/kg diet, and 40 mg Zn as well as 240 mg vitamin C/kg diet, respectively). The components and composition of the basal diet are shown in Table 1. Small amounts of the basal diet were first mixed with the calculated amounts of vitamin C and Zn as a small batch and then with a larger amount of the basal diet until the total amount of the experimental diets were homogeneously mixed. Zinc sulfate (German Merck products) with a purity of 98% was used as a Zn source. Vitamin C was provided by pharmaceutical company Osvah Tehran, Iran, with a purity of 98%.

Productive performance and egg quality

Egg production (EP) was calculated daily and was determined on hen day basis. Egg weight (EW) was recorded daily and egg mass (EM) was obtained based on grams of egg/hen/day. Feed conversion ratio (FCR) was created as feed consumed per egg mass produced. The feed intake (FI) was obtained by subtracting the residual feed, collected at the end of the month, from the total feed offered.

In week 78 of the trial, 36 eggs randomly per each treatment during three frequent days were collected to measure egg quality in terms of yolk colour, eggshell thickness, eggshell weight (%), egg index, yolk index, white weight egg, yolk weight, specific gravity and Haugh unit. Egg index was obtained as the ratio of egg width to egg length. Yolk index was calculated by dividing the yolk height into the yolk width. Eggshell weight was measured after drying. The shell thickness was a mean value of measurement at three locations on the egg (air cell, equator and sharp end); also, yolk colour was measured using the Roche fan scale. Haugh units were calculated using the HU order (Eisen et al. 1962). The unit based on the height of albumen was determined by a micrometer and egg weight.

Determination of the blood biochemical parameters

At the end of the experiment, six hens were randomly selected from each treatment and blood samples were collected from the brachial vein by 5-ml syringe into non heparinized tubes and centrifuged at 3000×g for 15 min to obtain sera (SIGMA 4–15 Lab Centrifuge, Germany). Individual serum samples were analysed for glucose, cholesterol, triglyceride, albumin and total protein by automatic analyser (Abbott alcyon 300, USA) using Pars Azmoon kit package (Pars Azmoon Co, Tehran, Iran). Vitamin C was provided by pharmaceutical company Osvah Tehran, Iran, with a purity of 98%.

| Table 1. Composition of the basal diet. |
|----------------------------------------|
| Ingredient                               | Amount (%) |
| Corn                                    | 67.64       |
| Soybean meal                            | 21.01       |
| Wheat Bran                              | 0.15        |
| Soybean oil                             | 0.07        |
| Lime stone                              | 3.00        |
| Oyster Shells                           | 5.47        |
| Dicalcium phosphate                     | 1.64        |
| NaHCO3                                  | 0.18        |
| Common salt                             | 0.19        |
| Min. Premixa                            | 0.25        |
| Vit. Premixa                            | 0.25        |
| DL-Methionine                           | 0.15        |
| Nutrient composition (as fed basis)     |             |
| ME (Kcal/kg)                            | 2750        |
| Crude protein (%)                       | 14.69       |
| Calcium (%)                             | 3.64        |
| Available phosphorus (%)                | 0.37        |
| Sodium (%)                              | 0.15        |
| Crude fibre (%)                         | 2.32        |
| (Na + K)-Cl (meg/kg)                    | 207         |
| Lysine (%)                              | 0.71        |
| Methionine (%)                          | 0.37        |
| Methionine + Cystine (%)                | 0.63        |
| Tryptophan (%)                          | 0.54        |
| Zinc analysed (mg/kg)                   | 36.8        |

*a*Each 2.5 kg of mineral supplement contains: 33,000 mg iron, 66,000 mg zinc, 8800 mg copper, 6600 mg manganese, 900 mg iodine and 300 mg selenium.

*b*Each 2.5 kg of vitamin supplement contains: 7,700,000 (IU) of vitamin A, 3,300,000 (IU) of vitamin D3, 6600 mg of vitamin E, 550 mg of vitamin K3, 2200 mg of vitamin B1, 4400 mg Vitamin B2, 4400 mg of vitamin B6, 5500 mg of calcium pentotenate, 2200 mg of niacin, 110 mg of folic acid, 275,000 mg of choline chloride, 125 mg of antioxidant, 55,000 µg of biotin and 8800 µg of B12.
Tehran, Iran). T3 and T4 hormones were measured by the ELISA method.

**Blood leukocyte subset counts**

In week 78 of the trial, one bird was randomly selected from each replicate and blood samples were collected from the brachial vein into EDTA anticoagulant-treated vials. Blood leukocyte counts were evaluated by the same person using an optical microscope (BX51; Olympus, Tokyo, Japan); then the heterophile (H) ratio to lymphocyte (L) was calculated.

**Determination of zinc in feed and plasma**

At the end of the experiment period, from each replication, a bird was randomly selected and blood samples placed into 7 mL trace-mineral-free tubes with 100 USP units of sodium heparin, and then centrifuged for 15 min at 3000×g at 4°C. After centrifugation, the fresh plasma was collected and analysed for zinc. Plasma zinc levels were analysed by using atomic absorption spectrophotometer (PerkinElmer, AA 600, USA). Zinc concentrations of feed were measured using an atomic absorption spectrometer containing a graphite furnace and graphite tubes (PerkinElmer, AA 600, USA). Chemical analysis of the diet samples was run using international procedures of AOAC (1990). Calibrations for the zinc assay were conducted with a series of mixtures containing graded concentrations of standard solutions of zinc.

**Statistical analysis**

The experiment was done as a 2 × 2 factorial arrangement of treatments based on a completely randomized design with four dietary treatments consisting Zn and vitamin C. The experimental unit differed conforming to the measured parameters. For performance characteristics, and egg quality parameters, the experimental unit was cage, whereas exclusive laying hen data were used for blood biochemical parameters. Data were analysed via using the General Linear Model procedure of SAS institute (2003). Duncan’s multiple range test was used to acquire the differences (P ≤ 0.05) among different group means (P ≤ 0.05). This experiment analysed for the main effects of Zn and vitamin C and the interactions between Zn and vitamin C. All of the measured parameters were analysed as follows: Yijk = μ + (Zi) + (Ci) + (ZCij) + (eijk), where Yijk is the measured characteristic, μ is the overall mean, (Zi) is the main effect of Zn, (Ci) is the main effect of vitamin C, (ZCij) is the interaction between Zn and vitamin C, and (eijk) is the residual error. The effects of the main factors were not considered, whenever the interaction was significant.

**Results**

**Productive performance**

The effects of dietary supplemental Zn and vitamin C on performance parameters of the laying hens are shown in Tables 2–5. Under cold stress condition in the present study, in the first month, supplementation of vitamin C and Zn significantly improved egg weight, egg mass, food conversion ratio

| Table 2. The effect of diet supplementation by vitamin C and Zn on feed intake (FI) and food conversion ratio (FCR) of the laying hens. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Groups | Mon1 | Mon2 | Mon3 | total | Mon1 | Mon2 | Mon3 | total |
| Vitamin C (mg/kg) | FI (g/hen/day) | | | | FCR (g/g) | | | |
| 0 | 119.81 ± 0.27 | 120 ± 0 | 120 ± 0 | 119.93 ± 0.09 | 2.47 ± 0.29 | 2.51 ± 0.35 | 2.40 ± 0.24 | 2.45 ± 0.24 |
| 240 | 119.83 ± 0.40 | 120 ± 0 | 120 ± 0 | 119.94 ± 0.13 | 2.37 ± 0.35 | 2.62 ± 0.30 | 2.38 ± 0.24 | 2.44 ± 0.30 |
| Zn (mg/kg) | | | | | | | | |
| 40 | 119.78 ± 0.78 | 120 ± 0 | 120 ± 0 | 119.92 ± 0.14 | 2.56 ± 0.27 | 2.75 ± 0.43 | 2.45 ± 0.26 | 2.58 ± 0.27 |
| SEM | 0.102 | 0 | 0 | 0.034 | 0.082 | 0.118 | 0.074 | 0.074 |
| P value | Vitamin C | 0.89 | 0 | 0 | 0.88 | 0.42 | 0.51 | 0.88 | 0.93 |
| Zn | 0.65 | 0 | 0 | 0.62 | 0.02 | 0.03 | 0.28 | 0.02 |
| Vitamin C × Zn | 0.70 | 0 | 0 | 0.72 | 0.02 | 0.23 | 0.68 | 0.17 |

Means (± SD) within columns with different lower case letters differ significantly (P ≤ 0.05). SEM standard error of the mean for main effects.

| Table 3. The effect of diet supplementation by vitamin C and Zn on egg weight (EW) and egg mass (EM) of the laying hens. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Groups | Mon1 | Mon2 | Mon3 | total | Mon1 | Mon2 | Mon3 | Total |
| Vitamin C (mg/kg) | EW (g) | | | | EM (g/hen/day) | | | |
| 0 | 66.99 ± 8.18 | 65.14 ± 1.77 | 65.05 ± 1.88 | 69.83 ± 3.73 | 49.13 ± 6.00 | 48.56 ± 6.61 | 50.36 ± 4.75 | 49.3 ± 34.82 |
| 240 | 69.371 ± 1.33 | 65.81 ± 3.04 | 65.70 ± 2.02 | 71.36 ± 4.49 | 51.40 ± 7.32 | 47.28 ± 8.85 | 50.72 ± 5.08 | 49.7 ± 16.08 |
| Zn (mg/kg) | | | | | | | | |
| 40 | 63.53 ± 7.93 | 64.89 ± 2.53 | 65.54 ± 2.44 | 67.34 ± 3.34 | 47.111 ± 4.60 | 44.63 ± 7.59 | 49.44 ± 5.38 | 46.9 ± 55.24 |
| SEM | 2.399 | 0.750 | 0.624 | 0.752 | 2.28 ± 0.32 | 2.38 ± 0.32 | 2.33 ± 0.18 | 2.31 ± 0.19 |
| P value | Vitamin C | 0.49 | 0.53 | 0.46 | 0.16 | 0.34 | 0.67 | 0.86 | 0.85 |
| Zn | 0.01 | 0.28 | 0.71 | 0.0001 > | 0.01 | 0.04 | 0.32 | 0.02 |
| Vitamin C × Zn | 0.01 | 0.44 | 0.82 | 0.42 | 0.02 | 0.18 | 0.73 | 0.13 |

Means (± SD) within columns with different lower case letters differ significantly (P ≤ 0.05). SEM standard error of the mean for main effects.
but in general dietary supplementation with zinc improved some productive performance parameters (EW, EM and FCR) of the laying hens compared to those fed the basal diet. Egg production, feed intake and body weight were not affected by dietary zinc and vitamin C ($P > 0.05$).

### Egg quality traits

Significant interactions between Zn and vitamin C on Haugh unit and shell thickness were found ($P < 0.05$). That in birds with a diet containing vitamin C, Haugh unit and shell thickness were higher than other diets. White weight increased significantly by zinc supplementation. Specific gravity and yolk weight were not affected by any supplements (Table 6 and 7).

### Blood biochemical parameters

Tables 8 and 9 show the effects of diet addition by Zn and vitamin C on the blood biochemical parameters of the laying hens. Adding Zn and vitamin C to the diet did have no significant effect on the serum concentrations of cholesterol, glucose, albumin, total protein, hormones T3, T4 and serum zinc concentration ($P > 0.05$). A significant interaction between Zn and vitamin C on serum concentration of triglycerides was detected ($P < 0.05$), so that the laying hens fed the diet added with Zn showed the lower serum level of triglycerides.

#### Heterophile to lymphocyte ratio

Zinc supplements significantly increased the proportion of heterophile to lymphocyte, but vitamin C supplements did not significantly affect this ratio (Table 9).

### Discussion

In the present study, the effects of vitamin C and zinc supplementation on some productive performance parameters (FI, BW, EP, EW, EM, and FCR) as well as egg quality (shape index, yolk index, Haugh unit, shell thickness, yolk color, eggshell weight, white weight egg, yolk weight, specific gravity) of the laying hens compared to those fed the basal diet in laying hens reared under a low ambient temperature (13–15°C) were investigated. It was found that dietary vitamin C and zinc alleviated the negative effects of cold stress. Based on previous works, Skrivan et al. (2013) found that ascorbic acid improved the productive performance and influenced some traits and egg quality of the laying hens. Consistent with our results, Sahin et al. (2002a) and Sahin and Sahin (2001) reported that 250 mg of L-ascorbic acid and 400 µg of Cr plus per kg diet supplementation on some productive performance parameters (FI, BW, EP, EW, EM, and FCR) as well as egg quality (shape index, yolk index, Haugh unit, shell thickness, yolk color, eggshell weight, white weight egg, yolk weight, specific gravity) of the laying hens compared to those fed the basal diet in laying hens reared under a low ambient temperature (13–15°C) were investigated. It was found that dietary vitamin C and zinc alleviated the negative effects of cold stress. Based on previous works, Skrivan et al. (2013) found that ascorbic acid improved the productive performance and influenced some traits and egg quality of the laying hens. Consistent with our results, Sahin et al. (2002a) and Sahin and Sahin (2001) reported that 250 mg of L-ascorbic acid and 400 µg of Cr plus per kg diet.
improved feed efficiency and egg weight of the laying hens reared under low ambient temperatures. Wang et al. (2015) reported that egg weight in the laying hens reared under heat stress condition in fed the diet containing 200 and 300 mg of ascorbic acid was higher compared to that in those fed the 0 and 100 mg ascorbic acid diet.

Cold stress compromised intestinal epithelial cell duplication and induced inflammation in the small intestine through a combined action of nitric oxide, neutrophils and mast cells (Kaushik and Kaur 2005; Zhao et al. 2013). Immune stress can break the homeostasis of cecal microflora and wreck intestinal mucosal immune function in chickens (Yang et al. 2011). Stress changed in inducible nitric oxide synthase-nitric oxide system activity in the duodenum of chicks, which was related to the intestinal damage process. Acute cold stress significantly decreased glutathione levels in blood and tissues (Siems et al. 1994; Kaushik and Kaur 2003) and pro-inflammatory cytokines and induced in the small intestine of rats (Spasić et al. 1993).

In addition, it is known that productive performance decreases when ambient temperature goes below or above the thermo-neutral zone. At temperatures upper or below the thermo-neutral region, corticosteroid secretion increases in response to stress (Sahin and Sahin 2001). Some antioxidant vitamins (C and E) and minerals (chromium and zinc) have been used to prevent the effects of environmental stress (Sahin et al. 2001; Sahin et al. 2002a; Sahin et al. 2002b; Panjwani et al. 2003). Several studies have demonstrated that dietary supplementation with vitamin E and/or vitamin C alleviates the negative effects of heat stress on apparent nutrient digestibility. McKee and Harrison (1995) also discover an improvement in FCR of broilers as a result of vitamin C supplementation during heat stress. It is well known that vitamin C improves iron assimilation by reduction of Fe$^{3+}$ to Fe$^{2+}$, which is better assimilated by the intestine, and there by vitamin C improves resistance to infections locally, oxidative damage leading to conformational changes of proteins could induce pancreatic enzyme inhibition and/or dietary protein resistance to digestion. Consequently, the attendance of antioxidants (vitamin E/or C) could partially interfere with oxidative protein denaturation and would improve digestibility of nutrients and FCR.

### Table 7. The effect of diet supplementation by vitamin C and Zn on egg quality traits of the laying hens.

| Groups | Haugh unit | Shell thickness (mm) |
|--------|------------|----------------------|
| Vitamin C | Zn | |
| 0 | 0 | 76.52 ± 2.25 $^b$ | 39.72 ± 2.02 $^b$ |
| 240 | 0 | 82.10 ± 5.29 $^b$ | 42.88 ± 0.98 $^a$ |
| 0 | 40 | 78.06 ± 2.32 $^b$ | 42.00 ± 2.14 $^a$ |
| 240 | 40 | 76.54 ± 3.99 $^b$ | 41.49 ± 1.84 $^a$ |

Means (±SD) within columns with different lower case letters differ significantly ($P \leq 0.05$).

### Table 8. The effect of diet supplementation by vitamin C and Zn on blood biochemical parameters of the laying hens.

| Groups | Glucose (mg/dl) | Cholesterol (mg/dl) | Albumin (g/dl) | Total protein (g/dl) | Triglycerides (mg/dl) |
|--------|----------------|---------------------|---------------|----------------------|----------------------|
| Vitamin C | Zn | |
| 0 | 0 | 19.57 ± 245.50 | 37.34 ± 176.50 | 0.99 ± 3.02 | 0.46 ± 5.25 | 95.93 ± 1885.25 |
| 240 | 0 | 13.20 ± 232.33 | 50.04 ± 167.25 | 1.57 ± 3.52 | 0.49 ± 5.17 | 237.66 ± 1621.25 |
| 0 | 240 | 13.09 ± 221.75 | 29.39 ± 150.33 | 0.45 ± 3.70 | 0.40 ± 5.43 | 123.57 ± 1456.00 |
| 40 | 40 | 11.84 ± 227.40 | 44.57 ± 176.20 | 1.29 ± 3.88 | 0.27 ± 3.54 | 254.38 ± 1634.60 |

Means (±SD) within columns with different lower case letters differ significantly ($P \leq 0.05$).

### Table 9. The effect of diet supplementation by vitamin C and Zn on blood biochemical and SEM.

| Groups | T3 (ng/ml) | T4 (μg/dl) | Zn concentration (μg/dl) | Heterophile to lymphocyte ratio |
|--------|------------|------------|--------------------------|-------------------------------|
| Vitamin C | mg/kg | |
| 0 | 2.78 ± 0.19 | 3.35 ± 1.33 | 12.57 ± 3.96 | 0.32 ± 0.19 |
| 240 | 2.47 ± 0.21 | 3.20 ± 0.73 | 12.71 ± 2.01 | 0.33 ± 0.18 |
| Zn | mg/kg | |
| 0 | 2.66 ± 0.63 | 3.33 ± 0.88 | 12.62 ± 2.78 | 0.23 ± 0.08 $^b$ |
| 40 | 2.60 ± 0.31 | 3.21 ± 1.19 | 12.66 ± 3.47 | 0.43 ± 0.20 $^a$ |

Means (±SD) within columns with different lower case letters differ significantly ($P \leq 0.05$).

SEM standard error of the mean for main effects haematological parameters of the laying hens.
phases to the aqueous compartment. In complement, ascorbate takes part in the rebuilding of reduced glutathione from the oxidized form in the cytoplasm and allows tocopherol regeneration through a non-enzymatic reaction (Ciftci et al. 2005). In relation to zinc similarly, in the present study Torki et al. (2015) showed that 40 mg/kg zinc supplementation causes decrease in feed conversion ratio and also increase in egg mass and egg weight of the laying hens reared under cold stress. On the other hand, dietary zinc supplementation did not affect the productive performance of quails reared at thermoneutral conditions (Sahin and Kucuk 2003). Several research reported that zinc supplementation improved performance parameters like the egg mass, egg weight and feed efficiency in the laying hens under stress conditions such as low or high environmental temperatures (Sahin et al. 2005; Kucuk et al. 2008; Koreneкова et al. 2007; Maciel et al. 2010). Zinc has a protective effect on pancreatic tissue against oxidative damage (Onderci et al. 2003), it may help the pancreas to function properly, including secretion of digestive enzymes, thus improving digestibility of nutrients. Onderci et al. (2003) reported that supplemental chromium and zinc ameliorated the decrease in digestibility of DM, CP and EE in laying hens reared under a low temperature. On the other hand, zinc is a cofactor of the main antioxidative enzyme Cu-Zn-superoxide dismutase; it may play a key role in suppressing free radicals and in inhibiting NADPH-dependent lipid peroxidation (Burke and Fenton 1985; Prasad 1997) as well as in preventing lipid peroxidation via inhibition of glutathione depletion (Gibbs et al. 1985). One of the proposed mechanisms of zinc’s operation is its capacity to replace transition metals (Fe, Cu) from binding sites. Zinc can compete with iron and copper to bind to the cell membrane and decrease the production of free radicals, thus exerting a direct antioxidant action (Burke and Fenton 1985; Girotti et al. 1985; Tate et al. 1999). Zinc induces the production of metallothionein, an effective scavenger of hydroxyl radicals and it has been suggested that Zn-metallothionein complexes in the islet cells provide conservation against immune-mediated free-radical attack (Burke and Fenton 1985; Shaheen and Abd El-Fattah 1995). Vitamin C prevent the activity of enzyme 21-hydroxylase and 11-beta hydroxylase (the key enzyme in biochemical direction creatocosterone) on the heat stress (Brake 1989). Consequently, this decrease in corticosteroid secretion by vitamin C is prevented from the negative effects of thermal stress on the function of the poultry (Pardue and Paul Thaxton 1986). El-Boushy et al. (1968) reported that dietary vitamin C supplementation increased egg production, eggshell strength and interior egg quality in stressed-laying hens. But, Wang et al. (2015) find the different amounts of 50, 100 mg per kg of vitamin C in the laying hens did not effect on the thickness and shell strength, yolk colour, Haugh unit, albumen weight, yolk weight, shell weight and skin colour. Several researches have documented a beneficial effect of ascorbic acid supplementation on egg quality like eggshell strength, shell thickness, and interior egg quality in poultry kept under environmental temperature stress (Sahin and Sahin 2001; Sahin et al. 2002a; El-Boushy et al. 1968). It has been presented that ascorbic acid plays a role in bone maturity by improving hydroxyproline production which is required for collagen formation. Accordingly, it was postulated that in birds, ascorbic acid stimulates production 1, 25-dihydroxy-cholecalciferol; which, in turn increase calcium mobilization from bones, calcium absorption from small intestine and thus eggshell quality (Sahin and Sahin 2001). Similar to the results of this study, Torki et al. (2014) reported in laying hens under cold stress with 40 mg/kg zinc diet improved Haugh unit and eggshell thickness compared to the control group. Also Sahin et al. (2003) found in quail under heat stress, the shell thickness and the Haugh unit were positively affected by zinc supplements. As well as Sahin et al. (2002b) reported that the supplementation of Zn sulfate increased egg weight, eggshell thickness, egg specific gravity and Haugh units when layers were subjected to low ambient temperatures. However, the addition of Zn has been reported to increase the utilization of Ca in hens and to improve the qualitative parameters of the eggshell (Klecker et al. 2002). Zinc supplementation also has been reported to improve eggshell quality because it is a component of the carbonic anhydrase enzyme, which supplies the carbonate ions during eggshell formation (Innocenti et al. 2004). Also it has been suggested that alkaline phosphatase secretion decreases under stress condition, and this enzyme interacts with zinc in the calcium storage of the bone (Brandão-Neto et al. 1995).

Also Irandoust and Ahn (2015) reported that there was no significant difference in triglycerides in laying hens with 250 mg/kg of vitamin C compared with control group. However, similar to this study Kucuk et al. (2003a) reported that in the laying hens under stress 250 mg/kg of vitamin C diet decreased serum triglyceride compared with control group. As well as, Bozakova et al. (2015) reported in the laying hens under heat stress total cholesterol, glucose and triglycerides decreased but increased the total protein with 10 mg per kg of arginine + 250 mg per kg of vitamin C diet. 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 1989a; Ensminger et al. 1990a; Siegel 1995; Teeter and Belay 1996). Actually, an increase in insulin activity can act as stimulator in anabolism and inhibitor in catabolism of lipids and subsequently increase the blood glucose uptake and utilization by cells (Cupo and Donaldson 1987).

Kucuk et al. (2008) found that zinc and pyridoxine addition to laying hens’ diet had no significant impact on the serum concentration of triglycerides. On the other, Torki et al. (2014) show that the addition of zinc and cinnamon essential oil to the diet has a significant reduction in the concentration of triglycerides due to the interaction between zinc and cinnamon essential oil. The relationship between cinnamon essential oil and zinc may be in a way that vitamins E and C appear to have important interactions on preventing lipid peroxidation and decreasing serum content of triglycerides (Khan et al. 2012a, b), which
could be attributed to the antioxidant and antistress effect of zinc and vitamin C limiting excessive corticosterone production and corticosterone-induced systemic biochemical changes (Sahin et al. 2002b; Kucuk et al. 2003b). Furthermore, zinc is a cofactor of the main antioxidative enzyme Cu Zn-superoxide dismutase; it may play a key role in repress free radicals and in inhibiting NADPH-dependent lipid peroxidation (Burke and Fenton 1985; Prasad 1997), as well as, in preventing lipid peroxidation via inhibition of glutathione depletion (Gibbs et al. 1985). In this study, cholesterol, albumin and total protein have not been affected by any of the vitamin C and zinc supplements. But the amount of glucose was somewhat reduced by the zinc and vitamin C supplementation, which was not statistically significant. Similar to our results, Irandoust and Ahn (2015) reported that 250 mg/kg of vitamin C in the diet of laying hens in comparison to the control group did not show any significant difference in total cholesterol and triglycerides. Asli et al. (2007) stated that the use of 0, 100, 200, 400 mg/kg of vitamin C and vitamin E in the diet did not find any difference in the concentration of cholesterol and triglyceride in laying hens. In relation to glucose, Sahin et al. (2002b) found that zinc supplements in the diet of laying hens under cold stress conditions reduced serum glucose levels. Salgueiro et al. (2001) reported a close relationship between zinc, glucose metabolism and insulin physiology. This is due to the ability of zinc in the Langerhans’ cells to produce and secrete insulin, also the integrity of the insulin structure in the hexamic form. Vitamin C and E supplements in the study of Kucuk et al. (2003a) reduced glucose concentrations. This result may be due to the effect of vitamin supplements on reducing corticosteroid (catabolic) and increased insulin concentration (anabolic).

Gross (1988) reported that number of white blood cells has increased due to the use of vitamin C in broiler diets, possibly due to the protection of vitamin C from lymphocytes and the prevention of damage to free radicals produced by thermal stress conditions in broiler chicks (Gross 1988). Asli et al. (2007) also reported that the use of vitamin C has a little positive effect on immune responses in laying hens at high temperature conditions, which may be due to the the short time heat stress conditions. This poor vitamin C effect is probably due to inadequate vitamin C intake and the inability to fully cover vitamin C under stress conditions. This weak vitamin C effect is probably because of inadequate doses of vitamin C and the inability to completely cover the need for vitamin C is under stress. Hosseini-Mansoub et al. (2010) have stated heterophile to lymphocyte ratio male broiler chickens who received 100 mg/kg of vitamin A and 50 g/kg of zinc under thermal stress conditions have decreased compared to the control group, but the ratio of heterophile to lymphocyte to control group there was no significant difference.

When the birds are exposed to heat stress, the immune response (antibody production) will increase (Heller et al. 1979). It has been shown that heat stresses have a repressive effect on the chicken immune system, andexposure to cold reduced the immune response (Regnier and Kelley 1981). In studies that have been conducted on cold stress along with our study, a number of researchers reported an increase in heterophile ratio and decrease in heterophile to lymphocyte ratio during a period of cold stress (Hangalapura et al. 2004; Campo et al. 2008; Yang et al. 2008). The effect of stress on the development of an immune response depends on the nature of specific stress (e.g. thermal extremes, diet, pollutants) and stress-modifiers (e.g. genetic make-up, duration or severity of the stressor) (Dohms and Metz 1991). Furthermore, the part of different results can be attributed to measurement method immunological parameters (hemorrhagic immunity, cellular immunity) specific to different measurement techniques and chicken breeds (Donker et al. 1990).

Conclusion

In this study, it can be concluded that supplementing diet of laying hens with 40 mg/kg zinc sulfate increased egg mass and improved FCR as whole data recording assessment (3-month experimental period). In addition, the significant interaction between dietary supplemental vitamin C and zinc sulfate was detected on egg Haugh unit and shell thickness, so that the best results were observed when the diet of laying hens was supplemented by 240 mg/kg vitamin C. Additionally, the concentration of triglyceride was reduced by the zinc supplementation. Moreover, the heterophile to lymphocyte ratio increased under the influence of Zn supplements. According to the data obtained, these two supplements can reduce the negative effects in low temperature conditions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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