Effects of dietary supplemental chromium methionine, zinc oxide, and ascorbic acid on performance, egg quality traits, and blood parameters of laying hens subjected to heat stress

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ABSTRACT

Based on a 2 × 2 × 2 factorial, the effects of three dietary supplemental including chromium methionine (Cr) (0 and 400 μg/kg diet), zinc oxide (Zn) (0 and 30 mg/kg diet), and vitamin C (VitC) (0 and 250 mg/kg diet) on egg production (EP) and mass (EM) and egg traits in heat-stressed (HS) Lohmann LSL-Lite laying hens from 30 to 45 weeks of age were evaluated. The house temperature was kept at 18°C (weeks 30–40 of age) and then increased to 32°C (weeks 41–45 of age). Dietary treatments had no significant effects on EP, egg weight, EM, and feed conversion ratio before and after exposure to heat stress (P > 0.05). Decreased feed intake intake was observed in group of VitC during exposing to HS (P < 0.05). Dietary supplemental Cr decreased serum glucose concentration before HS (P < 0.05). A combination of Cr and VitC increased serum glucose concentration before and during HS (P < 0.05). Decreased serum concentration of Zn was detected in hens fed the diets with VitC, Cr, or Zn (P < 0.05). Increased serum concentration of Cr was observed in hens fed the diets supplemented with Cr (P < 0.05).

Introduction

Heat stress is a great concern in the poultry industry. In laying hens, heat-stressed (HS) depresses body weight (Puthpongsiriporn et al. 2001; Mashaly et al. 2004; Khan, Naz and Dhama 2014; Chand et al. 2016), egg production (EP), egg weight (EW) (Mashaly et al. 2004), and eggshell quality (Mashaly et al. 2004), and is generally accompanied by suppression of feed intake (FI), which could be the cause of decline in production efficiency. In a previous study (Deng et al. 2012), a 12-day HS period caused a daily FI reduction of 28.58 g/birds, resulting in a 28.8% decrease in EP. However, in addition to decreased FI, it has been shown that HS leads to reduced diet digestibility and decreased plasma protein level (Donkoh 1989). Such an ambient temperature also disturbs oxidative status in vivo (Ghazi et al. 2012a), increases mineral excretion (Gorman and Balnave, 1994), decreases serum vitamin, mineral, and insulin and increases serum glucose, total cholesterol and corticosterone concentrations in poultry (Siegel 1995; Khan et al. 2011).

Various experiments have conducted to alleviate negative subsequence of high environmental temperature (e.g. reducing dietary protein level, supplementing diet with minerals and vitamins) (Laudadio et al. 2012); it has been reported that some minerals and vitamins such as Cr (Ghazi et al. 2012b; Torki et al. 2014), Zn (Salabi et al. 2011; Chand et al. 2014), and vitamins C (Roussan et al. 2008; Khan et al. 2012; Torki et al. 2014) and E (Rahman et al., 2017) can be supplemented to reduce the negative effects of HS.

In general, supplemental dietary Cr has been shown to improve effectively EP, feed efficiency, and eggshell strength, particularly in birds reared under cold or HS condition (Sahin, Ozbey, et al. 2002; Sahin et al. 2004; Khan, Naz, Dhama, Sami-nathan, et al. 2014); however equivocal results in improved performance (Esequi et al. 2010; Habibian et al. 2013) and serum insulin and glucose concentrations (Habibian et al. 2013) when birds fed supplemental dietary Cr under either thermo-neutral or HS conditions are reported. Cr is also thought to be essential for activating certain enzymes and for stabilization of proteins (Hayirli 2005).

Zn has multiple important functions because it is a cofactor for 200 enzymes (Oteiza et al. 1996; Naz et al. 2016). Serum, liver, and spleen levels of Zn are reduced in stressed birds (Sahin, Onderci, et al. 2002; Ghazi et al. 2012a). Supplemental Zn is used in poultry diets because of its reported benefits in laying hens during periods of environmental stress (Sahin, Onderci, et al. 2002; Onderci et al. 2003). Unlike humans, poultry is able to synthesize vitamin C (VitC). However, it has been reported that VitC synthesis is inadequate under stress conditions such as high or low environmental temperatures, high productive rate, and parasite infection (Khan et al. 2012). Whitehead and Keller (2003) have documented that particular environmental stressors can alter VitC utilization or synthesize in avian species. Numerous studies have documented the beneficial effects of VitC (Ciftci et al. 2005; Waseem et al. 2008; Torki et al. 2014) supplementation on EP and eggshell quality in stressed poultry. In a previous study, Sahin and Onderci (2002) observed that a dietary combination of 250 mg of VitC and 400 μg of Cr provides the highest positive effect on the
performance and egg quality traits of laying hens under a low ambient temperature. A similar study showed that the performance and egg quality traits of laying hens were improved by using a combination of 30 mg of Zn and 400 μg of Cr (Sahin, Onderci, et al. 2002); however, there is no information available about the probable interactions of (any probable synergic effects) dietary supplemental Cr, Zn, and VitC in HS laying hens. The objective of this study was to evaluate the effects of diet supplementation by Cr, Zn, and VitC, singly or in combined forms, on performance, egg quality traits and selected blood biochemical indices of laying hens reared under a high ambient temperature (32°C).

Materials and methods

Animals, treatments, and management

All experimental protocols adhered to the guidelines on, and were approved by, the Animal Ethics Committee of Razi University (Kermanshah, Iran) and were in accordance with the guidelines on animal welfare. A total number of 288 30-week-old Lohmann LSL-Lite laying hens was randomly divided into 48 cages and assigned to receive one of the 8 experimental diets of 6 replicates and 6 hens per replicate. Based on a 2 × 2 × 2 factorial arrangement of treatments, eight iso-caloric and iso-nitrogenous diets consisting two levels (0 and 400 μg/kg) of Cr as Cr methionine, two levels (0 and 30 mg/kg) of Zn as Zn, and two levels (0 and 250 mg/kg) of VitC were formulated. The experimental diets were mixed on an every 4-week basis to reduce the chance of loss of VitC activity during the feed storage period. The ingredients and chemical composition of the basal (control) diet is shown in Table 1. All hens were supplied with feed and water ad libitum in the 15-week trial period. The hen house temperature was kept at 18°C during the first 10-week (30–40 weeks of age) and then (41–45 weeks of age) was increased to 32°C to simulate thermal heating on a hot day. The relative humidity was kept as close as possible to a constant 50%, and hen house was lit for 16 h/day during the experimental period.

Performance of laying hens

Performance of the laying hens was measured from 30 to 45 weeks of age. Daily EP per replicate was recorded, and the total number of eggs laid per bird was calculated for before (30–40 weeks of age) and after (41–45 weeks of age) heat treatment. Similarly, the eggs laid per replicate were weighed daily, and the average egg mass (EM) per bird was calculated for before and after heat treatment. FI were measured on a weekly basis. EM (g egg/hen/day) and feed conversion ration (FCR) (g feed/g egg) were calculated from EP, EM, and FI.

Egg quality

Random samples of 12 eggs from each treatment (3 eggs per replicate) were collected on 36 (before heat treatment) and 44 (before heat treatment) weeks of age to measure egg quality traits. The eggs were broken on a glass plate to measure the albumen height using a micrometer, and Haugh units were calculated using the formula described by Eisen et al. (1962). The egg specific gravity was determined using 11 gradient saline solutions varying in specific gravity of 1.060–1.100 at 0.005-unit increments (Holder and Bradford 1979). The eggshell thickness was determined by taking the mean value of the thickness measured at three locations on the egg (air cell, equator, and sharp end) using an FHK device (Fujihira Industry Co. Ltd., Saitama, Japan). The yolk index was determined as the ratio of yolk height to yolk width and yolk colour was compared to the Roche yolk colour fan, which ranges from pale yellow at score 1 to dark orange at score 15 (Vuilleumier 1969).

Blood parameters

Blood samples were collected from the wing vein of 4 randomly selected birds per treatment (1 hen per replicate) on 36 (before heat treatment) and 44 (before heat treatment) weeks of age. The collected blood samples were centrifuged at 3000 rpm for 10 min, and the sera were frozen at −20°C until the analysis. The serum concentrations of total protein, albumin, glucose, triglycerides, total cholesterol, and insulin were measured calorimetrically using commercially available kits (Pars Azmun, Tehran, Iran), and the serum activity of glutathione peroxidase activity of GPx was measured according to Paglia and Valentine (1967). The serum concentrations of Cr, Zn, iron, and copper were determined using an atomic absorption spectrophotometer (Perkin Elmer HGA 500) with a graphite furnace atomizer in deuterium background correction method.

Statistical analysis

The data were subjected to ANOVA in a completely randomized design with a 2 × 2 × 2 factorial arrangement of treatments using GLM procedure of SAS software (SAS 2003). All

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**Table 1.** Ingredients and nutrient composition of the basal diet (%), unless stated otherwise.

| Ingredients          | Percentage |
|----------------------|------------|
| Corn                 | 63.10      |
| Soybean meal         | 22.91      |
| Soybean oil          | 0.69       |
| Oyster shell         | 8.78       |
| Dicalcium phosphate  | 1.39       |
| Common salt          | 0.34       |
| Vitamin premix<sup>a</sup> | 0.25 |
| Mineral premix<sup>b</sup> | 0.25 |
| DL-methionine        | 0.21       |
| Nutrient composition |            |
| Metabolizable energy | 2720 kcal/kg |
| Crude protein        | 16.62      |
| Lysine               | 0.59       |
| Methionine + Cysteine| 0.46       |
| Calcium              | 3.73       |
| Nonphytate phosphorus| 0.38       |
| Chromium (mg/kg)     | 5.43       |
| Zinc (mg/kg)         | 84.00      |

<sup>a</sup> Vitamin mixture per 2.5 kg of diet provides the following: vitamin A, 7,700,000 IU; vitamin D<sub>3</sub>, 3,300,000 IU; vitamin E, 6600 mg; vitamin K<sub>3</sub>, 550 mg; thiamine, 2200 mg; riboflavin, 4400 mg; vitamin B<sub>6</sub>, 4400 mg; pantothenate, 550 mg; nicotinic acid, 200 mg; folic acid, 110 mg; choline chloride, 275,000 mg; biotin, 55 mg; vitamin B<sub>12</sub>, 8.8 mg.

<sup>b</sup> Mineral mixture per 2.5 kg of diet provides the following: Mn, 66,000 mg; Zn, 66,000 mg; Fe, 33,000 mg; Cu, 8800 mg; Se, 300 mg; I, 900 mg. 
statements of significance are based on a P < 0.05. The mean values were compared by Duncan’s multiple-range tests.

Results

Performance of laying hens

The effects of dietary supplemental Cr, Zn, and VitC on the performance of laying hens are shown in Table 2. Dietary treatments had no significant (P > 0.05) effects on EP, EW, EM, and FCR either before (30–40 weeks of age) or after (41–45 weeks of age) exposure to HS. However, a significant (P < 0.05) decrease in FI was observed by VitC supplementation after hens were exposed to HS.

Egg quality

The effects of dietary supplemental Cr, Zn, and VitC on the measured egg quality traits of laying hens are shown in Table 3. As it is summarized in Tables 4 and 5, since significant interactions between dietary Cr, Zn, and VitC on yolk weight, shell thickness, specific gravity, egg shape index, Haugh unit, yolk colour, and yolk index were detected, the mean comparison of interactions has been done without considering the main effects of experimental factors, dietary supplemental Cr, Zn, and VitC. No effects of dietary treatments were found on albumen weight either before (35 weeks of age) or after (44 weeks of age) exposure to HS. The interaction between VitC and Cr on egg yolk weight before HS was significant, as shown in Table 4, the egg yolk weight in the experimental group Cr, VitC and control was the higher than the experimental group Cr, Zn, and VitC. No effects of dietary treatments were found on albumen weight either before (35 weeks of age) or after (44 weeks of age) exposure to HS. The interaction between VitC and Cr on egg yolk weight before HS was significant, as shown in Table 4, the egg yolk weight in the experimental group Cr, VitC and control was the higher than the experimental group containing two supplements. This effect was not observed after hens were exposed to HS. There was an interaction (P < 0.05) between VitC, Zn, and Cr on eggshell thickness before exposure to HS (Table 5), so that the eggshell thickness in control group was higher than other experimental groups and the lowest value was for the experimental group containing VitC (P < 0.05). A significant increase (P < 0.05) in eggshell thickness was found by dietary Zn supplementation after exposure to HS. There was an interaction (P < 0.05) between VitC and Zn on egg specific gravity before exposure to HS, as shown in the mean comparison table of their interactions (Table 4), the egg specific gravity was higher in the experimental group VitC, Zn and both than control group. Although egg specific gravity was increased by both VitC and Zn, the magnitude of the increase was lower by their combination. This effect was not observed after hens were exposed to HS.

There was an interaction (P < 0.05) between Zn and Cr on egg shape index before exposure to HS, as observe in the mean comparison table of their interactions (Table 4), the nature of this interaction show that egg shape index was increased by either Zn or Cr as a single supplement, but not with their combined treatment. There was an interaction (P < 0.05) between VitC, Zn, and Cr on egg shape index after exposure to HS (Table 5), so that egg shape index was increased (P < 0.05) by combined supplementation of Cr and Zn, VitC and Zn, and a combination of VitC, Cr, and Zn than other experimental groups (P < 0.05). The interaction (P < 0.05) between VitC and Zn on Haugh unit before exposure to HS was observed P < 0.05 (Table 4), so that the Haugh unit values in the control and experimental group containing Zn or VitC were higher than experimental group containing both Zn and VitC P > 0.05. There was also an interaction (P < 0.05) between VitC, Zn, and Cr on egg yolk colour before exposure to HS, so that yolk colour was decreased P < 0.05 by combination of Zn and Cr when no VitC was supplemented to diet (Table 5). However, yolk colour was increased (P < 0.05) by Zn, Cr, and VitC when they were alone in the diet. Dietary treatments had no significant (P > 0.05) effects on Haugh unit and yolk colour after hens were exposed to HS. There was an interaction (P < 0.05) between VitC, Zn, and Cr on egg yolk index before exposure to HS (Table 5), so that yolk index was increased (P < 0.05) by supplementation of Zn and Cr when no VitC was supplemented to diet. Also, yolk index was increased (P < 0.05) by Cr supplementation after hens were exposed to HS.
Table 3. Effects of dietary treatments VC, Cr, and Zn on egg quality traits of laying hens before (36 weeks of age) and after (44 weeks of age) exposure to heat stress.

| Treatments | Yolk weight (g) | Albumen weight (g) | Shell weight (%) | Shell thickness (mm × 10^{-2}) | Egg specific gravity | Egg shape index | Haugh unit | Yolk color (Roche) | Yolk index |
|------------|----------------|-------------------|------------------|-------------------------------|---------------------|----------------|------------|-----------------|------------|
| Vitamin C (mg/kg) | | | | | | | | | |
| 0 | 17.0a | 17.1 | 35.0 | 33.5 | 5.96a | 5.51 | 0.37 | 0.35 | 1.08 | 1.08 | 74.1 | 74.5b | 68.7 | 68.9 | 7.13a | 6.05 | 40.9 | 38.1 |
| 250 | 16.6b | 17.2 | 34.0 | 33.6 | 5.84b | 5.51 | 0.36 | 0.35 | 1.08 | 1.08 | 74.1 | 77.0a | 67.8 | 69.3 | 6.88b | 6.04 | 40.6 | 37.9 |
| Zinc (mg/kg) | | | | | | | | | | | | | | | | | | |
| 0 | 16.8 | 17.0 | 35.5 | 33.7 | 5.87 | 5.51 | 0.36 | 0.34b | 1.08 | 1.08 | 74.1 | 73.5b | 68.2 | 69.4 | 7.18a | 6.04 | 40.5 | 37.9 |
| 30 | 16.8 | 17.4 | 34.4 | 33.4 | 5.92 | 5.52 | 0.37 | 0.35a | 1.08 | 1.08 | 74.1 | 78.2a | 68.4 | 68.8 | 6.84b | 6.05 | 40.9 | 38.1 |
| Chromium (μg/kg) | | | | | | | | | | | | | | | | | | |
| 0 | 16.9 | 17.3 | 34.5 | 33.3 | 5.85 | 5.51 | 0.36 | 0.35 | 1.08 | 1.08 | 74.2 | 74.5b | 67.8 | 69.3 | 7.23a | 6.03 | 40.7 | 37.7b |
| 400 | 16.7 | 17.0 | 34.6 | 33.3 | 5.94 | 5.51 | 0.37 | 0.35 | 1.08 | 1.08 | 74.1 | 77.4a | 68.7 | 68.9 | 6.78b | 6.06 | 40.7 | 38.3a |
| Pooled SEM | 0.11 | 0.12 | 0.27 | 0.27 | 0.029 | 0.029 | 0.001 | 0.001 | 0.001 | 0.001 | 0.14 | 0.63 | 0.36 | 0.38 | 0.073 | 0.029 | 0.19 | 0.17 |
| Sources of variation | | | | | | | | | | | | | | | | | | |
| Vitamin C | 0.03 | 0.06 | 0.07 | 0.07 | 0.03 | 0.07 | 0.15 | 0.82 | 0.49 | 0.28 | 0.80 | 0.01 | 0.17 | 0.60 | 0.04 | 0.93 | 0.35 | 0.64 |
| Zinc | 0.80 | 0.12 | 0.89 | 0.71 | 0.27 | 0.85 | 0.39 | 0.01 | 0.49 | 0.53 | 0.86 | 0.01 | 0.72 | 0.63 | 0.01 | 0.83 | 0.30 | 0.44 |
| Chromium | 0.07 | 0.15 | 0.49 | 0.42 | 0.12 | 0.89 | 0.39 | 0.94 | 0.23 | 0.06 | 0.71 | 0.01 | 0.20 | 0.43 | 0.01 | 0.61 | 0.82 | 0.04 |
| Vitamin C × Zinc | 0.15 | 0.12 | 0.22 | 0.06 | 0.44 | 0.45 | 0.04 | 0.57 | 0.01 | 0.35 | 0.36 | 0.03 | 0.04 | 0.37 | 0.07 | 0.67 | 0.48 | 0.88 |
| Vitamin C × Chromium | 0.04 | 0.26 | 0.58 | 0.07 | 0.76 | 0.07 | 0.15 | 0.13 | 0.41 | 0.53 | 0.67 | 0.01 | 0.13 | 0.12 | 0.33 | 0.90 | 0.65 | 0.26 |
| Zinc × Chromium | 0.06 | 0.29 | 0.29 | 0.78 | 0.23 | 0.48 | 0.15 | 0.71 | 0.07 | 0.64 | 0.04 | 0.01 | 0.28 | 0.37 | 0.44 | 1.00 | 0.32 | 0.10 |
| Three-way interaction | 0.68 | 0.52 | 0.15 | 0.59 | 0.15 | 0.11 | 0.04 | 0.28 | 0.85 | 0.16 | 0.75 | 0.01 | 0.28 | 0.64 | 0.01 | 0.53 | 0.01 | 0.67 |

Note: Values in the same column with different superscripts are significantly different ($P < 0.05$). SEM, standard error of the mean.
Blood parameters

The effects of dietary treatments on serum concentrations of total protein, albumin, uric acid, triglycerides, total cholesterol, and glucose are presented in Table 6. As it is summarized in Tables 7, 8, 9, 11, 13, and 14, since significant interactions between dietary Cr, Zn, and VitC on total protein, albumin, triglycerides, total cholesterol, glucose, Zn, and iron were detected, the mean comparison of interactions has been done without considering the main effects of experimental factors, dietary supplemental Cr, Zn, and VitC. There were significant \( P < 0.05 \) interactions between VitC and Zn, and also between Zn and Cr on serum total protein concentration before exposure to HS Table 7, so that serum total protein concentration was decreased \( (P < 0.05) \) when Zn was used as single dietary supplement, but this effect was not observed \( (P > 0.05) \) when Zn was used in combination with VitC or Cr. Dietary treatments had no effect \( (P > 0.05) \) on serum total protein concentration after hens were exposed to HS. There was an interaction between VitC and Zn on serum albumin

Table 4. Mean comparison effects of Cr and VitC, VC, and Zn on egg yolk weight, egg specific gravity, egg shape index, and Haugh unit before HS.

| Treatment | Egg yolk weigh (g) | Treatment | Egg specific gravity | Treatment | Egg shape index | Treatment | Haugh unit |
|-----------|--------------------|-----------|---------------------|-----------|----------------|-----------|------------|
| ab        | 17.01 ± 0.46a      | ac        | 1.0833 ± 0.00b      | bc        | 73.88 ± 0.94a  | ac        | 68.00 ± 2.13a |
| ab₁       | 17.02 ± 0.60a      | a₀c₁      | 1.0856 ± 0.00b      | b₀c₁      | 74.42 ± 0.92a  | a₀c₁      | 69.65 ± 2.26b |
| a₁b₀      | 16.95 ± 0.60a      | a₁c₀      | 1.0856 ± 0.00b      | b₁c₀      | 74.36 ± 1.00b  | a₁c₀      | 68.48 ± 2.43b |
| a₁b₁      | 16.26 ± 0.68b      | a₁c₁      | 1.0842 ± 0.00a      | b₁c₁      | 73.76 ± 0.18b  | a₁c₁      | 67.31 ± 2.32b |

**Note:** Values in the same column having different superscripts are significantly different \( (P < 0.05) \).

Table 5. Mean comparison effects of VC, Cr, and Zn on eggshell thickness, egg yolk colour, egg yolk index before HS and on egg shape index after HS respectively.

| Treatment | Eggshell thickness (mm × \( 10^{-2} \)) | Egg shape index | Egg yolk index | Egg yolk colour (Roche) |
|-----------|-----------------------------------------|----------------|----------------|------------------------|
| a₁b₁c₀    | 0.3800 ± 0.000a                        | 72.20 ± 1.23c  | 41.80 ± 0.54a  | 7.16 ± 0.40a           |
| a₁b₀c₁    | 0.3666 ± 0.001b                        | 73.95 ± 1.03b  | 40.22 ± 1.05b  | 7.44 ± 0.34b           |
| a₁b₀c₀    | 0.3683 ± 0.000bc                       | 73.30 ± 1.09b  | 39.47 ± 1.02b  | 7.62 ± 0.27b           |
| a₁b₁c₁    | 0.3750 ± 0.012bc                       | 81.25 ± 0.47a  | 41.68 ± 1.67a  | 6.66 ± 0.36b           |
| a₁b₁c₀    | 0.3600 ± 0.010c                        | 74.16 ± 1.63b  | 39.73 ± 1.11c  | 7.60 ± 0.15b           |
| a₁b₁c₁    | 0.3700 ± 0.007abc                      | 80.19 ± 0.06a  | 41.34 ± 0.92ab | 6.77 ± 0.28b           |
| a₁b₀c₁    | 0.3683 ± 0.006bc                       | 73.41 ± 1.62b  | 40.82 ± 1.20abc | 6.77 ± 0.34b           |
| a₁b₀c₀    | 0.3750 ± 0.0104bc                      | 80.69 ± 0.75a  | 40.41 ± 1.39abc | 6.50 ± 0.28b           |

**Note:** Values in the same column having different superscripts are significantly different \( (P < 0.05) \).

Table 6 Effects of dietary treatments VC, Cr, and Zn on serum metabolite concentrations of laying hens before (36 weeks of age) and after (44 weeks of age) exposure to heat stress.

| Treatments | Total protein (g/dL) | Albumin (g/dL) | Uric acid (mg/dL) | Triglycerides (mg/dL) | Total cholesterol (mg/dL) | Glucose (mg/dL) |
|------------|----------------------|----------------|-------------------|-----------------------|---------------------------|-----------------|
| Before     | After                | Before         | After             | Before                | After                     | Before          | After      |
| Vitamin C (mg/kg) | 0 | 10.25 | 5.99 | 3.18 | 3.04 | 7.55 | 14.01 | 1199 | 1181 | 296 | 289 | 346b | 170 |
| 250        | 6.92                 | 2.88           | 2.98              | 3.44                  | 9.21a                     | 14.59           | 1216 | 1181 | 285 | 277 | 521 | 181 |
| Zinc (mg/kg)  | 0            | 9.65            | 5.99              | 2.98                  | 3.44                      | 9.21a            | 14.59 | 1216 | 1181 | 285 | 277 | 521 | 181 |
| 30         | 10.25               | 5.19           | 3.18              | 3.47                  | 6.82b                     | 12.72            | 1212 | 1154 | 292 | 298 | 414 | 203 |
| Chromium (µg/kg) | 0       | 9.38            | 5.34              | 3.11                  | 3.30                      | 7.21             | 14.65 | 1235 | 1152 | 249b | 297 | 499 | 200 |
| 400        | 10.58               | 5.44           | 3.05              | 3.59                  | 9.10                      | 12.75            | 1190 | 1183 | 328a | 280 | 427 | 183 |
| Pooled SEM | 0.579               | 0.121          | 1.156             | 2.171                 | 0.532                     | 0.640            | 18.0  | 12.8  | 10.3  | 9.4  | 54.6 | 22.3 |

**Note:** Values in the same column with different superscripts are significantly different \( (P < 0.05) \). SEM, standard error of the mean.

Table 7. Mean comparison effects of Cr and Zn on serum total protein concentration, VC, and Zn on serum total protein concentration and glucose concentration before HS.

| Treatment | Total protein (g/dL) | Treatment | Total protein (g/dL) | Treatment | Glucose (mg/dL) |
|-----------|----------------------|-----------|----------------------|-----------|-----------------|
| b₁c₀      | 10.18 ± 3.64a        | ac        | 11.19 ± 4.06c        | ac        | 222.33 ± 117.62a|
| b₁c₁      | 8.58 ± 2.96b         | a₁c₀      | 9.39 ± 3.85a         | a₁c₁      | 141.09 ± 88.36a |
serum triglycerides concentration was increased \( (P < 0.05) \) by Cr when used as a single supplement, but serum glucose concentration was increased \( (P < 0.05) \) when Cr was used in combination with VitC. This interaction also showed that serum glucose concentration was higher \( (P < 0.05) \) for hens given combination of VitC and Zn than that for hens given Zn alone. There was interaction \( (P < 0.05) \) between VitC and Zn on serum glucose concentration before hens were exposed to HS, so that serum glucose concentration was raised.

Table 8. Mean comparison effects of VC and Zn and Cr and Zn on serum albumin concentration and serum triglycerides concentration after HS respectively.

| Treatment                | Albumin (g/dL) | Triglycerides (mg/dL) |
|--------------------------|---------------|-----------------------|
| a                      | 2.55 ± 0.74a | 1138.36 ± 948.33ab    |
| aCb                     | 4.33 ± 0.59b | 2129.39 ± 67.8a       |
| aCc                     | 3.59 ± 0.46a | 1168.50 ± 59.46ab     |
| aCc                     | 3.30 ± 0.41a | 1144.38 ± 100.69bc    |

Note: Values in the same column having different superscripts are significantly different \( (P < 0.05) \).

Table 9. Mean comparison effects of VC, Cr, and Zn on serum triglycerides concentration and total cholesterol concentration after exposure to HS respectively.

| Treatment                | Triglycerides (mg/dL) | Total cholesterol (mg/dL) | Glucose (mg/dL) |
|--------------------------|------------------------|---------------------------|-----------------|
| aCb                     | 1312.00 ± 154.39a      | 193.45 ± 17.84a           | 598.16 ± 342.00a|
| aCb                     | 1128.66 ± 70.86bc      | 286.78 ± 388.06b          | 334.66 ± 249.65bc|
| aCb                     | 1106.16 ± 105.92c      | 353.28 ± 60.63a           | 276.60 ± 116.99f|
| aCb                     | 1250.54 ± 177.04f      | 351.03 ± 49.62a           | 281.41 ± 370.50bc|
| aCb                     | 1243.04 ± 67.33abc     | 286.03 ± 67.81bc          | 502.60 ± 369.37bc|
| aCb                     | 1257.83 ± 85.21abc     | 231.53 ± 29.96c           | 564.00 ± 412.97b|
| aCb                     | 1200.75 ± 69.36abc     | 307.45 ± 72.63bc          | 941.80 ± 275.45ab|
| aCb                     | 1211.25 ± 101.04abc    | 301.85 ± 61.47bc          | 286.66 ± 301.21bc|

Note: Values in the same column having different superscripts are significantly different \( (P < 0.05) \).

Table 10. Effects of dietary supplemental Cr, Zn, and VC on serum insulin concentration and serum glutathione peroxidase activity of laying hens after (44 weeks of age) exposure to heat stress.

| Items                  | Treatments | Insulin (μIU/mL) | Glutathione peroxidase (U/g protein) |
|------------------------|------------|------------------|-------------------------------------|
| Vitamin C (mg/kg)      | 0          | 24.9             | 609                                 |
|                        | 250        | 25.3             | 676                                 |
| Zinc (mg/kg)           | 0          | 16.9b            | 580                                 |
|                        | 30         | 33.4a            | 704                                 |
| Chromium (μg/kg)       | 0          | 19.1b            | 665                                 |
|                        | 400        | 31.1a            | 621                                 |
| Pooled SEM             |            | 2.64             |                                     |

Sources of variation: P values

Table 11. Mean comparison effects of Cr, VC, and Zn on serum glutathione peroxidase activity after HS.

| Treatment                | Glutathione peroxidase activity (U/g protein) |
|--------------------------|-----------------------------------------------|
| aCb                      | 520.14 ± 266.86ab                            |
| aCb                      | 803.34 ± 408.42ab                            |
| aCb                      | 467.33 ± 424.60ab                            |
| aCb                      | 613.37 ± 285.85ab                            |
| aCb                      | 881.15 ± 320.78ab                            |
| aCb                      | 459.15 ± 117.50ab                            |
| aCb                      | 424.10 ± 514.82ab                            |
| aCb                      | 942.84 ± 448.20a                             |

Note: Values in the same column having different superscripts are significantly different \( (P < 0.05) \).

Table 12. Effects of dietary supplemental Cr, Zn, and VC on serum zinc, chromium, iron, and copper concentrations of laying hens after (44 weeks of age) exposure to heat stress.

| Items                  | Treatments | Zinc (μg/mL) | Chromium (μg/mL) | Iron (μg/mL) | Copper (μg/mL) |
|------------------------|------------|--------------|------------------|--------------|----------------|
| Vitamin C (mg/kg)      | 0          | 11.4         | 1.29             | 281b         | 0.89b          |
|                        | 250        | 11.6         | 1.20             | 377a         | 0.99a          |
| Zinc (mg/kg)           | 0          | 11.4         | 1.18b            | 303          | 0.88b          |
|                        | 30         | 17.7         | 1.31a            | 355          | 1.00a          |
| Chromium (μg/kg)       | 0          | 11.1b        | 1.10b            | 293b         | 0.84b          |
|                        | 400        | 11.9a        | 1.38b            | 3649         | 1.034          |
| Pooled SEM             |            | 0.33         | 0.054            | 24.7         | 0.030          |

Sources of variation: P values

Note: Values in the same column with different superscripts are significantly different \( (P < 0.05) \). SEM, standard error of the mean.
The effects of dietary treatments on serum concentration and activity of glutathione peroxidase in serum of HS laying hens are presented in Table 10. Serum insulin concentration was increased \( \left( P < 0.05 \right) \) by both Zn and Cr supplementation. There was an interaction \( \left( P < 0.05 \right) \) between VitC, Zn, and Cr on serum glutathione peroxidase activity. Table 11 shows that the glutathione peroxidase activity of in hens given combination of VitC, Zn, and Cr was higher \( \left( P < 0.05 \right) \) than that in hens given combination of VitC and Cr.

Table 12 shows the effect of dietary treatments on serum concentrations of Zn, Cr, iron, and copper. All two-way interactions were significant \( \left( P < 0.05 \right) \) on serum Zn concentration. Table 13 shows that serum Zn concentration was slightly decreased \( \left( P < 0.05 \right) \) when each of VitC, Cr, or Zn were used alone; however, the serum Zn concentration was increased \( \left( P < 0.05 \right) \) when each of the two supplements were used together. The similar interactions (Table 14) show that the serum iron concentration was increased \( \left( P < 0.05 \right) \) by the addition of VitC, Cr, and Zn, and the effect of each supplement was similar to combination of them. The serum Cr concentration was increased \( \left( P < 0.05 \right) \) by Cr supplementation. Also, the serum copper concentration was increased \( \left( P < 0.05 \right) \) by the addition of VitC, Cr, and Zn to diets.

**Discussion**

**Performance of laying hens**

An ambient temperature of about 32°C or higher is considered to have an adverse effect on the performance of laying hens. Earlier findings have suggested that reduced FI, EP, EWs, and increased FCR are caused by high environmental temperatures (Puthpongsiriporn et al. 2001; Mashaly et al. 2004; Panda et al. 2008). In the present study the effect of environmental temperature was not included in the statistical model. In birds, it was postulated that VitC stimulates 1,25-dihydroxy-cholecalciferol and together with it increases calcium mobilization from bones, suggesting that VitC has an important role in eggshell formation (Dorr and Balloun 1976). It is reported that different levels of zinc oxide (30, 60, 90, and 120 mg/kg diet) did not significantly influence EP, number and weight of ovariian follicles, and weight of ovary and oviduct under normal temperature \( \left( 22 \pm 2\text{°C} \right) \) (Sharideh et al. 2016). Supplementing the diet with VitC (Ciftci et al. 2005; Waseem et al. 2008), Zn (Sahin and Kucuk 2003), or Cr (Sahin, Ozbey, et al. 2002; Sahin et al. 2004) can alleviate some of these adverse effects on production performance. However, along with the beneficial effects observed in these studies, several investigators reported no beneficial effects on performance (Kechik and Sykes 1974; Puthpongsiriporn et al. 2001; Saki et al. 2010) due to adding VitC to the diet under elevated environmental temperatures; besides, there are studies which show that diet supplementation with Zn (Cruz and Fernandez 2011), or Cr (Torki et al. 2014) cannot affect production performance under HS condition; similarly, our results showed that dietary supplementation of Cr, Zn, and VitC had no significant effects on EP, EW, EM, and FCR either before or after exposure to HS. A significant decrease in FI was observed by VitC supplementation when hens were exposed to HS. Such a decrease in FI due to VitC supplementation has not been reported in the literature. Definitely, several variables such as supplementation ways (in diet or drinking water), supplemented level, level in the basal diet, bioavailability, stress condition, degree of stress, and also duration of usage are, at least in part, probable reasons for different results in different experiments.

**Egg quality**

A significant decrease in egg yolk weight was found by combined supplementation of VitC and Cr before exposure to HS. This effect was not observed after hens were exposed to HS. No effects of dietary treatments were found on egg white and eggshell weight either before or after exposure to HS. These results are not consistent with those of Torki et al. (2014), who showed that eggshell weight was increased by the addition of either 250 mg/kg VitC or 200 μg/kg Cr to diet of HS laying hens. Similarly, Sahin, Onderci, et al. (2002) reported higher eggshell weight for HS hens given either 30 mg/kg Zn or 400 μg/kg Cr. In contrast, several investigators reported no beneficial effect of VitC supplementation on eggshell quality (Kechik and Sykes 1974; Puthpongsiriporn et al. 2001) under high environmental temperatures. The shell thickness was decreased by Zn supplementation before exposure to HS, but this effect was not observed when Zn was used in combination with either Cr or VitC. Otherwise, a significant increase in eggshell thickness was found by dietary Zn supplementation after exposure to HS, which is consistent with those reported by Sahin, Onderci, et al. (2002) and Sahin and Kucuk (2003).

| Table 14. Mean comparison effects of VC and Zn, VC and Cr, and Cr and Zn on serum Fe concentrations after HS. |
|-----------------|-----------------|-----------------|
| Treatment | Iron (μg/mL) | Treatment | Iron (μg/mL) | Treatment | Iron (μg/mL) |
| ac | 11.80 ± 1.86abc | ab | 11.58 ± 1.99abc | bc | 12.17 ± 1.86abc |
| a0c1 | 10.90 ± 1.53abc | a1b0 | 11.23 ± 1.40abc | b0c1 | 10.53 ± 1.13abc |
| a1c0 | 10.99 ± 1.46abc | a0b1 | 10.62 ± 1.46abc | b1c0 | 10.01 ± 1.72abc |
| a1c1 | 12.35 ± 2.42abc | a1b1 | 1263 ± 2.22abc | b1c1 | 13.32 ± 1.53abc |

Mean values in the same column having different superscripts are significantly different \( \left( P < 0.05 \right) \).
Specific gravity is an indirect measure of shell thickness and strength (Roberts 2004). Therefore, it is expected that an egg with a higher shell thickness has also a higher specific gravity. In the present study, the egg specific gravity was increased by VitC and Zn supplementation and also by their combination, but the magnitude of the increase was lower by combined treatment. This effect was not observed after hens were exposed to HS. At present, we have no explanation for these apparently contradictory findings on eggshell thickness and specific gravity.

The egg shape index was increased by either Zn or Cr as a single supplement but not with their combined treatment before exposure to HS. Aside from VitC supplementation, egg shape index was increased by combined supplementation of Zn or Cr after exposure to HS. Egg shape index was also increased by combined supplementation of VitC and Zn. Also, after exposure to HS, it was demonstrated that yolk index was decreased by single supplementation of Zn and Cr when no VitC was supplemented to diet. Conversely, yolk index was increased by single supplementation of Cr when diet was supplemented with VitC. Such changes in egg shape index due to dietary supplementation of VitC, Zn, and Cr have not been reported before. However, our results do not confirm the results of Lin et al. (2004), who found a positive correlation between egg shape index and EW. Haugh unit was decreased by combined supplementation of VitC and Zn before exposure to HS, but this effect was not observed by either VitC or Zn as a single supplement. Haugh unit is a measure of albumen quality that determines the quality of the egg. Therefore, our results suggest that using a combination of 250 mg/kg VitC and 30 mg/kg Zn in diet of laying hens under normal brooding temperature may have a deleterious effect on egg quality. Amem and Al-Daraji (2011) suggested that the effects of dietary treatments on Haugh unit is mediated by changes in serum concentrations of total protein and albumin, however, this is not consistent with the results of the present study.

The yolk colour was decreased by combination of Zn and Cr before exposure to HS. Also, yolk colour was decreased when Zn and Cr were used in combination with VitC. The yolk colour was not affected by dietary treatment after exposure to HS. In contrast, Torki et al. (2014) reported diet supplementation with Cr and VitC improved yolk colour in HS laying hens, and the highest egg yolk colour content was seen in the hens fed with 250 mg/kg supplemental VitC. The interaction between plant pigments and feed additives on yolk colour merits further research. There was an interaction between VitC, Zn, and Cr on egg yolk index. It was demonstrated that yolk index was decreased by single supplementation of Zn and Cr before exposure to HS. Conversely, yolk index was increased by single supplementation of Cr when diet was supplemented with VitC. Also, yolk index was increased by Cr supplementation after exposure to HS. The effects of these dietary supplements to yolk index have not been reported earlier.

**Blood parameters**

The effects of dietary treatments on serum biochemical indices were not consistent during the study, and, therefore, it is hard to make conclusions. The serum total protein concentration was decreased when Zn was used as single dietary supplement before exposure to HS, but this effect was not observed when Zn was used in combination with VitC or Cr. Also, no significant effect of dietary Cr and VitC alone was detected on serum total protein concentration either after or before exposure to HS. In other studies, Salim et al. (2012) reported that the serum total protein concentration was not affected by supplementation of 25 mg/kg Zn from an organic source, whereas Feng et al. (2010) showed that serum total protein concentration of broilers was increased by dietary addition of 30 mg/kg Zn either from zinc-glycine or zinc sulphate. Also, Kucuk et al. (2003) reported that serum total protein concentration of HS broiler chickens was increased by supplementation of 30 mg/kg Zn (as zinc sulphate) to diet. Similarly, Sahin, Onderci, et al. (2002) showed that serum total protein concentration of laying hens reared under low ambient temperature was increased by dietary Cr (400 μg/kg as Cr picolinate) and Zn (30 mg/kg as zinc sulphate) supplementation either alone or in combination. The same increase in serum total protein concentration was observed by Torki et al. (2014), who added 200 or 400 μg/kg Cr (as Cr picolinate) and 250 mg/kg VitC to diets of HS laying hens.

The serum albumin concentration was not affected by dietary treatments before exposure to HS. These results are in general agreement with those of Wang et al. (2011), Salim et al. (2012), and Ma et al. (2014). Wang et al. (2011) reported that the serum albumin concentration of laying hens was not affected by the addition of 500 mg/kg VitC, whereas Salim et al. (2012) reported that the serum albumin concentration of broiler chickens was not affected by the addition of 25 mg/kg Zn to the diet. Ma et al. (2014) also reported that the serum albumin concentration of laying hens was not affected by the addition of 200, 400, or 600 μg/kg Cr (as Cr propionate) to the diet. Also, after exposure to HS, our results showed that the serum albumin concentration was not affected by supplementation of Cr and VitC. In contrast, Torki et al. (2014) reported that serum albumin concentration was increased by supplementation of 200 or 400 μg/kg Cr, although VitC at 200 mg/kg had no effect. However, the serum albumin concentration was increased when Zn was used as single supplement after exposure to HS, but this effect was not observed by combination of Zn and VitC.

The serum uric acid concentration was decreased by Zn supplementation before exposure to HS, whereas no effect of dietary treatments was detected on serum uric acid concentration after exposure to HS. The lowering effect of Zn on serum uric acid level could be related to its antioxidant activity and is supported by the results of Kim and Patterson (2004), who evaluated the effects of Zn sulphate or Zn oxide supplementation of broiler diets on growth performance and loss of uric acid and total nitrogen from manure. They reported that the Zn treatments significantly reduced serum uric acid level and nitrogen loss in poultry manure, and Zn oxide could be a better Zn source to prevent nitrogen loss to the atmosphere without any detrimental effect on growth performance. It is not clear why Cr and VitC did not affect serum uric acid levels, as these supplements also reported to have substantial antioxidant activity (Puthpungsiriporn et al. 2001; Onderci et al. 2003). Ma et al. (2014) showed that a 200 μg/kg Cr
supplementation decreased the uric acid concentration by 31%. Also, in another study (Saki et al. 2010), it has been reported that plasma uric acid level was decreased by supplementation of 250 mg/kg VitC to diet. However, similar to our results, Samanta et al. (2008) showed that serum uric acid concentration was similar for broilers receiving 500 or 1000 μg/kg Cr (as Cr picolinate) and those receiving the control diet.

The serum triglycerides concentration was decreased when Zn or Cr were used as single supplements before exposure to HS, but this effect was not observed when VitC was added to diets. Otherwise, the serum triglycerides concentration was increased by Zn supplementation after exposure to HS, but this effect was not observed when Zn was used in combination with Cr. Contrary to our results, Ma et al. (2014) reported that the serum triglycerides concentration of laying hens was not affected by the addition of 200, 400, or 600 μg/kg Cr to the diet. Mirfendereski and Jahanian (2015) showed that plasma concentrations of triglycerides were not influenced by dietary supplementation of 0, 500, and 1,000 μg/kg Cr (as Cr methionine, and 500 mg/kg VitC). Also, Kucuk (2008) showed that addition of 30 mg/kg Zn (as zinc acetate) lowered serum triglyceride concentration in HS broiler chickens. Similarly, Torki et al. (2014) showed that HS laying hens given the diet supplemented with 200 or 400 μg/kg Cr exhibited significantly lower serum triglycerides concentration compared to the control.

Cr was suggested to be biologically active mineral as part of a biomolecule called chromodulin, which is part from an insulin signalling pathway and appears to a biomolecule called chromodulin, which is part from an insulin storage, and secretion of insulin as well as conformational integrity of insulin in the hexameric form (Salgueiro et al. 2001). Zn deficiency has been reported to result in an impairment of glucose tolerance in rats because of the known role of Zn associated with stored insulin (Linder 1991).

The serum activity of glutathione peroxidase was higher in hens given combination of VitC, Zn, and Cr than that in hens given combination of VitC and Cr. One of the most important functions of Zn is its participation in the antioxidant defence system. The mechanism by which Zn exerts its antioxidant action is not well defined. However, it has been suggested that it increases the synthesis of metallothionein, a cysteine-rich protein, which acts as a free radical scavenger (Oteiza et al. 1996). Furthermore, Zn can occupy iron and copper binding sites on lipids, proteins and DNA thus exert a direct antioxidant action (Powell 2000; Prasad and Kucuk 2002). Preuss et al. (1997) reported that formation of hepatic malonaldehyde, a marker of lipid peroxidation, in liver was decreased by supplementation of Cr picolinate and Cr nicotinate in rats and Cr acted as an antioxidant. Similarly, results of the present study showed that Cr and Zn acted as antioxidants, as supplementation of them increased the serum activity of glutathione peroxidase. Anderson et al. (2001) reported that rats fed with supplemented diet with Cr and Zn had a significant reduction in malondialdehyde levels in serum and tissues. Zago and Oteiza (2001) defined Zn as an important component of an antioxidant network that prevents membrane damage from oxidation and also reported that both Zn and vitamin E partially inhibited malondialdehyde formation, but the simultaneous presence of both antioxidants had a higher protective action. Zn is a cofactor of the main anti oxidative enzyme Cu/Zn-superoxide dismutase. Zn may play a key role in the suppression of free radicals and in the inhibition of NADPH-dependent lipid peroxidation (Prasad 1997), as well as in the prevention of
lipid peroxidation via inhibiting glutathione depletion (Gibbs et al. 1985). Regeneration of Vitamin E from α-chromanol radical appears to be mediated by glutathione and VitC (Kagan et al. 1992).

The serum concentration of Cr was increased by Cr supplementation, which is consistent with those of Habibian et al. (2013). The serum Zn concentration was slightly decreased when VitC, Cr, or Zn used alone, but it was increased when each of the two supplements were used together. The serum concentration of iron was increased by the addition of VitC, Cr, and Zn, and the effect of each supplement was similar to combination of them. Also, the serum concentration of copper was increased by the addition of VitC, Cr, and Zn to diets. In other studies, Sirirat et al. (2013) reported that supplemental nano Cr picolinate could significantly improve retention of Cr and Zn. Also, Sahin, Sahin, et al. (2002) reported that both supplemental Cr at 400 μg/kg and VitC at 250 mg/kg increased serum concentrations of iron, Zn, manganese, and Cr, but decreased serum concentration of copper. Similar results were reported by Ghazi et al. (2012b), who added 600 and 1200 μg/kg Cr methionine to diet of HS broiler chickens.

Conclusion
In conclusion, addition of single or combination of Cr, Zn, and VitC can improve at least some production performance and egg quality parameters. In addition of these valuable positive effects, the more mentionable result of the current study could be the interaction effects of Cr, Zn, and VitC on enzyme activity (glutathione peroxidase) in laying hens during heat stress condition.

Disclosure statement
No potential conflict of interest was reported by the authors.

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