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EFFECT OF ASCORBIC ACID AND/OR ALPHA-TOCOPHEROL FORTIFICATION ON SEMEN QUALITY, METABOLIC PROFILE, ANTIOXIDANTS STATUS, AND DNA OF ROOSTERS EXPOSED TO HEAT STRESS

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ABSTRACT

This study investigates the influence of heat stress (HS) and vitamin C (VC) and/or vitamin E (VE) on semen quality, biochemical constituents of seminal and blood plasma, antioxidant status and jejunum and ilium deoxyribonucleic acid (DNA) of roosters. Forty-five, 32-week-old roosters were divided into five groups of nine replicates. One group was kept under a thermoneutral environment, whereas the other four were kept under HS. One of the four groups served as an unsupplemented HS group and was fed the basal diet only. The other three HS groups were supplemented with VC (200 mg/kg ascorbic acid and/or VE 150 mg/kg α-tocopherol acetate). Vitamins fortification completely restored sperm liveability , semen pH, fertility, seminal plasma total protein and globulin to the control level. Vitamins fortification completely recovered seminal plasma and blood plasma total antioxidant capacity (TAC) and malondialdehyde (MDA), red blood cell (RBCs), haemoglobin (Hgb), packed cell volume (PCV), white blood cells (WBCs), blood plasma glucose, lipid profiles, liver enzymes, and renal function. Heterophil/lymphocyte (H/L) ratio was totally restored following VE fortification. Vitamins fortification significantly increased jejunum and ileum DNA compared with the HS group, with VC inducing a complete recovery. In conclusion, either 200 mg /kg VC or 150 mg/kg VE improved semen quality, fertility, seminal plasma and blood biochemistry and haematology of HS roosters, with the VE group yielding greater immunity.

Keywords: Roosters. Heat Stress. Semen Quality. Vitamin C. Vitamin E.

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INTRODUCTION

Heat stress (HS) has been shown to have adverse effects on animal productivity and leads to increasing reactive oxygen species (ROS) production, stimulating the secretion of catecholamines and corticosterone and initiating the peroxidation of lipid in cell membranes (Khan, 2011; Attia *et al*., 2017; Conte *et al*., 2018). The balance between antioxidants reserves (biomolecules, enzymes, vitamins) and oxidants (production of free radicals) plays a major role in controlling the adverse impact of HS and limiting ROS production (Khan *et al*., 2012a;b; Kutlu *et al.,* 2019).

The HS has been shown to have a negative effect on testes and testicular function through inhibition of intracellular ion exchange (Ayo *et al*., 2011; Khan *et al*., 2014; Chen *et al.*, 2015), as well as influencing the ejaculate volume, concentration and motility of sperm (Turk *et al*., 2016). The adverse impact of HS on performance, semen quality, and fertility of animals was observed when the air temperature was increased above 27°C (Biswas *et al*., 2009). High levels of polyunsaturated fatty acids, including arachidonic and docosatetraenoic acids, have been reported in the cell membrane of avian spermatozoa (Surai *et al*., 2001). Hence, avian sperm are very sensitive to ROS, which cause male infertility (Zaniboni *et al*., 2006). Oxidative damage of spermatozoa can decrease motility and fertility (McDaniel *et al*., 2004). Moreover, HS was shown to damage the spermatozoal deoxyribonucleic acid (DNA), resulting in abnormal mitotic division during spermatogenesis. This could affect spermatozoal parameters including low sperm number, viability and motility with associated DNA impairment. Interestingly, these abnormal spermatozoal parameters were observed among spermatozoa developed from spermatids in testis exposed to HS (Ayo *et al*., 2011). This could lead to the development of abnormal spermatozoa, which

consequently may not have the ability to achieve fertilisation (McDaniel *et al*., 2004).

There are several approaches to relieve the negative influences of HS. Antioxidants including vitamin C (VC) and vitamin E (VE) play a crucial role in this context (Khan, 2011; Khan *et al*., 2012a; Attia *et al*., 2017). The efficacy of VC in eliminating the adverse impact of HS and increasing thermotolerance has been previously reported (Umar *et al*., 2010). Increasing thermotolerance can minimise mortality from HS and maintain animal productivity (Attia and Hassan, 2017). Generally, HS increases the metabolism and the excretion of minerals and vitamins, leading to aggravating a deficiency in vitamins and minerals (Khan *et al*., 2012a). Heat stress has also been shown to decrease blood ascorbic acid concentration (Khan, 2011). The metabolism of minerals and amino acids requires VC, which, in turn, is needed for the biosynthesis of adrenaline and testosterone, collagen and 1 25-dihydroxy vitamin D (Khan *et al*., 2012a). Ascorbic acid has been reported to be involved in the activation of immunity, body temperature regulation and production of leucocytes (Khan, 2011). Adequate dietary VC helps to alleviate the metabolic signs of stress and improve immunity and behaviour of birds and their productive performance (Daghir, 2008; Attia *et al*., 2009; Attia *et al*., 2011). Ascorbic acid also spares VE by transforming the oxidised form of α-tocopherol back to α-tocopherol (Attia *et al*., 2017; Attia *et al*., 2018). VC was shown to improve the semen quality of heat-stressed roosters (Nowaczewski and Knotecka, 2005).

Alpha-tocopherol (VE), the 'anti-sterility' vitamin, is a natural antioxidant that guards the cell membranes against oxidative injury caused by the destruction of lipoperoxides, and thus VE requirements must be met (Traber and Atkinson, 2007; Tufarelli and Laudadio 2016). VE is not only essential for the prevention of lipid peroxidation (Khan, 2011) but also boosts the function and proliferation of macrophages, lymphocytes and plasma cells against oxidative injury (Traber, 2007); thereby improving the quality of semen and fertility of boars during the high temperature season (Khan, 2011). Consequently, enriching semen and spermatozoa with antioxidants is a prerequisite for improving male fertility (Tran, *et al*., 2017). Interestingly, administering a diet of 100-400 mg/kg of VE has been shown to significantly elevate semen quality of roosters (Keskes-Ammar*et al*., 2003; Al-Zahrani *et al*., 2012; Alm El-Dein *et al.*, 2013). Supplementation with 100 mg/kg VE (Biswas *et al*., 2009) and 200 mg/kg VE (Ebeid, 2012) has been shown to enhance the antioxidant status and reduce the peroxidation of lipids in the seminal plasma of chicken males exposed to HS (Khan *et al*., 2012a;b). Vitamin C significantly raised the serum total protein and globulin of laying hens exposed to HS, but significantly reduced serum glucose, cholesterol, HDL and triglycerides (Ezzat *et al*., 2011).

The VE and/or VC were shown to equally elevate lymphocyte numbers and leucocytes, reducing the heterophil/lymphocyte ratio ($p<0.05$) in quails, suggesting that VC or VE alone is adequate (Sahin *et al*., 2002). A combination of both VC and VE showed a greater effect on physiological traits than administering only one of the vitamins (Attia *et al*., 2017). The same authors added that vitamins C+E induced a further increase in basophil, serum total protein and albumin than VC alone. Moreover, the in vivo antioxidant influence of VE may be greater than that of VC (Hoehler and Marquardt, 1996). There is insufficient literature on the influence of HS on semen quality when a combination of different antioxidants is administered and whether this would improve chickens' tolerance to HS and improve semen quality and fertility. Hence, this study aimed to determine the impact of HS and VC and/or VE fortification in feed on semen quality and fertility, biochemical and haematological profile, antioxidants status and the DNA of the jejunum and ilium of male chickens.

MATERIALS AND METHODS

The experimental protocol was approved by the scientific committee of Animal Production Research Institute under registration code no: 9-2-4-3-10-1. The committee recommended the care and good handling of the animals, keeping in mind their rights and their welfare and causing them minimal stress.

Forty-five, 32-week-old male chickens of the Mandarah a native breed of Egypt classified as dual-purpose breed [a cross between ♂Alex × ♀Dokki-4 (Abd- El- Gawad *et al*., 1981)], with similar initial body weights, were allocated randomly amongst five treatment groups of nine males in each group, between 32 and 52 weeks of age. The males were individually housed in galvanised wire cages (30 × 50 × 60 cm), in batteries with standard dimensions, in an environmentally-controlled lightproof house (close system-controlled for temperature, humidity and light). Each cage was provided with a manual feeder and with nipple drinkers. Free access to a mash basal diet and to water was provided. Common veterinary care practices were used for vaccination and for the medical programme. The roosters were illuminated with 16:8 h light-dark cycles.

The health care, housing conditions, indoor and outdoor temperature and relative humidity and light schedule were as reported by Attia *et al*. (2016). The roosters were reared (indoors), either at optimal environmental conditions of 22°C–24°C with a relative humidity (RH) of 45% – 55%, serving as a thermoneutral group and fed a basal diet (Table 1), or under heat stress

(38°C ± 1°C; 55% – 65% relative humidity) for 3 successive days a week, from 11.00 am to 15.00 pm and returned to thermoneutral conditions thereafter. The outdoor temperature was 30.7ºC ± 0.47ºC, with a RH of 70.9% ± 0.9%. The aim of heat stress scheme (3 days only per week), is to keep similar heat stress condition to that naturally occurs as 2-5 days a wave usually seen followed by more or less period of thermoneutral, thus this heat stress design is similar to what is happening in nature. Roosters exposed to HS were allocated to four groups: roosters kept under HS and fed with a basal diet without VC, VE and VC + VE serving as unsupplemented HS control (Control). In the second group, roosters were kept under HS and fed with a basal diet (Table 1) supplemented with 200 mg/kg ascorbic acid (VC) (L-ascorbic acid; a heat-stabilised product, Hoffmann-La Roche, Switzerland). In the third group, roosters were kept under HS and were fed a basal diet, supplemented with 150 mg/kg α-tocopherol acetate (VE) (α-tocopherol acetate, Hoffmann-La Roche, Switzerland). In the fourth group, roosters were kept under HS and were fed a basal diet, fortified with 200 mg/kg VC and 150 mg/kg VE (VC + VE). The vitamins content of the basal diet particularly the total VE by calculation (20.5 mg/kg) were in according to the NRC (1994).

The roosters were individually weighed, at 32 and 52 weeks of age, and the body weight gain was calculated on the basis of the differences between the initial and the final body weight. Daily feed intake (g/cock) and mortality were recorded for each treatment.

Semen was collected weekly from all roosters after 10 weeks on the treatments, at 42 weeks of age and maintained for another 10 weeks, to determine the quality of the semen. Collection of semen was performed utilising the procedure of abdominal massage. The internal temperature of the semen at collection time was kept around 41°C – 44°C, using a water bath, and then the semen samples were transferred immediately to the laboratory after collection, to determine the quality of the spermatozoa. Moreover, attention was given so that the semen would be guarded from cold shocks and from direct light. Throughout the course of the semen collection, the time, place of collection and collector were constant. Semen quality included ejaculate volume (ml), sperm concentration ($\times 10^9$sperm), sperm concentrate/ejaculate ($\times 10^9$sperm), sperm motility (%), sperm liveability (%), total live sperm/ejaculate ($\times 10^9$sperm), and semen pH were determined as outlined by Attia *et al*. (2015) and Attia and Kamel (2012), whereas semen quality factor was estimated according to Liu*et al*. (2008).

At 44, 48 and 52 weeks of age, the fertility of the roosters was measured. Semen was artificially collected by abdominal massage and was used for the artificial insemination of hens. Semen was used after 1:1 dilution, using a 0.9% saline solution as a diluent (Bootwalla and Miles, 1992). The semen of each male was used to inseminate ten hens. Each hen was inseminated with 0.5 mL of semen, over 2 successive days. After 2 days of insemination, the eggs were collected over 10 days, stored at room temperature (22°C – 24°C with 45% – 55% RH), incubated (37.6ºC, 55% RH) and hatched (36.8ºC, 65% RH) in an automatic incubator, and the fertility was estimated by dividing the number of fertile eggs by the total number of eggs set.

At 52 weeks of age, blood samples (5 mL) were withdrawn from the brachial vein of each treatment. Blood samples (n=5) at each treatment were collected from the overnight-fasted roosters in the morning in tubes, with heparin as an anticoagulant agent. Blood plasma and seminal plasma were obtained by the centrifugation of the blood and semen, at 1500× g for 20 minutes and kept at −20ºC until used for analysis.

Plasma and seminal plasma metabolites, seminal and blood plasma total antioxidant capacity (TAC) and malondialdehyde (MDA) were determined, as outlined by the manufacturer's recommendation, using diagnostic kits (Diamond diagnostics, 23 EL-Montazah St. Heliopolis, Cairo, Egypt, http://www.diamonddiagnostics.com). Blood plasma creatinine was measured using special kits delivered from N.S. BIOTEC (http://www.nsbiotec.com). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as (U/L) in the blood and seminal plasma were measured using available kits manufactured by Pasteur Lab (http://www.pasteurvetlab.com). Blood plasma alkaline phosphatase (ALP) was measured according to the method of Yan *et al.* (DGKC), (1972). Seminal plasma α-, β- and γ-globulin were determined using commercial ELISA, according to Bianchi *et al.* (1995).

Blood haemoglobin (Hgb; %), were determined according to the method by Tietz (1982). Red blood cells (RBCs) were determined according to the method of Helper (1966), as well as that of Hawkeye and Dennett (1989). Packed cell volume (PCV; %) was measured according to Wintrobe's method (1965). The mean cell volume (MCV), the mean cell haemoglobin (MCH) and the mean cell haemoglobin concentration (MCHC) were estimated as absolute values, as reported by Attia *et al*. (2015). Phagocytic activity (PA) and phagocytic index (PI) were determined according to Kawahara*et al*. (1991). White blood cells (WBCs) were determined as reported by Helper (1966) and Hawkeye and Dennett (1989), using a light microscope at 100× magnification. Blood film was prepared according to the method by Lucky (1977), to determine different leucocytes.

High molecular weight DNA was extracted from intestinal parts (jejunum and ileum), according to the method by Sambrook *et al.*(1989), with some modification following Abdel-Fattah (1995). The concentration of DNA was calculated on the basis of the optical density (O.D.) reading of the UV

spectrophotometer, at a wave length of 260 nm (1.0 O.D. = 50 µg DNA/mL of solution), according to Charles (1970).

The data were tested using the GLM procedure of SAS® (SAS, 2011), and a one-way ANOVA, according to the following model: $yij = \mu + \tau j + \varepsilon ij$, where μ = the general mean, τj = the effect of treatment and εij = the experimental error. A $P \leqslant 0.05$ value of significance of student Newman Klaus' test was used for testing mean differences amongst the experimental groups, at $P \leqslant 0.05$. Before the analysis, all the percentages were subjected to a logarithmic transformation to normalise the distribution of the data.

RESULTS

The HS significantly decreased body weight (BW) gain and feed intake of roosters, compared with roosters in the thermoneutral group (Table 2). The fortification with vitamins resulted in a similar partial recovery in BW gain. Vitamins induced a partial recovery in feed intake, with VC + VE causing a greater response in feed intake than either VC or VE alone.

The exposure of roosters to HS without added vitamins significantly reduced ejaculate volume, sperm concentration, sperm concentration per ejaculate, sperm motility, sperm liveability, total live sperm per ejaculate, semen quality factor and fertility, but increased semen pH (Table 2).

The addition of vitamins resulted in a complete recovery in terms of sperm liveability, semen pH and fertility, compared with the thermoneutral group (Table 2). In addition, VE with or without VC caused a complete recovery of sperm concentration, compared with the thermoneutral group, but VC alone induced a partial recovery; however, the differences among the three vitamin groups were not significant.

A partial recovery due to the addition of vitamins to the HS group was shown in terms of the volume of the ejaculate, the concentration of sperm per ejaculate, the motility of the sperm, the total live sperm per ejaculate and the semen quality factor, compared with the thermoneutral group. The HS significantly modified seminal plasma total protein, the globulin, AST, ALT, TAC and MDA, compared with the thermoneutral group. On the other hand, seminal plasma albumin, different immunoglobulins, albumin/globulin ratio and AST/ALT ratio were not affected (Table 2).

Vitamin fortifications resulted in a complete recovery in terms of seminal plasma total protein, globulin, TAC and MDA, compared with the thermoneutral group, but only the combination of VC + VE resulted in a significantly higher plasma total protein and globulin, than the unsupplemented HS group (Table 2). In addition, vitamins fortifications resulted in a significantly higher TAC than in the thermoneutral group. Additional vitamins induced a partial recovery in ALT, but VE with or without VC resulted in a complete recovery in plasma AST, compared with the thermoneutral group, showing a stronger effect of VE than VC, which induced a partial recovery in seminal plasma AST (Table 2).

Exposure to HS significantly impaired most of the biochemical constituents and jejunum and ilium DNA, except for blood globulin and the urea/creatinine ratio (Table 3).

Additional vitamins caused a complete recovery in plasma glucose, total lipids, triglycerides, cholesterol, ALT, AST/ALT ratio, creatinine, urea, TAC and MDA. VC and VC + VE resulted in a complete recovery in the total plasma protein, thus the difference from the thermoneutral group disappeared, while VC + VE had a greater effect than VE alone (Table 3). Vitamins C + E induced a complete recovery in plasma albumin, A/G ratio, AST and alkaline phosphatase, while VC and VE induced a partial recovery. VC + VE resulted in a higher TAC than the VC or VE separately and the thermoneutral group (Table 3).

Table 1. Ingredients and nutrient compositions of the experimental basal diet.

Ingredients and composition		g/kg
Yellow corn, ground		663.3
Soybean meal (48% CP)		242.0
Wheat bran		65.0
Limestone		10.0
Dicalcium phosphate		13.2
Vit+Min Premix[1]		2.5
NaCl		2.5
DL-methionine		1.5
Total	1000	
Calculated[2] and determined[3] composition		
Metabolisable energy(MJ/kg[2])		11.98
Dry matter (g/kg)[3]		917.3
Crude protein (g/kg)[3]		179.6
Ether extract (g/kg)[3]		28.5
Crude fibre (g/kg)[3]		4.78
Methionine (g/kg)[2]		4.1
Methionine + Cystine (g/kg)[2]		6.70
Lysine (g/kg)[2]		8.8
Ash (g/kg)[3]		63.7
Nitrogen free extract (g/kg)[3]		609.8
Calcium (g/kg)[2]		1.10
Available P (g/kg)[2]		3.94
Vitamin E (mg/kg)		20.5

1Vit+Min mixture provides per kilogram of diet: vitamin A, 12000 IU; vitamin E (all-rac-α-tocopheryl acetate) 4.5 mg or 10 IU; menadione, 3 mg; Vit. D3, 2200 ICU; riboflavin, 10 mg; Ca pantothenate, 10 mg; nicotinic acid, 20 mg; choline chloride, 500 mg; vitamin B12, 10 µg; vitamin B6, 1.5 mg; vitamin B1, 2.2 mg; folic acid, 1 mg; biotin, 50 µg. Trace mineral (milligrams per kilogram of diet): Mn, 55; Zn, 50; Fe, 30; Cu, 10; Se, 0.10; Anti oxidant, 3 mg.

[2]Calculated from NRC (1994) table values. [3]Deterimed values based on AOAC (2004).

Additional vitamins significantly increased the DNA concentrations in both intestinal segments, compared with the unsupplemented HS group (Table 4). Additional VC resulted in a complete recovery in jejunum and ilium DNA, and VC + VE caused a complete recovery in the ileum DNA. VC had a greater effect than VE on jejunum and ilium DNA.

Exposing roosters to HS significantly impaired most of the haematological constituents of blood, excluding MCV, MCH, MCHC, basophils and eosinophils (Table 4). Vitamin fortification resulted in a complete recovery in RBCs, Hgb, PCV and WBCs. fortification with VC and VE induced a complete recovery in blood pH, thus the difference with the thermoneutral group was diminished, but VC + VE resulted in a partial recovery (Table 4). Phagocyte activity and monocytes were totally recovered when VE was supplemented, but VC and VC + VE had a lower effect on monocytes than VE. Additional intake of vitamins resulted in incomplete PI and lymphocytes. Additional VE and VC + VE resulted in a total recovery in the heterophil and H/L ratio, thus the differences from the thermoneutral group was diminished (Table 4).

Table 2. Effects of dietary vitamin C (VC), vitamin E(VE), and their combined fortification on performance, semen quality and some seminal plasma constituents in roosters reared under heat stress condition (mean±SEM).

Parameters	Thermoneutral	Heat stress treatments				SEM	p-value
		Heat stress	+VC	+VE	+ VC+VE		
Performance of roosters							
Initial BW (g)	2141	2128	2123	2118	2121	22.4	0.834
Body weight gain (g)	513^{a}	401^{c}	463^{b}	455^{b}	473^{b}	15.7	0.001
Feed intake (g/bird/d)	136^{a}	123^{d}	$131b^{c}$	129^{c}	133^{b}	1.11	0.001
Semen quality							
Ejaculate volume (ml)	0.561^{a}	0.431^{c}	0.525^{b}	0.532^{b}	0.523^{b}	0.011	0.001
Concentrate/ml ($\times10^{9}$sperm)	2.76^{a}	2.11^{c}	2.60^{b}	2.67^{ab}	2.68^{ab}	0.055	0.001
Concentrate/ejaculate ($\times10^{9}$sperm)	1.55^{a}	0.917^{c}	1.37^{b}	1.42^{b}	1.41^{b}	0.039	0.001
Sperm motility (%)	90.8^{a}	78.9^{c}	88.3^{b}	88.5^{b}	88.6^{b}	0.848	0.001
Sperm liveability (%)	87.2^{a}	79.1^{b}	86.1^{a}	86.3^{a}	86.5^{a}	0.527	0.001
Total live sperm/ejaculate ($\times10^{9}$sperm)	1.35^{a}	0.728^{c}	1.18^{b}	1.22^{b}	1.22^{b}	0.035	0.001
Semen pH	7.28^{b}	7.55^{a}	7.29^{b}	7.34^{b}	7.28^{b}	0.031	0.001
Semen quality factor	1349^{a}	728^{c}	1177^{b}	1206^{b}	1217^{b}	36.4	0.001
Fertility (%)	96.5^{a}	79.2^{b}	94.6^{a}	96.3^{a}	96.0^{a}	0.333	0.001
Seminal plasma constituents.							
Total protein (g/dl)	6.13^{a}	5.48^{b}	5.88^{ab}	5.78^{ab}	6.03^{a}	0.182	0.025
Albumin (g/dl)	2.30	2.28	2.33	2.10	2.28	0.097	0.205
Globulin (g/dl)	3.83^{a}	3.20^{b}	3.53^{ab}	3.68^{ab}	3.78^{a}	0.189	0.032
A/G ratio	0.604	0.651	0.662	0.576	0.602	0.052	0.091
α- globulin (g/dl)	1.625	1.475	1.625	1.775	1.500	0.102	0.065
β- globulin (g/dl)	1.20	1.175	1.225	1.175	1.225	0.108	0.626
γ- globulin (g/dl)	1.00	0.675	0.675	0.725	1.025	0.182	0.163
AST (U/L)	41.3^{c}	51.8^{a}	45.0^{b}	43.0^{bc}	43.3^{bc}	1.27	0.001
ALT (U/ L)	15.0^{c}	19.8^{a}	17.3^{b}	17.0^{b}	16.8^{b}	0.736	0.001
AST/ALT	2.77	2.65	2.60	2.54	2.60	0.154	0.653
TAC (Mmol/dl)	409^{b}	324^{c}	431^{a}	432^{a}	435^{a}	6.32	0.001
MDA (Mmol/dl)	0.925^{ab}	1.10^{a}	0.800^{b}	0.775^{b}	0.728^{b}	0.100	0.014

SEM: standard error of means; semen quality factor: ejaculate volume (mL) × sperm concentration ($\times10^{6}$/mL) × live and morphologically normal spermatozoa (%) (Liu *et al*., 20008); A/G ratio: albumin/globulin ratio; α- globulin: alpha globulin; β-globulin:Beta globulin, γ- globulin: gamma globulin; ALP: alkaline phosphatase, AST:aspartate amino transferase; ALT:alanine amino transferase; TAC:total antioxidant capacity; MAD: malondialdehyde. ^{a,b,c} Means in the same row having different superscripts are significantly different ($P<0.05$).

Table 3. Effects of dietary vitamin C (VC), vitamin E (VE), and their combined fortification on some blood biochemical constituents, antioxidants status, and deoxyribonucleic in jejunum and ilium of roosters reared under heat stress condition (mean±SEM).

Parameters	Thermoneutral	Heat stress treatment				SEM	P-value
		Heat stress	+VC	+VE	+VC+VE		
Glucose (mg/dl)	228[a]	210[b]	224[a]	219[a]	225[a]	3.20	0.001
Total protein (g/dl)	5.92[a]	5.13[c]	5.56[ab]	5.40[bc]	5.76[a]	0.127	0.001
Albumin (g/dl)	2.64[a]	1.82[c]	2.32[b]	2.24[b]	2.58[a]	0.057	0.001
Globulin (g/dl)	3.28	3.32	3.33	3.16	3.20	0.133	0.743
A/G ratio	0.803[a]	0.550[c]	0.698[b]	0.710[b]	0.815[a]	0.119	0.001
Total lipids (g/dl)	4.42[b]	5.54[a]	4.53[b]	4.49b	4.48[b]	0.191	0.001
Triglycerides (mg/dl)	150[b]	175[a]	155[b]	158[b]	156[b]	3.62	0.001
Cholesterol (mg/dl)	136[b]	154[a]	140[b]	142[b]	139[b]	2.87	0.001
AST (U/L)	40.1[c]	61.7[a]	44.1[b]	43.3[b]	41.7[bc]	1.13	0.001
ALT (U/ L)	17.3[b]	21.9[a]	18.3[b]	18.2[b]	17.7[b]	0.389	0.001
AST/ALT	2.32[b]	2.81[a]	2.40[b]	2.38[b]	2.33[b]	0.082	0.001
ALP (U/ l)	171[d]	192[a]	183[b]	179[bc]	176[cd]	2.04	0.001
Creatinine (mg/dl)	3.19[b]	3.30[a]	3.23[ab]	3.20[b]	3.22[ab]	0.032	0.058
Urea (mg/dl)	3.40[b]	3.58[a]	3.41[b]	3.51[ab]	3.42[ab]	0.046	0.034
Urea/ creatinine ratio	0.940	0.923	0.940	0.913	0.930	0.191	0.684
TAC (Mmol/dl)	431[c]	374[d]	447[b]	450[b]	458[a]	2.24	0.001
MDA (Mmol/dl)	0.915[b]	1.27[a]	0.888[b]	0.870[b]	0.858[b]	0.026	0.001
DNAJ (µg/ml)	26.99[a]	22.75[d]	26.90[a]	24.40[c]	24.89[b]	0.219	0.001
DNAI (µg/ml)	25.20[a]	20.20[c]	25.17[a]	23.00[b]	24.70[a]	0.316	0.001

SEM: standard error of means; A/G ratio: albumin/globulin ratio; AST:aspartate amino transferase; ALT:alanine amino transferase; TAC:total antioxidant capacity; MAD: malondialdehyde;DNAJ: deoxyribonucleic acid jejunum, DNAI: deoxyribonucleic acid ilium
[a,b,c]Means in the same row having different superscripts are significantly different (P<0.05).

Table 4. Effects of dietary vitamin C (VC), vitamin E (VE), and their combined fortification on some blood hematological parameters, some immunological indices, and differential leukocyte in roosters reared heat stress condition (mean±SEM).

p-Value	SEM	Heat stress treatments				SEM	p-Value
		Heat stress	+VC	+VE	+VC+VE		
Hematological parameters							
RBCs ($x10^6$/mm)^3	1.52[a]	1.25[b]	1.58[a]	1.42[a]	1.45[a]	0.070	0.001
Hgb(g/dl0	10.7[a]	8.50[b]	10.6[a]	10.5[a]	10.0[a]	0.427	0.001
PCV (%)	32.7[a]	26.5[b]	32.5[a]	30.7[a]	31.0[a]	1.09	0.001
MCV (µm^3/red blood cell)	215	214	206	218	216	14.14	0.919
MCH(pg/dl)	70.5	68.6	67.6	74.7	69.4	4.36	0.534
MCHC (%)	32.7	32.2	32.8	34.2	32.4	1.48	0.681
Blood pH	7.54[c]	7.76[a]	7.57[bc]	7.59[bc]	7.61[b]	0.021	0.001
Immunological traits							
PA(%)	20.0[a]	15.83[c]	17.50[bc]	19.17[ab]	17.67[bc]	0.837	0.001
PI (%)	1.73[a]	1.23[c]	1.48[b]	1.58[b]	1.42[b]	0.072	0.001
White blood cell counts and differential leukocyte							
WBCs ($x10^3$/mm)	26.3[a]	22.5[b]	24.7[a]	24.8[a]	26.0[a]	0.663	0.001
Lymphocyte (%)	47.8[a]	43.3[c]	45.5[b]	45.8[b]	46.5[b]	0.490	0.001
Monocyte (%)	6.83[ab]	7.50[ab]	6.50[b]	8.00[a]	6.50[b]	0.508	0.024
Basophil (%)	0.500	0.500	0.500	0.833	0.500	0.302	0.745
Eosinophil (%)	9.50	9.33	9.50	9.33	10.17	0.572	0.579
Heterophil(%)	35.3[c]	39.3[a]	38.0[ab]	36.0[bc]	36.3[bc]	0.909	0.001
H/L ratio	0.739[c]	0.909[a]	0.835[b]	0.786[bc]	0.782[bc]	0.025	0.001

SEM: standard error of means; RBC:red blood cells; PCV: packed-cell volume; Hgb :haemoglobin; MCV:mean corpuscular volume; MCH :mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBCs: white blood cell; PA: phagocytic activity; PI: phagocytic index; H/L ratio heterophil /lymphocyte ratio. [a,b,c] Means in the same row having different superscripts are significantly different (P<0.05).

DISCUSSION

HS has negative effects on both animal and human welfare. The use of antioxidants to relieve the negative effects of HS is important in animal and human nutrition (Attia *et al*., 2018; Kutlu*et al*., 2019). In this study, we showed that HS negatively affects fertility and semen quality, while vitamin fortification shows promising relieving effects. High H/L in roosters exposed to HS indicated the low welfare of roosters (McDaniel, 2004; Khan *et al.,* 2012a;b). Similarly, H/L appears to be a more reliable indicator for stress and welfare in poultry (Aberra, 2011), which has adverse effects on animal immunity (Attia *et al*., 2009; Attia *et al*., 2011). The decrease in TAC and the increase in MDA shown herein indicated the stressor effect of HS on the antioxidant status of roosters (Sahin *et al*. 2002). The MDA was higher in the spleen, thymus and bursa of the HS-exposed chicks than in those of the control one (Zhao *et al*., 2014).

Blood metabolites and immune indices were negatively affected by HS, showing a decrease in total protein in seminal and blood plasma, blood plasma albumin (non-specific immune protein), γ-globulin (innate immunity), PA and PI (non-specific immunity), WBCs and lymphocytes (cell-mediated immunity) and DNA of the intestinal segments. In addition, RBCs, Hgb concentration and PCV were adversely affected due to HS, showing low welfare and health of the animals. This could be attributed to the decline in the RBCs and thus oxygen carrying capacity causing less metabolic heat loss. Similarly, HS decreased Hgb and PCV and increased blood pH (Toyomizu *et al.,* 2005; Daghir, 2008). This was concurred with a loss of ionic Ca (Sandercock *et al*., 2001), low availability of nutrients that are essential for DNA synthesis, function and repair (Habashy *et al*., 2017a; Habashy *et al*., 2017b) and increase in water intake (Attia *et al.,* 2009; Attia *et al*., 2011).

There was a decrease in the semen quality and fertility traits of roosters exposed to HS, and this was associated with the reduction in feed intake of this group, as reported by Hood (1999) and McDaniel *et al*. (2004), who showed that HS of >31°C depressed rooster sperm motility, viability and fertilisation potential. The declining fertility of heat-stressed males could be attributed to a decrease in sperm motility and the number of spermatozoa stored in the sperm host gland of laying hens (Brillard, 2003; Khan *et al*., 2012a;). In addition, HS can negatively affect testosterone, causing hypertrophy and weakening of the Leydig cell function (Stone and Seamark, 1984). The damaged DNA of sperm can increase abnormality, which could cause low fertility and subsequently could lead to reduced embryo survival (Sutovsky, 2015; Peña, *et al*., 2016; Zheng *et al*., 2018).

The results indicate that the improved semen quality, along with metabolic and haematological improvements and that of DNA of the intestinal segments of roosters exposed to HS and receiving diets fortified with dietary VC, VE or VC+VE agrees with the improved TAC and MDA in vitamin- fortified groups. This could be attributed to 6.5%, 4.9% and 8.1% increase in the feed intake of VC, VE and VC + VE groups, respectively; hence increasing the intakes of VC and VE. Similarly, chickens receiving 200–400 mg/kg VC (Jacob, 1995) and VC and VC + VE (Attia *et al.,* 2017) increased survival rate and feed intake.

In the literature, VC improved chickens' thermotolerance (Umar *et al*., 2010; Khan, 2011; Attia *et al*., 2018) and this can mimic mortality from HS and can maintain animal productivity (Attia *et al*., 2011;Daghir, 2008). A marginal deficiency of vitamins and minerals may have occurred due to HS exposure, as a result of increasing utilisation and excretion of vitamins and minerals from tissues (Khan *et al*., 2012a;b), and decreasing blood ascorbic acid (Khan, 2011; Attia *et al*., 2016;). In addition, VC is essential for amino acids and mineral metabolism, collagen, 1, 25-dihydroxy vitamin D, biosynthesis of testosterone and adrenaline (Khan *et al*., 2012a;b), secretion of corticosterone, leukocyte production and immune system activation (Daghir, 2008; Attia *et al*., 2016;Attia *et al*., 2017).

Fortification with VE above the recommended dose (5 mg/kg diet; NRC, 1994) to 150 mg/kg diet shows a similar effect to VC on most of investigated traits except that VE has a stronger effect on monocyte (macrophages) and H/L ratio than VC, but a lower influence on the DNA of the jejunum and ilium. VE guards cells and tissues from lipoperoxidative injury caused by ROS and preclude the lipid peroxidation of spermatozoa from oxidative damage (Khan, 2011; Tufarelli and Laudadio, 2016). Interestingly, addition of 100 and 150 mg/ kg diet of VE significantly increased semen quality, compared with roosters on both 15 and 75 mg/kg VE groups (Al-Zahrani *et al*., 2012; Alm El-Dein *et al*., 2013). VE guards lymphocytes, macrophages and plasma cells against oxidative injury, boosting their function and proliferation (Traber, 2007). A dose of 250 mg/kg diet VE was found to be ideal for partly easing the negative effect of HS and improve productivity and immunity of chickens (Çiftçi *et al*., 2005; Mohiti-Asli *et al*., 2010;).

The enhancing impact of VE on fertility reflected the results of sperm motility, liveability and semen quality factor. In addition, fertility found herein was numerically higher (≈1.7%) in the VE and VC+VE groups, than in the VC group alone. In concert with the present results, a 100 mg/kg VE was found to be the best for maintained the fertility in male chickens (Keskes-Ammar *et al*., 2003). Similarly, Hoehler and Marquardt (1996) showed that the in vivo antioxidant influence of

VE might be greater than that of VC, but was adequate for relieving HS in quails (Ipek *et al*., 2007). In male chickens, however VC of 250 mg and VE up to 200 mg/kg can boost semen quality and fertility (Khan *et al*., 2012a).

A synergistic effect of VC + VE was observed in feed intake, blood plasma albumin (non-specific immune-protein), A/G ratio and blood plasma TAC, as VC + VE surpassed the effect of VC or VE alone, which can explain the superior performance of roosters on VC + VE treatment. In addition, VC + VE had lower monocytes than VE alone, but higher blood plasma total protein and intestinal DNA, and lower ALP and jejunum DNA than VC alone. Similarly, VC and VE had a synergistic influence for decreasing the negative impact of HS as VC defends VE from peroxidation (Sahin *et al*., 2002). Obviously, VC or VE were less effective than VC + VE (Attia *et al*., 2017), and VC + VE delayed the oxidation of myoglobin (Yin *et al*., 1993). Furthermore, VC enhanced VE antioxidant power by decreasing the tocopheroxyl radicals back to their active formula of VE, or by sparing the available VE (Jacob, 1995).

The increase in antioxidant intakes resulted in 33.0%, 33.3% and 34.3% increases in seminal plasma TAC, in the HS exposed roosters whose feed had been supplied with VC, VE and VC+VE and 19.5, 20.3 and 22.5% in blood plasma TAC, respectively, as compared with the unsupplemented HS group. The results demonstrated a symbiotic effect between the two vitamins in blood plasma TAC and in the concentration in blood levels which was higher than cellular levels. This may be due to the crucial role of antioxidants in the cell defence system (Attia *et al*., 2017; Kutlu *et al*., 20019). Further evidence was found in the reduction in the MDA in seminal plasma, which amounted to 27.3, 29.5 and 33.8% and in blood plasma, which amounted to 30.1, 31.5 and 32.4% respectively, due to VC, VE and VC+VE. These results are in line with those reported by Khan *et al*. (2012a), Attia *et al*. (2016) and Attia *et al*. (2017). Likewise, VC was found to boost the semen quality of heat-stressed roosters as reported by Nowaczewski and Knotecka (2005) and Ezzat *et al.* (2011). However, the beneficial of VC on semen quality remains contradictory (McDaniel *et al*., 2004), maybe due to the oxidative degradation of VC (Mckee and Harrison, 1995); thus Khan *et al*. (2012a;b) concluded that the effect of VC on semen quality is dose-specific.

Conclusions: Either VC of 200 mg/kg or VE of 150 mg/kg can significantly improve semen quality, fertility, or physiological of roosters reared under HS, with the VE group yielding greater immunity. In addition, VC + VE surpassed the influence of VC or VE alone on feed intake, blood plasma albumin, albumin/globulin ratio and blood plasma TAC, showing a synergetic effect of VC + VE. Moreover, VC + VE decreased monocytes compared with VE alone, but increased blood plasma total protein and DNA in jejunum and ilium and lowered blood alkaline phosphatase, compared with VC.

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