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Original Research Article

INFLUENCE OF DIETARY VITAMIN C SUPPLEMENTATION ON GROWTH PERFORMANCE, BLOOD BIOCHEMICAL PARAMETERS AND TRANSCRIPT LEVELS OF HEAT SHOCK PROTEINS IN HIGH STOCKING DENSITY REARED BROILER CHICKENS

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Abstract: This study was conducted to evaluate the effects of dietary supplementation of different levels of vitamin C (VC) in broiler chicken reared under high stocking density on the growth performance, blood biochemical parameters and the expression of heat shock protein genes. A total of 150, one day old mixed sex broiler chicks (*Cobb 500*) were randomly distributed in five equal groups. Group1 was reared in normal stocking density (10.6 birds/m^2) and fed on the basal diet (BD) without VC (control). While, chicks in other groups (2, 3, 4 and 5) reared in high stocking density (15.6 birds/m^2) and fed on BD supplemented with VC at 0, 200, 400 and 600 mg / kg diet, respectively. Birds reared in high stocking density showed a reduction of the final body weight and total feed intake, with high mortality (6.6%). Moreover, they revealed a significant up-regulation of *HSP70* mRNA and elevated *HSP90* and *HSF1* mRNA expression in heart and liver tissue. Graded dietary levels of VC provided variable protection against the hazard of high density through improved final body weight and total feed intake, decreased the mortality % and downregulated liver *HSP70* expression level. However, the best performance was observed in birds supplemented with 200 mg/kg VC (group 3).

Key words: chicks' growth performance; gene expression; stocking density; vitamin C

Introduction

Broiler production facing many forms of stressor including high ambient temperature, nutritional stress (imbalance or deficiency in the nutrient requirements) and vaccination programs stress as well as diseases. It is known that birds will perform better when grown in more space, hence, high stocking density (SD) is considered one of these stressful factors which have undesirable effects on broiler performance, livability, health and immune system response, as it reduces bird access to feed and water (1). It also leads to raising the environmental temperature dangerously where more metabolic heat will be added to the house air than was planned (2).

Different feed additives have a role in reducing stress in broiler feed are included in several studies (3-5). The ameliorative effects of ascorbic acid or vitamin C (VC) in many forms of

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stressors have been documented (6, 7). The heat shock proteins (*HSPs*) family is highly homologous chaperone proteins contributing to cellular protection, protein homeostasis and cell survival against a variety of environmental and metabolic stresses. *HSP70* is one of the most conserved and important protein which plays a deep role in enhancing tolerance to various stressors in broiler chickens (8,9). Different studies indicated that *HSP70* has an importance not only at high ambient temperatures but also in cell death mediated by free radicals and reactive oxygen species (10). Furthermore, in both physiological and stress conditions *HSP90* is a molecular chaperone involved in maturation and stabilization of a wide range of proteins to maintains cellular homeostasis and function (11). We hypothesized that VC could modulate the negative impacts of high stocking density as a stress factor in broilers farms

Therefore, this study aimed to investigate the effect of dietary VC supplementation with different concentrations on growth performance, some blood biochemical parameters, and heat shock protein genes expression in broiler chicks reared under high stocking density.

Material and methods

This experiment was approved by the local ethical committee of animal use from Faculty of Veterinary Medicine, Alexandria University.

Birds, experimental design and feeding Program

One hundred and fifty, one-day-old mixed sex *Cobb 500* broiler chicks were used in this study. The chicks were weighed at one day old and then randomly distributed into five groups (30 chicks/ group), each treatment has three replicates (10 chicks/replicate) in 15 compartments, every compartment was provided by a suitable feeder and waterer. All groups received their experimental diet for 6 weeks. The bird's compartments were bedded with fresh, clean chopped wheat straw forming a deep litter of 4 cm depth. The room temperature was adjusted on 33^{o}C in the first week of age after that decreased 3^{o}C/week until reaching 21^{o}C at the fifth week of age and the relative humidity was kept at 70 %. The chicks were vaccinated according to a normal regime (vaccination against Newcastle disease on days 7, 18 and 28 and infectious bursal disease (Gumboro) on day 12 of chicks age).

The broiler chicks were fed on the basal diet (BD) prepared from a corn-soybean meal based diet and were formulated to meet the nutrient requirements of poultry (12). Starter diet was given from 0-2 weeks of age, followed by grower diet (3-4 weeks) and finally finisher diet from 5-6 weeks of age. Chicks of group 1 (G1), were fed on BD without VC supplementation and reared in normal stocking density (10.6 birds/m^2), while the chicks of groups G2, G3, G4 and G5 distributed to be in high stocking density (15.6 birds/m^2) (13) and fed on the BD supplemented with VC (Introvit-C WS, Interchemie Co., Netherland) at levels of 0, 200, 400 and 600 mg/kg diet, respectively. The composition of experimental diet and its calculated analysis were presented in table (1). Data for final body weight (BWT) and feed intake (FI) were recorded. Feed conversion ratio (FCR) and body weight gain (BWG) were calculated.

Sample collection

At the end of the experiment, three blood samples from each replicate (n=9) were collected for analysis of some biochemical parameters, separation of the serum was done using centrifuge adjusted at 3000 rpm for 10 minutes then stored in -20°C. The serum was used for measuring serum total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, glucose, total protein, albumin, globulin, serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic amino-transferase (SGOT) using commercial kits (Biodiagnostic company, Egypt). The analysis was done by using a spectrophotometer according to the manufacture instructions. The liver and heart tissues from the same slaughter birds were collected and homogenized then snap frozen in liquid nitrogen immediately and stored at -80°C.

Table 1: Composition of experimental starter, grower and finisher diets (gm/kg diet) and calculated chemical analysis of the basal diet

Ingredients	Diet		
	Starter	Grower	Finisher
Yellow corn	542	558.8	606
Soybean meal (44%)	319	281	253.3
Corn gluten meal (60%)	71	81	48.1
Vegetable oil	29.8	41	54.4
Limestone [1]	15	15	15
Monocalcium phosphate	14	14	14
Common salt	3	3	3
Mineral Premix[2]	1.5	1.5	1.5
Vitamin Premix[2]	1.5	1.5	1.5
Methionine [3]	1	1	1
Lysine [4]	1	1	1
Anti Coccidial[5]	0.2	0.2	0.2
Antimold[6]	1	1	1
Calculated Analysis			
Crude protein (CP) %	23.1	22.18	19.39
Metaboliziable Energy (ME) Kcal / kg diet [7]	3053	3160.7	3252.6
Calorie / protein ratio [8]	132.16	142.5	167.7

[1]Limestone (contain 36% calcium). Monocalcium phosphate: contain 22 % Phosphorus and 16 % Calcium. [2]Mineral and Vitamin premix produced by Heropharm and composed (per 3 kg) of vitamin A 12000000 IU, vitamin D32500000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B1210 mg, niacin 30000mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10000 mg, manganese 60000 mg, zinc 50000 mg, iron30000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg and cobalt 100 mg. [3] DL-Methionine (Produced by Evonic Co and contain 99 % methionine), [4] Lysine = lysine hydrochloride (contain 98 % Lysine). [5] Kill cox, Produced by Arabian company for pharmaceutical industries, 6 Produced by EL TOBA CO. For Premixes & Feed El-Sadat city Egypt. [7] ME calculated according to NRC (1994), [8] Calorie /protein ratio = ME Kcal /CP%

Table 2: Primers used for quantitative real-time PCR

Gene and ID	Primer Sequence (5`-3`)	References
HSP70 (EU747335)	F: CCAAGAACCAAGTGGCAATGAA R: CATACTTGCGGCCGATGAGA	(15)
HSP90 (NM_206959)	F: GAGTTTGACTGACCCGAGCA R: TCCCTATGCCGGTATCCACA	(15)
HSF1 (L06098.1)	F:CAGGGAAGCAGTTGGTTCACTACACG R: CCTTGGGTTTGGGTTGCTCAGTC	(15)
GAPDH (NM_204305)	F: GGGCACGCCATCACTATCTTC R: ACCTGCATCTGCCCATTTGA	(16)

Table 3: Effect of different dietary VC supplementation on growth performance of broiler chickens

Parameters	G1	G2	G3	G4	G5
W0 (Initial wt.)	45.64±0.48	45.64±0.54	45.63±0.52	45.61±0.56	45.69±0.46
W6 (Final wt.)	2549.82±72.73	2470.45±80.91	2643.50±73.00	2575.18±64.14	2540.00±55.58
TBG (g)	2504.18±72.27	2424.82±80.39	2597.87±72.50	2529.57±63.60	2494.31±55.14
TBG, RTG2	103.27	100	107.14	104.32	102.87
TFI (g)	4747.17	4494.32	4590.43	4604.81	4507.83
TFI, RTG2	105.63	100	102.14	102.46	100.30
FCR	1.90	1.85	1.77	1.82	1.81
FCR, RTG	102.70	100	95.68	98.38	97.84
Mortality %	3.3	6.6	3.3	0	0

TBG = Total body gain, TFI= Total Feed intake, FCR=Feed conversion ratio (Feed intake/ body gain), RTG2= Relative to group 2

Table 4: Effect dietary VC supplementation on some blood biochemical parameters of broiler chickens

Parameters	G1	G2	G3	G4	G5
Total protein (g/dL)	6.12±0.02	6.14±0.14	6.04±0.10	6.14±0.08	6.12±0.14
Albumin (g/dL)	5.23±0.08	5.27±0.02	5.23±0.05	5.24±0.02	5.27±0.02
Globulin (g/dL)	0.89±0.10	0.87±0.13	0.81±0.13	0.90±0.10	0.85±0.13
Cholesterol (mg/dL)	200.6±5.01	198.27±2.33	189.37±6.08	191.00±1.60	190.83±5.40
Triglyceride (mg/Dl)	200.43±2.42^{a}	200.70±0.64^{a}	199.73±0.65^{a}	201.07±1.85^{a}	184.53±8.48^{b}
HDL (mg/dL)	54.50±0.99^{a}	55.93±0.18^{a}	51.80±1.10^{ab}	52.87±0.94^{b}	50.80±1.38^{b}
LDL (mg/dL)	106.01±6.44	107.19±2.30	97.62±5.67	106.92±2.24	103.13±5.76
Glucose (mg/dL)	204.07±0.94^{b}	212.97±1.09^{a}	209.70±1.66^{ab}	206.93±3.90^{ab}	206.53±2.07^{ab}
SGOT (U/100 mL)	39.67±4.67a^{b}	43.67±1.76^{a}	39.33±4.67^{ab}	39.67±4.63^{ab}	32.33±1.33^{b}
SGPT(U/100 mL)	88.33±2.03	90.33±±3.18	88.671.20	88.33±2.33	88.33±2.96

Means with different letters in the same row differ significantly (P<0.05).
HDL= High density lipoprotein, LDL= Low density lipoprotein, SGOT= Serum glutamic pyruvic transaminase and SGPT = Serum glutamic oxaloacetic amino-transferase

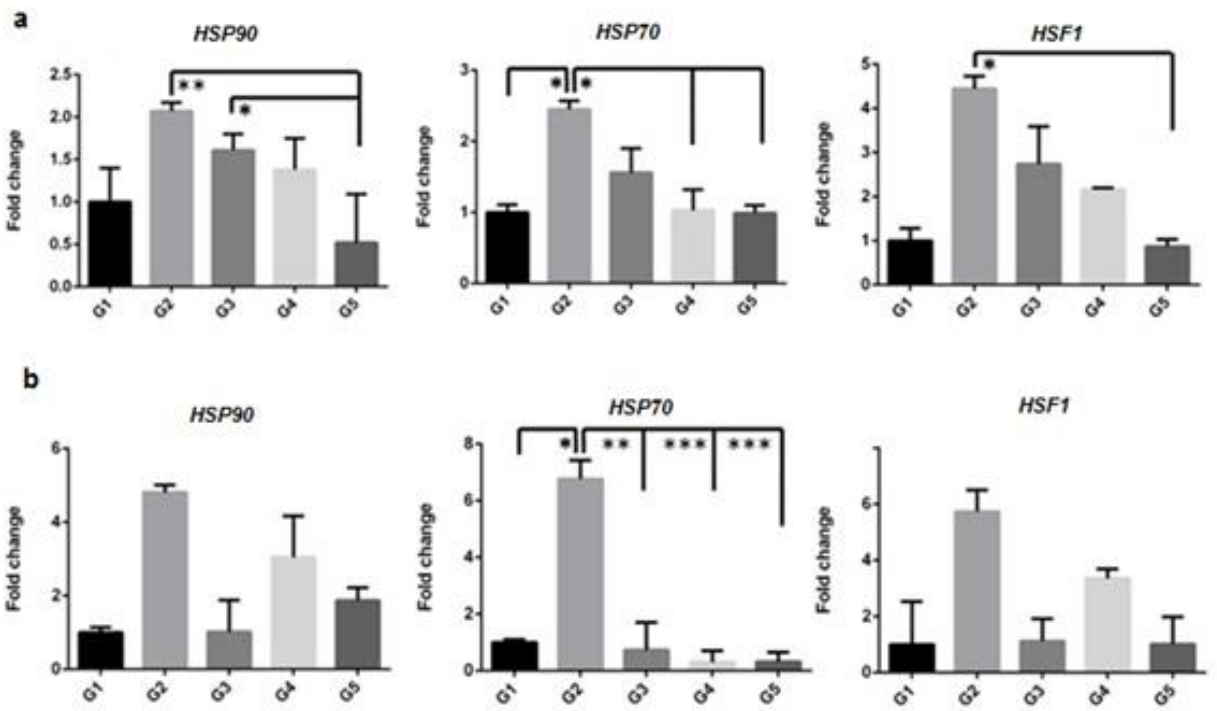

Figure 1: The mRNA expression level of heat shock proteins (*HSP90, HSP70 and HSF1*) genes in heart tissue (a) and liver (b). Asterisks on the data bars indicated when P<0.05 (*), P<0.005 (**), and P<0.0005 (***). G1 (chicks were reared in normal stocking density and fed on the basal diet), G2 (chicks were reared in high stocking density and fed on the basal diet), G3, G4, and G5 (chicks were reared in high stocking density and fed on the basal diet with VC supplementation (200, 400, 600 mg/kg diet) respectively

Total RNA isolation and cDNA synthesis

Total RNA of liver and heart tissues was isolated using the Biozol (Bioflux, Japan) according to the manufacturer instructions. The cDNA was synthesis from isolated RNA using the SensiFAST™cDNA Synthesis Kit (Bioline, United Kingdom) according to the manufacturer instructions. Briefly, 4 µl of total RNA mixed with 4 µl 5X buffer, 1 µl reverse transcriptase and 11 µl RNase\ DNase free H2O was added. The reaction was incubated at 25°C for 10 min, 42°C for 15 min (reverse transcription) and 4°C hold. The obtained synthesis cDNA was checked by glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* gene and stored at -20°C until further use.

Quantitative real-time PCR and data analysis

Quantitative Real-Time PCR (qRT-PCR) was performed for detecting the expression levels of *HSP70, HSP90* and *HSF1* genes in heart and liver tissues using specific primers for each gene (Table 2) and SensiFAST™Syber green master mix with low Rox (Bioline, United Kingdom) in the Mx3000P®System (Stratagene, USA). The amplification reaction was 20 µl consisting of 2 µl cDNA, 0.8 µl primers (50 nm), 10 µl Syber green master mix and up to 20 µl RNase\ DNase free H2O. The PCR thermal program started as 95ºC for 10 min followed by 40 cycles of 95ºC for 15 sec then 60ºC for 60 sec. The dissociation curve was carried at the end of the last cycle. The housekeeping gene (*GAPDH*) used to normalize threshold cycle value (Ct). The relative expression values were determined using comparative threshold cycle method $2^{-\Delta\Delta CT}$(14) and the results were reported as fold change differences relative to the control group (G1).

Statistical analysis

The obtained data were analyzed using the statistical analysis system (17). The significance designated at P<0.05 for the differences

among the different experimental groups for all studied parameters. The significance designated as ($P<0.05$) using one-way ANOVA between all genes in studied groups.

Results

Final BWT was improved in all groups which reared under high SD and fed diet supplemented with different levels of VC compared to G2 (Table 3). The G3 which received VC (200 mg/kg diet) showed the highest final BWT compared to G2. The G5, which received the highest level of VC (600 mg/kg) showed lower final BWT. The total body gain (TBG) of birds was improved in all studied groups supplemented with VC when compared with G2 with the highest TBG of chicks was found in G3 which subjected to high SD and supplemented with VC at 200 mg/kg diet. The lowest TBG was found in G2 that was in high SD without VC supplementation. Additionally, High SD in G2 decreased total feed intake (TFI) compared to G1. While the addition of VC in G3, G4, and G5 increased the TFI when compared with G2. The overall chick mortality percentage during the experiment was the highest in G2 (6.6 %), but it was decreased in G3 **(**3.3 %) with 200 mg/kg diet VC supplementation. Also, no mortality was observed with increased VC supplementation in G4 and G5 (400 and 600 mg/kg diet) (Table 3).

The addition of VC in G3, G4 and G5 alleviated the stress effect induced by high SD through decreasing the level of serum glucose, cholesterol, SGOT and SGPT compared with those reared in high SD without VC supplementation (Table 4).

In the present study, rearing broiler chicken in high SD leads to an alteration in the expression of *HSPs* (*HSP70* and *HSP90*), and *HSF1* in heart and liver tissue (Figure 1a and b). The expression level of *HSP70* mRNA in both heart and liver tissue revealed a significant up-regulation ($P<0.05$) in the G2 (2.35 ± 0.12 and 6.76 ± 0.66 fold) relative to the control. While *HSP70* mRNA expression in both tissues markedly lowered in chicken reared in high SD and supplemented with different concentration of VC than G2. The G2 showed an increase in expression level of *HSP90* and *HSF1* (2.07 ± 0.10 and 4.45 ± 0.28) in the heart tissue than G3, G4, and G5 supplemented with VC (200, 400 and 600 mg/kg ration, respectively). The *HSP90* expression level also showed a significant increase ($P<0.05$) in G3 than G5 (Fig. 1a). In the liver tissue, *HSP90*, and *HSF1* showed nearly similar expression pattern where they were higher in all groups especially G2 relative to the control (Fig. 1b).

Discussion

Managemental factors like SD had a significant effect on bird growth performance, health and welfare. In the present study, high SD in G2 decreased the final BWT compared with birds reared in normal SD. Similarly, Dozier et al. (18) showed that increasing the density produced some negative effects on the live performance of broilers. Addition of VC (200 mg/kg diet) to birds reared under high SD ameliorated these negative impacts on growth while failed with the higher levels of VC (600 mg / kg diet). Likewise, Elagib-Hind and Omer (19) reported that BWT was improved by the low and moderate levels (150 and 350 mg/kg) of VC compared with the higher level. Also, SabahElkheir et al. (20) observed that VC supplementation at higher doses (500 mg and 750 mg/kg) resulted in lower final weight and weight gain.

Previous studies reported that VC supplementation increased TBG (21, 22). On the other hand, others found that broiler feed intake was not affected by the VC supplementation (23, 24). The FCR was improved in all groups reared in high SD with VC supplementation (G3, G4, and G5) compared to G2, and G1. McKee and Harrison (25) also, observed an improvement in FCR of broilers as result of VC supplementation during heat stress. Decreased mortalities with VC supplementation may confirm the relationship between adding VC and its protective effect as reported by other studies (26, 27) which noticed that VC under stress and disease conditions protects the birds by improving the immune response.

Blood serum biochemical parameters are important diagnostic indicators especially under

stress conditions such as high SD. In the present study, high SD altered the activity of liver function enzymes (SGPT and SGOT), glucose, and, HDL serum concentrations (increased). The increased serum SGPT and SGOT activity in birds reared under high SD indicate that high SD might cause oxidative lesions which are in accordance with the findings of Simsek et al. (28). Dietary inclusion of VC improved these negative impacts of high SD by lowering the levels of the above-mentioned serum parameters. Similarly, Al-Darajji et al. (29) showed that plasma glucose and cholesterol concentration and plasma SGOT and SGPT activities were significantly lowered in both male and female broiler breeder reared under hot climate and supplementation with ascorbic acid. Moreover, Kucuk et al. (30) reported that supplementation with VC decreased MDA, glucose, cholesterol, and triglyceride concentrations in laying hen reared under cold stress.

The significant effect of high stocking density on *HSP70* and α1-acid glycoprotein (AGP) indicated that it was physiologically stressful condition to broiler chickens (31). Furthermore, Beloor et al. (32) reported that the expression of *HSP70* mRNA could be proper biomarkers to evaluate the stress induced by increased SD. As, *HSPs* help the stressed cells to manage the stressors, especially those affecting the protein machinery (33). Similarly, the previous study in other species (rainbow trout and sea bass) showed that *HSP70* expression level elevated in high SD (34, 35). Furthermore, Higher levels of *HSP70* gene in different tissues of birds after exposure to environmental stressors is important in the acquisition of stress tolerance (36, 37).

As other study demonstrated that supplementation of VC plays an important role in the prevention of the heat stress in poultry and improvement of their performance (38). Moreover, there is a significant ($P<0.05$) difference in *HSP70* mRNA expression in heart tissue between G2 versus G4 and G5 which fed on basal diet with 400 and 600 mg/kg ration VC supplementation. Also, a highly significant difference ($P<0.0005$) between G2 versus G4 and G5 and ($P<0.005$) between G2 versus G3 in the liver tissue were observed (Fig.1b). Similarly, Mahmoud et al. (39) demonstrated that chickens fed a diet supplemented with VC and exposed to cyclic high temperatures showed a significant decreased the expression level of *HSP70* compared with control chickens. Furthermore, Jang et al. (40) reported that the mRNA expression of *HSP70* in the liver of birds fed a diet containing VC significantly decreased compared with those birds fed basal diet under summer diurnal heat stress. In quail, *HSP70* expression in ovary and brain was decreased as the dietary VC or vitamin E supplementation increased in stressed groups (41). Hence, we suggest that VC supplementation act as an antioxidant and helps to prevent the growth of free radicals, which damage cells and subsequently reduced expression of *HSPs* in chicken reared under high SD.

As the *HSF1* is a master regulator of the heat shock genes, through activating the *HSPs* transcription by binding to heat shock element in the upstream promoter region of *HSP* genes (42). It has the ability to mediate up-regulation of *HSP70* and *HSP90* which act a critical role in survivability providing to the organisms subjected to stress (43, 44). In both heart and liver tissue in the present study, the *HSP1* expression showed more increased in G2 whish reared under high SD than G3, G4 and G5 relative to control. Its expression showed the same pattern of *HSP70* and *HSP90* expression in heart tissue and *HSP90* expression in liver tissue. Moreover, Beloor et al. (32) demonstrated that the expression levels of *HSP90* in the liver samples were higher in high density group compared with the low and standard groups but, didn't showed significant differences. The current study also showed that stress-induced due to high stocking density increases the expression of *HSP70* and *HSP90*, which play essential protective roles in maintaining the metabolic and structural integrity of the cells and organs against stress-induced injury (45-47).

Conclusions

Increasing the stocking density from 11.6 to 15.6 birds/m^2 caused stress in broiler chicks

which tended to reduce their performance, increase mortality rate, affect liver function enzymes (SGPT and SGOT) and change the expression level of heat shock protein genes. Supplementation of VC especially at 200 mg/kg chick's diet may offer a suitable nutritional strategy to overcome the disadvantageous effects of increased stocking density.

Conflicts of Interest

The authors declare no conflicts of interest

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