

PAPER

The effect of grape seed extract and vitamin C feed supplementation on some blood parameters and *HSP70* gene expression of broiler chickens suffering from chronic heat stress

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Abstract

In this experiment, the effect of hydroalcoholic grape seed extract (GSE) and vitamin C feed supplementation on some blood parameters and heat shock protein 70 (*HSP70* gene) expression of broiler chickens suffering from chronic heat stress was investigated. Experimental diets included control diet (with no additive), 3 levels of GSE (150, 300, 450 mg/kg), and one level of vitamin C (300 mg/kg). Each diet was fed to 5 replicates of 12 male chicks each, from d 1 to 42. The birds suffered from chronic daily heat stress under $34 \pm 1^\circ\text{C}$ temperature with 65 to 70% relative humidity for 5 h from 29 to 42 d of age. Results showed that 300 mg/kg GSE supplementation increased body weight of broilers both before and after heat stress condition (at 28 and 42 d, respectively). Also, birds fed 300 mg GSE/kg diet had higher European production efficiency factor during the whole period of the experiment. Supplementation of GSE decreased the concentration of serum glucose at 28 and 42 d; at 42 d (during heat stress condition) and at 450 mg/kg diet it decreased cholesterol, triglyceride, low- and very low density lipoprotein concentration of serum blood. Vitamin C supplementation decreased serum cholesterol concentration of broilers suffering from heat stress. *HSP70* gene expression in heart and liver of broilers reduced by GSE and vitamin C supplementation pre- and during chronic heat stress condition.

Introduction

Heat stress is one of the most challenging

environmental conditions especially on summer days in many areas of the world (Attia *et al.*, 2006, 2011). Regarding the finishing period, the suitable ambient temperature for poultry is between 16 and 25°C (Sahin *et al.*, 2001). It has been well documented that exposing broiler chickens to continuously high temperature during the finisher period caused chronic heat stress (Sahin *et al.*, 2003; Ahmad *et al.*, 2008). Previous studies have shown that heat stress suppressed performance and immunity system in broiler chickens (Quinteiro-Filho *et al.*, 2010). Broilers show disturbance in the acid-base balance and panting can lead to a respiratory alkalosis under heat stress condition (Syafwan *et al.*, 2011). Heat stress also changes blood metabolites and hormones (Attia *et al.*, 2009a, 2009b). It is reported that endocrinological changes caused by chronic heat stress in broilers stimulate lipid accumulation through increased *de novo* lipogenesis, reduced lipolysis, and enhanced amino acid catabolism (Ayasan *et al.*, 2009; Lara and Rostagno, 2013). Heat shock proteins (HSPs), called stress proteins, are a group of proteins that are present in all cells in all life forms but are expressed at high levels when cells are exposed to high or low temperature or other stressors (Figueiredo *et al.*, 2006). They are also important for both intra- and extracellular immune functions, and play a role in protein folding and unfolding, assembling and disassembling, and translocation (Sahin *et al.*, 2009). Ascorbic acid synthesis decreases at elevated environmental temperature and makes it an essential dietary supplement during summer days (Attia *et al.*, 2009a, 2011; Sejian *et al.*, 2012). Today, ascorbic acid in organic and herbal form is being preferred by poultry producers because of public concerns. Thus, to overcome the detrimental effects of heat stress using new plant derived additives as a natural antioxidant in poultry diets is being researched. Grape (*Vitis Vinifera*) seeds are considered as a good source of polyphenolic compounds which have been shown to have various beneficial pharmacological effects, including anti-hyper lipidemic (Moreno *et al.*, 2003), anti-inflammatory (Terra *et al.*, 2009), and anti-bacterial activities (Mayer *et al.*, 2008). Shi *et al.* (2003) reported that the antioxidant potential of grape seed is twenty and fifty fold greater than vitamins E and C, respectively, arising from increased levels of polyphenols proanthocyanidins and oligomers of flavan-3-ol units, especially catechin and epicatechin. Thus, the object of the present study was to evaluate the effects of grape seed extract (GSE) and vitamin C feed supplementation on some blood parameters and *HSP70*

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Key words: Blood parameters, Chronic heat stress, Grape seed extract, *HSP70* gene, Heat shock protein.

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gene expression of broiler chickens suffering from chronic heat stress.

Materials and methods

Animals, diets, and management

This experiment was carried out using a total of 300 Cobb-500 male broiler chicks. One-day-old chicks (with initial weights of 36.28 ± 0.38 kg) were obtained from a local hatchery and divided into 25 groups of 12 birds each. All procedures for the use and care of animals were conducted after approval by the Ferdowsi University of Mashhad, Iran. There were 5 experimental diets including control, 150, 300, 450 mg GSE/kg diet, and 300 mg vitamin C/kg diet. The feeding programme consisted of a starter (1 to 10 d), grower (11 to 22 d), and finisher diet (23 to 42 d). The basal diet was fed in mash form and prepared with the same batch of ingredients for starter, grower, and finisher periods and was formulated to meet the nutrient requirements according to Cobb-500 rearing guidelines (Cobb-Vantress, 2012). All birds had free access to feed and water during the whole rearing period. The ingredients and chemical composition of the basal diets are shown in Table 1. Each desired level of GSE and also ascorbic acid was added to 100 mL water, well mixed and sprayed on the

basal diet. Feed was prepared weekly and stored in airtight containers. Temperature was initially set at 34°C on day 1 and decreased linearly by 0.5°C per day up to 28 d. A chronic heat stress under 34±1°C temperature with 65 to 70% relative humidity for 5 h was imposed on birds from 29 up to 42 days of age. During the study, the birds received a lighting regimen of 23L:1D from d 1 to 42.

Grape seed analysis

Black grape (*Vitis Vinifera*) samples were collected in September 2012 from Sari, Mazandaran, Iran. After collection, berries were snipped from the cluster. The seeds from berries were manually separated from pulp, washed with tap water and air dried. The composition of grape seeds was measured following the AOAC procedures (AOAC, 1990).

Grape seed extract preparation and analysis

Grape seeds were grounded, and extracted with acetone:methanol:water (60:30:10 v/v/v) for 12 h with shaker incubator. Solvents were removed by rotary evaporator. Then, the extract was dried in vacuum oven and kept in freezer under -20°C (Salari et al., 2009).

The chromatographic analysis was carried out on a Knauer (Berlin, Germany) high-performance liquid chromatography (HPLC) system equipped with a Triathlon auto sampler, a K-1001 pump and a UV-vis detector (K-2600). A reversed-phase C18 Nucleosil 100 (12.5 cm×5.0 mm×5.0 µm) column was used for the separation of sample components. Analysis of catechin, epicatechin, procyanidin B1, B2, and C1 was performed according to the method of Iacopini et al. (2008) with some modifications. Standards of catechin, epicatechin, procyanidin B1, B2, C1 were purchased from Sigma Aldrich (St. Louis, MO, USA). Before injection, each sample was centrifuged in an eppendorf tube (4 min at 5000 rpm) and the centrifuged supernatant was allowed to pass through a 0.45 µm pore size membrane filter. Injection volume was 20 µL and the flow rate was 0.8 mL/min. The HPLC grade solvents used were formic acid/water (5:95 v/v) as solvent A, and acetonitrile/formic acid/water (80:5:15 v/v) as solvent B. The elution gradient was linear as follows: from 0 to 10 min, 0.0% B, from 10 to 28 min, 10.0% B, from 28 to 35 min, 25% B, from 35 to 40 min, 50% B, from 40 to 45 min, 80% B, and for last 10 min again 0% B. The different polyphenolic compounds were identified by comparing their retention times and spectral characteristics with data of original reference standard compounds. All analyses were done in duplicate.

European production efficiency factor

At the end of the experiment, European production efficiency factor (EPEF) of broiler chickens was calculated with the following formula (Marcu et al., 2013).

$$\text{EPEF} = \frac{\text{viability (\%)} \times \text{BW (kg)} \times 100}{\text{age (d)} \times \text{FCR (kg feed/kg gain)}}$$

Blood biochemical

At 28 and 42 d, two birds per each replicate were selected and their blood samples were collected using sterile syringes (2 mL) to draw blood from the wing vein. Blood samples were centrifuged at 3000xg for 10 min. Serum of the samples stored at -20°C until metabolite analyses were carried out. Plasma glucose concentration was determined as mg/dL using commercial laboratory kits (parsazmoon) with god-pap method at 546 nm wavelengths (Datar et al., 2006). Triglyceride, cholesterol, low- (LDL) and high-density lipoprotein (HDL) cholesterol were measured using commercial laboratory kits (Friedewald et al., 1972; Gordon and Amer, 1977).

HSP70 gene expression

At 42 d, heart and liver samples of birds washed with normal saline, put into the liquid nitrogen tank and transferred to -80°C freezer. Relative real-time polymerase chain reaction (RT-PCR) was performed to assess HSP70 gene expression in the heart and liver of broiler chickens. Total RNAs were extracted from the homogenised tissues using high pure RNA isolation kit (Roche, Basel, Switzerland). RNA con-

centration was assayed by spectrophotometer nano-drop (MD-1000) in wavelength of 260/280 nm. Synthesis of cDNA was done by gene PAK RT universal kit (Fermentas, Hanover, MD, USA), with reverse specific primer and hexanucleotide random primer. Genotype and sequence of the primers of GAPDH and HSP70 was collected from the National Center for Biotechnology Information (Bethesda, MD, USA). Then, specific primers were designed by primer-5 software and examined by BLAST for checking the specificity of primers. Synthesis of the primers was done by Sigma company. The primers for HSP70 (GU980869.1) and GAPDH (NM_204305.1) were as follows, respectively:

Forward 5' ATCAAGCGTAACACCACCATTCC3',
Reverse 5' GTTGTCCTTTGTCATAGCCCTCTC3',
Forward 5' CTGCCAGAACATCATCCCA 3',
Reverse 5' GCAGGTCAGGTCAACAACAGAGAC 3'

Qualitative PCR showed that primers designed well and there was no non-specific band or primer dimer (Figures 1 and 2). Optimisation of annealing temperature was examined with melting curve by applied biosystems-7300 RT-PCR system. The highest ΔRn and the lowest Ct considered to determine the optimum annealing temperature, which was 62°C for both genes. The optimum level of primers was 0.15 µL. Real time PCR was executed in triplicate. Reaction conditions were 45 cycles of a three phase PCR (denaturation at 95°C for 15 s; annealing at 62°C for 30 s; extension at 72°C for 30 s) after an initial denaturation step (95°C for

Table 1. Ingredients and nutrient composition of basal experimental diets.

	Starter diet (1-10 d)	Grower diet (11-22 d)	Finisher diet (23-42 d)
Ingredient, %			
Corn, ground	56.2	59.9	63.34
Soybean meal	37.11	32.55	28.71
Soybean oil	2.26	3.3	3.94
Dicalcium phosphate	1.92	1.86	1.74
Oyster shell	1.16	1.12	1.06
Common salt	0.3	0.3	0.3
Minerals mix ^o	0.25	0.25	0.25
Vitamins mix [†]	0.25	0.25	0.25
DL-Methionine	0.31	0.26	0.23
L-Lysine hydrochloride	0.24	0.21	0.18
Nutrient composition			
ME, kcal/kg	3000	3105	3180
CP, %	21.23	19.46	18
Ca, %	1	0.96	0.9
AP, %	0.50	0.48	0.45
Lysine, %	1.32	1.19	1.06
Methionine+cystine, %	0.98	0.89	0.82

ME, metabolisable energy; CP, crude protein; AP, available phosphorus. ^oMineral mix supplied the followings per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg. [†]Vitamins mix supplied the followings per kg of diet: vitamin A, 18,000 U; vitamin D₃, 4000 U; vitamin E, 36 mg; vitamin K₃, 4 mg; vitamin B₁₂, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg.

10 min). In real-time assay, a melt curve analysis, performed at the end of the PCR cycles, will confirm specificity of primer annealing. The thermal profile for melting curve is 95°C for 15 s, 60°C for 1 min; 95°C for 15 s and 60°C for 15 s. The efficiency calibrated model is a more generalised $\Delta\Delta Ct$ model. In this model, Ct is the sign of the first cycle that amplification curve begins to rise. The model considers both Ct of target gene and also Ct of reference gene or housekeeping gene. ΔCt for each target gene is then calculated by subtracting the Ct number of target gene from that of housekeeping gene for each sample. $\Delta\Delta Ct$ for each gene was calculated by subtracting the ΔCt of target sample from that of control sample (Pfaffle *et al.*, 2004; Bustin *et al.*, 2009).

Statistical analysis

Birds' livability data were subjected to arcsine transformation prior to analysis. Data of production index and haematological parameters were analysed by analysis of variance using GLM procedures (SAS, 2001). Means were compared using Duncan's new multiple range test (Duncan, 1955). The level of significance was reported at $P < 0.05$. Data of *HSP70* expression were analysed by Excel software.

Results

Grape seed composition and extract analysis

The chemical composition (dry matter, crude fat, crude protein, nitrogen free extract, crude fibre, calcium, total phosphorus and ash) of the grape seed and the content of catechin, epicatechin and procyanidins of GSE are shown in Table 2.

European production efficiency factor

Results showed that there was no significant difference among birds livability percentages ($P > 0.05$). Grape seed supplementation at the levels of 150 or 300 mg/kg diet increased body weight and EPEF of broiler chickens compared with control group pre-heat stress condition. Also, birds fed with GSE at the level of 300 mg/kg diet had higher body weight at the end of the experiment (42 d). Supplementation of 300 mg GSE/kg diet improved EPEF of broilers after chronic heat

Table 2. Grape seed composition and grape seed extract analyses.

Grape seed composition		GSE, mg/100 g DM	
Item	Value	Item	Value
DM, %	90.93	Catechin	142
Gross energy, kcal/kg	3292	Epicatechin	108
Crude fat, %	24.83	Procyanidine B1	83
Crude protein, %	10.17	Procyanidine B2	77
Nitrogen-free extract, %	17.86	Procyanidine C	53
Crude fibre, %	35.39		
Calcium, %	0.56		
Phosphorus, %	0.31		
Ash, %	2.68		

GSE, grape seed extract; DM, dry matter.

Table 3. Effects of grape seed extract and vitamin C on European production efficiency factor and haematological parameters of broilers at d 28 (pre-heat stress condition) and 42 (under heat stress condition).

Parameter	Control	GSE, mg/kg			Vitamin C	SEM	Pr>F
		450	300	150			
Livability (d 1-42), %	91.66	96.66	95.00	96.66	95.00	0.679	0.3221
BW, kg							
d 28	1.285 ^c	1.328 ^c	1.404 ^a	1.396 ^{ab}	1.336 ^{bc}	0.0431	0.003
d 42	2.323 ^b	2.418 ^b	2.731 ^a	2.479 ^b	2.485 ^b	0.0747	0.0028
EPEF							
d 1-28	283.02 ^b	307.84 ^{ab}	325.01 ^a	327.95 ^a	298.70 ^{ab}	0.9184	0.0165
d 29-42	396.29 ^b	470.21 ^b	582.73 ^a	438.50 ^b	456.28 ^b	1.526	0.0010
d 1-42	257.33 ^c	302.13 ^b	346.95 ^a	302.28 ^b	290.61 ^{bc}	1.014	0.0006
Haematology (d 28), mg/dL							
Glucose	220.7 ^a	193.3 ^b	192.0 ^b	197.6 ^b	212.6 ^a	0.585	<0.0001
Triglyceride	118.2	106.5	113.1	115.7	114.9	0.709	0.6558
Cholesterol	141.4	132.2	136.9	139.8	137.3	0.485	0.1782
HDL	62.3	65.8	66.4	65.2	64.7	0.493	0.8622
LDL	55.3	45.1	47.9	51.4	49.5	0.553	0.3175
VLDL	23.6	21.3	22.6	23.1	22.9	0.317	0.6558
Uric acid	4.38	4.25	4.31	4.42	4.40	0.106	0.8566
Haematology (d 42), mg/dL							
Glucose	228.6 ^a	181.0 ^c	194.2 ^{bc}	203.8 ^b	218.6 ^a	0.639	<0.0001
Triglyceride	122.2 ^a	109.0 ^b	115.8 ^{ab}	118.3 ^a	114.2 ^{ab}	0.490	0.0306
Cholesterol	156.2 ^a	136.2 ^d	148.3 ^{bc}	153.8 ^{ab}	144.8 ^c	0.432	<0.0001
HDL	65.6	66.2	67.2	66.7	66.4	0.559	0.9986
LDL	66.1 ^a	48.2 ^b	58.0 ^{ab}	63.4 ^a	55.5 ^{ab}	0.566	0.0179
VLDL	24.4 ^a	21.8 ^b	23.1 ^{ab}	23.6 ^a	22.8 ^{ab}	0.219	0.0306
Uric acid	4.68	4.42	4.36	4.49	4.52	0.084	0.1072

GSE, grape seed extract; BW, body weight; EPEF, European production efficiency factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein. ^{a-d}Means within the same row with different superscript differ significantly ($P < 0.05$).

stress condition (29 to 42 d). During the whole period of the experiment, GSE supplementation at the levels of 150, 300, or 450 mg/kg diet improved EPEF of broilers (Table 3).

Haematological parameters

As shown in Table 3, different levels of GSE supplementation decreased the concentration of serum glucose, however, GSE or vitamin C supplementation did not affect serum triglyceride, cholesterol, HDL, LDL, very-LDL (VLDL) and uric acid at 28 d. Different levels of GSE decreased glucose concentration of serum blood, also GSE supplementation at 450 mg/kg diet decreased cholesterol, triglyceride, LDL and VLDL concentration of serum blood at 42 d. Vitamin C supplementation decreased serum cholesterol concentration of broilers at 42 d. Concentration of serum HDL and uric acid did not affected by GSE or vitamin C supplementation (Table 3).

HSP70 gene expression

Table 4 shows the average C_T results for

treatments and how these C_T s are manipulated to determine ΔC_T and $\Delta\Delta C_T$ and the relative amount of *HSP70* mRNA. Results showed that GSE and vitamin C supplementation reduced the relative amount of *HSP70* expression in heart and liver of broilers compared to the control group at 42 d (Table 4).

Discussion

European production efficiency factor

In the present study, live body weight and EPEF improved by GSE supplementation before and after exposure to heat stress condition. It was reported that phytogetic additives from plant extracts are an alternative to antibiotic performance enhancer because they may promote higher nutrient digestibility, increase digestive enzyme activity and gastric and pancreatic juice secretion, protect the intestinal

microvilli and improve bird performance by antimicrobial activity (Hernandez *et al.*, 2004; Toledo *et al.*, 2007). Seven *et al.* (2008) found that high doses of propolis rich in phenolics and vitamin C could partially overcome the depression in growth caused by heat stress in broilers which is similar to the present study. Brenes *et al.* (2010) demonstrated that the inclusion of concentrations of GSE up to 3.6 g/kg did not change the growth performance (0 to 3 weeks and 3 to 6 weeks of age). Hughes *et al.* (2005) and Lau and King (2003) reported a growth depression with the use of GSE containing 90.2% of total phenolics, expressed as gallic acid equivalent by the Folin method, and incorporated in the diet at 30 g/kg.

Grape seeds are a major source of condensed tannins. Tannins are subdivided into two groups: hydrolysable and condensed. Hydrolysable tannins are compounds containing a central core of glucose or another poly-olesterified with gallic acid, called gallotannins, or with hexahydroxydiphenic acid, called

Table 4. Effects of grape seed extract and vitamin C on *HSP70* gene expression in heart and liver of broiler chickens at 42 d (under heat stress condition).

Parameter	Control	GSE, mg/kg			Vitamin C	SEM	P
		150	300	450			
Heart							
<i>HSP70</i> (average C_T)	31.59±0.55	33.86±0.74	38.61±0.5	31.81±1.41	33.96±2.23	-	-
<i>GAPDH</i> (average C_T)	30.39±0.08	27.55±0.18	29.18±0.37	22.53±1.15	26.78±0.31	-	-
ΔC_T°	1.20±0.55 ^c	6.30±0.76 ^b	9.43±0.62 ^a	9.27±1.82 ^a	7.17±2.24 ^{ab}	0.647	<0.0001
$\Delta\Delta C_T^{\ddagger}$	0.00±0.55	5.10±0.76	8.24±0.62	8.07±1.82	5.97±2.24	-	-
Fold change	1.00 ^a	0.033 ^b	0.000 ^b	0.003 ^b	0.040 ^b	0.090	<0.0001
Liver							
<i>HSP70</i> (average C_T)	29.13±1.41	30.98±0.55	35.87±0.45	32.61±0.55	30.42±0.64	-	-
<i>GAPDH</i> (average C_T)	26.37±1.52	23.45±1.18	26.51±0.61	24.80±0.69	22.73±0.55	-	-
ΔC_T°	2.75±2.07 ^c	7.53±1.30 ^b	9.35±0.75 ^a	7.811±0.88 ^{ab}	7.68±0.85 ^b	0.870	<0.0001
$\Delta\Delta C_T^{\ddagger}$	0.00±2.07	4.78±1.30	6.60±0.75	5.06±0.88	4.93±0.85	-	-
Fold change	1.00 ^a	0.063 ^b	0.011 ^b	0.032 ^b	0.034 ^b	0.107	<0.0001

GSE, grape seed extract. ^{a-c}Means within the same row with different superscript differ significantly ($P < 0.05$). [°] ΔC_T is C_T HSP70- C_T GAPDH; [†] $\Delta\Delta C_T$ is ΔC_T ample- ΔC_T control.



Figure 1. Polymerase chain reaction products of heart of broiler chickens: GAPDH with 137 bp (1, 2, 3 columns) and HSP70 with 120 bp (4, 5, 6 columns).



Figure 2. Polymerase chain reaction products of liver of broiler chickens: GAPDH with 137 bp (1, 2, 3 columns) and HSP70 with 120 bp (4, 5, 6 columns).

ellagitannins. They are found in pomegranate, strawberries, peels of walnuts, *etc.* Condensed tannins are oligomers or polymers of flavan-3-ol units such as (+) - catechin and (-) - epicatechin and flavan-3, 4-diols, such as leucoanthocyanidins or a mixture of the two (Kumari and Jain, 2012). They are found in green tea, grape seed, blueberries, *etc.* They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalysed oxidation reaction upon heating in acidic alcohol solutions (Dai and Mumper, 2010). In general, tannins bind protein through H-bonds and hydro-phobic interactions. So, they may reduce the digestibility of protein and carbohydrates including starch and fibres. Another important property is their bitter and astringent taste, which in many cases reduces palatability, so the animal will not eat it. In poultry, tannin levels 0.5 to 2.0% in the diet can depress growth, while levels from 3 to 7% can cause death. Condensed tannins are usually not toxic, but hydrolysable tannins can cause liver and kidney damage, and death (Makkar, 2007). Conversely, tannins are anti-oxidants and can improve resistance to heat stress (Liu *et al.*, 2011). However, it seems that the amount of GSE supplementation in this study was lower to decrease birds' body live weight. On the other hand, phenolic compound of GSE such as catechin, epicatechin, epicatechin-3-o-galate, and procyanidins has antioxidant, antimicrobial, anti-proliferative, anti-mutagenic, and anti-aging activities (Shi *et al.*, 2003), and may help birds to perform better in normal situation or to overcome the detrimental effects of heat stress. Thus, it seems that not only may GSE supplementation at proper dosage have positive effects on birds' livability and live weight, but it may also improve the profitability of broiler rearing industry by increasing the production index at normal or under chronic heat stress conditions.

Blood biochemical

Grape seed extract supplementation decreased the concentration of serum glucose at 28 d (pre-heat stress condition), which is in agreement with the findings of Maghrani *et al.* (2005). They reported that flavonoid or plant extract with high content of flavonoid have potent inhibition of renal glucose re-absorption through inhibition of the sodium-glucose symporters located in the proximal renal tubule. Flavonoids are divided into at least six different structural families: flavonols, flavones, flavanones, isoflavones, catechins, and anthocyanins (Beecher, 2003). Tayer *et al.* (2012) stated that the beneficial effects of

flavonoids in medicinal plants such as grape leaves related to the hypoglycaemic and hepatic glucokinase activity of liver. It is reported that stress hormones (corticosteroid, adrenocorticotrophic and glucocorticoid hormones) change energy, protein, lipid and mineral metabolisms, blood gases, acid-base and electrolyte balances, as well as haemoglobin concentration (Siegel, 1995). Mobilisation or production of glucose for the energy required to maintain homeostasis in the presence of the stressor is an important function (Viriden and Kidd, 2009) that increases blood glucose (Puvadolpirod and Thaxton, 2000). It is reported that insulin signalling is impaired under conditions of oxidative stress (Rudich *et al.*, 1998) and polyphenols have been shown to reverse oxidative stress-induced impairments in insulin signalling (Hininger-Favier *et al.*, 2009). *In vitro* cell culture studies show that oxidative stress increases serine (Ser-307) phosphorylation in insulin receptor substrate 1 (IRS-1) protein molecules. Serine phosphorylation in IRS-1 is known to inhibit insulin-mediated downstream signalling resulting in impaired glucose uptake (Werner *et al.*, 2004; Tanti *et al.*, 2004). Absorption of glucose in intestine is mediated by active transport via the sodium-dependent glucose transporter SGLT1 and by facilitated sodium-independent transport via the glucose transporter GLUT2 (Drozdowski and Thomson, 2006). Previous investigations showed that flavonoids decreased glucose uptake by a sodium-dependent pathway via the sodium-dependent glucose transporter 1 SGLT1 (Hossain *et al.*, 2002; Cermak *et al.*, 2004; Aoshima *et al.*, 2005). Kwon *et al.* (2007) reported the inhibition of the intestinal glucose transporter GLUT2 (independent facilitative glucose transporter) by flavonoids. Ding *et al.* (2013) reported that grape seed proanthocyanidins treatment increased normal insulin content and decreased the number of apoptotic cells in diabetic islets. Also, they stated that GSPs treatment partially alleviated endoplasmic reticulum (ER) stress by decreasing some ER stress markers. In general, polyphenolic compounds inhibit carbohydrate digestion (α -amylase and α -glucosidase) and glucose absorption in the intestine (glucose transporters), stimulate insulin secretion from the pancreatic β cells, modulate glucose release from the liver, activate insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulate intracellular signalling pathways and gene expression (Hanhineva *et al.*, 2010). On the other hand, heat stress effects on blood glucose are not entirely conclusive as decreases in blood glucose levels have been observed in a

variety of species including ruminants (O'Brien *et al.*, 2010) and chickens (Rahimi, 2005; Attia *et al.*, 2009a, 2009b). Alnaimy *et al.* (1992) indicated that the phenomenon could be attributed to an increase in total body water or a decrease in acetate concentration, which is the primary precursor for the synthesis of cholesterol. Also, Sahin *et al.* (2002) and Nazifi *et al.* (2003) reported that heat stress resulted in hypoglycemia. The researchers attributed the decline in blood glucose concentration during heat stress to a decrease in concentration of thyroxine, which is closely associated with energy metabolism during heat stress. The reasons for the inconsistencies above may be due to the type of experimental model, plane of nutrition, and timing/severity of heat stress.

Stressors increase the cholesterol level in chicken plasma (Attia *et al.*, 2009a, 2009b, 2011; Dozier *et al.*, 2006). Higher activity of hypothalamic-pituitary-adrenal axis has been found in stress conditions characterised by higher circulating levels of cortisol and adrenocorticotrophic hormone (ACTH) or corticotropin-releasing hormone (Eutamene and Bueno, 2007). Hypercholesterolemia is caused by hyperactivity of the adrenal gland (Siegel, 1995). Stress hormones, such as epinephrine, normally induce lipolysis and increase circulating non-esterified fatty acids concentrations (Pearce, 2011). It is stated that share of food expenditure to reproduction (30%), growth (30%), health (10%) and survival (30%) is directed according to basal requirements but it can only be directed to health (80%) and survival (20%) during stress (Siegel and Gross, 2000). High ambient temperature impairs absorption of vitamin C and increases the dietary requirement of this vitamin (Klasing, 1998; Attia *et al.*, 2009a, 2009b, 2011). At temperatures above thermoneutral zone, corticoid secretion increases as a response to stress. It has been reported (Kutlu and Forbes, 1993) that ascorbic acid supplementation reduces the synthesis of corticoid hormones in birds under heat stress. As corticoids induce gluconeogenesis from non-carbohydrate precursors such as lactate, amino acids and glycerol (Linne and Ringsrud, 1999), decrease of glucocorticoids secretion could limit lipid and protein catabolism (Kucuk *et al.*, 2003). Sahin *et al.* (2002) found that heat stress tended to elevate plasma corticosterone concentrations which were significantly reduced with vitamin E supplementation in a diet of Japanese quails. In addition, increasing concentrations of ACTH were parallel to increases in serum glucose, uric acid, and triglycerides concentrations. These results were probably due to the greater catabolic effect (or concentration) of

ACTH, yielding more glucose, uric acid, and triglycerides in the serum. Catecholamine production (e.g. epinephrine) inhibits insulin release, increases gluconeogenesis/glycogenolysis (to provide glucose for extrahepatic tissues), and adipose tissue lipolysis (to provide fatty acids for skeletal muscle) to meet energy requirements (Pearce, 2011). It has been demonstrated that during an increased heat-load, dietary carbohydrates are not able to reduce glucose production by the liver (Angus *et al.*, 2001).

Results of the present study showed that GSE supplementation decreased glucose concentration of serum blood, also GSE supplementation at 450 mg/kg diet decreased cholesterol, triglyceride, LDL and VLDL concentration of serum blood of broilers under heat stress condition. Vitamin C supplementation decreased serum cholesterol concentration of broilers. This is in agreement with the findings of previous studies (Ferit Gursu *et al.*, 2004; Ngamukote *et al.*, 2011; Hosseini-Vashan *et al.*, 2012; Çiftçi *et al.*, 2013).

Ferit Gursu *et al.* (2004) reported that serum cholesterol, triglyceride, HDL cholesterol, and glucose concentrations decreased, whereas total protein and albumin concentrations increased with dietary vitamin C and folic acid supplementation compared with the heat stressed Japanese quails. It is stated that a likely mechanism by which vitamin C causes a reduction in corticosterone concentration is through inhibitory effect of vitamin C on glucocorticoid synthesis, and decrease in protein-derived gluconeogenesis (Ferit Gursu *et al.*, 2004). Hosseini-Vashan *et al.* (2012) reported that dietary turmeric rhizome decreased the concentration of blood cholesterol and LDL, while increased the level of blood HDL in pre- and post-heat stressed birds.

Ngamukote *et al.* (2011) reported that three major polyphenolic compounds (gallic acid, catechin, and epicatechin) present in a particular grape seed have cholesterol-lowering activity by inhibiting pancreatic cholesterol esterase, binding of bile acids, and reducing solubility of cholesterol in micelles which may result in delayed cholesterol absorption. In general, pancreatic cholesterol esterase plays an important role in hydrolysing dietary cholesterol esters which liberates free cholesterol in the lumen of the small intestine (Brod-Eppley *et al.*, 1995). Furthermore, it enhances the incorporation of cholesterol into mixed micelles and aids transport of free cholesterol to the enterocyte (Myers-Payne *et al.*, 1995). Therefore, the inhibition of cholesterol esterase is expected to limit the absorption of dietary cholesterol, resulting in reduced cho-

lesterol concentration. Ariana *et al.* (2011) reported that the decrease in total lipid and cholesterol may be attributed to the diminishing effect of herbal extracts on hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that is needed for cholesterol synthesis in liver.

The concentration of uric acid in broilers serum was not affected by dietary treatments before and during heat stress condition. Uric acid is a metabolite that can function in the plasma as an antioxidant due to its capacity to neutralise the peroxy radical. The conversion of hypoxanthine into xanthine and uric acid, catalysed by xanthine oxidase, is associated with the production of superoxide anions contributed by ubiquinone from the electron transport chain (Chevion *et al.*, 2003; Santos *et al.*, 2004), and according to Speranza *et al.* (2007), uric acid is a good indicator of the stress. Very short-term exposure to high temperatures (15 min at 42°C) results in a reduction in protein synthesis, a depletion of essential and non-essential plasma free amino acids, an increased plasma uric acid levels, maybe reflecting active protein catabolism, and lower N retention (Ostrowski-Meissner, 1981). In contrast, chronic exposure to heat stress depresses protein synthesis in various muscles, reduces protein breakdown and decreases the levels of most plasma free amino acids, especially sulphur and branched-chain amino acids. Uric acid levels are maintained at concentrations comparable to that seen in control birds (Temim *et al.*, 1998, 2000). Thus, protein breakdown may initially increase rapidly but then decrease as thermal stress continues. Protein synthesis and N deposition seem to be depressed throughout these processes.

HSP70 gene expression

It is reported that heat stress causes increased oxidative stress as reflected by increasing lipid peroxidation and HSP expression (Sahin *et al.*, 2009) and concomitantly lowers the concentrations of antioxidant vitamins in serum and tissues (Sahin and Kucuk, 2003). Increases in HSP70 in liver (Mahmoud and Edens, 2003), lungs and heart (Mahmoud *et al.*, 2004), and brain (Sahin *et al.*, 2009) is an atypical response in heat-stressed broilers. In this study, GSE and vitamin C supplementation reduced HSP70 gene expression in broilers suffering from chronic heat stress, which is in agreement with previous research (Sahin *et al.*, 2009; Tran *et al.*, 2010). The multiple mechanisms of grape seed antioxidative activity are expressed in its ability of radical scavenging, metal chelation, and synergism with other antioxidants (Lu and Foo, 1999). Sahin

et al. (2009) reported that the induction of HSP70 was significantly decreased by the combined supplementation of vitamin C and E. Tran *et al.* (2010) reported that epigallocatechin-3-gallate (EGCG) significantly decreased the levels of heat shock transcription factor 1 and 2 in a dose dependent manner, so decreased the expression of HSP70 and HSP90. Also, they stated that EGCG competes with ATP for binding to the ATPase domain of HSP70 and HSP90.

Conclusions

In conclusion, hydroalcoholic GSE supplementation in Cobb mail broilers decreased serum glucose of the birds before heat stress condition, this may be beneficial for the animals and human who suffered from diabetic disease. Grape seed supplementation at the level of 300 mg/kg diet could improve live weight and EPEF, and suppress the detrimental effect of heat stress on blood metabolites such as the levels of glucose, cholesterol, and HSP70 gene expression in birds suffering from chronic heat stress condition. Thus, it seems that further investigations are needed to introduce hydroalcoholic GSE as a beneficial additive under chronic heat stress condition to broiler industry.

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