IJAS Iranian Journal of Applied Animal Science

The Effect of Grape Seed Extract Supplementation on Performance, Antioxidant Enzyme Activity, and Immune Responses in Broiler Chickens Exposed to Chronic Heat Stress

Research Article

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Received on: 1 Jan 2017
Revised on: 19 Apr 2017
Accepted on: 15 May 2017
Online Published on: Mar 2018

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Online version is available on: www.ijas.ir

ABSTRACT

The effect of grape seed extract (GSE) feed supplementation on performance, antioxidant enzyme activity, and immune responses in broiler chickens suffering from heat stress were investigated in this study. Experimental diets including control diet (with no additive), 3 levels of GSE (150, 300, 450 mg/kg), and one level of vitamin C (300 mg/kg) as a positive control were fed to the birds from 1 to 42 d of age. The chronic heat stress (34±1 ˚C temperature for 5 hours per day) was provided from 29 to 42 d of age. The GSE supplementation up to 300 mg/kg diet increased the average daily gain of broiler chickens compared to the control group prior to heat stress (1-28 d). During the heat stress condition, dietary GSE at the rate of 300 and 450 mg/kg diet improved feed conversion ratio. The GSE supplementation at 300 mg/kg diet increased IgG titer as a primary and secondary response to sheep red blood cell (SRBC) injection. In addition, birds fed diet with GSE (150, 300, 450 mg/kg diet), or vitamin C (300 mg/kg diet) had higher levels of IgG titers as a secondary responses to SRBC. The GSE supplementation at the rate of 300 and 450 mg/kg diet reduced heterophil percent, heterophil/lymphocyte ratio, and increased the percentage of lymphocyte of broilers under heat stress. Supplementation of diet with GSE (300, 450 mg/kg diet) increased glutathione peroxidase (GPx) activity in birds under heat stress condition. The GSE or vitamin C supplementation did not affect the results of cutaneous basophil hypersensitivity (CBH) response, and relative weights of spleen and bursa of fabricius in chickens under heat stress. However, supplementation of diet with GSE (300, 450 mg/kg diet) or vitamin C (300 mg/kg diet) increased relative weight of thymus in birds under heat stress condition. Thus, GSE supplementation could alleviate the detrimental effects of heat stress in broiler chickens better than vitamin C and it is preferable for the health and economic goals since it is a natural waste by-product.

KEY WORDS antioxidant enzyme, broiler chicken, grape seed extract, heat stress, immunity.

INTRODUCTION

Heat stress is one of the most challenging environmental conditions especially on summer days in many countries. Regarding the finishing period, the suitable ambient temperature for poultry is between 16 ˚C and 25 ˚C (Sahin *et al*. 2001). It has been well documented that exposing broiler chickens to continuously high temperature during the finisher period causes chronic heat stress (Sahin *et al*. 2003; Ahmad *et al*. 2008). Previous studies have shown that heat stress suppresses performance and immune system in broiler chickens (Sohail *et al*. 2010; Quinteiro-Filho *et al*. 2010). The hypothalamic–pituitary–adrenal (HPA) and the sympathetic–adrenal-medullar (SAM) axes constitute the main pathways through which the immune response can be altered (Lara and Rostango, 2013). In a recent study, Sohail

et al. (2010) reported that broilers subjected to chronic heat stress had significantly reduced feed intake (-16.4%), lower body weight (-32.6%), and higher feed conversion ratio (FCR) (+25.6%) at d 42. Exposure to high temperatures may depress the activity of the mitochondrial respiratory chain in broiler chickens.

This results in overproduction of reactive oxygen species, which caused oxidative injury and significant difference in the activities of superoxide dismutase and glutathione peroxidase enzymes (Tan *et al*. 2010). High ambient temperature not only has negative effects on livability, performance, and product quality of birds, but it also may suppress welfare and health status of birds.

It is well known that nutrition has a strong effect on heat stress. In addition, insufficient intake of antioxidants, a high intake of pro-oxidants, or both, may lead to oxidative stress (Voljc *et al*. 2011). In this regard, using proper herbal additives with antioxidant activity may help broiler chickens to overcome the condition in an organic manner to respect animal welfare.

Grape seed extract is a by-product derived from the grape seeds (*Vitis vinifera*) originating from grape juice and wine processing that is extracted, dried and purified to produce a polyphenolic compound-rich extract (Lau and King, 2003). The total extractable phenolics present in grape are about 10% or less in the pulp, 60-70% in the seeds, and 28-35% in the skin. The phenol content of seeds may range from 5% to 8% by weight. The most abundant phenolics isolated from grape seeds are catechins (catechin, epicatechin, and procyanidins) and their polymers (Shi *et al*. 2003). The benefits derived from phenolic compounds in grape seeds are closely related to their antioxidant and singlet oxygen quenching ability. These phenolic compounds are able to trap and quench free radicals, and it has been shown that their antioxidant potentials to be four to five fold higher than that in vitamin C or E (Shi *et al*. 2003). They are also very potent metal chelating agents (Shi *et al*. 2003). Some researcher reported that 300 mg ascorbic acid/kg of broiler chickens feed gave the best result in terms of birds performance and total revenue. Therefore, the objective of the present study was to evaluate the effects of GSE supplementation in feed on performance, antioxidant enzyme activity, and immune response in broiler chickens suffered from 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 (initial weight (g), 36.28±0.38) were obtained from a local hatchery and were divided into 25 groups of 12 birds each. All procedures for the use and the care of animals were conducted after approval by the Ferdowsi University of Mashhad. There were 5 experimental diets including negative (0), and 150, 300, 450 mg GSE/kg diet, and 300 mg vitamin C/kg diet as a positive control. The vitamin C (L-ascorbic acid, 99%) was purchased from Sigma Aldrich Company. The feeding program consisted of a starter (1 to 10 d), grower (11 to 22 d), and finisher diet (23 to 42 d).

The basal diet was 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, 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 ascorbic acid was added to 100 mL water, well mixed and sprayed on the basal diet. The feed was prepared weekly and stored in airtight containers. The temperature was initially set at 34 ˚C on d 1 and decreased linearly by 0.5 ˚C per day up to 28 d. A chronic heat stress under 34 ± 1 ˚C temperature with 65-70% relative humidity for 5 hours was imposed on birds from 29 to 42 d of age. During the study, the birds received a lighting regimen of 23 L:1 D from 1 to d 42.

Grape seed analysis

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

Preparation of grape seed extract

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

Grape seed extract analysis

The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon auto sampler, a K-1001 pump and a UV–visdetector (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, C1 performed according to the method of Iacopini *et al*. (2008). Standards of catechin, epicatechin, procyanidin B1, B2, C1 were purchased from Sigma-Aldrich (St. Louis, USA).

Table 1 Ingredients and nutrient composition of basal experimental diets

Ingredient (%)	1-10 d	11-22 d	23-42 d
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 premix[1]	0.25	0.25	0.25
Vitamins premix[2]	0.25	0.25	0.25
DL-methionine	0.31	0.26	0.23
L-lysine hydrochloride	0.24	0.21	0.18
Calculated composition			
Metabolizable energy (ME) (kcal/kg)	3000	3105	3180
Crude protein (CP) (%)	21.23	19.46	18
Ca (%)	1	0.96	0.9
Available phosphorus (%)	0.50	0.48	0.45
Lysine (%)	1.32	1.19	1.06
Methionine + cystine	0.98	0.89	0.82

[1] Mineral premix supplied the following per kg of diet: Cu: 20 mg; Fe: 100 mg; Mn: 100 mg; Se: 0.4 and Zn: 169.4 mg.
[2] Vitamins premix supplied the following per kg of diet: vitamin A: 18000 IU; vitamin D_3: 4000 IU; vitamin E: 36 mg; vitamin K_3: 4 mg; vitamin B_{12}: 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 and Choline chloride: 500 mg.

Birds performance

The experimental period lasted 42 d. On d 1, 28, and 42, birds were pen weighed, and feed consumption was recorded. The FCR was calculated for each period.

Antioxidant enzyme activity

Blood hemolysate of chicken was prepared on d 28 and 42. The methodology of Paglia and Valentine (1967) was used for measurements of GPx activity in blood hemolysate. Samples were assayed with commercially available GPx kits (Randox, Crumlin, UK) following the instructions of the kit manufacturer and absorbance was monitored at 340 nm wavelength using a spectrophotometer.

Immunological measurements

Toe web swelling test

The cutaneous basophilic hypersensitivity response to phytohemagglutinin P (PHA-P; Sigma Chemical Co., St. Louis, MO), as an indicator of a T-cell-induced delayed-type hypersensitivity reaction, was assessed as described previously (Corrier and DeLoach, 1990).

The CBH response to PHA-P was measured in 2 birds from each pen at d 42 which received 100 µg of PHA-P in 0.1 mL of sterile phosphate-buffered saline (0.15 M at pH=7.4), that was injected intra-dermally in interdigital skin between the second and third toes of the right foot. The left foot was injected with 0.1 mL of phosphate-buffered saline (PBS) as a sham control.

The thickness of each injection site was measured using a pressure-sensitive micrometer before injection and at 4, 8, 12 and 24 h after injection.

The CBH response to PHA-P was calculated using the following formula: swelling index= [(thickness of left toe web after PHA-P injection−initial thickness of left toe web) − (thickness of right toe web after PBS injection−initial thickness of right toe web)].

Antibody response to SRBC

Sheep red blood cells (SRBC) were used as T-dependent antigens to quantify the antibody response. Two broiler chickens from each pen (marked with a red color) were injected with SRBC (5% suspension in PBS, 0.5 mL/bird) intramuscularly at 28 d of age, followed by the second injection 7 d later. Blood samples were collected 7 d after the first and second injections. The serum of samples was collected, heat inactivated at 56 ˚C for 30 min and then analyzed for total antibody, IgG (mercaptoethanol-resistant), IgM (mercaptoethanol-sensitive) anti-SRBC antibodies as described by Cheema *et al.* (2003).

Hematological parameters

At d 28 and 42, two birds from each replicate were selected and their blood samples were collected using sterile syringes (2 mL) to draw blood from the wing vein. Samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Blood smears were prepared on slides and painted by Giemsa method. The white blood cell counts were determined by an improved Neubauer hemocytometer method (Jain, 1986).

Immune organs

Two birds per pen were selected at random and humanely

killed by cervical dislocation for carcass yield and organ sampling (spleen, bursa, and thymus) on 28 or 42 d. Organ weights were expressed as a percentage of body weight (BW).

Statistical analysis

Data were analyzed by analysis of variance using GLM procedure (SAS, 2001). Means were compared using Duncan's new multiple range test (Duncan, 1955). The level of significance was reported at $P < 0.05$.

RESULTS AND DISCUSSION

Grape seed composition and extract analysis

The chemical composition (crude fat, crude protein, total carbohydrate, fiber, calcium, total phosphorus and ash) and extract analysis (catechin, epicatechin and procyanidins) of the grape seed are shown in Table 2.

Growth performance

The effects of dietary supplementation of GSE or vitamin C on growth performance of broiler chickens before or under chronic heat stress condition are shown in Table 3. Prior to heat stress (1-28 d), GSE at the level of 300 mg/kg diet increased average daily feed intake (ADFI) (75.5 g) compared to the control birds (72.3 g).

Besides, GSE at the level of 150 and 300 mg/kg diet increased average daily gain (ADG) of broiler chickens (48.5 and 48.8 g, respectively) compared to the control birds (44.6 g; $P<0.05$).

However, there was not any difference between FCR of the birds. During the heat stress condition (29-42 d), there was not a significant difference between AFDI of the broilers with the different diets ($P>0.05$). The GSE supplementation at the level of 300 mg/kg diet increased ADG of broilers ($P<0.05$).

Dietary GSE at the level of 300 and 450 mg/kg diet decreased FCR of birds compared to control group. The GSE supplementation at the level of 300 and 450 mg/kg diet improved broilers FCR, during the whole rearing period (1-42 d; $P<0.05$).

Antioxidant enzyme activity

Data of the glutathione peroxidase activity in blood hemolysate of birds is shown in Table 4. There was not a significant difference between the GPx activities of birds prior to heat stress (at 28 d), however, dietary supplementation of GSE (150, 300 mg/kg diet) increased GPx activity of broilers compared to control group during the chronic heat stress (at 42 d) ($P<0.05$).

Immunological measurements

Toe web swelling test

Dietary GSE or vitamin C did not affect the CBH response (4 h, 8 h, 12 h, 24 h after PHA-P injection) in broilers at 42 d of age (Table 4).

Antibody response to SRBC

The GSE supplementation at the level of 300 mg/kg diet increased IgG titer as a primary response to SRBC injection ($P<0.05$). Broiler chickens fed the diet with GSE at the rate of 300 or 450 mg/kg had higher total antibody titer against SRBC as a secondary response compared to the control birds. Also, birds fed diets contained GSE (150, 300, 450 mg/kg diet), or vitamin C (300 mg/kg diet) had higher levels of IgG titers as a secondary response to SRBC (Table 4).

Haematological parameters

As shown in Table 5, dietary GSE or vitamin C did not affect white blood cell number, the percentages of heterophil, lymphocyte, monocyte, eosinophil, basophil, and heterophil/lymphocyte ratio of birds prior to heat stress (28 d of age, Table 5). However, the GSE supplementation at the levels of 300 and 450 mg/kg diet reduced heterophil percent, heterophil/lymphocyte ratio, and increased the percentage of lymphocyte of broilers under heat stress (42 d of age, Table 5).

Immune organs

As shown in Table 6, the GSE or vitamin C supplemented diet did not have a significant effect on spleen, bursa of fabricius, and thymus relative weight of birds prior to heat stress (28 d). Dietary GSE or vitamin C did not affect the relative weights of spleen and bursa of fabricius of broilers during heat stress condition (42 d, Table 6). However, supplementation of GSE (300 and 450 mg/kg diet) or vitamin C (300 mg/kg diet) increased the relative weight of thymus in birds under heat stress condition compared to the control birds (Table 6; $P<0.05$).

Growth performance

In the present study, GSE supplementation at the levels of 300 and 450 mg/kg diet improved average daily gain of broiler chickens compare to control group. Chamorro *et al.* (2013) investigated the effect of inclusion of GSE at 0.025, 0.25, 2.5 and 5.0 g /kg in a wheat soybean control diet on growth performance of broiler chickens. Chamorro *et al.* (2013) reported that incorporation of GSE in chicken diets up to 2.5 g/kg had no adverse effect on growth performance.

Table 2 Composition of the grape seed analyzed by AOAC methods and analysis of the grape seed extract by HPLC method

Grape seed		Grape seed extract	(mg/100 g)
Dry matter (%)	90.93	Catechin	1420
Gross energy (kcal/kg)	3292	Epicatechin	1080
Crude fat (%)	24.83	Procyanidine B1	830
Crude protein (%)	10.17	Procyanidine B2	770
Nitrogen free extract (%)	17.86	Procyanidine C	530
Crude fiber (%)	35.39	-	-
Calcium (%)	0.56	-	-
Phosphorus (%)	0.31	-	-
Ash (%)	2.68	-	-

Table 3 Effects of grape seed extract and vitamin C on growth performance of broilers at different periods

Performance	Control	Grape seed extract (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
1 to 28 d							
ADFI (g)	72.3^{bc}	74.0^{ab}	75.5^{a}	71.3^{c}	74.2^{ab}	0.163	0.0067
ADG (g)	44.6^{c}	48.5^{ab}	48.8^{a}	46.1^{c}	46.4^{bc}	0.257	0.0030
FCR (g/g)	1.62	1.52	1.54	1.54	1.59	0.047	0.0707
29 to 42 d							
ADFI (g)	177.2	181.9	191.4	171.2	189.9	0.776	0.2135
ADG (g)	74.1^{b}	77.3^{b}	94.7^{a}	77.8^{b}	82.0^{b}	0.610	0.0193
FCR (g/g)	2.40^{a}	2.36^{ab}	2.02^{c}	2.20^{b}	2.32^{ab}	0.070	0.0007
1 to 42 d							
ADFI (g)	107.3^{ab}	110.0^{ab}	114.1^{a}	104.6^{b}	112.8^{a}	0.446	0.0403
ADG (g)	54.4^{b}	58.1^{b}	64.1^{a}	56.7^{b}	58.3^{b}	0.364	0.0028
FCR (g/g)	1.97^{a}	1.89^{ab}	1.78^{c}	1.84^{bc}	1.93^{a}	0.047	0.0003

ADFI: average daily feed intake; ADG: average daily gain and FCR: feed conversion ratio.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Effects of grape seed extract and vitamin C on blood glutathione peroxidase (GPx) activity, cutaneous basophil hypersensitivity (CBH) response, and antibody titer (log_2) against sheep red blood cell (SRBC) in broiler chickens

Items	Control	Grape seed extract (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
GPx activity, U/L of hemolysate							
28 d	431	463	494	442	453	1.313	0.2233
42 d	470^{c}	547^{ab}	608^{a}	492^{bc}	503^{bc}	1.366	0.0012
Hypersensitivity (mm), d 42							
4 h after	0.808	0.810	0.816	0.814	0.810	0.095	1
8 h after	0.816	0.832	0.824	0.834	0.824	0.095	0.9999
12 h after	0.688	0.690	0.726	0.712	0.690	0.094	0.9982
24 h after	0.588	0.604	0.646	0.638	0.610	0.096	0.9939
SRBC injection, d 28							
7 d after injection							
Total anti-SRBC	2	3.4	3.8	2.4	2.4	0.205	0.0680
IgG	1^{b}	1.6^{ab}	2.6^{a}	1.2^{b}	1^{b}	0.186	0.0445
IgM	1	1.8	1.2	1.2	1.4	0.145	0.2027
SRBC injection, d 35							
7 d after injection							
Total anti-SRBC	2.6^{b}	4^{ab}	4.8^{a}	4.4^{a}	4^{ab}	0.207	0.0449
IgG	1^{b}	2.4^{a}	3.6^{a}	3^{a}	2.6^{a}	0.203	0.0112
IgM	1.6	1.6	1.2	1.4	1.4	0.157	0.8283

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

However, Brenes *et al.* (2010) reported that the supplementation of GSE (contained 45.5% extractable polyphenols) up to 3.6 g/kg did not affect growth performance (0 to 3, or 3 to 6 weeks of age).

In contrast with our results, 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 incorpo-

rated in diet at the rate of 30 g/kg. Quinteiro-Filho *et al.* (2012) stated that long-term heat stressors (31±1 ˚C and 36±1 ˚C for 10 h/d) applied to broiler chickens from d 35 to 42 of life decreased performance, also acute 31˚C heat stress applied for 10 h on the 35th day of life decreased performance variables. Seven *et al.* (2008) observed that high doses of propolis rich in phenolics and vitamin C could partially overcome the depression in growth and carcass quality caused by heat stress in broilers, which was similar to our study. Interestingly, GSE supplementation at the levels of 300 and 450 mg/kg diet improved FCR by 15.8 and 8.3%, respectively. Akbarian *et al.* (2013) reported that a mixture of herbal extracts at the level of 200 and 400 mg/kg diet did not affect broilers performance under chronic heat stress condition. In our study, the GSE supplementation at the level of 300 mg/kg diet improved average daily gain and FCR in the whole period (1-42 d). Xing *et al.* (2004) reported that four factors may affect the effectiveness of phytobiotic additives: 1) plant parts and their physical properties, 2) source, 3) harvest time, and 4) compatibility with the other ingredients in the feed, which may also explain why difference in body weight gain (BWG) and FCR could happen when different kinds of phytobiotics are used in chicken diet.

Antioxidant enzyme activity

Antioxidant nutrients and enzyme defenses are fundamental protectors against all forms of stress (Ojha *et al.* 2010). 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 present in GSE. Reactive oxygen species (ROS) are metabolic products of the respiratory chain. Under normal circumstances, ROS are eliminated by cellular enzymatic and non-enzymatic antioxidant defenses. Within the enzymatic mechanisms, GPx metabolizes hydrogen peroxide into water and oxygen. If ROS are not effectively eliminated, they can cause oxidative cell injuries, such as peroxidation of lipids (membranes and organelles), proteins (receptors and enzymes) and DNA (Zal *et al.* 2012). In the present study, GSE or vitamin C supplementation did not affect GPx activity in birds prior to heat stress (28 d), however, GSE supplementation at the level of 300 and 450 mg/kg diet increased GPx activity in birds under heat stress condition (42 d). This is in agreement with the findings of Brenes *et al.* (2010) and Zal *et al.* (2012), who reported an increase in antioxidant activity in diet and excreta of broilers fed diet contained GSE at 21 and 42 days of age. The principal defense systems against oxygen-free radicals are SOD, GSH, GPx, GR, catalase and antioxidant nutrients. Vitamins also directly scavenge ROS and up-regulate the activities of antioxidant enzymes (Zal *et al.* 2012). Bautista-Ortega and Ruiz-Feria (2010) reported that antioxidant vitamins might have played synergistic roles to increase NO bioavailability and reduce oxidative stress damage. Vitamin C is a cytosolic antioxidant that restores the antioxidant capability of oxidized Vitamin E (Guney *et al.* 2007). Vitamin C restores VE (Serbecic and Beutelspacher, 2005), most likely by recycling the tocopheroxyl radical (Nagaoka *et al.* 2007). Ojha *et al.* (2010) stated that the intracellular level of glutathione, which helps to maintain the redox potential of the cell decreased with the increased concentration of vitamin C. They also reported that the activities of other enzymes related to glutathione metabolism such as glutathione reductase, glutathione peroxidase, and glutathione-S-transferase also decreased. This is in contrast with the findings of the present study. Vitamin C functions either as an antioxidant or prooxidant is determined by at least 3 factors: 1) the redox potential of the cellular environment; 2) the presence/absence of transition metals; and 3) the local concentrations of ascorbate (Gonzalez *et al.* 2005).

Immunological measurements

Toe web swelling test

The GSE has many pharmacological and health benefits that include antioxidant, anti-microbial, cardio protective, hepatoprotective, neuroprotective, anti-inflammatory, effects (Mesquita and Nascimento, 2009; Xia *et al.* 2010). Results of the present study indicated that although dietary supplementation of GSE or vitamin C increased CBH response numerically, they did not have a significant effect on CBH response in birds (P>0.05). This is in contrast with the findings of Hashemipour *et al.* (2013), who reported that phenolic compound such as thymol and carvacrol supplementation increased hypersensitivity response of broiler chickens. However, some researcher reported that mice that received either 0.5 or 1.0% grape seed proanthocyanidins (w/w) in diet exhibited a significant reduction in UVB-induced suppression of the local contact hypersensitivity response. The various results of CBH responses reported by several researchers may be due to discrepancy in site of injection, PHA-P dose, and time of measuring the response of birds.

Antibody response to SRBC

In poultry production, it is very important to improve immunity to prevent infectious diseases. A variety of factors such as vaccination failure, infection by immune-suppressive diseases, and abuse of antibiotics can induce immunodeficiency (Hashemipour *et al.* 2013). Use of immune stimulators is one solution to improve immunity and to decrease susceptibility to infectious disease.

Table 5 Effects of grape seed extract and vitamin C on hematological parameters in broiler chickens

Items	Control	Grape seed extract (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
Hematology, d 28							
White blood cells (10^6/mm^3)	11	11.2	11.6	11	11.4	0.207	0.8790
Heterophil	31.6	29.4	28	28.6	29.4	0.301	0.1666
Lymphocyte	59	61.8	62.6	61.4	61	0.336	0.3743
Monocyte	3.8	3.4	3.3	3.7	3.6	0.207	0.9735
Eosinophil	3.4	3.2	3.5	3.5	3.4	0.164	0.9695
Basophil	2.2	2.2	2.6	2.8	2.6	0.2524	0.9646
Heterophil/lymphocyte (H/L)	0.53	0.47	0.44	0.46	0.48	0.0461	0.1470
Hematology, d 42							
White blood cells (10^6/mm^3)	17	16	15.8	16.4	16.4	0.230	0.6654
Heterophil	39.2^{a}	36.6^{ab}	34.3^{b}	33.8^{b}	37.2^{ab}	0.306	0.0129
Lymphocyte	50.6^{c}	53.8^{abc}	55.9^{ab}	56.2^{a}	52.4^{bc}	0.316	0.0136
Monocyte	4.4	4	4.2	4.4	4.2	0.209	0.9751
Eosinophil	3.6	3.2	3.2	3.4	3.4	0.164	0.8716
Basophil	2.2	2.4	2.4	2.2	2.8	0.241	0.9653
Heterophil/lymphocyte (H/L)	0.775^{a}	0.689^{abc}	0.624^{bc}	0.604^{c}	0.706^{ab}	0.052	0.0073

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 Effects of grape seed extract and vitamin C on relative weights (g/100 g of BW) of immune organs in broiler chickens at 28 or 42 d

Items	Control	Grape seed extract (mg/kg)			Vitamin C	SEM	Pr > F
		150	300	450			
28 d							
Spleen	0.195	0.192	0.199	0.191	0.198	0.024	0.8921
Bursa of fabricius	0.225	0.214	0.218	0.212	0.227	0.023	0.3738
Thymus	0.364	0.361	0.365	0.358	0.362	0.038	0.9983
42 d							
Spleen	0.127	0.131	0.150	0.146	0.129	0.027	0.2495
Bursa of fabricius	0.063	0.070	0.064	0.068	0.068	0.027	0.9738
Thymus	0.174^{b}	0.228^{ab}	0.292^{a}	0.313^{a}	0.308^{a}	0.053	0.0240

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Herbs that are rich in flavonoids extend the activity of vitamin C, act as antioxidants, and may therefore enhance immune functions (Acamovic and Brooker, 2005). In the present study, the GSE supplementation at the rate of 300 mg/kg diet increased primary titer of IgG in birds. The secondary total antibody response against SRBC antigen increased in broilers fed diet with GSE at the rate of 300 or 450 mg/kg was higher than control group. In addition, birds fed diets containing GSE (150, 300,450 mg/kg diet), or vitamin C (300 mg/kg diet) had higher levels of IgG titers as a secondary responses to SRBC. Thus, it seems that GSE could help to improve bird's response to SRBC antigen and it is more effective than vitamin C, which used as a positive control. This is in agreement with the previous reports of improving antibody response caused by herbal depravities (Khaksar *et al*. 2012; Hashemipour *et al*. 2013), but contrasted the findings of Khalaji *et al*. (2011).

Haematological parameters

As mentioned, dietary GSE or vitamin C did not affect white blood cell numbers and their differentiation, in birds prior to heat stress (28 d of age). This is in agreement with (Hashemipour *et al*. 2013). Jain (1993) reported that corticosteroid induced lymphopenia attributed to lympholysis in blood and lymphoid tissue, increased shift of lymphocytes from blood to other body compartments.

Ojha *et al*. (2010) reported that vitamin-C boosts immunity by keeping disease-fighting white blood cells increased, thus, the body is better able to stave off infections. Heckert *et al*. (2002) reported that heterophil/lymphocyte (H/L) ratio is a common indicator of stress in poultry, and the blood leukocyte profile is influenced by stress. Reduction of lymphocytes and monocytes and enhancement in the numbers of heterophils, which leads to a higher H/L ratio, have been reported for stressed animals.

In the present study, GSE supplementation at the level of 300 and 450 mg/kg diet reduced heterophil percent, H/L ratio, and increased the percentage of lymphocyte in broilers under heat stress. This is in contrast with the results of Tayer *et al*. (2012). The researchers stated that green grape leaves increased heterophil percent and H/L ratio in broilers.

Immune organs

Data of the present study showed that supplementation of GSE or vitamin C did not affect the relative weight of immune organs prior to impose heat stress (28 d), however, the GSE (300, 450 mg/kg diet) or vitamin C (300 mg/kg diet) increased the relative weights of thymus in birds under chronic heat stress condition (42 d).

This is in agreement with the findings of Hosseini-Vashan *et al*. (2012). They reported that the relative weight of spleen in broiler chickens fed diet with different levels of turmeric rhizome powder revealed no significant differences among treatments pre and post heat stress. Regarding the present study, it seems that GSE may be helpful to broilers by suppressing the atrophy effect of heat stress on lymphatic organs.

CONCLUSION

In conclusion, GSE improved growth performance, antioxidant enzyme activity, and immune response of birds exposed to chronic heat stress. We recommend the addition of 300 mg GSE/kg diet to improve performance of broiler chickens exposed to chronic heat stress during finisher period. The heat stress alleviating effects of GSE may be associated with increased antioxidant enzyme activity, elevated immunoglobulin production and immune organ development. It seems that further study is needed to introduce grape seed extract as an efficient antioxidant feed additive for poultry suffering from chronic heat stress condition.

ACKNOWLEDGEMENT

The authors are grateful to the office of the vice president in research at Ferdowsi University of Mashhad, Iran, for approving (3/22886) and providing the facilities and financial support of the experiment.

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