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Lipid Peroxidation in the Plasma, Lungs, Heart and Liver of Broilers Fed a Grape Seed Extract and Raised at 2278 m of Altitude

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ABSTRACT

The aim of this research was to evaluate the effect of partial substitution of vitamin E (VE) by grape seed extract (GSE) on glutathione peroxidase activity (GPxA), nitric oxide (NO) concentration and lipid peroxidation [malondialdehyde (MDA) concentration] in the plasma, lungs, heart and liver, and live performance of broilers raised at 2278 m of altitude. One-d-old Ross 308 male chickens (n = 420) were randomly distributed into three treatments: Control-AL (basal diet containing 40 IU of VE and fed *ad libitum*; AL), Control-FR (basal diet and feed restriction; FR) and GSE-AL [basal diet containing 10 mg of GSE (equivalent to 30 IU VE) plus 10 IU of VE and fed AL]. The data were analyzed using one-way analysis of variance. Glutathione peroxidase activity, and NO and MDA concentrations in the plasma were evaluated on d 46. On d 47, MDA was evaluated in the lungs, heart and liver. Live performance parameters were recorded weekly. There were no significant differences (p>0.05) in live performance and NO concentration among treatments. Birds from the GSE-AL treatment presented the lowest (p<0.05) GPxA, the highest (p<0.05) MDA concentration in the plasma, heart and liver, and intermediate MDA concentration in the lungs. Our results suggest that GSE may partially replace VE in broiler diets without impairment of live performance. However, further research is required to determine the optimal level of dietary GSE inclusion to reduce lipid peroxidation in the plasma, lungs, heart, and liver of broilers raised at 2278 m of altitude.

INTRODUCTION

Ascites syndrome (AS) is a metabolic disorder (Bautista-Ortega & Ruiz-Feria, 2010) that mostly occurs in fast-growing chickens (Arab *et al.,* 2006). High altitude, poor ventilation (Julian, 2000), low temperature (Fathi *et al.,* 2011), and *ad libitum* feeding (Rodríguez-Ortega *et al.,* 2014) are predisposing factors for AS. High altitude reduces the partial pressure of oxygen (PO_2) in the inspired air (hypoxia, Wideman *et al.,* 2013), which in turn produces hypoxic broilers (Ruiz-Feria & Wideman, 2001). The central etiology of AS is a hypoxemic condition (hypoxemia refers to blood in the systemic arteries that is under saturated with oxygen; Wideman *et al.,* 2013) resulting from an imbalance between demand and supply of oxygen (Kalmar *et al,* 2013). Bakonyi & Radak (2004) reported that exposure to high altitude increases the formation of reactive oxygen species (ROS) and reactive nitrogen species, and ROS cause lipid peroxidation in heart and liver of broilers (Díaz-Cruz *et al.*, 1996). Bottje *et al.* (1995) reported that oxidative stress is associated with AS. Rodríguez-Ortega *et al.* (2014) observed that *ad libitum* feeding decreases antioxidant activity in lungs, heart, and liver of broilers.

Vitamin E is a well-recognized natural antioxidant used for the prevention of biological oxidation, and was shown to reduce mortality due to ascites (Bottje *et al.,* 1995). However, the addition of high concentrations of VE increases the cost of the diet (Kennedy *et al.,* 1992), leading to the search of new more affordable sources of antioxidant compounds, such as grape seed extract (GSE).

Grape seed extract has antioxidant effects as it contains polyphenolic compounds (Brenes *et al.,* 2010), such as procyanidins, catechins, epicatechins, gallocatechins, and epigallocatechins (Chamorro *et al.,* 2013). However, grape seed extract has been seldom evaluated in broiler feeding. El-Damrawy (2014) found that supplementation with GSE (100 or 200 mg kg^{-1} of diet) increased glutathione concentration and superoxide dismutase activity, and decreased malondialdehyde (MDA) concentration in the liver of broilers reared under heat stress conditions. Hao *et al.* (2015) reported that supplementation with GSE (100 or 150 mg kg^{-1} of feed) increased glutathione peroxidase activity and decreased MDA concentration in the serum of pigs.

The high altitude (2278 m) in Texcoco, State of Mexico, Mexico (atmospheric pressure, 581.1 mm Hg and PO_2, 122 mm Hg; Vázquez-García & Pérez-Padilla, 2000) and *ad libitum* feeding are predisposing factors that increase the ascites mortality. The average worldwide incidence of AS was approximately 4.7% (Maxwell & Robertson, 1997). In Mexico, 333,435,192 broilers were raised in 2014 (SIAP, 2014), and therefore, it can be estimated that at least 15,671,454 chickens died due to AS that year.

We hypothesized that grape seed extract may partially replace vitamin E without impairing live performance, increase glutathione peroxidase activity and nitric oxide concentration in plasma, and decrease malondialdehyde concentration in the plasma, lungs, heart, and liver of broilers. Therefore, the aim of this research was to evaluate the effect of partial substitution of VE by GSE on glutathione peroxidase activity and nitric oxide concentration in the plasma, and on lipid peroxidation in the plasma, lungs, heart and liver, live performance and mortality due to ascites in broilers raised at 2278 m of altitude.

MATERIALS AND METHODS

Chickens were raised at 2278 m of altitude (PO_2 of 122 mm Hg; Vázquez-García & Pérez-Padilla, 2000) and fed *ad libitum* (AL) to amplify ascites mortality risk and lipid peroxidation in the plasma, lungs, heart, and liver. The experiment was performed at the poultry facilities of Colegio de Postgraduados, Texcoco, Mexico.

Four hundred and twenty one-d-old male Ross 308 chickens were raised to study the effect of partial substitution of vitamin E (VE) by grape seed extract (GSE) on plasma glutathione peroxidase activity (GPxA) and nitric oxide (NO) concentration; lipid peroxidation, as malondialdehyde (MDA) concentration, in the plasma, lungs, heart, and liver; live performance; and ascites mortality. Chicks were randomly allocated into three dietary treatments (seven replicates per treatment, 20 birds per replicate): Control-AL [basal diet containing 40 IU of VE (dl-α-tocopheryl acetate) and fed *ad libitum*; AL], Control-FR [basal diet containing 40 IU of VE and feed restriction; FR, (feed was offered 16 h d^{-1} starting at d 14 until the end of the experiment)], GSE-AL [basal diet containing 10 mg of GSE (equivalent to 30 IU of VE or 75% of substitution) plus 10 IU of VE and fed AL]. The birds were housed in pens (3 m^2) with wood-shavings litter, each equipped with a feeder and a waterer. Water was offered *ad libitum.* During the entire experimental period, a lighting program of 23 h light, 1 h dark was applied. House temperature during the first week was 32 °C and was reduced in 2 °C per week until the third week. Birds were fed diets based on corn and soybean meal. The starter diet was fed from 1 to 21 d of age, and contained 3025 kcal metabolizable energy (ME) kg^{-1} of diet and 22% crude protein (CP). The finisher diet was fed from 22 to 47 d of age, and contained 3100 kcal ME kg^{-1} of diet and 19% CP. The antioxidant activity of all diets was evaluated.

Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were recorded from d 1 to 47. Ascites mortality was recorded daily and reported as percentage.

Antioxidant activity (AOA) of the diets

Antioxidant activity (AOA) of each diet was measured in triplicate and reported as percent *in-vitro* inhibition of the radical 1, 1-diphenyl-2 picrylhydrazyl (DPPH), according to Brand-Williams *et al.* (1995). Briefly, 1 g of feed was mixed with 10 mL of methanol (Sigma Aldrich) and incubated at 30 °C for 30 min; the mixture was stirred in a vortex every 10 min for 20 s; and then centrifuged at 1342 × *g* for 10 min. The methanol extract was filtered through Whatman number 4 paper. Two hundred µL of the extract were mixed with 3 mL of a methanolic DPPH solution (0.11 mM) and stirred for 10 s. The mixture was kept in the

darkness for 20 min, and the absorbance was read in a spectrophotometer (Thermo Scientific, 10S Vis model) at 515 nm; a blank (without extract) was simultaneously run. The antioxidant activity of the diets was calculated using the following equation:

$$AOA = \frac{\text{absorbance of DPPH without extract - absorbance of DPPH with extract}}{\text{absorbance of DPPH without extract}} \times 100$$

Plasma, heart, liver, and lung samples

At d 46 of age, 14 birds per treatment were randomly selected for blood collection. Three mL of blood were collected per bird in vacuum tubes containing EDTA, and centrifuged at 1000 × *g* for 10 min at 4 °C. Plasma was stored at -80 °C for subsequent analysis of GPxA, and NO and MDA concentrations using commercial kits (Cayman Chemical, Ann Arbor, MI, USA). After addition of the corresponding amount of reactants, the absorbance of the plasma samples was read using a microplate reader (Biotek, Sinergy 2, BioTek Instrument, Inc., model Vermont, USA).

At 47 d of age, 42 chickens (14 birds per treatment selected at random) were humanely sacrificed by cervical dislocation, according to the Mexican Norm NOM-033-ZOO-1995. The lungs, heart, and liver were removed, and tissue samples were collected and stored at -46 °C until lipid peroxidation analysis.

Lipid peroxidation (MDA) analysis of the lungs, heart and liver

Lipid peroxidation as MDA concentration was determined according to the methodology of Buege & Aust (1978). Briefly, 10 g of tissue (lungs, heart and liver; each sample was tested individually in triplicate) were mixed with 30 mL of HPLC-grade water (Sigma Aldrich) and 0.2 mL of butylhydroxytoluene (BHT, Sigma Aldrich, 7.2% m/v). The mixture was homogenized in a mortar and allowed to stand for 15 min in darkness at room temperature. Subsequently, one mL of the sample was taken and mixed with two mL of thiobarbituric acid (TBA, 0.02 M) in 15% trichloroacetic acid (TCA, Sigma Aldrich). The mixture was incubated at 80 °C for one h (Ulu, 2004), kept in a cold place, and centrifuged at 1342 × *g* for 10 min. Finally the supernatant was read in a spectrophotometer (Thermo Scientific, Model 10S Vis, USA) at 530 nm. Lipid peroxidation was expressed as nmol MDA per g tissue.

Statistical analysis

The experimental design was completely randomized with three treatments. The variables BWG, FI, FCR, ascites mortality, GPxA and NO concentration in plasma, and MDA concentration in the plasma, lung, heart, and liver were analyzed by one-way analysis of variance (ANOVA) using the MIXED procedure of SAS v. 9.0 (SAS, 2006). For BWG, FI, FCR, and ascites mortality, the experimental unit was the pen replicate. For GPxA and NO concentration in the plasma, and MDA concentrations in the plasma, lungs, heart, and liver, the experimental unit was one bird.

RESULTS

The grape seed extract diets had lower AOA than the control diets (starter: 11 *vs.* 14; finisher: 11 *vs.* 17% inhibition of DPPH; respectively). There were no significant differences in BWG, FI, or FCR among treatments (Table 1). Ascites mortality was higher ($p<0.05$) in the GSE-AL treatment than in the others, and birds from the Control-FR and Control-AL treatments showed similar ascites mortality (Table 1).

Table 1 – Body weight gain (BWG, g), feed intake (FI, g), feed conversion ratio (FCR), and ascites (AS) mortality (%) of broilers fed vitamin E and grape seed extract (GSE), with *ad libitum* or restricted feeding at 47 d of age.

Treatment	BWG	FI	FCR	AS mortality
Control-AL	3360	6030	1.79	10.71 [b]
Control-FR	3324	5929	1.79	10.71 [b]
GSE-AL	3392	5953	1.76	25.00 [a]
*SEM	47.0	80.0	0.02	3.7
		Significance		
Treatment	0.6034	0.6574	0.5444	0.0198

[a,b] Means with different letter in each column are significantly different ($p<0.05$).

*Standard error of the mean. AL = *Ad libitum*, FR = Feed restriction.

The birds from the GSE-AL treatment had the lowest ($p<0.05$) GPxA, whereas those from the Control-AL treatment had intermediate ($p<0.05$) values and from the Control-FR treatment presented the highest ($p<0.05$) GPxA in the plasma (Table 2).

Table 2 – Glutathione peroxidase activity (GPxA, nmol min^{-1} mL^{-1}); nitric oxide (NO, μM) and malondialdehyde (MDA, μM) concentrations in the plasma of broilers fed vitamin E or grape seed extract (GSE), with *ad libitum* or restricted feeding at 47 d of age.

Treatment	GPxA	NO	MDA
Control-AL	840 [b]	13	6 [b]
Control-FR	1025 [a]	12	5 [b]
GSE-AL	665 [c]	13	8 [a]
*SEM	43.50	0.60	0.40
	Significance		
Treatment	0.0001	0.2261	0.0017

[a,b,c] Means with different letter in each column are significantly different ($p<0.05$).

*Standard error of the mean. AL = *Ad libitum*, FR = Feed restriction.

Nitric oxide concentration in plasma was similar in all treatments (Table 2). The GSE-AL treatment resulted in a higher (p<0.05) MDA concentration than the other treatments, while the plasma MDA concentrations Control-AL and Control-FR birds were not different (Table 2). The lowest (p<0.05) MDA concentrations in the lungs, heart and liver were determined in the Control-FR birds, and the GSE-AL birds presented the highest (p<0.05) MDA concentrations in the heart and the liver (Table 3). The Control-AL treatment resulted in a higher (p<0.05) MDA concentration in the lungs than GSE-AL. The MDA concentrations in the heart and the liver of broilers from the Control-AL treatment was higher compared with those of Control-FR birds, but lower than those observed in the GSE-AL treatment (Table 3).

Table 3 – Lipid peroxidation (nmol MDA g^{-1} tissue) of the lungs, heart, and liver of broilers fed vitamin E or grape seed extract (GSE), with *ad libitum* or restricted feeding at 47 d of age.

Diet	Lungs	Heart	Liver
Control-AL	20 [a]	16 [b]	18 [b]
Control-FR	14 [c]	14 [c]	12 [c]
GSE-AL	17 [b]	19 [a]	22 [a]
*SEM	0.70	0.50	0.50
		Significance	
Treatment	0.0001	0.0001	0.0001

[a,b,c] Means with different letter in each column are significantly different (p<0.05).

*Standard error of the mean. AL = *Ad libitum*, FR = Feed restriction.

DISCUSSION

The results of this research showed that grape seed extract (GSE) may partially replace vitamin E (VE) in broiler diets with no impairment of their live performance. The thyroid gland is the body's primary regulator of metabolism through hormones such as triiodothyronine (T3) and thyroxine (T4), which are essential for the normal development of body organs (Malik & Hodgson, 2002; Peepre *et al.,* 2014). Peepre *et al.* (2014) found that vitamin C, VE, and turmeric extract increase the plasma T3 and T4 concentrations of Wistar rats. Grape seed extract is a concentrated source of polyphenols, which have antioxidant capacity (Brenes *et al.,* 2010). The live performance results observed in the present study may be attributed to the antioxidant capacity of VE and GSE, protecting thyroid hormones from oxidative degradation. There are inconsistent reports on GSE inclusion levels in broiler diets. Brenes *et al.* (2010) did not find any significant differences in broiler body weight gain, feed intake, or feed conversion ratio when GSE was included in the diet (at 0.6, 1.8, and 3.6 g kg^{-1} of diet). On the other hand, Hughes *et al.* (2005) observed a decrease in the feed intake and live weight of broilers fed 30 g GSE kg^{-1} of diet (the GSE product contained 90.2% of total extractable polyphenols); in contrast, the GSE used in the present study contained 85% of total extractable polyphenols, which added at only 10 mg kg^{-1} of diet. Apparently, high dietary GSE supplementation levels impair the live performance of chickens (Hughes *et al.,* 2005; Chamorro *et al.,* 2013). This is probably because polyphenols bind to proteins (Brenes *et al.,* 2010), and as consequence of this interaction, protein digestibility is reduced (Ortiz *et al.,* 1993). In the present study, when GSE was added to the diet (GSE-AL), live performance results were similar to those observed with the Control-AL and Control-FR treatments.

Birds from the GSE-AL treatment presented the highest ascites mortality, the highest MDA concentrations in the plasma, heart, and liver, and the lowest plasma GPxA values. The high altitude and *ad libitum* feeding may have induced the high ascites mortality rate observed with the GSE-AL treatment; which is in agreement with Rodríguez-Ortega *et al.* (2014), who observed that *ad libitum* feeding increased ascites mortality in chickens raised at 2278 m of altitude. High feed intake can trigger ascites mortality because the metabolic rate for feed digestion and absorption increases oxygen requirements at tissue level, as well as mitochondrial production of reactive oxygen species (ROS; Kalmar *et al.,* 2013).

The production of ROS due to *ad libitum* feeding and high altitude explains the low GPxA and high MDA concentration in plasma, lungs, heart, and liver of GSE-AL and Control-AL broilers. We did not find any information in literature on GPxA and lipid peroxidation evaluated in the plasma, lungs, heart, and liver of broilers fed GSE and raised at high altitude. However, Imai *et al.* (1995) found that people residing at high altitude (4000 m) had low levels of glutathione peroxidase activity in the serum. El-Damrawy (2014) reported a decrease in MDA concentration and an increase in glutathione concentration and superoxide dismutase activity in the liver of broilers fed 100 or 200 mg of GSE kg^{-1} of diet and raised under heat stress conditions at low altitude (11 m, approximately). Those GSE levels are greater than those included in this study (100 or 200 mg *vs.* 10 mg kg^{-1} of feed). Therefore, another possible explanation of the low GPxA in the plasma and the high MDA concentrations in the plasma, lungs, heart, and liver of broilers observed in the present study is the low GSE inclusion level applied.

The low AOA observed in the GSE diet compared with the control diets (starter: 11 *vs.* 14; finisher: 11 *vs.* 17% inhibition of DPPH; respectively) may also explain the low plasma GPxA values and the high MDA concentrations in the plasma, lungs, heart, and liver of the broilers fed GSE. The results of this study showed that the GSE-AL treatment did not decrease the MDA concentration in the plasma, lungs, heart and liver of broilers as much as the Control-FR treatment.

Vitamin E functions as a peroxyl radical scavenger that terminates chain reactions of polyunsaturated fatty acid oxidation (Traber, 2007). In addition, feed restriction reduces the metabolic rate (Malan *et al.,* 2003; Singh *et al.,* 2011), and therefore, reduces oxygen requirements for metabolic processes as well as the formation of the $O_2^{\cdot-}$ radical (Kalmar *et al.,* 2013). This could be a possible explanation of the low MDA concentration observed in broilers from the Control-FR treatment. Also, it is possible that less $O_2^{\cdot-}$ radical is converted into hydrogen peroxide (H_2O_2), which could result in higher availability of glutathione peroxidase.

Nitric oxide is a potent vasodilator of the cardiovascular system that has a short half-life, in the order of seconds. Thomas *et al.* (2006) estimated the half-life of extravascular NO in a range of 0.09 to < 2 s. A possibility of the disappearance of NO is its rapid reaction with the $O_2^{\cdot-}$ radical, at an estimated rate of 6.7 x 10^9 M^{-1} s^{-1}, to form peroxynitrite ($ONOO^-$) that, contrary to NO, is a powerful vasoconstrictor (Goldstein & Czapski, 1995). Kojda & Harrison (1999) mentioned that the reaction of NO with the $O_2^{\cdot-}$ radical is approximately three times faster than the dismutation of the $O_2^{\cdot-}$ radical by superoxide dismutase; thus, an increased generation of the $O_2^{\cdot-}$ radical in the vascular wall may inhibit the physiological functions of NO throughout its transformation in $ONOO^-$. This may explain the low and similar concentrations of NO among treatments. Although Bowen *et al.* (2007) reported values as high as 47 and 85 µM, the concentration of NO in this study was kept in a range of 12 to 13 µM. Bautista-Ortega & Ruiz-Feria (2010) observed that arginine supplementation (2.35% kg^{-1} of feed) increased bioavailability of NO in the plasma of broilers; in contrast, the arginine level in our research was 1.5% kg^{-1} in the starter and finisher diets. In addition, these similar dietary arginine levels may explain the lack of NO differences among treatments in the present study.

In conclusion, the evaluated grape seed extract in partial replacement (75%) of vitamin E in the diets of broilers raised at 2278 m of altitude did not negatively affect live performance, although it decreased glutathione peroxidase activity and increased lipid peroxidation in the plasma, heart, and liver.

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