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RESPUESTA A LA COMBINACIÓN DE ARGININA, SELENIO, VITAMINAS E Y C EN POLLOS CON SÍNDROME ASCÍTICO CRIADOS EN EL VALLE DE MÉXICO

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RESPONSE TO THE COMBINATION OF ARGININE, SELENIUM, VITAMINS E AND C IN BROILERS WITH ASCITIC SYNDROME RAISED IN THE VALLEY OF MEXICO

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ABSTRACT

The objectives of this research was to evaluate the effect of occlusion of a primary pulmonary bronchus (BO), feed *ad libitum* (AL) and feed restriction (FR), the combination of arginine (Arg), selenium (Se), vitamins E (vit E) and C (vit C) and the partial substitution of vit E by grape seed extract (GSE) on productive performance, the concentration of nitric oxide (NO), glutathione peroxidase activity (GPx) in plasma, antioxidant activity (AOA) and lipid oxidation measured as concentration of malondialdehyde (MDA) in the heart, lung and liver of broilers raised to 2278 m of altitude (Experiments 1, 2 and 3). The results in the first experiment showed that BO and LA increased ascites mortality, while FR improved AOA in the heart, lung and liver of broilers chickens, OB is not necessary to induce ascites in broiler chickens 2278 m of altitude. In the second experiment, supplementation with Arg, Se, vit E and vit C increased AOA in the lung, heart and liver. Feed restriction decreased the concentration of MDA in the lung and liver. In the third experiment the GSE could partially replace vit E in diets for broilers without affecting the productive performance; however, more research is required to find the optimal level of GSE inclusion in the broilers' diet that may decrease ascites mortality, concentrations MDA in plasma, lungs, heart and liver, and increase GPx and NO concentration in plasma of broilers raised at 2278 m of altitude.

Key words: arginine, grape seed extract, occlusion of a pulmonary bronchus, selenium, vitamins E and C.

RESPUESTA A LA COMBINACIÓN DE ARGININA, SELENIO, VITAMINAS E Y C EN POLLOS CON SÍNDROME ASCÍTICO CRIADOS EN EL VALLE DE MÉXICO

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RESUMEN

Los objetivos de esta investigación fueron evaluar el efecto de la oclusión de un bronquio pulmonar primario (OB), la alimentación *ad libitum* (AL) y la restricción alimenticia (RA), la combinación de arginina (Arg), selenio (Se), vitaminas E (vit E) y C (vit C), y la sustitución parcial de vit E por extracto de semilla de uva (ESU) sobre el comportamiento productivo, la concentración de óxido nítrico (NO), actividad de la glutatión peroxidasa (GPx) en el plasma, la actividad antioxidante (AAO) y la oxidación lipídica medida como concentración de malondialdehido (MDA) en el corazón, pulmón e hígado de pollos de engorda criados a 2278 m de altitud. Los resultados en el primer experimento demostraron que la OB y la AL incrementaron la mortalidad de ascitis, mientras que la RA mejoró la AAO en el corazón, pulmón e hígado de pollos de engorda, la OB no es necesaria para inducir ascitis en pollos criaos a 2278 m de altitud. En el segundo experimento la suplementación con Arg, Se, vit E y vit C incrementaron la AAO en el pulmón, corazón e hígado. La restricción de alimento disminuyó la concentración de MDA en el pulmón e hígado. La hipoxia hipobárica y la alimentación AL tuvieron un efecto aditivo que disminuyó GPx e incremento la concentración de MDA en el plasma de pollos alimentados con a dieta control. En el tercer experimento el ESU pudo parcialmente reemplazar la vit E en dietas para pollos sin afectar el comportamiento productivo, sin embargo es necesaria más investigación para encontrar la concentración óptima de ESU que pueda disminuir la mortalidad de ascitis, la concentración de MDA en plasma, pulmón, corazón e hígado e incrementar la GPx y la concentración de NO.

Palabras clave: arginina, extracto de semillas de la uva, oclusión de un bronquio pulmonar, selenio, vitaminas E y C.

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CONTENT

LIST OF TABLES

CHAPTER I. ANTIOXIDANT ACTIVITY IN THE HEART, LUNGS AND LIVER OF BROILER RAISED AT 2278 m ALTITUDE WITH SIMULTANEOUS BRONCHUS OCCLUSION AND FEED *Ad libitum*

CHAPTER II. SUPPLEMENTATION OF ARGININE, SELENIUM AND VITAMINS E AND C INCREASED THE ANTIOXIDANT ACTIVITY IN BROILERS RAISED AT 2278 m OF ALTITUDE

CHAPTER III. LIPID PEROXIDATION IN PLASMA, LUNGS, HEART AND LIVER OF BROILERS FED A GRAPE SEED EXTRACT AND RAISED AT 2278 m OF ALTITUDE

LIST OF FIGURES

CHAPTER I. ANTIOXIDANT ACTIVITY IN THE HEART, LUNGS AND LIVER OF BROILER RAISED AT 2278 M ALTITUDE WITH SIMULTANEOUS BRONCHUS OCCLUSION AND FEED *Ad libitum*

CHAPTER II. SUPPLEMENTATION OF ARGININE, SELENIUM AND VITAMINS E AND C INCREASED THE ANTIOXIDANT ACTIVITY IN BROILERS RAISED AT 2278 M OF ALTITUDE

INTRODUCCIÓN GENERAL

Wideman *et al.,* (2013) definen la hipoxia como la menor presión parcial de oxígeno en el aire inspirado, y a la hipoxemia como la menor PO_2 de oxígeno en la sangre. Kalmar *et al.,* (2013) indican que la etiología central del síndrome ascítico, es un estado de hipoxemia. La elevada altitud sobre el nivel del mar reduce la presión parcial de oxígeno (PO_2) ocasionando hipoxia hipobárica (Wideman *et al.,* 2013). El Municipio de Texcoco, Estado de México tiene una altitud de 2278 m, con una presión atmosférica de 581.1 mm Hg y una PO_2 de 122 mm Hg (Vázquez y Pérez, 2000). Wideman *et al.* (1997) observaron que la oclusión de un bronquio primario es un método experimental efectivo para generar ascitis en los pollos de engorda, de forma inmediata ocasiona hipertensión pulmonar, hipoxemia, hipercapnia (aumento de la presión parcial de dióxido de carbono en sangre), acidosis (disminución del pH sanguíneo), incrementa la relación peso ventricular derecho entre peso ventricular total (RV:TV). Julian y Mirsalimi (1992) reportan que el incremento de la demanda de oxigeno generado por el rápido crecimiento ocasiona hipoxemia, porque la digestión y el metabolismo tienen un alto requerimiento de oxígeno (la alimentación *ad libitum* incrementa la tasa metabólica). Cuando la demanda de oxigeno aumenta, incrementa la frecuencia cardiaca y el gasto cardiaco, lo cual incrementa el flujo de sangre a través del pulmón y la presión requerida para forzar la sangre a través de las arteriolas y capilares del pulmón. El objetivo del primer experimento fue evaluar el efecto de la oclusión de un bronquio pulmonar, la alimentación *ad libitum* y la hipoxia hipobárica causada por la elevada altitud en la actividad antioxidante y oxidación lipídica medida como concentración de MDA en pollos criados a 2278 m de altitud.

Bottje y Wideman (1995) mencionan que el estrés oxidativo está asociado con el síndrome ascítico (SA). Bakonyi y Radak (2004) reportaron que la exposición a elevada altitud incrementa

la formación de especies reactivas de oxígeno (radical superóxido) y de especies reactivas de nitrógeno (peroxinitrito; $ONOO^-$). La hipoxia genera vasoconstricción de la arteria pulmonar lo que conduce a hipertensión pulmonar (Basnyat, 2005). Belik *et al.* (2010) observaron que el radical $ONOO^-$ es un vasoconstrictor responsable de la hipertensión pulmonar en ratas expuestas a hipoxia crónica.

La L-arginina es el substrato de la enzima endotelial óxido nítrico sintasa (eNOS), una enzima que sintetiza óxido nítrico (NO), un potente vasodilatador pulmonar (McConell, 2007). Ruiz-Feria (2009) observó que la suplementación de 2.2% de L-arginina 240 UI de vitamina E y 500 mg de vitamina C kg^{-1} de alimento en pollos de engorda desafiados con fenilefrina incrementó los niveles de óxido nítrico (NO) en el plasma.

La vitamina E (α-tocoferol) actúa como un antioxidante evitando la oxidación de los ácidos poliinsaturados o lipoproteínas de las células endoteliales. Reacciona donando un H del carbono 6 a un radical peroxil (ROO), sin embargo al evitar la oxidación, esta vitamina (α-tocoferol) queda en estado oxidado como radical libre de menor impacto (α-tocoferoxil; Combs, 2008). Mientras que la vitamina C tiene la capacidad de reciclar la vitamina E, reducir el α-tocoferoxil y convertirlo nuevamente en α-tocoferol (Combs, 2008). El selenio (Se) es parte de la enzima glutatión peroxidasa. La enzima Glutatión Peroxidas (GPx) tiene Se cómo un componente esencial (Combs y Gray, 1998). La GPx participa de forma importante como antioxidante, cataliza la reducción de H_2O_2 o hidroperóxidos orgánicos a agua. Por otra parte, Sies y Arteel (2000) mencionan que la GPx puede reducir el peroxinitrito (vasonstrictor) a nitrito usando glutatión (GSH). El objetivo del segundo experimento fue evaluar la combinación de L-arginina, selenio, vitaminas E y C en la actividad antioxidante y concentración de MDA en el corazón, pulmón e hígado de pollos criados a 2278 m de altitud.

La vitamina E es reconocida no solo como antioxidante natural para la prevención de la oxidación biológica, sino también como un nutriente esencial para disminuir la mortalidad debida a síndrome ascítico (Bottje *et al.,* 1995). Sin embargo, la adición de altas concentraciones de vitamina E aumenta el costo de la dieta (Kennedy *et al.,* 1992), esto justifica la búsqueda de nuevas fuentes de antioxidantes como el extracto de semilla de uva (ESU). El extracto de semilla de uva tiene un efecto antioxidante debido a su contenido de compuestos polifenólicos (Brenes *et al.,* 2010), tales como procianidinas, catequinas, epicatequinas, gallocatequinas y epigallocatequinas (Chamorro *et al.,* 2013), sin embargo, ha sido poco evaluado en la alimentación de los pollos de engorda. El objetivo del tercer experimento fue evaluar la sustitución parcial de vitamina E por extracto de semilla de uva sobre el comportamiento productivo, la actividad antioxidante y la oxidación lipídica medida como concentración de malondialdehido en el corazón, pulmón e hígado de pollos de engorda criados a 2278 m de altitud.

REFERENCIAS

Bakonyi, T., and Z. Radak. 2004. High altitude and free radicals. J. Sports Sci. Med. 3: 64-69.

Basnyat, B. 2005. High Altitud cerebral and pulmonary edema. Travel Med. Infect. Dis. 3: 199-211.

Belik, J., D. Stevens, J. Pan, B. A. S. McIntyre, C. Kantores, J. Ivanovska, E. Z. Xu, C. Ibrahim, B. K. Panama, P. H. Backx, P. J. McNamara, R. P. Jankov Pulmonary vascular and cardiac effects of peroxynitrite decomposition in newborn rats. 2010. Free Radic. Biol. Med. 49: 1306-1314.

Bottje, W., B. Enkvetchakul, and R. More. 1995 Effect of α-tocopherol on antioxidants, lipid peroxidation, and the incidence of pulmonary hypertension syndrome (ascites) in broilers. Poult. Sci. 74: 1356-1369.

Bottje, W. G., and R. F. Wideman. 1995. Potential role of free radicals in the pathogenesis of pulmonary hypertension syndrome. Poult. Avian Biol. Rev. 6:221–231.

Brenes, A, A. Viveros, I. Goñi, C. Centeno, F. Saura-Calixto, and I. Arija. 2010. Effect of grape seed extract on growth performance, protein and polyphenol digestibilities, and antioxidant activity in chickens. Span. J. Agric. Res 8(2): 326-333.

Chamorro S, Viveros A, Centeno C, Romero C, Arija I, Brenes A. Effects of dietary grape seed extract on growth performance, amino acid digestibility and plasma lipids and mineral content in broiler chicks. Animal 2013; 7(4): 555-561.

Combs, G. F. 2008. The vitamins Fundamental Aspects in Nutrition and Health. Third Edition. ISBN 13: 978-0-12-183493-7.

Combs, G. F. Jr, and W. P. Gray. 1998. Chemopreventive agents: selenium. Pharmacol. Ther. 79 (3): 179-192.

Julian, R. J., and S. M. Mirsalimi. 1992. Blood oxygen concentration of fast-growing and slow-growing broiler chickens, and chickens with ascites from right ventricular failure. Avian Dis. 36:730-732.

Kalmar, I. D., D. Vanrompay, and G. P. J. Janseens. 2013. Broiler ascites syndrome: collateral damage from efficient feed to meat conversion. Vet. J.; 197:169-174.

McConell, G. K. 2007. Effects of L-arginine supplementation on exercise metabolism. Curr. Opin. Clin. Nutr. Metab. Care. 10:46-51.

Ruiz-Feria, C. A. 2009. Concurrent supplementation of arginine, vitamin E, and vitamin C improve cardiopulmonary performance in broilers chickens. Poult. Sci. 88: 526-35.

Sies, H., and Arteel G. E. 2000. Interaction of peroxynitrite with selenoproteins and glutathione peroxidase mimics. Free Radical Biology & Medicine, (28) 10: 1451-1455.

Vázquez-García, J. C., and R. Pérez-Padilla. 2000. Valores gasométricos estimados para las principales poblaciones y sitios a mayor altitud en México. Rev. Inst. Nal. Enf. Resp. Mex. 13(1): 06-13.

Wideman, R. F., Y. K. Kirby, M. Ismail, W. G. Bottje, R. W. Moore, and R. C. Vardeman. 1995. Supplemental L-arginine attenuates pulmonary hypertension syndrome (ascites) in broilers. Poult. Sci. 74: 323-330.

Wideman R. F., Y. K. Kirby, R. L. Owen, and H. French. 1997. Chronic unilateral occlusion of an extrapulmonary primary bronchus induces pulmonary hypertension syndrome (ascites) in male and female broilers. Poultry Science 76: 400-404.

Wideman, R. F., D. D. Rhoads, G. F. Erf , and N. B. Anthony. 2013. Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. Poult. Sci. 92:64-83.

CHAPTER I. ANTIOXIDANT ACTIVITY IN THE HEART, LUNGS AND LIVER OF BROILER RAISED AT 2278 m ALTITUDE WITH SIMULTANEOUS BRONCHUS OCCLUSION AND FEED *Ad libitum*

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ABSTRACT

The effect of occlusion of a primary bronchus (BO), and *ad libitum* feeding (AL) or feed restriction (feed offered 12 h/day; FR) in the antioxidant activity (AOA) of the heart, lungs and liver, and ascites related variables, were evaluated in male broilers chickens reared at 2278 m above sea level. Chickens (Ross 308; n = 85) were subjected to BO or not (noBO), and were fed on an *ad libitum* basis (AL) or FR (2 × 2 factorial). The left extrapulmonary bronchus was surgically occluded at d 21 in 43 chickens and the rest remained intact. Hematocrit (Hct%) was measured 16 d after occlusion (10 birds/treatment). Chickens were euthanized at d 37, the heart was dissected to calculate the right ventricular weight: total ventricular weight ratio (RV:TV), and samples from the heart, lung and liver tissue were collected and stored at -46 °C until assay for *in vitro* AOA. The interaction feeding × occlusion was significant ($P \leq 0.05$) in the Hct%, RV:TV, and AOA. The noBO-FR chickens had the highest AOA in the heart (78.3 ± 0.6), lung (63.6 ± 0.6) and liver (83.2 ± 0.8). The lower AOA in the heart (43.6 ± 0.6) and lung (31.1 ± 0.6) was observed in noBO-AL, and in the liver (43.3 ± 0.8) of birds in BO-AL. Birds in the BO-AL had the highest ascites mortality (52%), followed by birds in the noBO-AL (29%), BO-FR group (18%) and noBO-FR group (5%). The Hct% was lower in noBO-FR (37.0 ± 1.4) than in BO-FR (45.1 ± 1.4) or BO-AL (43.9 ± 1.4) birds. The RV:TV ratio was lowest in noBO-FR birds (0.22 ± 0.02) without difference among the other treatments (0.30, 0.33, and 0.33 ± 0.02 for BO-FR, BO-AL, and noBO-AL, respectively). The results of this experiment demonstrated that BO and AL decreased AOA in the liver, lung and heart. The AL increased ascites mortality, whereas FR improved the AOA in the studied organs and decreased ascites mortality. Occlusion of a bronchus is not necessary to induce ascites in chickens reared at 2278 m of altitude.

Key words: Occlusion of a primary bronchus, ascites syndrome, lipid oxidation, feed restriction

RESUMEN

Fue evaluado el efecto de la oclusión de un bronquio primario (OB), y la alimentación *ad libitum* (AL) o restricción alimenticia (alimento ofrecido 12 h/d, RA) sobre la actividad antioxidante (AAO) del corazón, pulmones e hígado, y variables relacionadas a ascitis en pollos de engorda machos criados a 2278 m sobre el nivel del mar. Pollos (Ross 308, n=85) fueron sujetos a OB o no (noOB), y fueron alimentados con una dieta basal *ad libitum* o RA (2 × 2 factorial). El bronquio extra pulmonar izquierdo fue ocluido quirúrgicamente al día 21 en 43 pollos y el resto permaneció intacto. Hematocrito (Hct%) fue medido a día 16 después de la oclusión (10 aves/tratamiento). Los pollos fueron eutanizados al día 37, el corazón fue diseccionado para calcular el peso ventricular derecho: peso ventricular total (RV:TV), y las muestras de tejido del corazón, pulmón e hígado fueron colectadas y almacenadas a -46°C hasta su análisis *in vitro* de AAO. La interacción alimentación × oclusión fue significativa ($P \leq 0.05$) en el Hct%, RV:TV y AAO. Los pollos noOB tuvieron la mayor AAO en el corazón (78.3± 0.6), pulmón (63.6 ± 0.6) e hígado (83.2 ± 0.8). La menor AAO en el corazón (43.6 ± 0.6) y pulmón (31.1 ± 0.6) fue observada en noOB-AL y en el hígado (43.3 ± 0.8) en las a ves BO-AL. Las aves OB-AL tuvieron la mayor mortalidad de ascitis (52%), seguidas por las aves en el noOB-AL (29%), OB-RA (18%) y noOB-RA (5%). El Hct% fue menor en noOB-RA (37.0 ± 1.4) que in OB-RA (45.1 ± 1.4) o OB-AL (43.9 ± 1.4) aves. El RV:TV fue menor en las aves noOB-RA (0.22 ± 0.02) sin diferencia entre los otros tratamientos (0.30, 0.33, and 0.33 ± 0.02 para OB-RA, OB-AL, y noOB-AL, respectivamente). Los resultados de este experimento demostraron que la OB y AL disminuyeron la AAO en el hígado, pulmón y corazón. La AL incrementó la mortalidad de ascitis, mientras que la RA mejoró la AAO en los órganos estudiados y disminuyó la mortalidad de ascitis. La OB no es necesaria para inducir ascitis en pollos criados a 2278 m de altitud

Palabras clave: Oclusión de un bronquio primario, síndrome ascítico, oxidación lipídica.

INTRODUCTION

Pulmonary arterial hypertension (PAH), pulmonary hypertension syndrome (PHS), and ascites syndrome (AS) are commonly used synonymously (Wideman et al., 2013). The PHS is a metabolic disease caused by a wide variety of factors (e. g. high altitude, cold temperature, fast-growth, *ad libitum* feeding). The central etiology of PHS is a hypoxemic condition resulting from an imbalance between demand and supply of oxygen (Kalmar et al, 2013); caused by pulmonary vasoconstriction (Ruiz-Feria and Wideman, 2001) leading to an increased hematocrit (Julian, 2000), pulmonary hypertension, right ventricular hypertrophy (Kalmar et al., 2013) and accumulation of fluid in the abdominal cavity (Wideman et al., 2013). High altitude is a powerful predisposing factor for the incidence of PHS (Owen et al., 1995). At high altitudes the partial pressure of oxygen (PO_2) is low, generating tissue hypoxia (Maiti et al., 2006). Reduced levels of inspired O_2 trigger acute pulmonary vasoconstriction and pulmonary hypertension in broilers (Ruiz-Feria and Wideman, 2001). The Municipality of Texcoco, State of Mexico, is located at 2278 m above sea level, with an atmospheric pressure of 581.1 mm Hg and a PO_2 of 122 mm Hg (Vázquez and Pérez, 2000). Thus, hypoxia is a key environmental stressor that contributes significantly to the incidences of PHS when broilers are reared the high altitudes of Mexico State. Paddenberg et al. (2003) demonstrated that hypoxia induces the generation of reactive oxygen species (ROS) by cells of the pulmonary vasculature. Oxidative stress due to increased production of ROS has been implicated in the pathophysiology of PHS (Bowers et al., 2004; Nain et al., 2008). Hypobaric hypoxia, the occlusion of a primary bronchus (BO), or the occlusion of a pulmonary artery are effective methods to induce PHS (Wideman et al. 1997; Bautista-Ortega and Ruiz-Feria, 2012).

The combined effect of environmental hypoxia caused by high altitude, the occlusion of a primary bronchus and *ad libitum* feeding (AL) have not been evaluated regarding PHS incidence and their effect on the antioxidant activity (AOA) in the heart, lung and liver in broilers raised to 2278 m above sea level.

We hypothesized that under the high altitude conditions prevailing in Texcoco, State of Mexico, the occlusion of primary bronchus and access to feed *ad libitum* would increase the mortality due to PHS, hematocrit (Hct%), right ventricular weight: total ventricular weight ratio (RV:TV) and to reduction of the AOA in the heart, lung and liver tissue**.**

MATERIALS AND METHODS

Animals and management

One-day-old male Ross 308 broiler chicks (n=100) were housed in 4 pens (3 m x 1 m) with clean wood shaving litter. The chicks were brooded conventionally with temperature starting at 32°C and decreasing 2°C weekly until week 3, under a constant lighting program; incandescent light was used to provide 23 h of light and 1 h darkness per day throughout entire experimental period. All birds were fed a pelletized diet containing 3,100 kcal of ME/kg of feed and 21% CP, formulated to meet or exceed the requirements for broilers specified by the NRC (1994), water was provided *ad libitum*. Chickens were grown at an altitude of 2278 m (7473.75 ft) above sea level (PO_2 of 122 mm Hg; Vázquez and Pérez, 2000) to amplify the incidence of PHS.

Treatments

The treatment arrangement was a 2 x 2 factorial design. At day 21, chickens had an extrapulmonary bronchus surgically occluded (BO) or remained intact (noBO), and were offered feed continuously (AL) or were offered feed 12 h / d (feeder was removed from 8 pm to 8 am;

FR). The four treatments were: BO-FR, n = 22; BO-AL, n = 21; noBO-FR, n = 21; noBO-AL, n = 21 chickens.

Occlusion of a primary bronchus

The primary bronchus of chickens was occluded according to the method described by Wideman *et al*. (1996). Briefly, birds were anesthetized to a surgical plane with intramuscular injections of a 1:1 mixture of ketamine HCl (Anesket® 100 mg/mL; Laboratorio PISA, S. A. de C. V. Guadalajara Jalisco, México) and xylazine (Andozine® 100 mg/mL, Laboratorio ANDOCI, S. A. México), at a dose of 0.01 mL of the mix / 100 g of BW (Harvey et al., 1985). Anesthetized chickens were fastened in a supine position with the neck extended. Feathers of the thoracic inlet were removed, and the skin was swabbed with iodine (YODO-VET 5; Laboratorios AGRO-VET S. A. de C. V. México). Lidocaine (Pisaina® 2%; Laboratorio PISA, S. A. de C. V. Guadalajara Jalisco, México) was infiltrated subcutaneously along the midline of the thoracic inlet as a supplemental local anesthetic. A midline incision was made, the crop was retracted, and the left extra-pulmonary bronchus was located and clamped (completely occluded) with a silver vascular clip fashioned from 0.50 mm diameter silver wire (World Precision Instruments, Sarasota, FL). The incision was closed with stainless steel surgical wound clips and sprayed with a topical antibacterial powder (TOPAZONE®, Laboratorio PISA, S. A. de C. V. Guadalajara Jalisco, México). The chickens were placed under a heat lamp for up to 2 h to recover from anesthesia, then returned to their pens.

***Hematocrit and RV:TV* ratio**

At 10 and 16 d after occlusion (31 and 37 d of age) 3 mL samples of blood were collected in EDTA tubes from 10 chicks per treatment. The samples were centrifuged at 1000 × g for 10 min and the percentage of hematocrit (Hct%) was measured. Thirty-seven-d-old chickens were

weighed (BW), euthanized by cervical dislocation and subsequently the heart, lungs and liver, were removed and weighed. The weight of these organs was expressed as relative weight (percentage of body weight). The heart was dissected to determine the right ventricle weight: total ventricle weight ratio (RV:TV). The organs were stored at -46 °C to measure the lipid oxidation by malondialdehyde (MDA) and antioxidant activity by inhibition of the radical 1,1-diphenyl-2-picrylhydrazyl (AOA).

Relative weight of internal organs

The relative weight of the heart, lung (weight of both lungs) and liver was calculated in the same manner as Bautista-Ortega and Ruiz-Feria (2012), that is: weight of each organ divided by the corresponding live weight of each chicken multiplied by 100.

***Lipid oxidation* (MDA)**

The method of thiobarbituric-2 acid is the test most often used to measure the degree of oxidation of lipids in tissues and foods (Karatas et al., 2002). This method is based on the reaction of two molecules of thiobarbituric acid with one molecule of malondialdehyde (MDA) and the probable removal of two water molecules, generating a red pigment (Sinnhuber et al., 1958). Lipid oxidation of organs is proportional to the concentration of MDA (Buege and Aust (1978) in the tissue (MDA nmol per g of tissue). Each organ was tested in triplicate. The liver (20 g) was mixed with 15 mL of HPLC grade water and 0.2 mL of butylhydroxytoluene 7.2% (BHT, Sigma Aldrich). Samples of lung or heart (4 g each) were mixed with 3 mL of HPLC grade water and 0.2 mL of BHT 7.2%. Each sample was homogenized in a mortar and allowed to stand for 15 min in a dark place at room temperature. Subsequently, the homogenized samples of liver (2 mL), heart (1 mL) or lung (1 mL) were mixed with 2 mL of thiobarbituric acid (TBA, 0.02 M) in trichloroacetic acid (TCA, Sigma Aldrich, 15%). 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) at 530 nm, lipid peroxidation was expressed as nmol MDA per g tissue.

***Antioxidant activity* (AOA)**

The antioxidant activity (AOA) of an organ is its ability to accept free radicals (Asghar *et al.,* 1990). The technique of Brand-Williams *et al.* (1995) was used to measure the antioxidant activity in heart, lungs and liver of the chickens. This technique is based on the inhibition of the DPPH radical (1,1-diphenyl-2-picrylhydrazyl), where the greater the percentage of inhibition of DPPH, the higher the antioxidant activity in the samples (Molyneux, 2004). Each organ was tested in triplicate as follows: liver (5 g), lung (1 g) and heart (1 g) tissue were mixed with 5 mL of methanol (Sigma Aldrich). The tissue was triturated in a mortar, incubated for 30 min at 30 °C, and stirred in a vortex every 10 min for 20 s. After incubation, the samples were centrifuged at 1342 × g for 10 min and filtered through a Whatman No. 4 filter. Filtered aliquots of liver (20 µL), lung (100 µL) or heart (180 µL) were mixed with 500 µL of methanol plus 1.5 mL of methanolic DPPH solution (0.11 mM), stirred vigorously for 10 s and then allowed to stand in darkness room at room temperature for 20 min. Finally, the absorbance was read in a spectrophotometer (Thermo Scientific, model 10S VIS) at 515 nm. The antioxidant activity of the diets was calculated using the following equation:

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

Statistical analysis

The Hct%, RV:TV, relative organ weight, antioxidant activity and oxidative damage data were analyzed as a completely randomized two-way ANOVA and the means were compared with the

Tukey test ($P \leq 0.05$) using the GLM procedure of SAS 9.0 (2006). The individual chicken was the experimental unit. Ascites mortality was analyzed by logistic regression, and means were compared with the following orthogonal contrasts: BO-FR *vs* noBO-AL; BO-AL *vs* noBO-FR; noBO-FRT x 3 *vs* BO-FR + BO-AL + noBO-AL. The following analyses of Pearson coefficients of correlation were performed: Hct% at 16 d after BO and RV:TV (r= 0.41054), AOA and MAD in the heart tissue (r= -0.5427), and liver tissue (r= -0.51074).

RESULTS

Hematocrit and RV:TV ratio

The Feeding × Occlusion interaction was significant ($P < 0.05$) for Htc% and RV:TV (Table 1). The noBO-FR birds had the lowest Hct% 10 d after BO, and the lowest RV:TV ratio of all treatments. The Hct% at 10 and 16 d after BO and the RV:TV ratio was higher in BO-FR and BO-AL than in noBO-FR chickens. At 16 d after BO, chickens from the noBO-AL treatment also showed higher Hct% than those from the noBO-FR. The noBO-FR birds had the lowest RV:TV ratio. Significant correlations (r = 0.41054) were observed between Hct% at 16 d after BO and RV:TV (when increasing Hct% increases the RV:TV).

Table 1. Percentage of hematocrit (Hct%) at 10 and 16 d after a primary bronchus occlusion (31 and 37 d of age), right ventricle weight between to total ventricular weight ratio (RV:TV) 37 d.

Treatment	Hct (%), Day 31	Hct (%), Day 37	RV:TV
BO-FR	46.1 a	45.1 a	0.30 a
BO-AL	42.2 ab	43.9 a	0.33 a
noBO-FR	33.1 c	37.0 b	0.22 b
noBO-AL	40.0 b	42.0 ab	0.33 a
* S. E.	1.414	1.364	0.017
	P-value		
Feeding	0.3024	0.1697	0.0002
Occlusion	<0.0001	0.0007	0.0202
Feeding × Occlusion	0.0005	0.0305	0.0383

Means in columns with different letter are statistically different (Tukey, $P < 0.05$). *S. E. = standard error. Feeding is the first factor with two levels: *feed ad libitum*; AL or feed restriction, feed was offered 12 h/day; FR, and occlusion is the second factor with two levels: occlusion of a primary bronchus; BO or without occlusion of a primary bronchus; noBO. Hct (%), Day 31: Percentage of hematocrit (Hct%) at 31 d of age or 10 d after occlusion of a primary bronchus. Hct (%), Day 37: Percentage of hematocrit (Hct%) at 37 d of age or 16 d after occlusion of a primary bronchus. RV:TV: right ventricle weight between to total ventricular weight ratio evaluated at d 37 age or 16 d after occlusion of a primary bronchus.

Body weight, PHS mortality and relative organ weight

The feeding × occlusion interaction was significant ($P < 0.05$) in BW and relative liver weight (Table 2). The noBO-AL birds were the heaviest, the BO-FR birds weighed the less, whereas

chickens from the BO-AL and noBO-FR treatments had intermediate and similar BW. The relative liver weight was lower in BO-FR chickens than in BO-AL chickens, but the relative liver weight of noBO chickens was not different from that of the BO-FR or BO-AL chickens. The relative weight of the heart and lung was not affected by treatment (data not shown). The noBO-FR chickens had the lowest PHS mortality (5%) compared to chickens in the other treatments (18, 52 and 29% for BO-FR, BO-AL, and noBO-AL, respectively). The BO-AL chickens had significantly higher PHS mortality than the noBO-FR chickens. However, PHS mortality was not different between BO-FR and noBO-AL chickens (Table 2).

Table 2. Body weight (BW), relative liver weight (liver, %) and mortality due to PHS.

Treatment	BW (g)	Liver (%)	PHS mortality (%)
BO-FR	1301 c	2.35 b	18(4/22)
BO-AL	1872 b	2.78 a	52 (11/21)
noBO-FR	1877 b	2.56 ab	5 (1/21)
noBO-AL	2121 a	2.51 ab	29 (6/21)
*S. E.	44	0.085	0.57
	P-value		
Feeding	<0.0001	<0.0001	0.0101
Occlusion	<0.0001	<0.0001	0.0887
Feeding × Occlusion	0.0119	0.0001	0.5494

The interaction (Feeding × Occlusion) was ($P < 0.05$) significant in Body weight (BW), relative liver weight (liver, %). Means with different letter are statistically different (Tukey, $P < 0.05$). Feeding is the first factor with two levels: *feed ad libitum*; AL or feed restriction, feed was offered 12 h/day; FR and occlusion is the second factor with two levels: occlusion of a primary

bronchus; BO or without occlusion of a primary bronchus; noBO. *S. E. = standard error. BW: Body weight was evaluated at 37 d age or16 d after occlusion of a primary bronchus. Liver, %: relative liver weight was evaluated at 37 d age or16 d after occlusion of a primary bronchus. PHS mortality (%): chickens deaths due to PHS divided by total birds in each treatment multiplied by 100. The contrasts and significance for mortality were: BO-FR *vs* noBO-AL it was not significant (P = 0.8589), BO-AL *vs* noBO-FR it was significant (P = 0.0119), noBO-FR *vs* BO-FR BO-AL noBO-AL it was significant (P = 0.0514).

Lipid oxidation (MDA)

The Feeding x Occlusion interaction was significant ($P < 0.05$) for lipid oxidation in heart and lung tissue (Figures 1). The noBO-AL birds had the highest heart lipid oxidation whereas the BO-AL and noBO-FR had the lowest lipid oxidation, and the BO-FR had intermediate levels of lipid oxidation (Figure 1). On the other hand, BO-FR birds had the highest lung lipid oxidation, whereas chickens in the other treatments had comparable levels of lung lipid oxidation (Figure 1). Liver lipid oxidation was higher in BO birds than in noBO birds (6.2 *vs* 5.5 ± 0.1904 nmol of MDA g^{-1} tissue), and higher in AL birds than in FR birds (7.7 *vs* 4.0 ± 0.1904 nmol of MDA g^{-1} tissue).

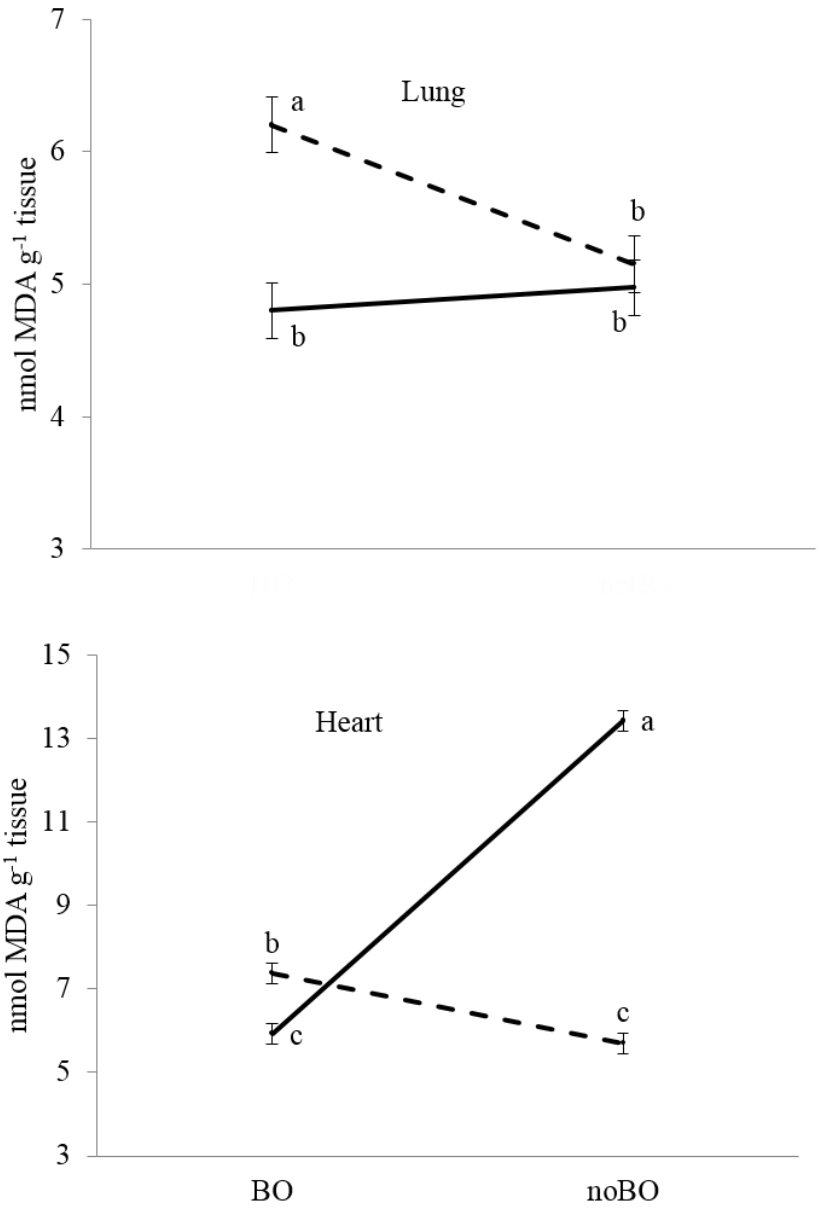

Figure 1. Lipid oxidation *in vitro* in the heart and lungs (nmol MDA g^{-1} tissue) of broilers (37 d age or 16 d after occlusion) raised to 2278 m altitude distributed in four treatments (2x2 factorial). Feeding is the first factor with two levels: feed ad libitum; AL (—) or feed restriction, feed was offered 12 h/day; FR (---) and occlusion is the second factor with two levels: occlusion of a primary bronchus; BO (21 d old) or without occlusion of a primary bronchus; noBO. The

interaction (Feeding × Occlusion) was ($P < 0.05$) significant. Means with different letter are statistically different (Tukey, $P < 0.05$).

***Antioxidant activity (*AOA)**

The Feeding × Occlusion interaction was significant ($P < 0.05$) for antioxidant activity in all tissues (AOA; Figure 2). The highest AOA in the heart, lung and liver was consistently observed in the noBO-FR chickens compared with the organ AOA of chickens in the other treatments. The noBO-AL chickens had the lowest AOA in the heart and the lungs. In the heart, the BO-AL broilers showed lower AOA than BO-FR. In the lung BO-FR had lower AOA than BO-AL broilers. The BO-AL broilers had the lowest liver AOA whereas the noBO-FR chickens had the highest liver AOA. Birds in the BO-FR and in the noBO-AL groups had intermediate and similar liver AOA values (Figure 2). Significant correlations (r = -0.54527 and -0.51074) were observed between AOA and MAD of the heart, and liver.

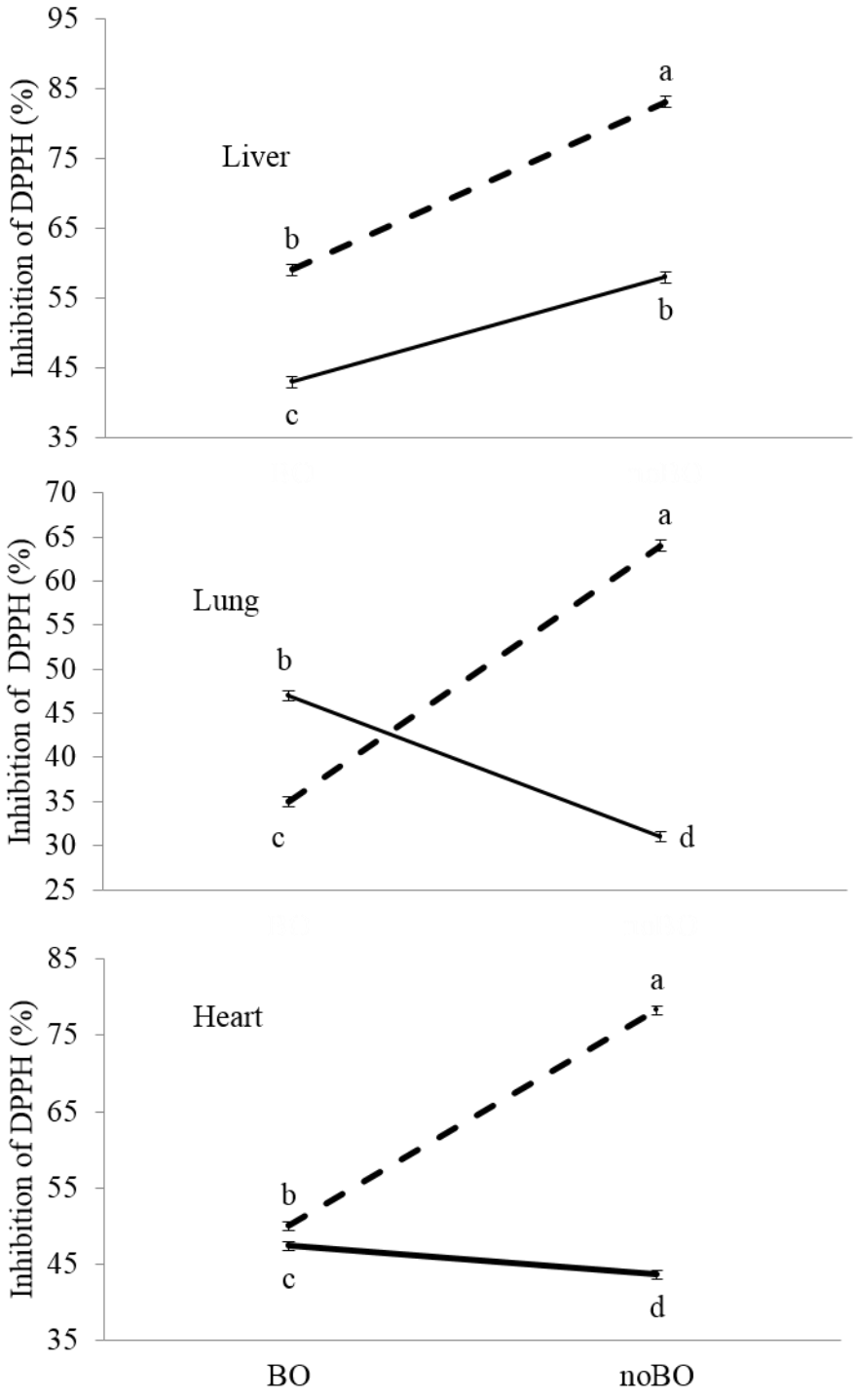

Figure 2. Antioxidant activity *in vitro* in the liver, lung and heart (inhibition of DPPH, %) of broilers (37 d age or 16 d after occlusion) raised to 2278 m altitude distributed in four treatments (2x2 factorial). Feeding is the first factor with two levels: feed ad libitum; AL (—) or feed

restriction, feed was offered 12 h/day; FR (---) and occlusion is the second factor with two levels: occlusion of a primary bronchus; BO (21 d old) or without occlusion of a primary bronchus; noBO. The interaction (Feeding × Occlusion) was ($P < 0.05$) significant. Means with different letter are statistically different (Tukey, $P < 0.05$).

DISCUSSION

In the present study, male broilers chickens were raised at 2,278 m above sea level and were subjected, or not, to a surgical occlusion of a primary bronchus, and were either fed *ad libitum* (continuous access to feed) or were feed restricted (feed was offered 12 h / day), to evaluate the additive effects of hypobaric hypoxia and hypoxemia and fast growth on ascites mortality, ascites characteristic, and antioxidant activity in the heart, lungs and liver.

The BO-AL and BO-FR broilers had higher Hct, RV:TV ratio and PHS mortality than noBO-FR chickens. The occlusion of a primary bronchus effectively triggers pulmonary hypertension, hypoxemia, hypercapnia, and acidosis (Wideman et al., 1997), and the hypoxemia stimulate erythropoiesis, which increases the hematocrit (Ruiz-Feria and Wideman, 2001). We observed a correlation (r = 0.41054) significant between Htc at day 16 after occlusion and RV: TV, this correlation confirmed that chickens with high hematocrit have right ventricular hypertrophy.

Both the *ad libitum* feeding and the high altitude are predisposing factors to trigger PHS. The high metabolic rate elicited by the *ad libitum* feeding increases the cardiac output and the demand for oxygen, whereas the hypobaric hypoxia due to the high altitude contributes to the hypoxemia in the broiler (Julian and Squires, 1995; Balog, 2003), explaining why noBO-AL chickens, which had the highest body weight, had similar Hct at d 37 (16 d after occlusion) and similar RV:TV ratio to birds with bronchus occlusion. The noBO-AL birds had lower mortality (29%) than the BO-AL birds (52%) but higher than the BO-FR birds (18%), whereas noBO-AL

birds were the heaviest of all groups (Table 2). The hypoxemia caused by occlusion of a primary bronchus and the hypoxia of the high altitude and increased cardiac output generated by *ad libitum* feeding explain the high mortality due to PHS observed in BO-AL, despite growing slower than noBO-AL chickens.

Feed restriction reduces growth rate and consequently, metabolic demands and cardiac output. The noBO-FR broilers had lower body weight than noBO-AL, and the lowest Hct, RV:TV ratio and mortality due to PHS. However, the lowest body weight was observed in chickens with BO-FR, which could be due to stress generated by the hypoxia caused by the BO and the FR. In Mexico, the FR is one of the main feeding strategies to reduce mortality due to PHS (Salinas-García et al., 2004).

The relative weight of the lung was no significantly affected. The volume of the non-inflating lung is dictated by the skeletal size of the bird, and there has been little selection on increasing lung volume along with selection for increased body weight and breast yield (Balog, 2003). The BO-AL chickens had higher relative liver weight with respect to BO-FR birds. Chronic hypertension results in right ventricular hypertrophy and causes malfunction of the right atrioventricular valve, allowing blood to flow backwards into the vena cava, leading to liver congestion and seepage of liquid from the liver surface (Wideman et al., 2013).

In the myocardium the hypoxia predisposes to oxidative stress. Chen et al. (2005) reported that rats exposed to chronic hypoxia (nadir O_2, 4 - 5%) had higher lipid oxidation in left ventricular tissue compared to rats in a normoxic state. On the other hand, in the metabolism of chicken mitochondrial electron transport chain is responsible for the formation of ATP and electron leak that react with oxygen to form superoxide radical (Cawthon *et al.,* 2001). Tang et al. (2002) observed that chickens with PHS (chicken exposed to 10 and 15 ° C as from week three with

feeding *ad libitum*) had higher leak of electrons in the mitochondria of the heart with respect to Control chickens and the heart muscle of chickens with PHS present increased production of hydrogen peroxide (H_2O_2) this contributed to oxidative stress and systemic hypoxia that develops in this metabolic disease. The results of previous research could be a possible response to the results of our research, the birds of the BO-FR and noBO-AL treatments had higher lipid peroxidation (MDA) and lower antioxidant activity (AOA) in heart tissue regarding chickens from noBO-FR treatment, this may be caused by the additive effect of occlusion of a primary bronchus and *ad libitum* feeding (interaction: feeding × occlusion was significant, $P < 0.05$). We observed a negative correlation (r= -0.54527) between antioxidant activity (AOA) and lipid peroxidation (MDA), this confirms that the higher the MDA produced in the heart tissue the AOA is reduced.

Hypoxia induces oxidative stress in lung tissue, Hoshikawa et al. (1995) reported that oxidative stress may play a role in the development of pulmonary hypertension induced by chronic hypoxia in rats. Hoshikawa et al. (2001) observed that chronic hypoxia (10% oxygen) increases pulmonary arterial hypertension and pulmonary activity xanthine oxidase in male Sprague-Dawley rats. Xanthine oxidase is responsible for the formation of superoxide radical (Tkaczyk and Vizek, 2007), it reacts with nitric oxide to form peroxynitrite (Tabima *et al.,* 2012). Bowers et al (2004) found that the lung tissue of patients with severe pulmonary hypertension is under oxidative stress and suggest that peroxynitrite, hydroxyeicosatetraenoic (HETE) and 5-oxo-eicosatetraenoic acid (5-oxo-ETE) are formed by the reaction of reactive oxygen species in this tissue. The above statements are consistent with the results observed in the lung tissue of this research, the chickens were in chronic hypoxemia by occlusion of a primary bronchus (BO) and feeding *ad libitum* (AL): broilers from the BO-FR, BO-AL and noBO-AL treatments had lower

antioxidant activity with respect to chickens from noBO-FR treatment. Interaction (feeding × occlusion) was significant in the lipid peroxidation and antioxidant activity in the lung tissue, probably the additive effect of *ad libitum* feeding and occlusion of a primary bronchus decreased antioxidant activity in the lung tissue. We observed a correlation (r= -0.08507) between MDA concentration and AOA in the lung tissue, this indicates that the higher concentration of MDA in the tissue antioxidant activity is lower.

The liver receives up to 25% of cardiac output, and therefore is highly sensitive to reduction in blood flow (Moller and Bernardi, 2013). Occlusion of a primary bronchus and *ad libitum* feeding are factors that increase cardiac output causing pulmonary hypertension syndrome (Wideman *et al.,* 1997). Diaz-Cruz *et al.* (1996) reported that chickens with pulmonary hypertension syndrome had higher damage oxidative in the liver compared to healthy birds. These data are consistent with the results of this research, the occlusion of pulmonary bronchus and *ad libitum* feeding increased lipid oxidation in the liver. Lipid oxidation in this organ could be caused by the formation of reactive oxygen species generated by the leak of electrons in the complex II and ubiquinone in the mitochondrial respiratory chain (Hamanaka and chamdel, 2009). Cawthon et al., (2001) reported that the complex II, ubiquinone, or both in the liver mitochondria of chickens with pulmonary hypertension (PHS) syndrome are specific sites responsible for lipid oxidation of this organ. The interaction (feeding × occlusion) was not significant, only the main effects were significant ($P < 0.05$). Occlusion of a bronchus primary causes tissue hypoxia, pulmonary hypertension and hypoxemia in broilers (Wideman et al., 1997). De Groot and Littauer (1989) mention that a state of hypoxia increases the formation of reactive oxygen species in the liver, coupled with this in posterior reoxygenation of the tissue also generate reactive oxygen species. Arteel et al. (1999) observed that peroxynitrite is involved in the formation of free radicals in the

liver under hypoxic conditions in the hepatic lobe caused by methanol. The possible cause of the formation of peroxynitrite in a hypoxic state is because mitochondria are unable to produce ATP, this leading to the accumulation of degradation products such as xanthine and hypoxanthine (Ponton, 2010). After reintroduction of oxygen, anion superoxide reacts rapidly with nitric oxide and form peroxynitrite: which is vasoconstrictor and free radical (Chirino et al., 2006). We observed a negative correlation between antioxidant activity and MDA concentration liver (-0.57104), which means that while the concentration is greater MDA less will be the antioxidant activity in the liver. The results of this research are consistent with those reported by Nakanishi et al. (1995) who observed higher MDA in the liver, heart and lung exposed to hypobaric hypoxia (5500 m simulated altitude) for 21 days with *ad libitum* feeding Wistar rats compared to rats exposed to sea level. This suggests that the heart, liver and lung of broilers are vulnerable to oxidative stress during a state of hypobaric hypoxia (2247 m), hypoxemia generated by occlusion of a primary bronchus and a high metabolic rate caused by feeding *ad libitum*.

CONCLUSIONS

Feed restriction improved the antioxidant activity in the heart, lungs and liver and reduces ascites mortality in broilers raised at 2247 m above sea level. The occlusion of a primary bronchus and *ad libitum* feeding had effect additive that increased ascites mortality, lipid oxidation and lowered the antioxidant activity in the heart, lungs and liver tissue. The BO does not amplify the incidence of ascites in broilers reared at 2278 m altitude when fed *ad libitum*.

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REFERENCES

Asghar, A., C. F. Lin, J. I. Gray, D. J. Buckley, A. M. Booren, and C. J. Flegal. 1990. Effects of dietary oils and α-tocopherol supplementation on membranal lipid oxidation in broiler meat. J. Food Sci. 55: 46-51.

Arteel, G. E., kadiiska M. B., Rusyn I., Bradford B. U., Mason R. P., Raleigh J. A., and Thurman R. G. 1999. Oxidative Stress Occurs in Perfused Rat Liver at Low Oxygen Tension by Mechanisms Involving Peroxynitrite. Mol. Pharmacol. 55:708-715.

Balog, J. M. 2003. Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: ¿Are we seeing the light at the end of the tunnel?. Avian Poult. Biol. Rev. 14: 99 -126.

Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensmittel-Wissenschaft and Technologie 1995; 28:25-30.

Bautista-Ortega, J., and C. A. Ruíz-Feria. 2012. Pulmonary vascular remodeling in broiler and Leghorn chickens after unilateral pulmonary artery occlusion. Poult. Sci. 91:2904-2911.

Bowers, R., C. Cool, R. C. Murphy, R. M. Tuder, M. W. Hopken, S. C. Flores, and N. F. Voelkel. 2004. Oxidative stress in severe pulmonary hypertension. Am. J. Respir. Crit. Care Med. 169:764-769.

Buege J, Aust SD. 1978. Microsomal lipid peroxidation. Methods Enzymol. 52:302-310.

Cawthon, D. F., K. Beers, and W. G. Bottje. 2001. Electron transport chain defect and inefficient respiration may underlie pulmonary hypertension syndrome (ascites)-associated mitochondrial dysfunction in broilers. Poult. Sci. 80:474-484

Chen L., E. Einbinder, Q. Zhang, J. Hasday, C. W. Balke, and S. M. Scharf. 2005. Oxidative Stress and Left Ventricular Function with Chronic Intermittent Hypoxia in Rats. Am. J. Respir. Crit. Care. Med. 172: 915–920.

Chirino Y. I., M. Orozco-Ibarra, and J. Pedraza-Chaverri. 2006. Evidencia de la participacion del peroxinitrito en diversas enfermedades. Revista de Investigación Clinica. 58 (4):350 – 358.

De Groot, H., and A. Littauer. 1989. Hypoxia, relative oxygen, and cell injury. Free Radic. Biol. Med. 6: 541 - 551.

Díaz-Cruz, A., C. Nava, R. Villanueva, M. Serret, R. Guinzberg, and E. Piña. 1996. Hepatic and cardiac oxidative stress and other metabolic changes in broilers with the ascites syndrome. Poult. Sci. 75: 900-903.

Hamanaka, R. B., and N. S. Chandel. 2009. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem. Sci. 35: 505-5013.

Harvey, R. B., L. F. Kubena, S. L. Lovering, and T. D. Philips. 1985. Ketamine/xylazine anesthesia for chickens. Avian Exotic. Pract. 2:6-7.

Hoshikawa, Y., S. Ono, S. Suzuki, T. Tanita, M. Chida, C. Song, M. Noda, T. Tabata, N. F. Voelkel, and S. Fujimura. 2001. Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. J. Appl. Physiol. 90: 1299 - 1306.

Hoshikawa Y , S. Ono, T. Tanita, T. Sakuma, M. Noda, T. Tabata, S. Ueda, Y. Ashino, and S. Fujimura. 1995. Contribution of oxidative stress to pulmonary hypertension induced by chronic hypoxia. Nihon Kyobu Shikkan Gakkai Zasshi. 33(11):1168 - 1173.

Julian, R.J., and E. J. Squires. 1995. Suggestions for reducing ascites in meat-type chickens. Proceedings of the 44th Western Poultry Diseases Conference (pp. 19–20). Sacramento, CA, USA.

Julian, R. J. 2000. Physiological, management and environmental triggers of the ascites syndrome: A review. Avian Pathol. 29:519-527.

Kalmar, I. D., D. Vanrompay, and G. P. J. Janssens. 2013. Broiler ascites syndrome: Collateral damage from efficient feed to meat conversion. Vet. J. 197: 169-174.

Karatas, F., M. Karatepe, and A. Baysar. 2002. Determination of free malondialdehyde in human serum by high-performance liquid chromatography. Anal. Biochem. 311: 76-79.

Nain, S., B. Ling, B. Bandy, J. Alcorn, C. Wojnarowicz, B. Laarveld, and A. A. Olkowski. 2008. The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth. Avian Pathol. 37:367-373.

Nakanishi, K., F. Tajima, A. Nakamura, Shigeyuki Yagura, Tomomi Ookawara, Hitoshi Yamashita, Keiichiro Suzuki t, Naoyuki Taniguchi t and Hideki Ohno. 1995. Effects of hypobaric hypoxia on antioxidant enzymes in rats. J. Physiol. 489 (3): 869-876.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

Maiti, P., S. B. Singh, A. K. Sharma, S. Muthuraju, P. K. Banerjee, and G. Ilavazhagan. 2006. Hypobaric hypoxia induces oxidative stress in rat brain. Neurochem. Int. 49: 709-716.

Molyneux, P. 2004. The use of the stable free radical diphenylpicrilhydrayl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26: 211-219.

Moller, S., M. Bernardi. 2013. Interactions of the heart and the liver. Eur. Heart J. 34: 2804 - 2811.

Owen, R. L., R. F. Wideman, and B. S. Cowen. 1995. Changes in pulmonary arterial and femoral arterial blood pressure upon acute exposure to hypobaric hypoxia in broiler chickens. Poult. Sci. 74:708-715.

Paddenberg, R., B. Ishaq, A. Goldenberg, P. Faulhammer, F. Rose, N. Weissmann, R. C. Braun-Dullaew, and W. Kummer. 2003. Essential role of complex II of the respiratory chain in hypoxia-induced ROS generation in the pulmonary vasculature. Am. J. Physiol. Lung Cell. Mol. Physiol. 284: L710–L719.

Poyton, O., R. K. A. Ball, and P. R. Castellano. 2009. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinolo. Metab. 20: 332-340.

Ruiz-Feria, C. A., and R. F. Wideman Jr. 2001. Taurine, cardiopulmonary hemodynamics, and pulmonary hypertension syndrome in broilers. Poult. Sci. 80:1607-1618.

SAS Institute. 2006. Language Guide for Personal Computers release 9.0 Edition. SAS Institute Cary N. C. USA. 1028 p.

Salinas-Garcia, I., A. Pro-Martínez, C. M. Becerril, J. M. Cuca-García, R. García-Mata, y E. Sosa-Montes. 2004. Restricción alimentaria en pollo de engorda para la prevención del síndrome ascítico y su efecto en el ingreso neto. Agrociencia 38: 33-41.

Sinnhuber, R. O., I.C. Yu, and T. C. Yu. 1958. Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. Food Res. 23: 624-634.

Tabima D. M., S. Frizzell, and M. T. Gladwin. 2012. Reactive oxygen and nitrogen species in pulmonary hypertension. Free Radic. Biol. Med. 52: 1970-1986.

Tang, Z., M. Iqbal, D. Cawthon, W.G. Bottje. 2002. Heart and breast muscle mitochondrial dysfunction in pulmonary hypertension syndrome in broilers (*Gallus domesticus*). Comp. Biochem. Physiol Part A 132: 527-540.

Tkaczyk J. and M. Vízek. 2007. Oxidative Stress in the Lung Tissue - Sources of Reactive Oxygen Species and Antioxidant Defence. Prague Med. Rep. 108 (2): 105 - 114.

Ulu, H. 2004. Evaluation of three 2-thiobarbituric acid methods for the measurement of lipid oxidation in various meats and meat products. Meat Sci. 67: 683-687.

Vázquez, G. J C., y R. Pérez P. 2000. Valores gasométricos estimados para las principales poblaciones y sitios a mayor altitud en México. Rev. Inst. Enf. Resp. Mex. 13: 06-13.

Wideman, R. F., Y. K. Kirby, C. D. Tackett, N. E. Marson, C. J. Tressler, and R. W. McNew.1996. Independent and simultaneous unilateral occlusion of the pulmonary artery and extra-pulmonary primary bronchus in broilers. Poult. Sci. 75: 1417-1427.

Wideman, R. F., Y. K. Kirby, R. L. Owen, and H. French. 1997. Chronic unilateral occlusion of an extrapulmonary primary bronchus induces pulmonary hypertension syndrome (ascites) in male and female broilers. Poult. Sci. 76:400-404.

Wideman, R. F., D. D. Rhoads, G. F. Erf , and N. B. Anthony. 2013. Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. Poult. Sci. 92:64-83.

CHAPTER II. SUPPLEMENTATION OF ARGININE, SELENIUM AND VITAMINS E AND C INCREASED THE ANTIOXIDANT ACTIVITY IN BROILERS RAISED AT 2278 m OF ALTITUDE

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ABSTRACT

The effect of L-arginine (Arg), selenium (Se) and vitamins E (vit E) and C (vit C) was evaluated on the antioxidant activity (AOA) in the lung, heart and liver in broilers chickens raised at 2278 m of altitude. One d-old male Ross 308 (n = 560) were randomly allocated into four dietary treatments: control diet with a nutrient content of: 1.5% Arg, 0.15 mg Se, 40 IU vit E kg^{-1} of feed and the supplemented diet had a nutrient content of: 2.0% Arg, 0.30 mg Se, 160 IU vit E, 1.0 g vit C kg^{-1} of feed, both diets were given *ad libitum* (AL) or restricted (FR, 8 h/d starting at 14 d age), the statistical design was 2 × 2 factorial with 7 replicates/treatment. At d 46, the glutathione peroxidase activity (GPx; nmol/min/mL) and malondialdehyde (MDA; μM) in plasma (14 birds/treatment) was analyzed. At d 47, 14 birds/treatment were killed to assess *in vitro* the AOA by the percentage inhibition of the 1,1-difenyl-2-picilhydrazyl radical. Control-AL chickens showed the lowest ($P < 0.05$) GPx (840 ± 35) and the highest MDA (6 ± 0.26) concentrations. The AOA was higher ($P < 0.05$) in broilers fed with the supplemented diet than in chickens fed with the basal diet (liver 44 *vs* 34 ± 1.77; lung 46 *vs* 38 ± 2.14; heart 53 *vs* 47 ± 1.23). The supplementation of L-arginine, selenium and vitamins E and C increased the antioxidant activity in the lung, heart and liver of chickens broilers raised at 2278 m of altitude.

Key words: Antioxidant activity, arginine, lipid peroxidation, selenium, vitamin E and C.

RESUMEN

El efecto de L-arginina (Arg), selenio (Se), vitaminas E (vit E) y C (vit C) fue evaluado sobre la actividad antioxidante (AAO) en el pulmón, corazón e hígado de pollos de engorda ciados a 2278 m de altitud. Pollos Ross 308 de un d de edad (n=560) fueron asignados a cuatro dietas tratamiento: dieta testigo con un contenido de nutrientes de: 1.5% Arg, 0.15 mg Se, 40 IU vit E, 1g vit C kg^{-1} alimento y una dieta suplementada 2.0% Arg, 0.30 mg Se, 160 IU vit E, 1.0 g vit C kg^{-1} alimento, ambas dietas fueron suministradas con alimentación *ad libitum* (AL) o restringida (RA, 8 h/d inició a los 14 días de edad) (factorial 2 × 2 con 7 repeticiones/tratamiento). Al día 46, la actividad de la glutatión peroxidasa (GPx; nmol/min/mL) y malondialdehído (MDA; μM) en el plasma (14 aves/tratamiento) fueron analizadas. Al día 47, 14 aves/ tratamiento fueron eutanizadas para evaluar la AAO por el porcentaje de inhibición del 1,1-difenil-2-picilhidrazyl radical. Los pollos del tratamiento Testigo-AL mostraron ($P < 0.05$) la menor GPx (840 ± 35) y la mayor concentración de MDA (6 ± 0.26) que las aves de los otros tratamientos. La AAO fue mayor en pollos alimentados con la dieta suplementada que en los pollos alimentados con la dieta basa (hígado 44 *vs* 34 ± 1.77; pulmón 46 *vs* 38 ± 2.14; corazón53 *vs* 47 ± 1.23). La suplementación de L-arginina, selenio y vitaminas E y C incrementó la actividad antioxidante en el pulmón, corazón e hígado de pollos de engorda criados a 2278 m de altitud.

Palabras clave: Actividad antioxidante, arginina, oxidación lipídica, selenio Vitaminas E y C.

INTRODUCTION

The central etiology of pulmonary hypertension syndrome (PHS) is a hypoxemic condition resulting from an imbalance between demand and supply of oxygen (Kalmar *et al,* 2013). Wideman *et al.* (2013) mentioned that the PHS can be attributed to an imbalance between cardiac output, as well as to an inappropriately elevated tone (degree of constriction) maintained by the pulmonary arterioles. The high altitude decreases the partial pressure oxygen (PO_2), generating hypobaric hypoxia (Peacock, 1998) which elicits pulmonary arterial hypertension in broiler chickens exposed to simulated altitudes of 2000 and 4000 m (Owen *et al.,* 1994). Fresquet *et al*. (2006) reported that chronic hypoxia increased the concentration of reactive oxygen species and limited the concentration of nitric oxide in the pulmonary arteries of mice. Paddenberg *et al.* (2003) demonstrated that hypoxia induces the generation of reactive oxygen species (ROS) in the pulmonary vasculature. Superoxide radical ($O_2^{\cdot-}$) and nitric oxide (NO) react very rapidly to form peroxynitrite ($ONOO^-$) a powerful vasoconstrictor can damage endothelial cells (Pacher *et al.,* 2007). The L-arginine is the substrate of the endothelial enzyme nitric oxide synthase (eNOS), an enzyme that synthesizes nitric oxide (NO), a potent pulmonary vasodilator (McConell, 2007). Previous research has shown that supplemental L-arginine improves cardiovascular performance in broilers (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009) and has been reported to reduce PHS in broiler chickens (Wideman *et al.,* 1995).

Vitamin E (Vit E) and vitamin C (Vit C) are important antioxidants that prevent lipid oxidation in the cells (Serbecic and Beutelspacher, 2005). The Vit C is a cytosolic antioxidant that restores the antioxidant capability of oxidized Vit E; α-tocopheroxyl radical to α-tocopherol (Combs, 2008). In previous studies we have shown that the combination of arginine and antioxidant vitamins (Vit E and Vit C) increased bioavailability of NO and the concentration in plasma

(Ruiz-Feria 2009; Bautista-Ortega y Ruiz-Feria, 2010). However, it has not been evaluated the effect combined of L-argine, antioxidant vitamins and selenium in *ad libitum* feeding broilers reared at 2278 m above sea level. Selenium is an essential nutrient involved in antioxidant defense via glutathione peroxidase activity (GPx) (Combs and Gray, 1998). This is an enzyme which reduces hydrogen peroxide and organic hydroperoxides, thereby limiting lipid oxidation in the cytosol, membranes, and extracellular space (Daniels, 1996).

The modern broiler has high growth rate and high meat yield, the former results from an increased feed intake per unit of time. This rapid growth implies a higher metabolic which consequently leads to a higher demand for oxygen (O_2) (Julian, 2000). However, not all the O_2 is completely reduced to water, it has been estimated that 1 to 2% of oxygen consumed by mitochondria is partially reduced to $O_2^{\cdot-}$ (Poyton et al., 2009) due to leakage of electrons from the respiratory chain (Turrens, 2003). Rodríguez-Ortega *et al.* (2015) found that the *ad libitum* feeding decreased the antioxidant activity in the heart, lungs and liver in broilers raised at 2278 m above sea level. Feed restriction is a more common commercial treatment used to reduce the incidence of ascites in broilers. Feed restriction decreases growth and metabolic rate, and reduces the incidence of metabolic diseases such as PHS (Sahraei, 2012). In previous research, we found that feeding *ad libitum* decreased antioxidant activity in the heart, lungs and liver of chickens to 2278 m above sea level, occlusion of a primary bronchus is not necessary to induce PHS at this altitude, *ad libitum* feeding induces PHS (Rodríguez-Ortega *et al.,* 2014). It was hypothesized that supplementation of arginine in combination with Se, vitamins E and C may increase activity antioxidant in the heart, lungs and liver of broilers raised to 2278 m above sea level subjected to feeding *ad libitum*. The hypobaric hypoxia caused by high altitude and feeding *ad libitum* would have additive effects that would lead to a reduction of the antioxidant activity in the heart, lungs

and liver. Supplementation of arginine, Se, vitamins E and C in the diet of broilers may contribute to increased GPx activity, NO and reduce malondialdehide (MDA) concentrations in plasma.

MATERIALS AND METHODS

Treatments

Chickens were raised at an altitude of 2278 m above sea level (PO_2 of 122 mm Hg; Vázquez-García and Pérez-Padilla, 2000) and *ad libitum* feeding (AL) to amplify the ascites mortality and the lipid oxidation in plasma, lungs, heart and liver. The experiment was performed in the poultry facilities of Postgraduate College, Texcoco, Mexico. Five hundred and sixty one-d-old male Ross 308 chickens were used to study the effect of the supplementation of L-arginine, selenium, vitamin E (vit E) and vitamin (vit C) on glutathione peroxidase activity (GPx), nitric oxide (NO) and lipid peroxidation as MDA concentration in plasma, antioxidant activity (AOA) and MDA in the lungs, heart and liver, productive performance and ascites mortality.

The chicks were randomly allocated into four dietary treatments (seven replicates per treatment): Control diet with a nutrient content of: 1.5% Arg, 0.15 mg Se, 40 IU vit E kg^{-1} and the supplemented diet had a nutrient content of: 2.0% Arg, 0.30 mg Se, 160 IU vit E, 1.0 g vit C kg^{-1} feed, both diets were given *ad libitum* (AL) or restricted (FR, 8 h/d starting at 14 d age of 6:00 am at 10: 00 pm) (2 × 2 factorial; 7 replicates/treatment).

The birds were housed in pens of 3 m^2 with wood-shavings litter; each pen had a feeder and a waterer, the water was offered *ad libitum*. The lighting program was 23 h of light and one hour of darkness during the entire experimental period. The temperature during the first day was 32 °C and decreased 2 °C each week until the third week. Birds were fed diets based on corn and soybean meal. Feeding was divided into two stages: starter from 1 to 21 d of age; containing

3025 kcal metabolizable energy (ME) kg^{-1} of diet and 22 % of crude protein (CP); finisher from 22 to 47 d of age, containing 3100 kcal of ME kg^{-1} of diet and 19 % CP (Table 1).

Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were recorded weekly, but only productive performance accumulated from d 1 to 47 is reported. Mortality due to ascites syndrome was recorded daily and reported as percentage of total birds per treatment.

Table 1. Composition of experimental diets (g/kg) used in the stages starter and finisher.

	Starter		Finisher	
Ingredients	Control	Supplemented	Control	Supplemented
Corn	533.800	533.820	616.700	616.700
Soybean meal	396.900	396.880	312.700	312.700
Soybean oil	26.400	26.440	28.000	28.000
Calcium carbonate	16.600	16.640	13.400	13.400
Dicalcium phosphate	16.800	16.790	15.700	15.700
L-Arginine	0.000	5.706	2.538	7.614
L-Lysine HCl	0.286	0.286	0.770	0.770
L-Threonine	0.665	0.660	0.850	0.850
DL-Methionine	1.217	1.217	0.880	0.880
L-Tryptophan	0.000	0.000	0.000	0.000
[1]Minerals	1.000	1.000	1.000	1.000
[2]Vitamins	0.500	0.500	0.500	0.500
Yeast of selenium	0.050	0.100	0.050	0.100
Vitamin E	0.073	0.2909	0.0730	0.2909

Vitamin C	0.000	1.026	0.000	1.026
Choline	2.130	2.130	2.130	2.130
Coccidiostat	0.500	0.500	0.500	0.500
Pigment	0.000	0.000	3.700	3.700
Salt	3.000	3.000	3.000	3.000
Nutrient content calculated				
Metabolizable energy (kcal/kg)	3025.000	3025.000	3100.000	3100.000
Crude protein (%)	22.000	22.000	19.000	19.000
Arginine (%)	1.500	2.000	1.500	2.000
Lysine (%)	1.280	1.280	1.090	1.090
Methionine (%)	0.480	0.480	0.410	0.410
Methionine + Cystine (%)	0.880	0.884	0.750	0.750
Threonine (%)	0.900	0.900	0.800	0.800
Tryptophan (%)	0.290	0.286	0.240	0.240
Calcium (%)	1.000	1.000	0.850	0.850
Available phosphorus	0.450	0.450	0.420	0.420
Sodium (%)	0.160	0.164	0.160	0.160
Chlorine (%)	0.220	0.224	0.220	0.220
Linoleic acid (%)	1.130	1.130	1.270	1.270
Selenium (mg)	0.150	0.300	0.150	0.300
Vitamin E (UI)	40.000	160.000	40.000	160.000
Vitamin C (g)	0.000	1.000	0.000	1.000

Choline (mg)	1600.000	1600.000	1600.000	1600.000

[1]Mineral premix containing: Zn, 100 g; Fe, 50 g; Cu, 10 g; Mn, 100 g; I, 1 g, as a basis of 1000 g per ton of feed.

[2]Vitamin premix containing: retinol, 24 000 000 IU; cholecalciferol, 8000000 IU; pyridoxine, 8 g; thiamine, 6 g; riboflavin, 16 g; niacin ,100 g; cyanocobalamin, 60 mg; menadione, 10 g; calcium pantothenate, 28 g; folic acid, 3 g, as a basis of 1000 g per ton of feed.

Plasma and organs (heart, liver and lungs) samples

At d 46 of age 3 ml of blood collected in EDTA vacutainer tubes from 42 chickens (14 birds per treatment taken at random) were centrifuged at 1000 × *g* for 10 min at 4 °C and stored at -80 °C for subsequent analysis of glutathione peroxidase activity (GPx), nitric oxide (NO) and malondialdehyde concentration (MDA). A kit (Cayman Chemical, Ann Arbor, MI) was used for the determination of these metabolites. After addition of the corresponding amount of reactants, plasma samples were read in a microplate reader Biotek, Sinergy 2 (BioTek Intrument, Inc., model Vermont, USA). At 47 days of age, 42 chickens (14 birds per treatment taken at random) were humanly killed by cervical dislocation according to the Mexican Norm NOM-033-ZOO-1995; lungs, heart and liver were removed and tissue samples were taken and stored at -46 °C until analysis of lipid peroxidation as malondialdehyde (MDA) concentration.

Antioxidant activity of the diets, lung, heart and liver

The antioxidant activity (AOA) expressed as percent of inhibition DPPH (1,1-Diphenyl-2-Picrylhydrazyl) was measured in triplicate according to the method described by Brand-Williams *et al.* (1995): One g of diet and tissue (lungs, heart, and liver), was mixed with 10 mL of methanol (Sigma Aldrich) was ground in a mortar and incubated for 30 min at 30 ° C, stirred a vortex every 10 min for 20 s. After incubation the samples were centrifuged at 1342 × *g* for 10 min and filtered on Whatman number 4. Each filtered sample was taken: 1000 µl for heart and

lung and 200 µl for liver and each mixed with 3 mL of DPPH (0.11 mM). They were stirred vigorously for 10 s and the extract was left in a dark room for 20 min. Finally reading was taken from the mixture in a spectrophotometer (Thermo Scientific®, modelo 10S VIS) at 515 nm, simultaneously a blank was run without extract. The antioxidant activity of the diets was calculated using the following equation:

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

Total phenols content in the diets, lung, heart and liver

The total phenolic content was measured in triplicate according to the methodology described by Jang et al. (2008) in the following manner: 1.67 g of tissue (lung, heart, and liver, each organ was analyzed individually and in triplicate) and diets were homogenized with 5 mL of distilled water and triturated in a mortar, the mixture was centrifuged at 1342 × *g* for 10 min and filtered through a Whatman number 4. To the filtrate was added 3 mL of chloroform to remove lipids. The mixture was centrifuged at 1342 × *g* for 10 min to separate lipid and aqueous supernatant. An aliquot of 100 µl of supernatant was mixed with 200 µl of Folin - Ciocalteum (1N) and allowed to stand 1 min. Sodium carbonate (3 mL 5%) was added and stirred for 15 s. The mixture was incubated at room temperature in a dark room for 1 hour, after incubation the absorbance was measured at 765 nm in a spectrophotometer (Thermo Scientific®, modelo 10S VIS), the quantification was done based on the standard curve generated with gallic acid.

Lipid oxidation measured as malondialdehyde concentration (MDA)

Lipid peroxidation measured as malondialdehyde concentration (MDA) was determined in triplicate according the methodology described by Buege and Aust (1978) with slight modifications; 10 g of tissue (heart, lung and liver, each individually), was 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 a dark room at room temperature. Subsequently, one mL of the sample was taken and mixed with two ml of thiobarbituric acid (TBA, 0.02 M) with trichloroacetic acid (TCA, Sigma Aldrich, 15%). 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) at 530 nm, lipid peroxidation was expressed as nmol MDA per g tissue.

Statistical analysis

The experimental design was completely randomized with factorial arrangement 2 × 2 (Supplementation × Feeding) with seven replicates per treatment with 20 broilers per replicate. Body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), ascites mortality, RV:TV ratio, hematocrit (Hct), glutathione peroxidase activity (GPx), nitric oxide (NO) and malondialdehyde (MDA) concentrations in plasma lung, heart and liver were analyzed with tow-way ANOVA using the MIXED procedure of SAS v. 9.0 (SAS, 2006). For BWG, FI, FCR and ascites mortality the experimental unit was the replicate (20 broilers). For GPx, NO, MDA concentrations in plasma in lungs, heart and liver the experimental unit was one bird (14 broilers per treatment). Ascites mortality was analyzed by logistic regression, using the PROC LOGISTIC procedure of SAS v. 9.0 (SAS, 2006).

RESULTS

The supplemented diet showed the highest antioxidant activity and total phenolic content in started and finisher compared to the control diet (Table 2).

Table 2 Antioxidant activity (% inhibition DPPH) and total phenols content (μg gallic acid per g of feed) diets in starter (1-21 d) and finisher (22-47 d).

	Antioxidant activity		Total phenols content	
Diets	Starter	Finisher	Starter	Finisher
Control	14	17	587	457
Supplemented	41	45	794	715

Control diet with a nutrient content of:1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or Supplemented diet with a nutrient content of: 2.0% L-arginine, 0.30 mg of Se, 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed.

Hct, RV:TV, BWG, FI and FCR

Percentage of hematocrit (Hct%) and RV:TV, body weight gain (BWG, g), feed intake (FI, g) and feed conversion (FRC) were not different among treatments (Table 3). Ascites mortality was not different among treatments (the ascites mortality in each treatment was following: Control-AL; 11% (15/140), Supplemented-AL; 19% (27/140), Control-FR; 10% (15/140), Supplemented-FR; 16% (23/140; chickens deaths due to ascites divided by total birds in each treatment multiplied by 100).

Table 3 Percentage of hematocrit (Hct, %), RV:TV ratio, body weight gain (BWG, g), feed intake (FI, g), feed conversion (FRC) at 47 days of age.

Treatments	Hct	RV:VT	BWG	FI	FCR
Control-AL	37	0.25	3360	6030	1.79
Supplemented-AL	35	0.25	3429	5952	1.76
Control-FR	39	0.26	3324	5929	1.77
Supplemented-FR	36	0.29	3346	5881	1.77
*S. E. M.	2	0.016	46	74	0.02
			P-value		
Supplementation	0.1426	0.1418	0.3367	0.4034	0.5522
Feeding	0.237	0.0799	0.2056	0.259	1.0000
Supplementation × Feeding	0.7643	0.3165	0.6144	0.8425	0.5522

*Standard error of the mean. Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2.0% L-arginine, 0.30 mg of organic Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction; feed was restricted 8 h/day; FR, beginning at 14 d of age.

GPx, MDA y NO en el plasma

The interaction (Supplementation × Feeding) was significant in the plasma GPx and MDA concentrations ($P<0.05$) (Table 4). The Control-AL birds had the lowest GPx and the highest MDA concentration in the plasma compared with other treatments. The Supplemented-AL, Control-FR and Supplemented-FR treatments were not different in GPx and MDA concentration

in the plasma. The nitric oxide concentration was not different in any treatment; the range of concentration of nitric oxide was 12 to 13 µM (Table 4).

Table 4 Glutathione peroxidase activity (GPx), malondialdehyde (MDA) and nitric oxide concentration (µM) in the plasma.

Treatments	GPx	MDA	NO
Control-AL	840 b	6 a	13 a
Supplemented-AL	989 a	5 b	13 a
Control-FR	1025 a	5 b	12 a
Supplemented-FR	1000 a	5 b	12 a
*S. E. M.	35.3	0.3	0.93
		P-value	
Supplementation	0.0204	0.0202	0.7891
Feeding	0.0006	0.0459	0.1298
Supplementation × Feeding	0.0019	0.0202	0.7891

*Standard error of the mean. Means in columns with different letter are statistically different (Tukey, $P < 0.05$). Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2.0% L-arginine, 0.3 mg of organic Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction, feed was restricted 8 h/day; FR, beginning at 14 d of age.

Antioxidant activity of the heart, lung and liver

The interaction (Supplementation × Feeding) was not significant in the antioxidant activity in the lung, heart and liver (Table 5). The main effect supplementation was significant in the antioxidant activity of the lung, heart and liver. The broilers fed with supplemented diet had higher AOA in lung, heart and liver with respect to the chickens fed with basal diet or Control (Table 5). The main effect feeding was not significant (Table 5).

Table 5. Antioxidant activity (% inhibition of DPPH) of the lungs, heart and liver.

Treatments	Lung	Heart	Liver
Control	38 b	47 b	34 b
Supplemented	46 a	53 a	44 a
S. E. M.	2.14	1.23	1.77
Fed Restricted	43 a	49 a	40 a
ad libitum feeding	40 a	51 a	38 a
S. E. M.	2.14	1.23	1.77
		P-value	
Supplementation	0.0014	<0.0001	<0.0001
Feeding	0.3531	0.0835	0.3227
Supplementation × Feeding	0.2671	0.6182	0.2477

*Standard error of the mean. Means in columns with different letter are statistically different (Tukey, $P < 0.05$).

Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2.0% L-arginine, 0.30 mg of organic

Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction; feed was restricted 8 h/day; FR, beginning at 14 d of age.

Total phenols content* (TPC) *in the lungs, heart and liver

The interaction (Supplementation × Feeding) was not significant in the TPC of the lung, heart and liver (Table 6). In lung and heart the main effect supplementation was significant in the TPC. The chickens fed supplemented diet had the highest TPC in the lung and heart compared with broilers fed Control die (basal diet) (Table 6). In the lungs the chickens with FR showed similar TPC than broilers with a*d libitum* feeding. In the heart and liver the broilers *ad libitum* feeding had higher TPC than broilers with Fed Restricted. Chickens fed with Control and Supplemented diet showed similar phenols content in the liver.

Table 6. Total phenols content (µg gallic acid; AG/g of tissue) in the lungs, heart and liver.

Treatments	Lung	Heart	Liver
Control	628 b	483 b	776 a
Supplemented	657 a	520 a	787 a
*S. E. M.	8.69	8.33	7.37
Feed Restriction	648 a	472 b	760 b
Ad libitum feeding	637 a	531 a	802 a
*S. E. M.	8.69	8.33	7.37
		P-value	
Supplementation	0.0241	0.0047	0.2864
Feeding	0.387	<0.0001	0.0004
Supplementation × Feeding	0.6794	0.1928	0.4952

*Standard error of the mean. Means in columns with different letter are statistically different (Tukey, $P < 0.05$). Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2.0% L-arginine, 0.30 mg of organic Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction; feed was restricted 8 h/day; FR, beginning at 14 d of age.

***Lipid oxidation measured as malondialdehyde* (MDA) concentrations**

The interaction (Supplementation × Feeding) was not significant in the MDA concentration of the heart and liver. In the heart the main effect supplementation was significant ($P<0.05$). The chickens fed with Control diet had higher MDA concentration in the heart whit respect to

broilers fed Supplemented diet. In the liver the main effect Feeding was significant ($P<0.05$). The broilers with *ad libitum* feeding showed higher MDA concentration in the liver than chickens with feed restriction (Table 7).

Table 7. Lipid oxidation measured as MDA (nmol of MDA/g of tissue) in the heart and liver.

Treatements	Heart	Liver
Control	15 a	15 a
Supplemented	12 b	14 a
*S. E. M.	0.38	0.45
Feed restriction	13 a	12 b
Ad libitum feeding	14 a	18 a
*S. E. M.	0.38	0.45
	P-value	
Supplementation	<0.0001	0.225
Feeding	0.0836	<0.0001
Supplementation × Feeding	0.1424	1.000

*Standard error of the mean. Means in columns with different letter are statistically different (Tukey, $P < 0.05$). Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2.0% L-arginine, 0.30 mg of organic Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction; feed was restricted 8 h/day; FR, beginning at 14 d of age.

In the lung the interaction (Supplementation × Feeding) of the MDA concentration was significant ($P<0.05$). The chickens from Control-AL treatment had the highest MDA concentration than the other treatments (Figure 1). Broilers from Control-FR, Supplemented-FR and Supplemented-AL treatments had similar MDA concentration.

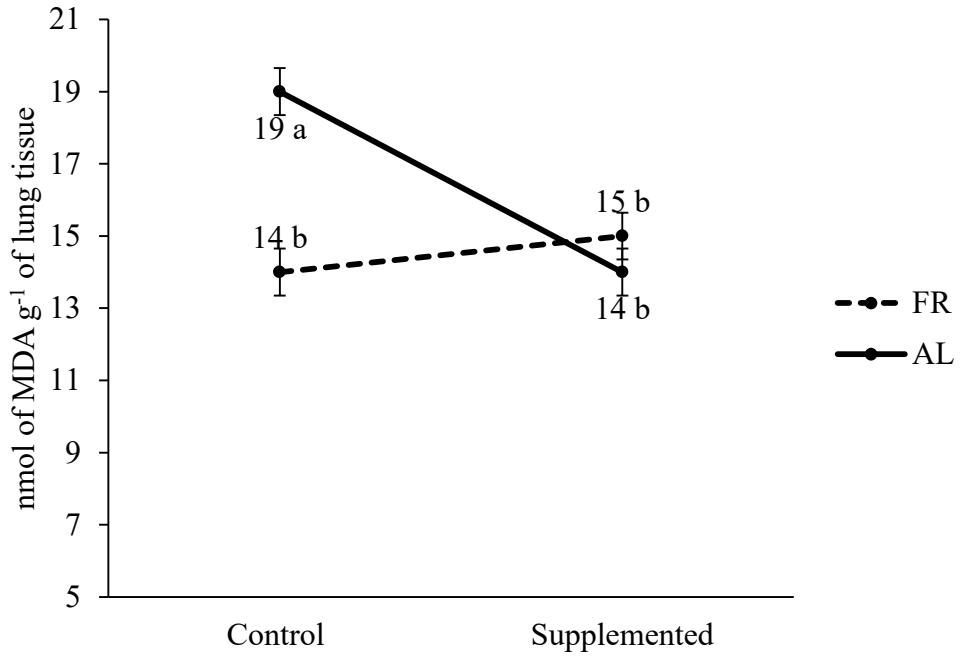

Figure 1. Lipid oxidation measured as malondialdehyde (MDA) concentrations *in vitro* in the lungs (nmol MDA g^{-1} lung tissue) of broilers raised to 2278 m altitude distributed in four treatments (2 × 2 factorial). Supplementation was the first factor with two levels: control diet with a nutrient content of: 1.5% L-arginine, 0.15 mg organic selenium (Se) and 40 IU of vitamin E (vit E; α-tocopherol acetate) kg^{-1} of feed or supplemented diet with a nutrient content of: 2% L-arginine, 0.30 mg of organic Se (Se yeast), 160 IU of vit E (dl-α- tocopherol acetate) and 1.0 g of vit C (ascorbic acid) kg^{-1} of feed. Feeding was the second factor with two levels: *ad libitum* feeding; AL (—) or feed restriction, feed was restricted 8 h/day; FR (---).

DISCUSSION

In the present study, male broilers chickens were raised at 2,278 m above sea level to evaluate the additive effects of hypobaric hypoxia caused by high altitude and rapid growth caused by fed *ad libitum* on supplementation or no of arginine (Arg), selenium (Se), vitamins E (vit E) and vitamin C (vit C) in the diet and the *ad libitum* feeding (continuous access to feed) or feed restricted (feed was restricted 8 h/day). We hypothesized that arginine in combination with selenium, vitamins E and C may increase activity antioxidant (AOA) in the heart, lungs and liver of broilers raised to 2278 m of altitude. High altitude and *ad libitum* feeding would have additive effects that would lead to a reduction of the antioxidant activity in the heart, lungs and liver.

Supplementation of Arg, Se, vit E and vit C and the type of feeding did not affect Hct%, RV:TV ratio, body weight gain (BWG), feed intake (FI), feed conversion (FCR) ratio and the mortality due to ascites. The birds fed a mash diet had an average of body weight gain of 3365 g at 47 d of age, an average of BWG close to that proposed by Ross 308 (3494 g) at 47. The Hct% and RV:TV ratio were used to determine the degree of PHS in broilers. The Hct% is an indicator of hypoxemia (low oxygen tension in the blood) because when chickens are exposed to hypoxia there is an increase in the production of red blood cells. Wang *et al.* (2012) indicated the following intervals for Hct: healthy chickens Hct <36% and chickens with PHS Hct> 36%. The RV:TV ratio is used as an indicator of pulmonary hypertension, which directly causes right ventricular hypertrophy in broiler chickens. Wideman *et al.* (2001) established that healthy chickens have RV:TV ratio from 0.15 to 0.27. In contrast, the RV:TV ratio of broilers with PHS is higher than 0.28. We did not find differences in the Hct% and RV:TV ratio. However, with respect to values observed in this research the birds were susceptible to PHS.

The interaction (Supplementation × Feeding) was significant ($P < 0.05$) in the GPx and lipid oxidation measured as MDA concentration in the plasma and in the lung. The high altitude decreases the partial pressure of O_2, this generates hypoxia in broilers (Ruiz-Feria and Wideman, 2001). Hypoxia decreases the rate of electron transport and increases the probability to generate superoxide radical ($O_2^{\cdot -}$) (Solani *et al.,* 2010). Added to hypoxia, the increased metabolic rate caused by *ad libitum* feeding increased the formation of reactive oxygen species. The above conditions may be the response to the lowest GPx and the highest MDA concentration in the plasma and the lung of birds fed the Control-AL diet *vs*. the supplemented-AL (Table 4 and Figure 1). An additive effect between hypobaric hypoxia and *ad libitum* feeding may be responsible for the results observed in the broilers from Control-AL treatment. Another probable cause of the lower GPx and the higher MDA concentration in the plasma and lung of broilers Control-AL could be the lower AOA and TPC of the control diet (Table 2), is important to remember that Supplementation was the first factor with two levels: control diet or supplemented diet and feeding was the second factor with two levels: *ad libitum* feeding; AL or feed restriction; FR.

It is important to note that in the four treatments evaluated in all variables presented in Table 3 did not show significant differences ($P > 0.05$); However, in the variables presented in Table 4, it was observed that the birds of Control-AL treatment showed the lowest ($P < 0.05$) GPx and the highest ($P < 0.05$) MDA concentration with respect to the rest treatments. We expected that feed restriction would reduce BWG, FI, Hct and RV:TV ratio. However, there were no significant differences in the BWG, FI and FRC ratio among treatments. One possibility is that chickens with FR eat the largest amount of feed when it was available, to support the period without feed. Nielsen et al., (2003) observed that chickens fed a mash diet (Fed twice a day) appeared to learn

about food availability and adjusted their behaviour accordingly. On the other hand, the possible response to high GPx and low MDA concentrations in treatments Supplemented-FR and Supplemented-AL (Table 4), could be due to the combination of arginine, selenium and vitamins E and C in the diet. Selenium is part of the enzyme glutathione peroxidase (GPx; Arteel y Sies, 2001), the vitamin C ensures α-tocopherol regeneration from the α-tocopherol radical (Combs, 2008).

We did not find significant differences ($P > 0.05$) in the concentration of nitric oxide (NO) in the plasma (Table 4). Our results do not agree with those found by Ruiz-Feria (2009) who observed that the supplementation with a combination of 2.2% L-arginine, 240 IU vit E and 500 mg vit C increased the concentration of NO in the plasma of broilers raised at 112 m above sea level. They used a higher concentration of L-arginine and vit E than the used in our experiment, added to this we raised the chickens to a higher altitude (Twenty times more; 2278 *vs* 112 m of altitude) than they. It would be motivated by future research to evaluate the concentrations of L-arginine, vitamins E and C used by Ruiz-Feria (2009) or higher concentrations in chickens reared in the Valley of Mexico. Another possible cause to our results could be due to the half-life of NO. Thomas *et al.* (2014) estimated that the extravascular half-life of NO will range from 0.09 to > 2 s, depending on O_2 concentration. One possibility for the NO disappearance was its rapid reaction with superoxide.

The interaction (Supplementation × Feeding) was not significant in AOA and TPC (Tables 5 and 6). The broilers fed with the supplemented diet had higher AOA in the lung, heart and liver with respect to chickens fed with control diet (Table 5). This coincides with the higher TPC in the lung and heart of the chickens fed the supplemented diet (Table 6). These results could be due to the higher AOA and TPC of the supplemented diet in started and finisher (Table 2).

The heart of the broilers fed with supplemented diet showed lower MDA concentrations than birds fed with the control diet, while the liver of the broilers with *ad libitum* feeding had higher MDA concentration with respect to the bird with feed restriction (Table 7). This could be due to *ad libitum* feeding and hypobaric hypoxia caused by high altitude. Rodríguez-Ortega *et al.* (2014) observed that *ad libitum* feeding increase the MDA concentration in broilers chickens reared at 2278 m altitude, due to a higher metabolic activity and hypobaric hypoxia that increased the formation of free radicals. Hoshikawa *et al.* (2001) observed that chronic hypoxia increases pulmonary xanthine oxidase activity in male Sprague-Dawley rats. Xanthine oxidase is responsible for the formation of superoxide radical (Tkaczyk and Vizek, 2007), it reacts with nitric oxide to form peroxynitrite (Tabima *et al.,* 2012).

CONCLUSIONS

The supplementation with arginine, selenium and vitamins E and C increased the antioxidant activity in the lung, heart and liver of chickens reared at 2278 m altitude. Feed restriction decreased lipid oxidation in the lungs and liver. Hypobaric hypoxia and *ad libitum* feeding had an additive effect that decreased the activity of glutathione peroxidase and increased lipid oxidation in the plasma of broilers fed with a control diet raised at 2278 m altitude.

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REFERENCES

Bautista-Ortega J., and C. A. Ruiz-Feria. 2010. L-Arginine and antioxidant vitamins E and C improve the cardiovascular performance of broiler chickens grown under chronic hypobaric hipoxia. Poult. Sci. 89: 2141-2146.

Brand-Williams, W., M. E. Cuvelier, and C. Berset. 1995. Use of a Free Radical Method to Evaluate Antioxidant. Lebensm.-Wiss. u.-Technol. 28: 25-30.

Buege J, and Aust SD. 1978. Microsomal lipid peroxidation. Methods in Enzymol. 52:302-310.

Combs, G. F. 2008. The vitamins Fundamental Aspects in Nutrition and Health. Third Edition. ISBN 13: 978-0-12-183493-7.

Combs, G. F. Jr, and W. P. Gray. 1998. Chemopreventive agents: selenium. Pharmacol. Ther. 79 (3): 179-192.

Daniels, L. A. 1996. Selenium metabolism and bioavailability. Biol. Trace Elem. Res. 54: 185 - 198.

Fresquet, F., F. Pourageaud, V. Leblais, R. P. Brandes, J.-P. Savineau, R. Marthan, and B. Muller. 2006. Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia. Br. J. Pharmacol. 148: 714-723.

Hoshikawa, Y , S. Ono, T. Tanita, T. Sakuma, M. Noda, T. Tabata, S. Ueda, Y. Ashino, and S. Fujimura. 1995. Contribution of oxidative stress to pulmonary hypertension induced by chronic hypoxia. Nihon Kyobu Shikkan Gakkai Zasshi. 33(11):1168-1173.

Jang, A., X. D. Liu, M. H. Shin, B. D. Lee, S. K. Lee, J. H. Lee, and C. Jo. 2008. Antioxidative Potential of Raw Breast Meat from Broiler Chicks Fed a Dietary Medicinal Herb Extract Mix. Poult. Sci. 87:2382–2389.

Julian, R. J. 2000. Physiological, management and environmental triggers of the ascites syndrome: A review. Avian Pathol. 29:519-527.

Kalmar, I. D., D. Vanrompay, and G. P. J. Janseens. 2013. Broiler ascites syndrome: collateral damage from efficient feed to meat conversion. Vet. J.; 197:169-174.

Lorenzoni, A. G., and C. A. Ruiz-Feria. 2006. Effects of vitamin E and L-arginine on cardiopulmonary function and ascites parameters in broiler chickens reared under subnormal temperatures. Poult. Sci. 85:2241-2250.

McConell, G. K. 2007. Effects of L-arginine supplementation on exercise metabolism. Curr. Opin. Clin. Nutr. Metab. Care. 10:46-51.

Molineux, P. 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26(2): 211-219.

Nielse, B. L., M. Litherland, F. Noddegaard. 2003. Effects of qualitative and quantitative feed restriction on the activity of broiler chickens. Appl. Anim. Behav. Sci. 83: 309-323.

NOM-033-ZOO-1995. Norma Oficial Mexicana. Sacrificio humanitario de los animales domésticos y silvestres. Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) 1995; 12 p.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. ed. Natl. Acad. Press, Washington, DC.

Owen R. L., R. F. Wideman Jr, and B. S. Cowen. 1995. Changes in pulmonary arterial and femoral arterial blood pressure upon acute exposure to hypobaric hypoxia in broiler chickens. Poult. Sci. 74 (4): 708-715.

Paddenberg, R., B. Ishaq, A. Goldenberg, P. Faulhammer, F. Rose, N. Weissmann, R. C. Braun-Dullaew, and W. Kummer. 2003. Essential role of complex II of the respiratory chain in

hypoxia-induced ROS generation in the pulmonary vasculature. Am. J. Physiol. Lung Cell. Mol. Physiol. 284: L710–L719.

Pacher, P., J. S. Beckman, and L. Liaudet. 2007. Nitric oxide and peroxynitrite in health and disease. Physiol. Rev. 87 (1): 315-424.

Peacock, A. J. 1998. Oxygen at high altitude. B. M. J. 317 (7165): 1063-1066.

Poyton, O., R. K. A. Ball, and P. R. Castellano. 2009. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinolo. Metab. 20: 332-340.

Rodríguez-Ortega, L. T., E. Sosa-Montes, A. Pro-Martínez, C. A. Ruiz-Feria, J. Bautista-Ortega, A. J. Vargas-Galicia, D. Chan-Díaz, P. Pérez-Hernández. 2014. Free access to feed reduces the antioxidative capacity in broilers raised at high altitudes. Poult. Sci. 93(E-Supp 1): 77.

Rodríguez-Ortega L. T., E. Sosa-Montes, A. Pro-Martínez Arturo, C. A. Ruiz-Feria, J. Bautista-Ortega, A. Vargas-Galicia, V. Valdes-Narvaez, and D. Chan-Díaz. 2015. Supplementation of arginine, selenium, and vitamines E and C increased the antioxidant activity in broilers raised at high altitude. Poult. Sci. 94(E-Supp 1): 98.

Ruiz-Feria, C. A., M. T. Kidd, and R. F. Wideman. 2001. Plasma levels of arginine, ornithine, and urea and growth performance of broilers fed supplemental L-arginine during cool temperature exposure. Poult. Sci. 80:358-369.

Ruiz-Feria, C. A. 2009. Concurrent supplementation of arginine, vitamin E, and vitamin C improve cardiopulmonary performance in broilers chickens. Poult. Sci. 88: 526- 35.

Sahraei, M. 2012. Feed restriction in broiler chickens production. Biotechnol. Anim. Husb. 28 (2): 333-352.

SAS Institute. Language guide for personal computers, release 9.0 Edition. SAS Institute Cary N. C. USA 2006. 1028 p.

Serbecic, N., and S. C. Beutelspacher. 2005. Anti-oxidative vitamins prevent lipid- peroxidation and apoptosis in corneal endothelial cells. Cell Tissue Res. 320:465-475.

Solaini, G., A. Baracca, G. Lenaz, and G. Sgarbi. 2010. Hypoxia and mitochondrial oxidative metabolism. Biochim. Biophys. Acta 1797: 1171-1177.

Tabima D. M., S. Frizzell, and M. T. Gladwin. 2012. Reactive oxygen and nitrogen species in pulmonary hypertension. Free Radic. Biol. Med. 52 (9): 1970-1986.

Tkaczyk J. and M. Vízek. 2007. Oxidative stress in the lung tissue-sources of reactive oxygen opecies and antioxidant defence. Prague Med. Rep. 108 (2): 105 - 114.

Tan, X., W. D. Sun, J. C. Li, J. Q. Pan, Y. J. Liu, J. Y. Wang, and X. L. Wang. 2007. L-arginine prevents reduced expression of endothelial nitric oxide synthase (NOS) in pulmonary arterioles of broilers exposed to cool temperatures. Vet. J. 173 (1): 151-157.

Turrens, J. F. 2003. Mitochondrial formation of reactive oxygen species. J. Physiol, 552.2:335-344.

Thomas SR, Schulz E, Keanney JF. 2006. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity-Role for iron-dependent oxidative stress. Free Radic. Biol. Med. 41: 681-688.

Ulu, Hasret. Evaluation of three 2-thiobarbituric acid methods for the measurement of lipid oxidation in various meats and meat products. Meat Sci. 67: 683-687.

Vázquez-García, J. C., and R. Pérez-Padilla. 2000. Valores gasométricos estimados para las principales poblaciones y sitios a mayor altitud en México. Rev. Inst. Nal. Enf. Resp. Mex. 13(1): 06-13.

Wideman RF. 2001. Pathophysiology of heart/lung disorders: pulmonary hypertension syndrome in broiler chickens. World's Poult Sci J. 57: 289-307.

Wang, Y., Y. Guo, D. Ning, Y. Peng, H. Cai, J. Tan, Y. Yang and D. Liu. 2012. Changes of hepatic biochemical parameters and proteomics in broilers with cold-induced ascites. J. Anim. Sci. Biotechnol. 3 (41): 1-9.

Wideman, R. F., Y. K. Kirby, M. Ismail, W. G. Bottje, R. W. Moore, and R. C. Vardeman. 1995. Supplemental L-arginine attenuates pulmonary hypertension syndrome (ascites) in broilers. Poult. Sci. 74:323–330.

Wideman, R. F., D. D. Rhoads, G. F. Erf , and N. B. Anthony. 2013. Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. Poult. Sci. 92:64-83.

CHAPTER III. LIPID PEROXIDATION IN 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), lipid peroxidation as malondialdehyde (MDA) concentration in plasma, lungs, heart, liver and productive performance in 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. Productive performance was recorded on a weekly basis. Glutathione peroxidase activity and MDA in plasma were evaluated at d 46, and at d 47, MDA was evaluated in lungs, heart and liver. There was no significant difference ($p>0.05$) in productive performance among treatments. The birds from GSE-AL treatment had the lowest ($p<0.05$) GPxA, the highest ($p<0.05$) MDA concentration in plasma, heart and liver, and the intermediate MDA concentration in lungs. Our results suggest that GSE may partially replace VE in diets of broilers without impairment of productive performance; however, more research is required to find the optimal level of inclusion of GSE that decrease lipid peroxidation in plasma, lungs, heart and liver of broilers raised at 2278 m altitude.

Key words: grape seed extract, lipid peroxidation, vitamin E.

RESUMEN

El objetivo de esta investigación fue evaluar el efecto de la sustitución parcial de la vitamina E (VE) por extracto de semilla de uva (ESU) en la actividad de la glutatión peroxidasa (AGPx), la concentración de óxido nítrico (ON) y la peroxidación lipídica medida como concentración de malondialdehído (MDA) en plasma, pulmones, corazón e hígado y el rendimiento productivo en pollos de engorda criados a 2278 m de altitud. Pollos macho Ross 308 de un día de edad (n = 420) fueron distribuidos aleatoriamente en tres tratamientos: Testigo-AL; dieta base (maíz-pasta de soya) que contenía 40 UI de VE kg^{-1} de dieta, alimentados *ad libitum* (AL), Testigo-RA; dieta base con restricción alimenticia (RA) y ESU-AL; dieta base que contenía 10 mg de ESU más 10 UI de VE y alimentación AL. El rendimiento productivo se registró semanalmente. La actividad de la glutatión peroxidasa y MDA en plasma fueron evaluados al d 46. Al d 47, el MDA se evaluó en pulmones, corazón e hígado. No hubo diferencias significativas ($P > 0.05$) en el rendimiento productivo y en la concentración de ON entre los tratamientos. Las aves ESU-AL tuvieron la menor ($P < 0.05$) AGPx, el valor más alto ($P < 0.05$) de MDA en plasma, corazón e hígado, y un valor intermedio de MDA en pulmones. Los resultados sugieren que el extracto de semilla de uva puede sustituir parcialmente a la vitamina E en las dietas para pollos de engorda sin afectar el rendimiento productivo; sin embargo, se requiere más investigación para encontrar el nivel óptimo de inclusión del extracto para disminuir la peroxidación lipídica en plasma, pulmones, corazón e hígado de los pollos de engorda criados a 2278 m de altitud.

Palabras clave: elevada altitud, peroxidación lipídica, extracto de semilla de uva, vitamina E.

INTRODUCTION

High altitude reduces partial pressure of oxygen (PO_2; Visschedijk, 1985). Hypoxia refers to a low partial pressure of O_2 in the inspired air (Ruiz-Feria and Wideman, 2001). Wideman and Kirby (1995) report that the average partial pressure of oxygen in the blood from the pulmonary artery is 103 mm Hg in broiler chickens clinically healthy, however in a state of hypoxemia the partial pressure of oxygen in the blood from the pulmonary artery decreases to 79 mm Hg; Julian (2007) defines hypoxemia as a reduced oxygen level in the blood. The high altitude in Texcoco, State of Mexico, Mexico (2278 m altitude with an atmospheric pressure of 581.1 mm Hg and 122 mm Hg PO_2; Vázquez-García & Pérez-Padilla, 2000) and *ad libitum* feeding are predisposing factors that increase the incidence of the ascites syndrome (SA). Bakonyi and Radak (2004) reported that exposure to high altitude increases the formation of reactive oxygen species (ROS) and reactive nitrogen species, ROS cause lipid peroxidation in heart and liver (Díaz-Cruz *et al.*, 1996). Bottje *et al.* (1995) and Wideman and Kirby (1995) reported that oxidative stress is associated with SA. Rodríguez-Ortega *et al.* (2014) observed that *ad libitum* feeding decrease antioxidant activity (AOA) in lungs, heart and liver of broilers. Antioxidants such as vitamin E (VE), Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethoxyquine are used to preserve diets containing high level of fat (Jang *et al.,* 1999). Vitamin E is well recognized not only as a natural antioxidant for prevention of biological oxidation but also as an essential nutrient for decreasing the 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 products such as grape seed extract (GSE).

Grape seed extract has antioxidant effects due to its content of polyphenolic compounds (Brenes *et al.,* 2010), such as procyanidins, catechins, epicatechins, gallocatechins and epigallocatechins (Chamorro *et al.,* 2013), however, these have been scarcely evaluated in broiler feeding. El-Damrawy (2014) found that supplementation with GSE (100 or 200 mg kg^{-1} of diet) increased glutathione and superoxide dismutase activities, and decreased MDA concentration in the liver of broilers raised under heat stress. Hao *et al.* (2015) reported that supplementation with GSE (100 or 150 mg kg^{-1} of feed) increases glutathione peroxidase activity (GPxA) and decreases MDA concentration in serum of pigs.

We hypothesized that grape seed extract may partially replace vitamin E without impairment of productive performance, increase the activity of glutathione peroxidase and nitric oxide concentration, and decrease malondialdehyde in plasma, lungs, heart and liver.

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. The average incidence of AS is approximately 4.7% (Maxwell & Robertson, 1997); in Mexico 333,435,192 broilers were produced in the year 2014 (SIAP, 2014); thus it can be estimated that 15,671,454 chickens died due to AS in Mexico that year. The aim of the present research was to evaluate the effect of partial substitution of VE by GSE on glutathione peroxidase activity (GPxA), nitric oxide concentration (NO) and lipid peroxidation as malondialdehyde (MDA) concentration in plasma, lungs, heart and liver; as well as productive performance and ascites mortality in broilers raised at 2278 m altitude.

MATERIALS AND METHODS

Treatments

Chickens were raised at an altitude of 2278 m above sea level (PO_2 of 122 mm Hg; Vázquez-García & Pérez-Padilla, 2000) and fed *ad libitum* (AL) to amplify the ascites mortality and the lipid peroxidation in plasma, lungs, heart and liver. The experiment was performed in the poultry facilities of Postgraduate College, Texcoco, Mexico. Four hundred and twenty one-d-old male Ross 308 chickens were used to study the effect of partial substitution of vitamin E (VE) by grape seed extract (GSE) on glutathione peroxidase activity (GPxA), nitric oxide (NO) and lipid peroxidation as MDA concentration in plasma, lungs, heart and liver, productive performance and ascites mortality. The chicks were randomly allocated into three dietary treatments (seven replicates per treatment): 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 day 14 of 6:00 am at 10: 00 pm until the end of the experiment), GSE-AL; basal diet containing 10 mg of GSE (equivalent to 30 IU of VE) plus 10 IU of VE and fed AL. The birds were housed in pens of 3 m^2 with wood-shavings litter; each pen had a feeder and a waterer, the water was offered *ad libitum*. The lighting program was 23 h of light and one hour of darkness during the entire experimental period. The temperature during the first day was 32 °C and decreased 2 °C each week until the third week. Birds were fed diets based on corn and soybean meal. Feeding was divided into two stages: starter from 1 to 21 d of age; containing 3025 kcal metabolizable energy (ME) kg^{-1} of diet and 22 % of crude protein (CP); finisher from 22 to 47 d of age, containing 3100 kcal of ME kg^{-1} of diet and 19 % CP. The antioxidant activity (AOA) was evaluated in each diet. Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were recorded weekly, but only productive performance

accumulated from d 1 to 47 is reported. Mortality due to ascites syndrome was recorded daily and reported as percentage of total birds per treatment.

Antioxidant activity (AOA) in diets

The antioxidant activity (AOA) of each diet was measured as percent inhibition of the *in vitro* radical 1, 1-diphenyl-2 picrylhydrazyl (DPPH) according to Brand-Williams *et al.* (1995) with slight modifications. One 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; later on the mixture was centrifuged at 1342 × *g* for 10 min. The methanol extract was filtered on Whatman number 4. 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 dark for 20 min, and the absorbance was read in a spectrophotometer (Thermo Scientific, 10S Vis model) at 515 nm; simultaneously a blank was run without extract. The antioxidant activity of the diets was calculated using the following equation:

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

Plasma samples and organs (heart, liver and lungs)

At d 46 of age 3 ml of blood collected in EDTA vacutainer tubes from 42 chickens (14 birds per treatment taken at random) were centrifuged at 1000 × *g* for 10 min at 4 °C and stored at -80 °C for subsequent analysis of glutathione peroxidase activity (GPxA), nitric oxide (NO) and malondialdehyde concentration (MDA). A kit (Cayman Chemical, Ann Arbor, MI) was used for the determination of these metabolites. After addition of the corresponding amount of reactants, plasma samples were read in a microplate reader Biotek, Sinergy 2 (BioTek Intrument, Inc., model Vermont, USA). At 47 days of age, 42 chickens (14 birds per treatment taken at random)

were humanly killed by cervical dislocation according to the Mexican Norm NOM-033-ZOO-1995; lungs, heart and liver were removed and tissue samples were taken and stored at -46 °C until analysis of lipid peroxidation as malondialdehyde (MDA) concentration.

Lipid peroxidation (MDA) of lungs, heart and liver

Lipid peroxidation as malondialdehyde concentration (MDA) was determined according the methodology described by Buege & Aust (1978) with slight modifications; 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 the darkness at room temperature. Subsequently, one ml of the sample was taken and mixed with two ml of thiobarbituric acid (TBA, 0.02 M) with trichloroacetic acid (TCA, Sigma Aldrich, 15%). 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) at 530 nm, lipid peroxidation was expressed as nmol MDA per g tissue.

Statistical analysis

The experimental design was completely randomized with three treatments (seven replications per treatment). The body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), ascites mortality, glutathione peroxidase activity (GPxA), nitric oxide (NO) and malondialdehyde (MDA) concentrations in plasma lung, heart and liver were analyzed with one-way ANOVA using the MIXED procedure of SAS v. 9.0 (SAS, 2006). For BWG, FI, FCR and ascites mortality the experimental unit was the replicate (20 broilers). For GPxA, NO, MDA

concentrations in plasma in lungs, heart and liver the experimental unit was one bird (14 broilers per treatment).

RESULTS

The grape seed extract (GSE) diet had lower antioxidant activity (AOA) than the control one (starter: 11 *vs.* 14; finisher: 11 *vs.* 17% inhibition of DPPH; respectively). There were not significant differences in body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) among treatments (Table 1). Ascites mortality (%) was higher ($p<0.05$) in the GSE-AL treatment than in the others. 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]
*S. E. M	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, broilers from Control-AL had intermediate ($p<0.05$) values and broilers from the Control-FR treatment had the

highest ($p < 0.05$) GPxA in plasma (Table 2). Nitric Oxide (NO) 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 others, while the chickens from the Control-AL and Control-FR treatments had similar MDA concentrations in plasma (Table 2).

Table 2. Glutathione peroxidase activity (GPxA, nmol min-1 ml-1); nitric oxide (NO, μM) and malondialdehyde (MDA, μM) concentrations in 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]
*S. E. M.	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.

Birds from the Control-FR treatment had the lowest ($p<0.05$) MDA concentrations in lungs, heart and liver, whereas, birds from GSE-AL had the highest ($p<0.05$) concentration of MDA in heart and liver (Table 3). The Control-AL treatment resulted in a higher ($p<0.05$) MDA concentration in lungs than GSE-AL. The heart and liver of broilers from the Control-AL treatment had higher MDA concentration than the same tissues from the Control-FR birds, but had lower values than those observed in the GSE-AL treatment (Table 3).

Table 3. Lipid peroxidation (nmol MDA g^{-1} tissue) of 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]
*S. E. M.	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 diets of broilers without impairment of the productive performance. The thyroid gland is the body's primary regulator of metabolism, through hormones as triiodothyronine (T3) and thyroxine (T4); thyroid hormones 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 T3 and T4 concentrations in plasma of Wistar rats; GSE is a concentrated source of polyphenols that have antioxidant capacity (Brenes *et al.,* 2010). The similar results in productive performance of broilers observed in the present research may be explained by the antioxidant capacity of VE and GSE that protects thyroid hormones from oxidative degradation. In the consulted literature, the GSE concentration included in the diet of broilers has not produced consistent results. Brenes *et al.* (2010) found no significant differences

in body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) in broilers when GSE was included in the diet (0.6, 1.8 and 3.6 g kg^{-1} diet); however, Hughes *et al.* (2005) observed a decrease in FI and live weight of broilers fed with 30 g GSE kg^{-1} diet. Hughes *et al.* (2005) used a product that contained 90.2% of total extractable polyphenols; in contrast, the GSE used in our research contained 85% total extractable polyphenols, but only were added 10 mg kg^{-1} diet. Apparently, high dietary supplementation of GSE reduced the performance of chickens (Hughes *et al.,* 2005; Chamorro *et al.,* 2013). Probably because the polyphenols bind to proteins (Brenes *et al.,* 2010) and as consequence of this interaction the digestibility of protein and amino acids are reduced (Ortiz *et al.,* 1993). However in this study, when GSE-*ad libitum* was added in low quantities (10 mg kg^{-1} diet) the results in productive performance were similar to those observed with the Control-AL and Control-FR treatments.

Birds from the GSE-AL treatment had the highest ascites mortality, the highest concentration of MDA in plasma, heart and liver, and the lowest GPxA in plasma. The high altitude and *ad libitum* feeding may have induced the high ascites mortality observed in birds fed GSE-*ad libitum*; Rodríguez-Ortega *et al.* (2014) observed that *ad libitum* feeding increased ascites mortality in chickens raised at 2278 m of altitude. High feed intake can trigger ascites mortality, due to an increased metabolic rate that augments oxygen requirements at tissue level and mitochondrial production of reactive oxygen species (ROS) (Kalmar *et al.,* 2013).

The production of ROS due to *ad libitum* feeding and high altitude may possibly explain the low GPxA and high MDA concentration in plasma, lungs, heart and liver of broilers fed with GSE and of birds from the Control-AL treatment. We did not find information about lipid peroxidation evaluated in lungs, heart and liver of broilers fed with GSE and raised at 2278 m of altitude; however, El-Damrawy (2014) found a decrease in MDA concentration and an increase

in glutathione and superoxide dismutase activities in the liver of broilers fed with 100 or 200 mg of GSE kg^{-1} diet and raised under heat stress at low altitude (11 m above sea level, approximately). These amounts of GSE are greater than those included in the present research (100 or 200 mg *vs.* 10 mg kg^{-1} feed). Therefore, another possible explanation of the low GPxA in plasma and the high MDA concentrations in plasma, lungs, heart and liver of broilers is the low inclusion of GSE in the diet of the present study. The low AOA observed in the GSE diet compared with the control one (starter: 11 *vs.* 14; finisher: 11 *vs.* 17% inhibition of DPPH; respectively) may also explain the low GPxA in plasma and the high MDA concentrations in plasma, lungs, heart and liver of broilers fed with GSE. The results of this study showed that *ad libitum* feeding increase MDA concentrations in plasma, lungs, heart and liver of broilers raised at high altitude (2278 m of altitude).

The VE functions as a peroxyl radical scavenger that terminates chain reactions of the polyunsaturated fatty acids oxidation (Traber, 2007). Furthermore, feed restriction reduces the metabolic rate (Malan *et al.,* 2003; Singh *et al.,* 2011), and therefore, decreases the oxygen requirement and 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, is possible that less $O_2^{\cdot-}$ radical are converted into hydrogen peroxide (H_2O_2) which could result in a high availability of glutathione peroxidase.

Nitric oxide (NO) is a potent vasodilator of the cardiovascular system and has a short half-life, in the order of seconds. Thomas *et al.* (2006) estimated the half-life of extravascular NO is in a range of 0.09 to < 2 s. A possibility of the disappearance of NO, was its rapid reaction with the $O_2^{\cdot-}$ radical, at an estimated rate of 6.7×10^9 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 whit $O_2^{\cdot-}$ is approximately three times faster than the dismutation of the superoxide radical by superoxide dismutase, thus, an increased generation of $O_2^{\cdot-}$ in the vascular wall may inhibit the physiological functions of NO throughout its transformation in peroxynitrite . This could be a possible explanation of 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} feed) increased bioavailability of NO in plasma of broilers; in contrast, the concentration of arginine in our research was 1.5% kg^{-1} in the starter and finisher diets. A possible explanation of the similar NO levels observed among treatments in this research is because arginine levels were similar in all diets.

CONCLUSION

Our results suggest that grape seed extract may partially replace vitamin E in diets for broilers without affecting the productive performance; however, more research is required to find the optimal level of grape seed extract inclusion in the broilers' diet that may decrease ascites mortality, lipid oxidation in plasma, lungs, heart and liver, and increase glutathione peroxidase activity and nitric oxide concentration in plasma of broilers raised at 2278 m of altitude.

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REFERENCES

Arab HA, Jamshidi R, Rassouli A, Shams, Hassanzadeh MH. Generation of hydroxyl radicals during ascites experimental induced in broilers. British Poultry Science 2006; 47(02):216-222.

Bakonyi T, Radak Z. High altitude and free radicals. Journal of Sports Science and Medicine 2004; 3:64-69.

Bautista-Ortega J, Ruiz-Feria CA. L-Arginine and antioxidant vitamins E and C improve the cardiovascular performance of broiler chickens grown under chronic hypobaric hypoxia. Poultry Science 2010; 89:2141-2146.

Bottje W, Enkvetchakul B, More R. Effect of α-tocopherol on antioxidants, lipid peroxidation, and the incidence of pulmonary hypertension syndrome (ascites) in broilers. Poultry Science 1995; 74:1356-1369.

Bowen OT, Erf GF, Chapman ME, Wideman Jr RF. Plasma nitric oxide concentrations in broilers after intravenous injections of lipopolysaccharide or microparticles. Poultry Science 2007; 86:2550-2554.

Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensmittel-Wissenschaft and Technologie 1995; 28:25-30.

Brenes A, Viveros A, Goñi I, Centeno C, Saura-Calixto F, Arija I. Effect of grape seed extract on growth performance, protein and polyphenol digestibilities, and antioxidant activity in chickens. Spanish Journal of Agricultural Research 2010; 8(2):326-333.

Buege J, Aust SD. Microsomal lipid peroxidation. Methods in Enzymology 1978; 52:302-310.

Chamorro S, Viveros A, Centeno C, Romero C, Arija I, Brenes A. Effects of dietary grape seed extract on growth performance, amino acid digestibility and plasma lipids and mineral content in broiler chicks. Animal 2013; 7(4):555-561.

Díaz-Cruz A, Nava C, Villanueva R, Serret M, Guinzberg R, Piña E. Hepatic and cardiac oxidative stress and other metabolic changes in broilers with the ascites syndrome. Poultry Science 1996; 75:900-903.

El-Damrawy SZ. Effect of grape seed extract on some physiological changes in broilers under heat stress. Egyptian Poultry Science 2014; 34(I):333-343.

Fathi M, Naser adl K, Nezhad YE, Shahryar HA, Daneshyar M, Tanha T. The role of oxidative stress in the development of congestive heart failure (CHF) in broilers with pulmonary hypertension syndrome (PHS). Journal of Cell and Animal Biology 2011; 5(8):176-181.

Goldstein S, Czapski G. The reaction of $NO^{\cdot}$ with $O_2^{\cdot -}$ and $HO_2^{\cdot}$: A pulse radiolysis study. Free Radical Biology and Medicine 1995; 19:505-510.

Hao R, Li Q, Zhao J, Li H, Wang W, Gao J. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. Livestock Science 2015; 178:237-242.

Hughes RJ, Brooker JD, Smyl C. Growth rate of broiler chickens given condensed tannins extracted from grape seed. Australian Poultry Science Symposium 2005; 17:65-68.

Jang SI, Chae RK, Kang ST, Kim KY, Kim KC, Hwang HJ, Hwang YD, Choi BC, Jung KK, Cho SJ. Effects of long-term vitamin E and butylated hidroxytoluene supplemented diets on murine intestinal and hepatic antioxidant enzyme activities. Asian-Australasian Journal of Animal Sciences 1999; 12 (6):932-938.

Julian RJ. Review article: Physiological, management and environmental triggers of the ascites syndrome: a review. Avian Pathology 2000; 29:519-527.

Julian RJ. The response of the heart and pulmonary arteries to hypoxia, pressure, and volume. A Short Review. Poultry Science 2007; 86:1006-1011.

Kalmar ID, Vanrompay D, Janseens GPJ. Review: Broiler ascites syndrome: Collateral damage from efficient feed to meat conversion. The Veterinary Journal 2013; 197:169-174.

Kennedy DG, Rice DA, Bruce WD, Goodall EA. Economic effects of increased vitamin E supplementation of broiler diets on commercial broiler production. British Poultry Science 1992; 33:1015-1023.

Kojda G, Harrison D. Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovascular Research 1999; 43:562–571.

Malan DD, Scheele CW, Buyse J, Kwakernaak C, Siebrits FK, van der Klis JD, Decuypere E. Metabolic rate and its relationship with ascites in chickens genotypes. British Poultry Science 2003; 44(2):309-315.

Malik R, Hodgson H. The relationship between the thyroid gland and the liver. The Quarterly Journal of Medicine 2002; 95:559-569.

Maxwell MH, Robertson GW. World broiler ascites survey. Poultry International 1997; 36:16-30.

NOM-033-ZOO-1995. Norma Oficial Mexicana. Sacrificio humanitario de los animales domésticos y silvestres. Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) 1995; 12 p.

Ortiz LT, Centeno C, Treviño J. Tannins in faba bean seeds: effects on the digestion of protein and amino acids in growing chicks. Animal Feed Science and Technology 1993; 41:271-278.

Peepre K, Deshpandey U, Choudhary PS. Role of antioxidants on thyroid hormone in Wister rats. International Journal of Science and Research 2014; 3(1):34-38.

Rodríguez-Ortega LT, Sosa-Montes E, Pro-Martínez A, Ruiz-Feria CA, Bautista-Ortega J, Vargas-Galicia AJ, Chan-Díaz D, Pérez-Hernández P. Free access to feed reduces the antioxidative capacity in broilers raised at high altitudes. Poultry Science 2014; 93 (E-Supp. 1)77.

Ruiz-Feria CA, Wideman Jr RF. Taurine, cardiopulmonary hemodynamics, and pulmonary hypertension syndrome in broilers. Poultry Science 2001; 80:1607-1618.

SAS Institute. Language guide for personal computers, release 9.0 Edition. SAS Institute Cary N. C. USA 2006. 1028 p.

SIAP. Servicio de Información Agroalimentaria y Pesquera. 2014. Ave para carne, Población Avícola 2005-2014. Avaible in: http://www.siap.gob.mx/opt/poblagand/avecarn.pdf. Consulted on May 14th, 2016.

Singh PK, Shekhar P, Kumar K. Nutritional and managemental control of ascites syndrome in poultry. International Journal of Livestock Production 2011, 2(8):117-123.

Thomas SR, Schulz E, Keanney JF. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity— Role for iron-dependent oxidative stress. Free Radical Biology and Medicine 2006; 41:681–688.

Traber MG. Vitamin E Regulatory Mechanisms. Annual Review of Nutrition 2007; 27:347-362.

Ulu, Hasret. Evaluation of three 2-thiobarbituric acid methods for the measurement of lipid oxidation in various meats and meat products. Meat Science 2004; 67:683-687.

Vázquez-García JC, Pérez-Padilla R. Valores gasométricos estimados para las principales poblaciones y sitios a mayor altitud en México. Revista del Instituto Nacional de Enfermedades Respiratorias 2000; 13(1):06-13.

Visschedijk AHJ. Gas exchange and hatchability of chicken eggs incubated at simulated high altitude. Journal of Applied Physiology 1985; 58:416-418.

Wideman RF, Kirby YK. Evidence of a ventilation-perfusion mismatch during acute unilateral pulmonary artery occlusion in broilers. Poultry Science 1995; 74:1209-1217.