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EVALUACIÓN DE ALICINA SINTÉTICA EN LA PREVENCIÓN DEL SÍNDROME ASCÍTICO EN POLLOS DE ENGORDA

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EVALUATION OF SYNTHETIC ALLICIN IN THE PREVENTION OF ASCITES SYNDROME IN BROILERS

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ABSTRACT

A series of experiments were conducted to evaluate the effect of allicin on ascites-related traits [blood oxygen saturation (SaO_2), hematocrit content (Hct%) and the right-to-total ventricular weight ratio (RV:TV)], expression of angiotensin II type 1 receptor (ATR1) gene in lung, heart and liver, digestive organs variables, ascites mortality and productive performance in broilers raised under ascites-inducing conditions. In all experiments, 1-day-old "Ross 308" broilers were used and distributed in the treatments: 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW), 2.5-ALLI (2.5 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) or 10-ALLI (10 mg of allicin/kg of BW). The treatments were applied daily from 14 to 20 or 27 days of age; and they were administered using a pediatric catheter to ensure consumption of allicin. Results of these experiments showed that 1-ALLI or 2.5-ALLI treatments increase SaO_2, reduce Hct% and RV:TV, and modify the expression of ATR1 gene in heart and lung in broilers. No differences ($P > 0.05$) were found among treatments for ATR1 gene expression in the liver. Regarding to the digestive organs variables, only liver weight was affected by treatment ($P < 0.05$); broilers in the 2.5-ALLI group showed higher liver weight than broilers in the 0-ALLI and 1-ALLI groups. Ascites mortality and productive performance was not affected by treatments. In conclusion, allicin improves some ascites related traits in broilers without compromising productive performance in broilers.

Keywords: allicin, blood oxygen saturation, hematocrit content, right-to-total ventricular weight ratio, angiotensin II type 1 receptor.

EVALUACIÓN DE ALICINA SINTÉTICA EN LA PREVENCIÓN DEL SÍNDROME ASCÍTICO EN POLLOS DE ENGORDA

Artemio Jovanny Vargas Galicia, Dr.
Colegio de Postgraduados, 2019

RESUMEN

Se realizaron una serie de experimentos para evaluar el efecto de la alicina en las variables relacionadas con la ascitis [saturación de oxígeno en la sangre (SaO_2), contenido de hematocrito (Hct%) y relación ventrículo derecho/ventrículo total (RV:TV)], expresión del receptor tipo 1 de angiotensina II (ATR1) en pulmón, corazón e hígado, variables de los órganos digestivos, mortalidad por ascitis y comportamiento productivo en pollos de engorda criados en condiciones inductoras de ascitis. En los experimentos se utilizaron pollos "Ross 308"de un día de edad los cuales fueron distribuidos en cada uno de los tratamientos: 0-ALLI o testigo [(0 mg de alicina/kg de peso vivo (PV)], 1-ALLI (1 mg de alicina/kg de PV), 2.5-ALLI (2.5 mg de alicina/kg de PV), 5-ALLI (5 mg de alicina/kg de PV) o 10-ALLI (10 mg de alicina/kg de PV). Los tratamientos se proporcionaron diariamente desde los 14 hasta los 20 o 27 días de edad; y se administraron utilizando una sonda pediátrica para asegurar el consumo de alicina. Resultados de estos experimentos mostraron que los tratamientos 1-ALLI o 2.5-ALLI incrementan la SaO_2, reducen el Hct% y RV:TV, y modifican la expresión del gen ATR1 en corazón y pulmón en pollos de engorda. No se encontraron diferencias ($P > 0.05$) entre tratamientos para la expresión del gen ATR1 en el hígado. Con respecto a las variables de los órganos digestivos, solo el peso del hígado fue afectado por efecto de tratamiento ($P < 0.05$); los pollos del tratamiento 2.5-ALLI tuvieron mayor peso hepático que los pollos de los tratamientos 0-ALLI y 1-ALLI. La mortalidad por ascitis y el comportamiento productivo no fueron afectados por efecto de los tratamientos. En conclusión,

la alicina mejora algunos rasgos relacionados con la ascitis sin afectar el comportamiento productivo en los pollos de engorda.

Palabras clave: alicina, saturación de oxígeno en la sangre, hematocrito, relación ventrículo derecho/ventrículo total, receptor tipo 1 de angiotensina II.

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GENERAL INTRODUCTION

Ascites syndrome (AS) or pulmonary hypertension syndrome is a metabolic disorder in broilers (Closter *et al*., 2012). AS is initiated by factors that elevate the blood pulmonary pressure; this increase in pulmonary arterial pressure (hypertension) triggers the accumulation of fluid in the abdominal cavity of broilers. Contributing factors such as high altitude, temperature, lighting program, ventilation and nutritional management, all seem to influence the development of AS (Balog, 2003; Issac *et al*., 2010). But, the susceptibility of broilers to develop AS is favored whenever environmental conditions restrict the availability of O_2 or increase the tissue demand for O_2 (Khajali and Wideman, 2016). So, AS has been induced experimentally with combinations of low ambient temperatures and simulated high altitude (Beker *et al*., 2003). The high altitude of the Valley of Mexico (2278 m) is a predisposing factor that increases the development of AS; also, it is estimated that about 15 million broilers are lost per year in Mexico by this problem (Rodríguez-Ortega *et al*., 2017). The use of compounds or drug agents that increase the vascular capacity of the lungs or decrease the pulmonary vascular resistance may help to deal with AS in broilers (Aftab and Khan, 2005; Lorenzoni and Ruiz-Feria, 2006). Bioactive compounds of garlic like allicin may represent an alternative to delay the pathophysiological progression of AS, and it has been reported that garlic compounds have antibacterial, antifungal, antiparasite, antiviral, antioxidant, antithrombotic, anticancer, vasodilator and blood pressure-lowering activities (Raeesi *et al*., 2010; Ried and Fakler, 2014). The mechanisms for the blood pressure-lowering effects of garlic include vasorelaxation mediated through H_2S production, modulation of the production and function of both endothelium-derived relaxing and constricting factors, beta-adrenoceptor blocking action, reduction in angiotensin converting enzyme and angiotensin II activity (Nwokocha *et al*., 2011). Therefore, the use of allicin may represent an alternative to reduce the development of AS.

Hypothesis

The anti-hypertensive properties of allicin may reduce the development of AS and therefore ascites mortality without compromising productive performance of broilers.

Objective

To evaluate the effect of synthetic allicin on ascites-related traits [hematocrit content (Hct%), right-to-total ventricle weight ratio (RV:TV) and blood oxygen saturation (SaO_2)], expression of angiotensin II type 1 receptor (ATR1) gene in lung, heart and liver, productive performance [body weight (BW) gain, feed intake, feed conversion and breast skin pigmentation], digestive organs variables and ascites mortality in broilers reared under ascites-inducing conditions.

LITERATURE REVIEW

AS has been a major concern for the broiler industry worldwide (Druyan *et al*., 2009). Broilers genetically selected for fast muscle growth are more susceptible to AS than slow-growing strains (Aftab and Khan, 2005). The causes of this syndrome are multifactorial but diet, environmental and genetic factors play an important role; also, the imbalance between O_2 supply and the O_2 required to sustain rapid growth rate lead to development of AS in broilers (Baghbanzadeh and Decuypere, 2008). AS is characterized by a cascade of events that begins when the O_2 demands for maintenance and growth are not fully supplied by the cardiovascular system (Druyan *et al*., 2009); due to the fact that broilers have a metabolic rate of approximately 2.0-2.5 times higher than mammals of comparable body size (Khajali and Wideman, 2016). Also, it has been reported that both heart and lung size (as percentage of live BW) have been reduced with genetic selection for increased growth rate; thus, hypoxia and AS could be triggered if the lung of broilers grows less rapid than the rest of the body (Hassanzadeh *et al*., 2014). Lesions observed in broilers that die from AS include fluid accumulation in the pericardium and in the abdominal cavity, lung

edema, flaccid heart, hypertrophy and dilation of the heart, especially the right ventricle, liver changes, hypoxemia, pale comb and higher blood hematocrit (Baghbanzadeh and Decuypere, 2008; Druyan *et al*., 2009); but, the presence of mild cyanosis (indicative that arterial blood is less than 80% saturated with O_2) serves as an early visible symptom that an apparently healthy broiler may develop AS (Wideman, 2000). In the following sections of this paper some factors (altitude, temperature, diet and feed restriction) that initiate or promote AS are described.

High altitude

The main environmental factor that play a role in AS development in broilers is the high altitude (Balog, 2003). At sea level O_2 makes up 20.9% of the atmosphere (Julian, 2000). The effect of high altitude (natural or simulated) is reflected in hypoxia, that is the decrease in the partial pressure of O_2 (PPO_2) in the inspired air (Wideman *et al*., 2013; Hassanzadeh *et al*., 2014). The PPO_2 becomes lower with increasing altitude, and reduced concentrations of inspired O_2 trigger pulmonary vasoconstriction and AS in broilers (Julian, 2000; Wideman *et al*., 2013). Baghbanzadeh and Decuypere (2008) mentioned that poor oxygenation of tissues can be caused by increased metabolism from rapid growth of broilers or by a decreased availability of environmental O_2 due to high altitude; whereas, Julian (1993) mentioned that consequences of exposure of broilers to low atmospheric O_2 concentrations are: constriction of pulmonary blood vessels, restriction of blood-flow to the lungs and increase the work-load on the right ventricle. On the other hand, hypoxemia (blood under-saturated with O_2) stimulates erythropoiesis to increases the hematocrit concentration (also increases the blood's viscosity) as a mechanism to enhance the O_2 carrying capacity of blood (Wideman *et al*., 2013). Thus, hypoxia causes an increase in blood viscosity which in turn increase the resistance to blood flow through the pulmonary vessels (Julian, 1993; Hassanzadeh *et al*., 2014). Then, a low percentage of blood oxygen saturation (SaO_2) is a

reliable indicator for AS susceptibility, so that some breeding companies have selected breeder broilers with high SaO_2 at 5 weeks of age (Navarro *et al*., 2006; Druyan *et al*., 2007).

Cold temperature

The second environmental factor that causes pulmonary arterial hypertension is cold temperature. Broilers expose to low temperatures exhibit the same pathological signs as birds that develop ascites as the result of low O_2 levels, such as high hematocrit and right ventricle/total ventricle ratio (Balog, 2003). Cold temperatures increase AS by increasing metabolic O_2 requirements (Wideman, 2000; Baghbanzadeh and Decuypere, 2008). The susceptibility of broilers to develop AS increases as ambient temperatures decrease, because broilers need more O_2 in order to maintain their body temperature (Ipek and Sahan, 2006). It is known that in order to balance the body temperature, broilers are forced to increase feed intake under low ambient temperatures (Aksit *et al*., 2008). Also, the duration of cold stress is more critical than the minimum temperature reached at which broilers are exposed (Groves, 2002). In this context, it has been reported that ascites mortality may reach up to 25% in broilers reared under naturally cold conditions (Balog *et al*., 2003) and up to 50% under experimental acute cold exposure (Druyan *et al*., 2007).

Diet

A bird consumes approximately 1 L of O_2 during the catabolism of 1 g of protein, thus given a reduced-protein diet may spare the O_2 needed for the catabolism, and this reduction in O_2 demand may account for a lower incidence of AS (Khajali and Wideman, 2016). Diet form is also involved in the development of AS. Mash-form diet reduces growth rate, mortality and condemnations due to AS at the processing plant; whereas, pelleted diet fed to broilers increases the incidence of AS when compared to broilers fed with mash-form diet (Baghbanzadeh and Decuypere, 2008). This may be explained because broilers consume more feed when it is given as pellets as opposed to a

mash; therefore, growth rate is increased (Balog, 2003); so, mash diets are effective in the prevention of AS in broilers (Khajali and Wideman, 2016). On the other hand, male broilers have a higher BW, and therefore a higher O_2 demand, thus males are expected to be more prone to developing ascites than females (Closter *et al*., 2012). Also, high-density diets fed to broilers promote the development of AS compared with low-density diets, this is related to increased metabolic rate which in turn increases O_2 demand for metabolic processes (Khajali and Wideman, 2016).

Feed restriction

High feed intake can trigger the AS by increasing the O_2 requirement (Julian, 2000). Feed restriction (FR) is a technique used to manipulate broiler growth to alleviate the incidence of some metabolic disorders like AS (Sahraei, 2014), this method is thought to have an effect by slowing the growth rate of broilers (Balog *et al*., 2000). However, although ascites mortality may be significantly reduced in fed-restricted birds, there is a decrease in body weight and breast meat yield compared to full-fed birds (Sahraei, 2014). Then, the FR program required to control AS needs to be balanced with the time required to reach market weight of broilers. Another problem resulting from FR programs is poor skin pigmentation, which is directly related to the quantity of xanthophylls consumed. Skin pigmentation is important because it is perceived as a measure of quality by the consumers (Baghbanzadeh and Decuypere, 2008).

Renin angiotensin system

The Renin Angiotensin System (RAS) is a hormonal cascade that is involved in the pathogenesis of cardiovascular disorders; the RAS functions in maintaining the homeostasis of arterial pressure, tissue perfusion, and extracellular volume (Burks, 2011). The precursor angiotensinogen, is released from the liver and is cleaved in the circulation by the enzyme renin that is secreted from

the juxtaglomerular apparatus of the kidney to form angiotensin I (Ang I). Ang I is then converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE), which is predominantly expressed in high concentrations on the surface of endothelial cells in the pulmonary circulation (Paul *et al*., 2006; Shouk *et al*., 2014). Angiotensin II (Ang II) is the main active component of RAS that regulates the cardiovascular system; so, alterations of the RAS contribute to the development of hypertension, renal diseases and chronic heart failure (Hassanpour *et al*., 2016). Most of the well-known actions of ANG II such as vasoconstriction, increased blood pressure, increased cardiac contractility and cardiac hypertrophy are mediated by the angiotensin II type 1 receptors (ATR1), while ATR2 receptors has been considered to be an enigma. ATR1 and ATR2 receptors are expressed in the heart where they appear to be localized on cardiomyocytes, while ATR1 receptors localized on vascular smooth muscle cells mediate vasoconstriction (Paul *et al*., 2006; Burks, 2011). Some authors suggest that blocking the RAS with either angiotensin converting enzyme inhibitors or Ang II receptor antagonists may prevent pulmonary arterial hypertension (Sharma and McNeill, 2006). In this context, compounds that tend to increase the vascular capacity of the lungs or decrease the pulmonary vascular resistance would theoretically reduce the incidence of AS (Aftab and Khan, 2005).

Garlic

Garlic (*Allium sativum* L.) has acquired a reputation in different cultures as a prophylactic as well as therapeutic medicinal plant; also, garlic has attracted the attention of modern medicine because its effects in maintaining good health (Bayan *et al*., 2014). Some studies have shown that garlic has antioxidant, antithrombotic, antimicrobial, hypolipidemic, antihypertensive and natural growth promoter activities (Raeesi *et al*., 2010; Borlinghaus *et al*., 2014; Shouk *et al*., 2014). When garlic is chopped or crushed, natural allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate) is

produced by an enzymatic reaction; the precursor of allicin is alliin which is hydrolyzed by the enzyme alliinase. This reaction leads to production of dehydroalanine and allyl sulfenic acid, two molecules of allyl sulfenic acid condense spontaneously to one molecule of allicin (Bayan *et al*., 2014; Borlinghaus *et al*., 2014). Allicin represents about 70% of the overall thiosulfinates present in the cloves upon mechanical crushing (Rahman, 2007). Allicin, is a compound with different biological properties, it is responsible for the smell and taste of freshly cut or crushed garlic (Borlinghaus *et al*., 2014). It has been suggested that one mechanism by which garlic may elicit its antihypertensive effects is through inhibition of angiotensin-converting enzyme (Shouk *et al*., 2014); however, Bayan *et al*. (2014) mentioned that another possible mechanism of antihypertensive activity of garlic is due to its prostaglandin-like effects, which decrease peripheral vascular resistance.

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CHAPTER I. PRELIMINARY STUDY: SYNTHETIC ALLICIN REDUCES HEMATOCRIT CONTENT AND MIGHT IMPROVE RIGHT VENTRICLE WEIGHT/TOTAL VENTRICULAR WEIGHT RATIO IN BROILERS RAISED AT HIGH ALTITUDE

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ABSTRACT

The current study was conducted to evaluate the effect of synthetic allicin on ascites-related traits [hematocrit content (Hct%) and right-to-total ventricular weight ratio (RV:TV)] and body weight (BW) gain in broilers raised at 2278 m above sea level. Twenty 1-day-old male broilers "Ross 308" were distributed into four treatments: 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) and 10-ALLI (10 mg of allicin/kg of BW). The treatments were applied during 7 days, from 14 to 20 days of age. Synthetic allicin was administered by using a pediatric catheter. BW gain was recorded weekly. Hct% was evaluated at 21, 28 and 35 days of age. All chickens were humanely killed by cervical dislocation at day 35 to determine RV:TV. BW gain and RV:TV were not affected by treatment. Birds in 1-ALLI and 5-ALLI groups showed lower ($P < 0.10$) Hct% than birds in 0-ALLI. In conclusion, allicin improved hematocrit without compromising BW gain in broilers. Further studies are needed to find the optimal dose of synthetic allicin that could statistically improve RV:TV, an important ascites-related trait. Therefore, the use of synthetic allicin might be an alternative to reduce ascites syndrome in broilers.

Keywords: allicin, hematocrit, right ventricular hypertrophy, ascites syndrome, broilers.

INTRODUCTION

Pulmonary arterial hypertension, ascites syndrome or pulmonary hypertension syndrome (Wideman *et al*., 2013) is caused by the imbalance between oxygen delivery and oxygen demand in fast-growing chickens (Baghbanzadeh and Decuypere, 2008). Ascites is a complex problem caused by many interacting factors such as genetics, high altitude, environment (especially in cold environments) and management (Aftab and Khan, 2005; Wideman *et al*., 2013). This syndrome is of major concern in Mexico and other countries with farm facilities at altitudes above 2000 m. At high altitude, atmospheric pressure (hence oxygen pressure) is lower than that at sea level, thus exposure to a low atmospheric partial pressure of O_2 (hypoxia) triggers acute pulmonary vasoconstriction and pulmonary hypertension. Due to this fact, high altitude has been reported to be an important factor in ascites development (Wideman *et al*., 2013; Tekeli, 2014). High hematocrit concentration and vasoconstriction are consequences of hypoxemia that lead to elevated viscosity/pressure and low oxygen saturation of blood (Aftab and Khan, 2005). The high altitude where the Valley of Mexico (2278 m) is located is a predisposing factor that increases the ascites mortality in broilers; it is estimated that about 15 million broilers are lost per year in Mexico by this problem (Rodriguez-Ortega *et al*., 2017). Research has focused on the development of feeding strategies to modulate broiler's body weight (BW) gain in order to deal with the ascites problem. However, innovative strategies that may reduce ascites mortality could be alternatives to reduce economic losses for the poultry industry worldwide. Garlic (*Allium sativum*) has played an important dietary as well as medicinal role in human history. Garlic is considered to be one of the best disease-preventive foods given its high content of organosulfur compounds which are considered the responsible for most of garlic´s pharmacological activities. Allicin is the most biologically active compound of garlic (Qidwai and Ashfaq, 2013). In this context, it is has been

reported that the inclusion of 0.5% garlic bulbs in broiler diets has an antihypertensive effect, which could decrease ascites incidence without impairing broiler performance (Varmaghany *et al*., 2015); whereas other researchers have found that garlic powder prevents the increase in pulmonary pressure in rats with pulmonary hypertension (Fallon *et al*., 1998). We did not find any report on the use of synthetic allicin in broilers with ascites. We hypothesized that compounds with hypotensive effect, such as allicin, could be an option to reduce the pulmonary arterial hypertension syndrome. Therefore, the present study was conducted to evaluate the effect of synthetic allicin on ascites-related traits [hematocrit content (Hct%) and right-to-total ventricular weight ratio (RV:TV)] and BW gain in broilers raised at 2278 m of altitude.

MATERIALS AND METHODS

This study was carried out at the poultry facilities at Colegio de Postgraduados, Campus Montecillo, Mexico State, Mexico, located at 2278 m of altitude (Vázquez-García and Pérez-Padilla, 2000). A total of 20 1-day-old male broiler chicks "Ross 308" were distributed into four treatments: 0-ALLI or control (0 mg of allicin/kg of BW; using only distilled water at pH 6.5), 1-ALLI (1 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) and 10-ALLI (10 mg of allicin/kg of BW). All treatments were randomly assigned to the experimental groups (five replicates per treatment). Diets were formulated to meet or exceed the nutritional recommendations of Aviagen (Aviagen, 2009). The diets were given in mash form and the feeding program was divided in two phases: starter diet (1-21 days) containing: 3025 kcal of metabolizable energy (ME)/kg, 22% crude protein (CP), 1.05% Ca and 0.50% available P; and a grower diet (22-35 days) containing: 3150 kcal ME/kg, 21% CP, 0.90% Ca and 0.45% available P. Feed and water were offered *ad libitum* throughout the experimental period. A 23 hours light, 1 hour dark (23L:1D) schedule was used until day 14; and from day 15 to the end of the experimental period

a 12L:12D lighting program was used. During the first week of the experiment, room temperature was maintained at 32 °C and then it was reduced 2 °C per week until 21 °C. The treatments were applied daily from 14 to 20 days of age; and they were administered by oral-esophageal route using a pediatric catheter (1.67 mm diameter) to ensure consumption of synthetic allicin.

Allicin production

Allicin was produced according to the methodology described by Argüello-García *et al*. (2010). Briefly, 1 g diallyl disulphide was dissolved in 5 mL acetic acid under stirring in an ice bath. Hydrogen peroxide (1.5 mL, 30% v/v) was added stepwise and the reaction was allowed to proceed for 30 minutes. Afterwards, the reaction was kept at 13 °C for 20 minutes; then, it was put again under stirring for 5 hours, stopped with 15 mL distilled water at pH 6.5, and extracted with 30 mL dichloromethane. After 5 extractions with 5% (w/v) Na_2CO_3 (20 mL each) and 3 extractions with distilled water (20 mL each), the solvent was left to evaporate until yellowish oil (allicin) remained. For stabilization and storage, allicin was resuspended in water at 2.5% (w/v) and kept at -70 °C until used. The synthetic allicin dissolutions were administered at pH 6.5 and 5 °C, due to its environmental temperature instability.

Hematocrit content and body weight gain

BW gain was recorded weekly from 14 to 35 days of age and Hct% was evaluated at 21, 28 and 35 days of age. For Hct% determination, blood samples from each bird (1 mL; 5 birds per treatment) were collected from a wing vein in EDTA tubes. Hct% was determined in whole blood samples by centrifugation of microhematocrit capillary tubes at 1000 × g for 10 minutes.

Right-to-total ventricular weight ratio

All chickens were humanely killed at 35 days of age according to the Normal Oficial Mexicana (2015). The hearts were collected, and the pericardium, peripheral adipose tissues, and atria were dissected. The left and right ventricles were separated and their individual weights were recorded, and the RV:TV was calculated. Ascites was diagnosed if RV:TV was above 0.27 (Balog *et al*., 2003). This study was conducted in compliance with existing protocols for the use and care of animal intended for research approved by the General Academic Council of Colegio de Postgraduados, México.

Statistical analysis

Data on Hct% and BW gain were analyzed as repeated measures using the GLIMMIX procedure of SAS version 9.4 (SAS Institute, 2013) using two statistical models: The generalized linear mixed model (GLMM) with a beta distribution with parameters π_{ijk} and ϕ as the mean and scale parameter respectively for the percentage of hematocrit content. That is; $y_{ijk}|r_k \sim beta(\pi_{ijk}, \phi)$ and r_k as random effect assuming $r_k \sim N(0, \sigma_r^2)$. The *linear predictor* the percentage of hematocrit content is $\eta_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + r_k$ where μ is the overall effect, α_i is the $i-$th treatment effect, τ_j is the $j-$th time effect, $(\alpha\tau)_{ij}$ is the $ij-$th interaction effect between treatment and time, r_k is the random effect due to subject (chicken) with $r_k \sim N(0, \sigma_r^2)$. The *link function* that relates the mean with the *linear predictor* (η_{ijk}) is $\log(\pi_{ijk}) = \eta_{ijk}$. And the linear mixed model (LMM) $y_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + r_k + \varepsilon_{ijk}$ where y_{ijk} is the BW gain, $\mu, \alpha_i, \tau_j, (\alpha\tau)_{ij}, r_k$ described above, and ε_{ijk} is the error term assuming $\varepsilon_{ijk} \sim N(0, \sigma^2)$ for the BW gain. RV:TV data were analyzed as a one-way ANOVA using the GLM procedure of SAS 9.4. Each bird was

considered as the experimental unit. Statistical difference was set at $P < 0.05$, and mean comparison were separated using the LSD test.

RESULTS

Hematocrit and BW gain

The Hct% was affected by age ($P < 0.0001$); but was not affected by treatment nor interaction treatment × age at level of $P < 00.5$. However, differences in this variable were found at level of $P < 0.10$ by treatment effect. Birds in the 1-ALLI and 5-ALLI groups showed lower Hct% than birds in the 0-ALLI group. At 28 days of age birds showed lower values of Hct% than at 21 and 35 days of age (Figure 1. 1). On the other hand, no differences were observed ($P > 0.05$) for BW gain among treatments (Table 1. 1).

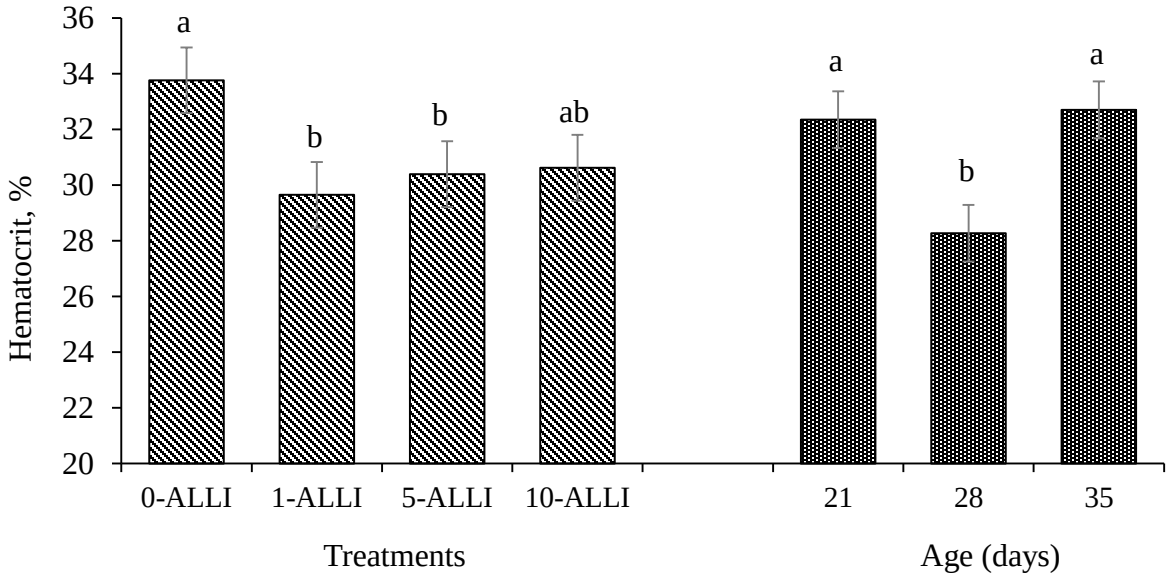

Figure 1. 1. Effect of synthetic allicin on hematocrit content (%) in broilers. 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) and 10-ALLI (10 mg of allicin/kg of BW). Treatment = 0.0212; Age = 0.0044; Treatment × Age = 0.6761. [a,b] Values with different superscript are statistically different ($P < 0.05$).

Table 1. 1. Effect of synthetic allicin on BW gain (g) in broilers.

	Age (days)			SEM	P-value		
Treatments	21	28	35		Treatment	Age	Treatment × Age
0-ALLI	513	588	778	50.61	0.7175	<0.0001	0.9504
1-ALLI	500	602	814				
5-ALLI	482	608	820				
10-ALLI	465	583	753				

SEM = Standard Error of the Mean.

0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) and 10-ALLI (10 mg of allicin/kg of BW). No statistical differences were found among treatments ($P > 0.05$).

Right-to-total ventricular weight ratio

In this assay, no significant ($P > 0.05$) differences were found in RV:TV among treatments. However, a trend of improvement in RV:TV was observed in birds from 1-ALLI (Figure 1. 2).

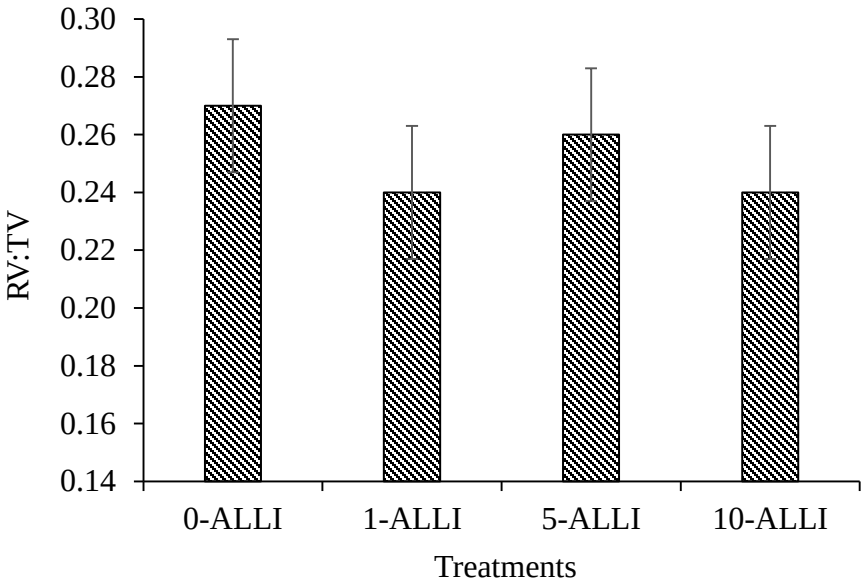

Figure 1. 2. Effect of allicin on right-to-total ventricular weight ratio (RV:TV) in broilers raised at 2278 m above sea level. 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW), 5-ALLI (5 mg of allicin/kg of BW) and 10-ALLI (10 mg of allicin/kg of BW). No statistical differences were found among treatments (P = 0.7093).

DISCUSSION

The high metabolic rate of modern broiler lines causes a high demand for O_2 (Aftab and Khan, 2005). At 2278 m of altitude, the partial pressure of O_2 is lower than that at sea level, which in turn reduces the levels of inspired O_2 triggering acute pulmonary vasoconstriction and pulmonary hypertension in broilers (Wideman *et al*., 2013). We hypothesized that the use of synthetic allicin could be an option to reduce the pulmonary arterial hypertension syndrome in broilers. In this study, the Hct% was not affected by treatment (P = 0.0795); however, differences were found at level of P < 0.10. Birds in the 1-ALLI and 5-ALLI groups showed lower Hct% than birds in the 0-ALLI group. On the other hand, age affected Hct%, and it was observed that birds at 28 days of age showed lower values of Hct% than at 21 and 35 days of age. Tekeli (2014) reported that the increase in hematocrit of broilers could be explained by increased oxygen demand of tissues due

to enhanced metabolic rate and decreased oxygen saturation. On the other hand, hypoxemia does stimulate erythropoiesis, which increases the Hct% (Wideman *et al*., 2013). Thus, the lower Hct% values observed in this study could be the result of the blood pressure-lowering properties of allicin. Allicin could had blocked angiotensin-II production, which in turn promotes vasodilatation and thus reduces the blood pressure (Ried and Fakler, 2014). Tekeli (2014) reported that broilers raised at high altitude showed an increased hematocrit concentration in blood; whereas Shlosberg *et al*. (1998) reported that dead ascitic birds show high hematocrit concentration that increases blood viscosity and augments the inability of right ventricle to pump blood through the pulmonary vessels. Baghbanzadeh and Decuypere (2008) mentioned that elevation in hematocrit can be caused by diminished plasma volume, as a result of fluid exudation out of the blood system to the abdominal cavity.

Balog *et al*. (2003) reported that RV:TV above 0.27 strongly indicates the start of ascites development. Although no significant differences were found in RV:TV ($P = 0.7093$) among treatments, broilers in 0-ALLI group (0.28 ± 0.02) showed a ratio above 0.27 which indicates the start of ascites development, whereas birds in the others treatments showed a ratio below 0.27. Thus, right ventricle hypertrophy can be attributed to an increased hematocrit concentration, which elevates blood viscosity that results in a higher cardiac workload. In this regard, it is has been reported that in ascitic chicks, the heart tries to pump more blood through the lungs to meet the body's O_2 requirement and right ventricle significantly enlarges in response to increased workload (Tekeli, 2014). Then, results found in this study may suggest that synthetic allicin is a compound that might dilate the pulmonary vasculature and thereby lower the resistance to blood flow which in turn could delay or inhibit the onset of pulmonary hypertension. On the other hand, no differences were observed for BW gain among treatments ($P = 0.7175$); however, a reduction in

BW was expected due to the pungent smell of allicin, that could lead to a reduction in feed intake. Likewise, Varmaghany *et al*. (2015) reported no significant differences in BW gain among groups during 1 to 42 days of age, under treatment with increasing garlic bulb levels at cold temperature conditions; however, Islam *et al*. (2017) found that garlic supplementation of 1% in broiler diet increased BW gain and improved feed conversion. In conclusion, allicin reduced Hct% without compromising BW gain in broilers. Further studies are needed to find the optimal dose of synthetic allicin that could improve the RV:TV in broilers and favor their survival in poultry farms located at high altitudes.

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CHAPTER II. EFFECT OF SYNTHETIC ALLICIN ON ANGIOTENSIN II TYPE 1 RECEPTOR GENE EXPRESSION IN LUNGS AND HEART, AND ASCITES-RELATED TRAITS IN BROILERS REARED UNDER ASCITES-INDUCING CONDITIONS

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Colegio de Postgraduados, 2019

ABSTRACT

The aim of this study was to evaluate the effect of synthetic allicin on ascites-related traits [hematocrit content (Hct%) and the right-to-total ventricular weight ratio (RV:TV)], angiotensin II type 1 receptor (ATR1) gene expression in lung, heart and liver, ascites mortality, and productive performance in broilers raised at 2278 m of altitude. Two hundred and ten 1-day-old male broiler chicks (strain: Ross 308) were randomly assigned to three treatments: 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). Seven replicates of 10 birds each per treatment were used. Ascites was induced by exposure to natural conditions of hypoxia and *ad libitum* provision of pelleted feed. Treatments were applied daily from 14 to 27 days of age. Synthetic allicin was administered using a pediatric catheter. At 21, 28, 35 and 42 days of age, 21 birds per treatment were randomly selected to assess Hct%. At 43 days of age eight birds per treatment were selected to determine RV:TV. Six chickens per treatment were randomly selected at 36 days to evaluate ATR1 expression in lung, heart and liver. Cumulative ascites mortality was recorded. Productive performance was recorded weekly. At 42 days of age, birds in 2.5-ALLI group showed the lowest Hct%. Similarly, birds in 2.5-ALLI group showed the lowest ($P < 0.05$) RV:TV values. The ATR1 expression in the lung and heart of broilers in the 1-ALLI and 2.5-ALLI groups, respectively was higher than the control group ($P < 0.05$). No significant differences among treatments were observed for ATR1 expression in the liver, ascites mortality and productive performance. In conclusion, synthetic allicin reduced

RV:TV and hematocrit content, and upregulates ATR1 gene in lung and heart without compromising broiler productive performance.

Keywords: allicin, angiotensin II receptor type 1, hematocrit, right ventricular hypertrophy, ascites syndrome.

INTRODUCTION

Pulmonary hypertension syndrome also known as ascites syndrome (AS), is a condition caused by an imbalance between the oxygen supply to the body tissues and the oxygen requirement of the tissues in fast-growing chickens. AS is characterized by an increased blood pressure within the pulmonary circulation (Baghbanzadeh and Decuypere, 2008; Wideman *et al*., 2013). Factors such as altitude, temperature (especially cold environments), lighting schedule, ventilation and nutritional approaches (such as feed form), all seem to influence the development of AS (Balog, 2003). Although ascites symptoms can be seen at low elevations, they generally become progressively worse with increasing altitude above 1200 m (Ranson, 2005). Some researchers mentioned that by reducing pulmonary vascular resistance it is possible to reduce the pulmonary arterial pressure and delay the pathophysiological progression leading to AS (Lorenzoni and Ruiz-Feria, 2006). In this context, it has been reported that the renin-angiotensin system (RAS) is involved in the pathogenesis of pulmonary arterial hypertension (Yuan *et al*., 2015). In RAS, angiotensin-converting enzyme (ACE) catalyzes the formation of angiotensin II (Ang II) from angiotensin I (Hao *et al*., 2014b). Ang II is the major biologically active compound of RAS and acts through Ang II type 1 (ATR1) and type 2 (ATR2) receptors, Ang II has been implicated in many cardiovascular diseases, such as pulmonary hypertension, atherosclerosis, coronary heart disease, and heart failure. The ATR1 receptor mediates most of the physiological and pathophysiological actions of Ang II (Lemarié *et al*., 2008; Hao *et al*., 2014a; Hassanpour *et al*., 2016). Thus, inhibition of ACE through drugs or food bioactives represents an attractive approach for dealing with AS (Shouk *et al*., 2014). Garlic is considered to be one of the best disease-preventive foods due to its high content of organosulfur compounds which are considered responsible for most of garlic´s pharmacological activities. Allicin is the most biologically active

compound of garlic (Rahman, 2007) and it has been suggested that the hypotensive effect of allicin is partially mediated by the inhibition of ACE (Shouk *et al*., 2014). Though the role of allicin on RAS in pulmonary arterial hypertension has been studied in rodents (Elkayam *et al*., 2013; Dubey *et al*., 2017), little is known about its role in pulmonary arterial hypertension in broilers. Therefore, we hypothesized that allicin could block the formation of Ang II (vasoconstrictor) through inhibition of ACE; consequently, could reduce hematocrit content, the right-to-total ventricular weight ratio and ascites mortality without compromising productive performance in broilers. Thus, the objective of this study was to evaluate the effect of allicin on, ascites-related traits [hematocrit content (Hct%) and the right-to-total ventricular weight ratio (RV:TV)], expression of angiotensin II type 1 receptor (ATR1) gene in lung, heart and liver, ascites mortality and productive performance in broilers raised under ascites-inducing conditions.

MATERIALS AND METHODS

This study was conducted at the experimental broiler facilities at Colegio de Postgraduados, Campus Montecillo, Mexico State, Mexico, located at an altitude of 2278 m above sea level, which is a natural predisposing factor for the development of AS (Vázquez-García and Pérez-Padilla, 2000).

Birds and treatments

Two hundred and ten 1-day-old male broiler chicks (Ross 308) were randomly assigned to one of three treatments: 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). Seven replicates per treatment were used (10 birds per replicate). AS in broilers was induced by exposure to natural conditions of hypoxia (2278 m of altitude), low ambient temperature (18.0-19.5 °C) from 14 to 42 days of age and feeding *ad libitum* of a pellet-form diet. Diets were formulated to meet or exceed the nutritional

recommendations for the Ross 308 broiler line (Aviagen, 2009). The diets were offered in pellet form and the feeding program was divided in two phases: starting diet (1-21 days) containing: 3025 kcal of metabolizable energy (ME)/kg, 22% crude protein (CP), 1.05% Ca and 0.50% available P; and a growing diet (22-42 days) containing: 3150 kcal ME/kg, 19% CP, 0.90% Ca and 0.45% available P. Water was offered *ad libitum* throughout the experimental period. The treatments were applied daily for two weeks (from 14 to 27 days of age) and were administered by oral-esophageal route using a pediatric catheter with diameter of 1.67 mm to ensure consumption of allicin. This study was conducted in compliance with existing protocols of the Guide for Care and Use of Experimental Animals approved by the General Academic Council of Colegio de Postgraduados (Mexico State, Mexico).

Allicin production

Synthetic allicin was produced by oxidation of diallyl disulphide according to the methodology described by Argüello-García *et al*. (2010). Briefly, 1 g diallyl disulphide was dissolved in 5 mL acetic acid under stirring in an ice bath. Hydrogen peroxide (1.5 mL, 30% v/v) was added stepwise and the reaction was allowed to proceed for 30 minutes. Afterwards, the reaction was kept at 13 °C for 20 minutes; then, it was put again under stirring for 5 h, stopped with 15 mL distilled water at pH 6.5, and extracted with 30 mL dichloromethane. After 5 extractions with 5% (w/v) Na_2CO_3 (20 mL each) and 3 extractions with distilled water (20 mL each), the solvent was left to evaporate until yellowish oil (allicin) remained. For stabilization and storage, allicin was resuspended in water at 2.5% (w/v) and kept at -70 °C until used. For utilization, synthetic allicin was thawed in ice bath and then was administered at pH 6.5 and 5 °C.

Hematocrit content and right-to-total ventricular weight ratio

At 21, 28, 35 and 42 days of age, 21 birds per treatment were randomly selected to assess hematocrit content (Hct%). Blood samples from each bird (1 mL) were collected from the right wing vein in EDTA tubes. Hematocrit volume was determined from whole blood samples by centrifugation using microhematocrit capillary tubes at 1000 × g for 10 minutes. To determine the right-to-total ventricular weight ratio (RV:TV), eight birds per treatment at 43 days of age were humanely sacrificed according to the Norma Oficial Mexicana (2015). To calculate RV:TV, the hearts were removed, and the pericardium, peripheral adipose tissue, and atria were dissected. The left and right ventricles were separated, their individual weights were recorded on an analytical balance (± 0.1 mg, model AE100, Mettler Instrument Co., Hightstown, NJ), and the RV:TV was calculated. Ascites was defined as having an RV:TV above 0.27 (Balog *et al*., 2003).

Expression of angiotensin II type 1 receptor

Six chickens per treatment were randomly selected and humanely killed at 36 days of age according to the Norma Oficial Mexicana (2015). Heart, lung and liver samples were collected and immediately frozen in liquid nitrogen and stored at -80 °C for subsequent RNA analysis.

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). The RNA purity and concentration were measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at -80 °C.

The RNA was purified using the Qiagen RNeasy kit, following the RNA cleanup protocol provided by the manufacturer. Total RNA with an absorbance ratio (260/280 nm) >1.9 was used for synthesis of complementary DNA (cDNA). Total RNA (2 µg) was subjected to reverse transcription using the High-capacity cDNA Reverse Transcription kit (Applied Biosystem, CA, USA) according to the manufacturer's instructions (thermal cycling conditions were: 25 °C for 10

minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes) using a Bio-Rad C100 thermal cycler (Bio-Rad, CA, USA) and cDNA samples were diluted 1:1 and stored at -25 °C.

Quantitative real-time PCR (qRT-PCR) analysis of gene expression was performed in triplicate, each reaction consisted of 0.5 µL diluted cDNA, 0.3 µL forward primer, 0.3 µL reverse primer, 8.9 µL DEPC-treated water and 10 µL Fast SYBR Green Master Mix (Applied Biosystems, CA, USA). Prior to qRT-PCR primers (Table 2. 1) were diluted to 10 µM in DEPC-treated water. The qRT-PCR conditions were as follows: 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. The melting curves were adjusted to 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. The samples were run using StepOnePlus machine (Applied Biosystem, CA, USA). The gene expression of ATR1 receptor gene was measured using the $2^{-\Delta\Delta C_T}$ method reported by Livak and Schmittgen (2001). Beta-actin gene was used as an internal control. The ATR1 mRNA expression was expressed relative to Beta-actin mRNA expression.

Table 2. 1. Primer sequences used in real-time PCR.

Gene symbol	Accession No.	Sequence (5´-3´)	Size (bp)
ATR1	NM_205157.3	Fw: GGAACAGCCTGGTCGTTATT Rv: CCCAGAGTGGCAGAGTTATTAG	120
ACTB	NM_205518.1	Fw: TCCCTGGAGAAGAGCTATGAA Rv: CAGGACTCCATACCCAAGAAAG	113

ATR1: Angiotensin II receptor type 1; ACTB: Beta-actin; Fw: forward; Rv: reverse.

Ascites mortality and productive performance

Cumulative ascites mortality in broilers reared to 42 days of age was recorded. All dead birds were examined to determine the cause of death. Ascites mortality was diagnosed when abdominal fluid

accumulation was observed and the RV:TV was higher than 0.27. Feed intake, BW gain and feed conversion ratio were recorded at 21, 28, 35 and 42 days of age.

Statistical analysis

Data for Hct%, RV:TV, expression of ATR1 gene and ascites mortality were analyzed as a one-way ANOVA using the GLM procedure in SAS 9.4 (SAS Institute, 2013), and for these variables each chicken was considered as the observational unit. Hematocrit and ascites mortality data were tested for deviations from a normal distribution using the Shapiro-Wilks W test. Feed intake, BW gain and feed conversion ratio were analyzed as repeated measures using the MIXED procedure in SAS, and each pen was considered as an experimental unit. Statistical differences were set at $P < 0.05$. Means of Hct%, RV:TV, ascites mortality, feed intake, BW gain and feed conversion were separated using the Tukey test; whereas, means of expression of ATR1 were separated using the t-test. Results are presented as mean ± standard error.

RESULTS

Hematocrit content and right-to-total ventricular weight ratio

Hematocrit was affected by treatment only at 42 days of age; birds in 2.5-ALLI showed lower ($P < 0.05$) Hct% than birds from the other treatments (Table 2. 2). Similarly, birds in 2.5-ALLI showed lower ($P < 0.05$) RV:TV than birds from the other treatments (Figure 2. 1).

Table 2. 2. Effect of synthetic allicin on hematocrit content (%) in broilers.

Age (days)	Treatment			SEM	P-value
	0-ALLI	1-ALLI	2.5-ALLI		
21	32.5	31.2	33.4	1.46	0.663
28	43.1	41.7	43.7	1.73	0.507
35	41.6	40.9	37.5	2.11	0.154
42	46.8 a	48.8 a	39.7 b	2.64	0.006

SEM = Standard Error of the Mean. 0-ALLI or control [(0 mg of allicin /kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Values with different superscripted letters within the same row are statistically different ($P < 0.05$).

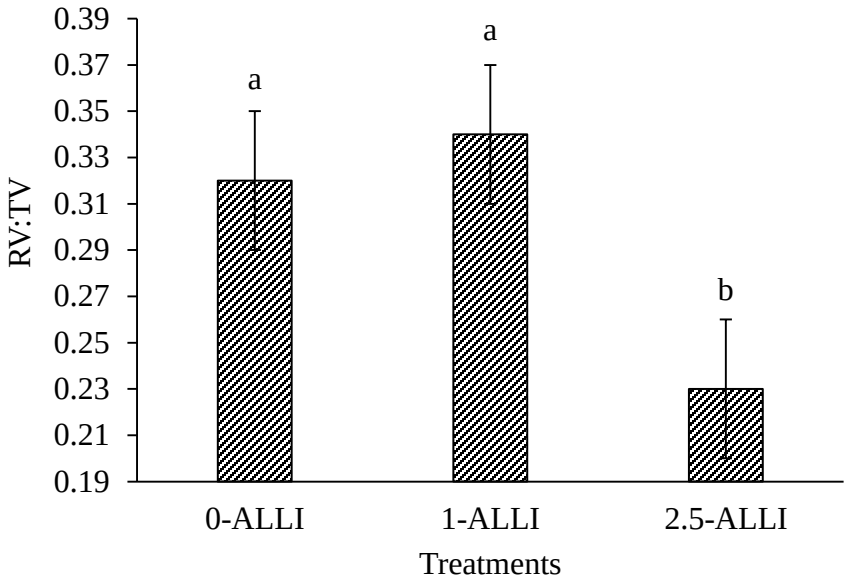

Figure 2. 1. Effect of allicin on right-to-total ventricular weight ratio (RV:TV) in broilers raised at 2278 m of altitude. 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Values with different superscripted letters are statistically different ($P < 0.05$).

Expression of angiotensin II type 1 receptor

The ATR1 mRNA expression in the lung of broilers in the 1-ALLI group was higher than the control group ($P < 0.05$; Figure 2. 2). However, in the heart the ATR1 mRNA expression was higher in broilers in the 2.5-ALLI group than the control birds ($P < 0.05$; Figure 2. 3). No differences ($P > 0.05$) were found among treatments for mRNA expression of this gene in the liver of broilers (Figure 2. 4).

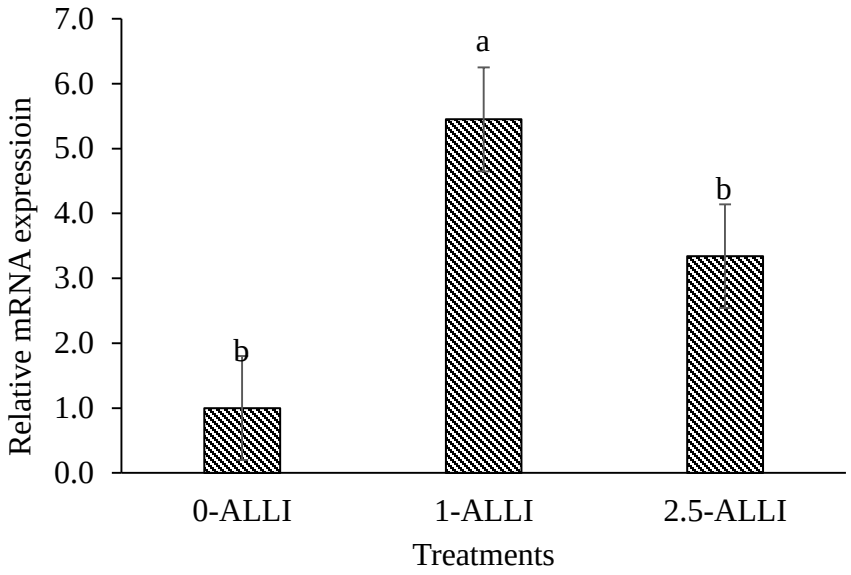

Figure 2. 2. Angiotensin II type 1 receptor mRNA expression in lungs of broilers treated with synthetic allicin. Treatments: 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Values with different superscript are statistically different ($P < 0.05$).

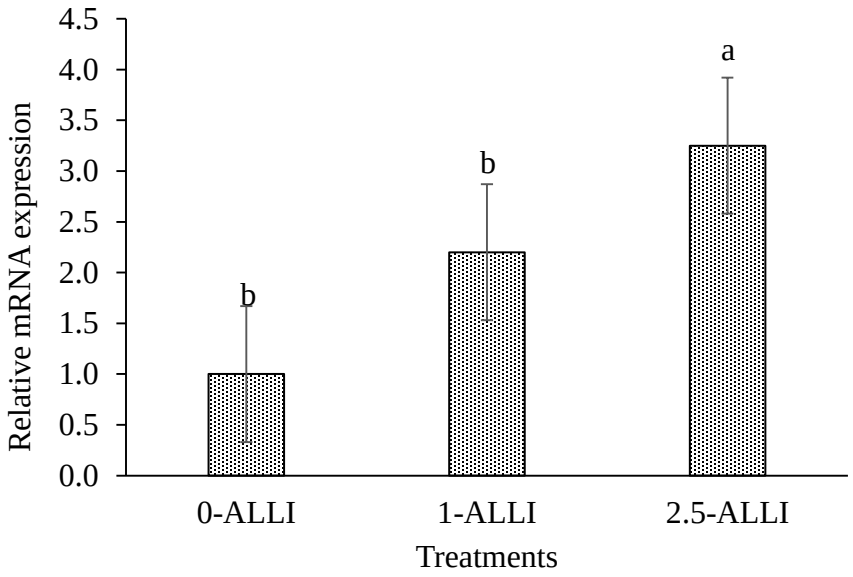

Figure 2. 3. Angiotensin II type 1 receptor mRNA expression in heart of broilers treated with synthetic allicin. Treatments: 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). ^{a,b} Values with different superscript are statistically different ($P < 0.05$).

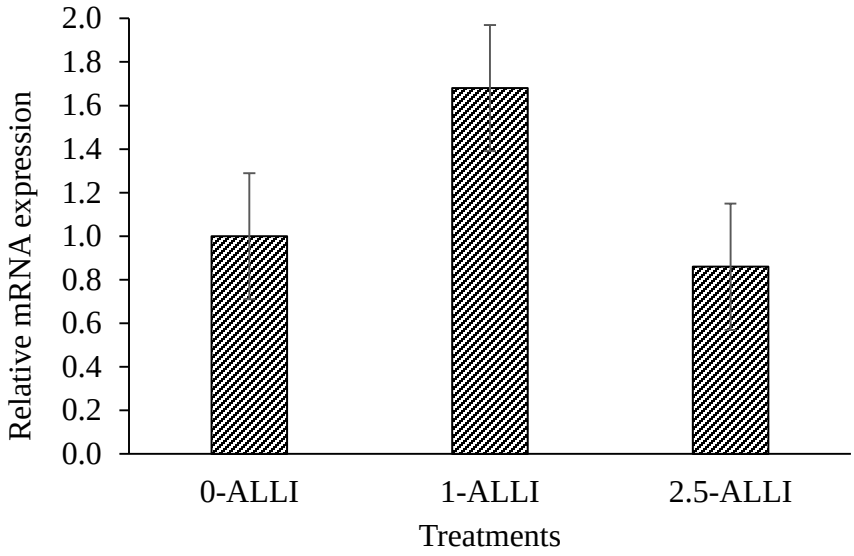

Figure 2. 4. Angiotensin II type 1 receptor mRNA expression in liver of broilers treated with synthetic allicin. Treatments: 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). No statistical differences were found among treatments ($P > 0.05$).

Ascites mortality and productive performance

Cumulative ascites mortality (from 14 to 42 days of age) was not affected ($P > 0.05$) by treatment (0-ALLI = 31.4%, 1-ALLI = 37.1% and 2.5-ALLI = 24.2%; data not shown in tables). Only 1.9% of mortality was produced by causes different from ascites syndrome. No significant differences ($P > 0.05$) among treatments were found for feed intake, BW gain and feed conversion (Table 2. 3).

Table 2. 3. Effect of synthetic allicin on productive performance in broilers.

Age (days)[1]	Treatment			SEM	P-value		
	0-ALLI	1-ALLI	2.5-ALLI		Treatment	Age	Treatment × Age
Feed intake (g/bird)							
21	740	734	728	83	0.582	<0.001	0.824
28	1040	1052	1068				
35	1141	1243	1130				
42	1310	1385	1379				
BW gain (g/bird)							
21	536	523	527	70	0.470	<0.001	0.060
28	592	623	621				
35	721	604	692				
42	764	976	754				
Feed conversion ratio							
21	1.37	1.41	1.37	0.11	0.905	<0.001	0.051
28	1.78	1.68	1.70				
35	1.62	1.90	1.65				
42	1.80	1.50	1.80				

SEM = Standard Error of the Mean. 0-ALLI or control [(0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [1]Number of surviving birds at each age were: 21 days = 70 birds for each treatment; 28 days (0-ALLI = 64, 1-ALLI and 2.5-ALLI = 65); 35 days (0-ALLI = 54, 1-ALLI = 51 and 2.5-ALLI = 57), and 42 days (0-ALLI = 47, 1-ALLI = 44 and 2.5-ALLI = 50). No statistical differences were found among treatments ($P > 0.05$).

DISCUSSION

At high altitude, the partial pressure of O_2 is lower than at sea level, which in turn reduces the levels of respired O_2, triggering acute pulmonary vasoconstriction and pulmonary hypertension in broilers (Wideman *et al*., 2013). We hypothesized that allicin could block the formation of Ang II through inhibition of ACE; and thus, could reduce hematocrit content, the right-to-total ventricular weight ratio and ascites mortality without compromising productive performance in broilers. In this study, the ascites-inducing conditions at which broilers were exposed could increase Hct%

and consequently RV:TV in broilers in the 0-ALLI and 1-ALLI treatments, since these birds showed a ratio above 0.27, indicating the beginning of ascites development (Balog *et al*., 2003). Birds in the 2.5-ALLI treatment showed lower Hct% than birds from the other treatments on day 42, which in turn could reduce RV:TV. Shlosberg *et al.* (1998) reported that birds dying of ascites showed high hematocrit levels that increase blood viscosity and the inability of the right ventricle to pump blood through the pulmonary vessels. Varmaghany *et al*. (2015) observed that birds fed 5 g of garlic/kg of diet under cold temperature conditions showed lower packed cell volume.

Healthy chickens exposed to high altitude cannot meet their oxygen demands due to hypoxic conditions, thus increasing their hematocrit level. On the other hand, hypoxemia (blood within the systemic arteries that is under-saturated with O_2) due to high altitude does stimulate erythropoiesis, which increases hematocrit content and blood viscosity (Wideman *et al*., 2013). Baghbanzadeh and Decuypere (2008) mentioned that the elevation in hematocrit can be caused by diminished plasma volume, because of fluid exudation out of the blood system into the abdominal cavity. Wideman *et al*. (2013) mentioned that large increases in hematocrit can increase blood viscosity and thereby increase resistance to blood flow that ultimately leads to terminal pulmonary arterial hypertension. In this regard, right ventricular hypertrophy observed in 0-ALLI and 1-ALLI groups in this study can be attributed to an increase in Hct%, which in turn elevates blood viscosity that results in a higher cardiac workload. The lower Hct% values observed in the 2.5-ALLI birds could have resulted from the blood pressure-lowering properties of allicin. On the other hand, allicin may have blocked angiotensin-II production, which in turn promotes vasodilation and thus reduces blood pressure (Ried and Fakler, 2014). In this regard, the RAS hormonal cascade begins with the biosynthesis of renin in the kidneys, which catalyzes the formation of angiotensin I (Ang I) from its precursor angiotensinogen. In turn, Ang I is converted to Angiotensin II (Ang II) through the

activity of ACE. Ang II acts via ATR1 receptors in the vasculature resulting in vasoconstriction (Mueller *et al*., 2014; Shouk *et al*., 2014). Hao *et al*. (2014a) suggested that low ambient temperature might activate lung tissue RAS and result in arterial remodeling, which contribute to pulmonary hypertension. Additionally, ACE activity in heart also plays an important role in hypertension development and ventricular hypertrophy (Sharifi *et al*., 2003).

In this study, AS in broilers was induced by exposition to natural conditions of hypoxia, low ambient temperature and feeding *ad libitum* of a pellet-form diet; and it was found that ATR1 gene expression was higher (4.45 fold) in lung of broilers in the 1-ALLI group than in the control group, whereas in the heart the ATR1 gene expression was higher (2.25 fold) in broilers in the 2.5-ALLI group than in the control birds. Therefore, results of this study may show that there is a possibility that synthetic allicin may act through the inhibition of ACE, which in turn decrease levels of circulating vasoconstrictor Ang II; and as consequence overexpression of ATR1 gene in lung and heart was found.

In the same line of thought, Saleem *et al*. (2010) mentioned that ACE inhibitors treatment results in upregulation of the ATR1 receptors, meanwhile El-Brolosy and Stainier (2017) reported that gene upregulation may be a direct consequence of the loss of protein function. In agreement with results found in this study, Ried and Fakler (2014) and Shouk *et al*. (2014) mentioned that one mechanism of action of garlic on hypertension is its potential of blocking Ang II production by inhibition of the ACE. Sharifi *et al*. (2003) found that the ACE activity in tissues and serum increases during the development of hypertension in rats, and this enhancement of ACE activity was reduced by administration of garlic extract, which is consistent with the inhibitory effect of garlic on ACE activity in aorta, clipped and normal kidney, lung, serum and heart of garlic-fed-treated animals. Thus, results found in this study may suggest that synthetic allicin might dilate

the pulmonary vasculature and thus lower the resistance to blood flow which can delay or inhibit the onset of pulmonary hypertension.

For cumulative ascites mortality, no differences were observed among treatments, although a statistically significant reduction in mortality was expected. Varmaghany *et al*. (2015) reported that ascites-related mortality rate was reduced in birds fed with 5 g of garlic/kg of diet under cold temperature conditions. Julian (2005) mentioned that the increase in metabolic rate at temperatures below the 'comfort zone' is a significant cause of increased mortality from pulmonary hypertension syndrome in broilers. Thus, the results of this study may be attributed to the extreme conditions (natural hypoxia, feeding *ad libitum* of a pelleted diet and low environmental temperature) to which birds were exposed, which in turn would increase ascites symptoms and mortality. After the second week, the temperature ranged from 18.0 to 19.5 °C, when normally it must be about 27 °C at this age.

Regarding productive performance, no differences among treatments were found for feed intake, BW gain and feed conversion ratio, but a reduction in BW was expected due to the pungent smell of allicin, that could lead to a reduction in feed intake. Varmaghany *et al*. (2015) reported no significant differences in BW gain among groups from 1 to 42 days of age, under treatment with increasing dietary garlic levels (0, 5, 10 or 15 g/kg) under cold temperature conditions. However, they observed a reduction in feed intake with increasing dietary garlic levels under standard temperature conditions, which may be related to diet palatability. Adjei *et al*. (2015) reported that the lowest inclusion level of allicin (0.10 g garlic oil/kg diet) was better ($P < 0.05$) for feed intake, average weight gain and average final BW. Also, the higher dietary levels of allicin reduced feed intake (0.15 and 0.20 g garlic oil/kg diet), probably due to the intense odor of allicin, which required a period of adaptation to this type kind of feed by the chickens.

The findings of this study show that synthetic allicin reduced Hct% and RV:TV, and upregulated ATR1 gene in heart and lung without compromising productive performance in broilers exposed to natural hypoxia, *ad libitum* feeding of pelleted diet and raised in low environmental temperatures. However, further studies are needed to find the optimal dose and administration period of synthetic allicin that could reduce ascites mortality and improve the productive performance of broilers.

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CHAPTER III. SYNTHETIC ALLICIN ON ANGIOTENSIN II TYPE 1 RECEPTOR GENE, ASCITES-RELATED TRAITS AND PRODUCTIVE PERFORMANCE IN BROILERS RAISED UNDER ASCITES-INDUCING CONDITIONS

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ABSTRACT

The present study was conducted to evaluate the effect of synthetic allicin on ascites-related traits [blood oxygen saturation (SaO_2), hematocrit content (Hct%) and right-to-total ventricular weight ratio (RV:TV)], angiotensin II type 1 receptor (ATR1) gene expression, productive performance [body weight (BW) gain, feed intake, feed conversion and breast skin pigmentation], digestive organs variables and ascites mortality in broilers reared under ascites-inducing conditions. A total of 900 1-day-old mixed broiler chicks "Ross 308" were randomly distributed among three groups: 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW); 10 replicates per treatment were used with 30 birds each. Ascites syndrome was induced in broilers by exposition to 2278 m above sea level and low ambient temperature (18.0-19.0 °C); birds were fed *ad libitum* with a mash-form diet. Synthetic allicin was given daily from 14 to 27 days of age, and was administered by oral-esophageal route using a pediatric catheter. At 35 and 42 days of age, 50 broilers per treatment were randomly selected to assess SaO_2 and breast skin pigmentation. While at 28, 35 and 42 days of age, 50 broilers per treatment were randomly selected for Hct% determination. For determination of RV:TV and digestive organs variables, 20 broilers per treatment were selected. Productive performance was recorded weekly and ascites mortality was registered daily. For ATR1 gene expression, eight chickens per treatment were randomly selected at 36 days. Results of this study show that allicin does not affect Hct%, RV:TV, ascites mortality, BW gain, feed conversion and ATR1 gene

expression in lung. However, administration of allicin improves SaO_2 and downregulates ATR1 gene expression in the heart; also increases liver weight and decreases feed intake.

Keywords: allicin, angiotensin II receptor type 1, oxygen saturation, ascites syndrome, broilers.

INTRODUCTION

Ascites syndrome (AS) or pulmonary hypertension syndrome (Wideman *et al*., 2013) is a metabolic disorder that affects fast-growing chickens; it is caused by an increase in either cardiac output due to a higher metabolic rate or vascular resistance to blood flow by exposure to environmental hypoxia (Julian, 2007; Bautista-Ortega and Ruiz-Feria, 2010). At high altitude, the incidence of AS increases because hypoxia causes pulmonary vasoconstriction by increasing vascular resistance and pulmonary arterial pressure (Bautista-Ortega and Ruiz-Feria, 2010). Also, AS development can be influenced by environment (Wideman, 2000), and it has been suggested that cold exposure is the secondary factor causing AS in temperate climates (Julian, 2000). Thus, it is possible to reduce the pulmonary arterial pressure and AS development by reducing pulmonary vascular resistance (Lorenzoni and Ruiz-Feria, 2006) by means of vegetal bioactives compounds. Allicin is the main bioactive compound in garlic, it is produced by the action of enzyme allinase on the precursor alliin (Shouk *et al*., 2014). Studies in animals and humans have confirmed the hypotensive effect of garlic (Rahman, 2007); moreover, other compounds of garlic have antibacterial, antifungal, antiviral, antioxidant, antithrombotic and vasodilator properties (Raeesi, *et al*., 2010). In a study, Nwokocha *et al*. (2011) found that garlic extract causes a reduction in blood pressure in normotensive and hypertensive rat models. On the other hand, it has been suggested that garlic extract can be used as growth promoter in broilers; Noman *et al*. (2015) reported that supplementation of 1% of aqueous extraction of garlic in broilers improves body weight (BW) gain and carcass quality. Besides, Atuahene *et al*. (2018) reported that garlic supplement in broiler diet (2.5, 5.0 and 7.5% of garlic/kg of diet) did not have adverse effect on productive performance. In a previous study, we found that allicin reduces hematocrit content and the right-to-total ventricular weight ratio in broilers raised under ascites-induced conditions; but

up to now scarce studies have reported the effects of synthetic allicin on ascites-related variables in broilers. Therefore, we hypothesized that allicin could inhibit the development of AS due to its antihypertensive property. Thus, the aim of this study was to evaluate the effect of synthetic allicin on ascites-related traits [blood oxygen saturation (SaO_2), hematocrit content (Hct%) and right-to-total ventricular weight ratio (RV:TV)], angiotensin II type 1 receptor (ATR1) gene expression, productive performance [body weight (BW) gain, feed intake, feed conversion and breast skin pigmentation], digestive organs variables and ascites mortality in broilers reared under ascites-inducing conditions.

MATERIALS AND METHODS

The research was conducted in the poultry facilities at Colegio de Postgraduados-Campus Montecillo (Mexico State, Mexico) located at 2278 m above sea level (Vázquez-García and Pérez-Padilla, 2000). Broilers were raised following the protocol for the use and care of animal intended for research approved by the General Academic Council of Colegio de Postgraduados.

Birds and treatments

Nine hundred 1-day-old mixed broiler chicks (Ross 308 strain) were distributed among three groups of three hundred broilers each. The treatments were randomly assigned to the experimental groups: 0-ALLI or control (0 mg of allicin/kg of BW), 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). Ten replicates per treatment were used with 30 birds each. AS was induced in broilers by exposing them to 2278 m above sea level (environmental hypoxia), low ambient temperature (18.0-19.0 °C) from 14 to 42 days of age; birds were fed *ad libitum* with a mash-form diet. Diets were formulated to meet or exceed broiler nutrient requirements recommended by Aviagen (2009a). The feeding program was divided in two phases: starter diet (offered from 1 to 21 days of age) containing 3025 kcal of metabolizable energy (ME)/kg, 22%

crude protein (CP), 1.05% Ca and 0.50% available P; and finisher diet (offered from 22 to 42 days of age) containing 3150 kcal ME/kg, 19% CP, 0.90% Ca and 0.45% available P. Water was offered *ad libitum* during the experimental period. Allicin was given daily from 14 to 27 days of age, and was administered by oral-esophageal route using a pediatric catheter with diameter of 1.67 mm.

Production of allicin

Synthetic allicin was produced according to the methodology described by Argüello-García *et al*. (2010). Allicin was resuspended in water at 2.5% (w/v) and kept at -70 °C until used. Before utilization, synthetic allicin was thawed in ice bath and then it was administered at pH 6.5 and 5 °C.

Blood oxygen saturation and skin pigmentation

Fifty broilers per treatment (five broilers per replicate) were randomly selected to assess blood oxygen saturation (SaO_2) and skin pigmentation at 35 and 42 days of age.

SaO_2 was measured with a pulse oximeter (Model AM1000A, Shanghai Berry Electronic Tech Co., Shanghai, China) and reported as percentage. SaO_2 was measured from the right ulnar artery using a clip sensor. The pigmentation of breast skin was measured in live birds using a colorimeter (Model CR-400, Konica Minolta, Inc., Tokyo, Japan). The CIELAB color profile (*L**: lightness, *a**: redness and *b**: yellowness) was determined on the cranial portion of the dorsal surface of the left breast skin.

Hematocrit content

At 28, 35 and 42 days of age, fifty broilers per treatment (five broilers per replicate) were randomly selected and blood samples from each bird (1 mL) were collected from the right wing vein in

EDTA tubes. Hct% was determined from whole blood samples by centrifugation using microhematocrit capillary tubes at 1200 × g for 5 minutes.

Right-to-total ventricular weight ratio and digestive organ variables

Twenty broilers per treatment with BW close to the average of the pen were selected, weighed and humanely killed by cervical dislocation according to the Norma Oficial Mexicana (2015). To determine the RV:TV, the heart was dissected, and the pericardium, peripheral adipose tissue, and atria were removed. The left and right ventricles were separated and weighed on an analytical balance (± 0.1 mg, model AE100, Mettler Instrument Co., Hightstown, NJ). RV:TV above 0.27 was considered as AS (Balog *et al*., 2003). The same birds were used for evaluation of digestive organ variables: The small-intestine and cecum lengths were measured on a wet cloth to avoid shrinkage using a tape. The fatty and mesentery tissues attached to each section of the digestive system were removed and the empty weight of proventriculus, gizzard, small intestine and cecum was recorded. Also, liver, spleen and pancreas weights were recorded.

Angiotensin II type 1 receptor gene expression

Eight chickens per treatment were randomly selected and humanely killed at 36 days of age according to the Norma Oficial Mexicana (2015). Heart and lung samples were collected and immediately frozen in liquid nitrogen and stored at -80 °C for later RNA analysis.

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). The RNA purity and concentration were measured with an absorbance ratio of 260/280 nm on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA samples were stored at -80 °C.

RNA cleanup and complementary DNA synthesis

The RNA was purified using the Qiagen RNeasy kit according to the manufacturer's instructions. Total RNA greater than 1.9 was used for synthesis of complementary DNA (cDNA). Two µg of RNA were subjected to reverse transcription using the High-capacity cDNA Reverse Transcription kit (Applied Biosystem, CA, USA) according to the manufacturer's instructions (thermal cycling conditions were 25 °C for 10 minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes) using a Bio-Rad C100 thermal cycler (Bio-Rad, CA, USA) and cDNA samples were diluted 1:1 and stored at -25 °C.

Quantitative real time PCR analysis

Quantitative real-time PCR (qRT-PCR) analysis of gene expression was performed in triplicate, each reaction consisted of 0.5 µL diluted cDNA, 0.3 µL forward primer, 0.3 µL reverse primer, 8.9 µL DEPC-treated water and 10 µL Fast SYBR Green Master Mix (Applied Biosystems, CA, USA). Prior to qRT-PCR primers (Table 3. 1) were diluted to 10 µM in DEPC-treated water. The qRT-PCR conditions were as follows: 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. The melting curves were adjusted to 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. The samples were run using StepOnePlus machine (Applied Biosystem, CA, USA). The gene expression of ATR1 receptor gene was measured using the $2^{-\Delta\Delta C_T}$ method reported by Livak and Schmittgen (2001). β-actin gene was used as an internal control. The ATR1 mRNA expression was expressed relative to β-actin mRNA expression.

Table 3. 1. Primer sequences used in real-time PCR.

Gene symbol	Accession No.	Sequence (5´-3´)	Size (bp)
ATR1	NM_205157.3	Fw: GGAACAGCCTGGTCGTTATT Rv: CCCAGAGTGGCAGAGTTATTAG	120
ACTB	NM_205518.1	Fw: TCCCTGGAGAAGAGCTATGAA Rv: CAGGACTCCATACCCAAGAAAG	113

ATR1: Angiotensin II receptor type 1; ACTB: Beta-actin; Fw: forward; Rv: reverse.

Ascites mortality and productive performance

Feed intake, BW gain and feed conversion ratio were recorded at 21, 28, 35 and 42 days of age. Cumulative AS mortality was recorded throughout the experimental period (from 14 to 42 days of age). AS mortality was considered when abdominal fluid accumulation was observed and the RV:TV was higher than 0.27 (Balog *et al*., 2003).

Statistical analysis

Data analysis was done using the SAS software (SAS Institute, 2011). The SaO_2, Hct%, breast skin pigmentation and productive performance (feed intake, BW gain and feed conversion ratio) variables were analyzed as repeated measures using the MIXED procedure. For productive performance variables pen was considered as the experimental unit. Data of RV:TV and digestive organs variables were analyzed as a 3 × 3 factorial arrangement (treatments and age) using the MIXED procedure. For SaO_2, Hct%, breast skin pigmentation, RV:TV and digestive organ variables each chicken was considered as the observational unit. Data reported as percentage were tested for deviations from a normal distribution using the Shapiro-Wilks W test. Gene expression and AS mortality variables were analyzed as a one-way ANOVA using the GLM procedure of

SAS. Statistical difference was set at $P < 0.05$. Means were separated using the Tukey's test and results are presented as mean ± standard error.

RESULTS

Blood oxygen saturation and skin pigmentation

SaO_2 was not affected by treatment ($P > 0.05$), but age and treatment × age interaction were significant ($P < 0.05$). At 35 days of age, birds in the 2.5-ALLI group showed higher values of SaO_2 than in broilers in the 1-ALLI group; however, at 42 days of age the opposite was observed (Figure 3. 1). The breast skin pigmentation was not affected by treatment ($P > 0.05$), but was affected by age. Broilers showed higher lightness and yellowness index at 42 days of age than at 35 days of age (Table 3. 2). Only redness index was affected by treatment × age interaction (Figure 3. 2).

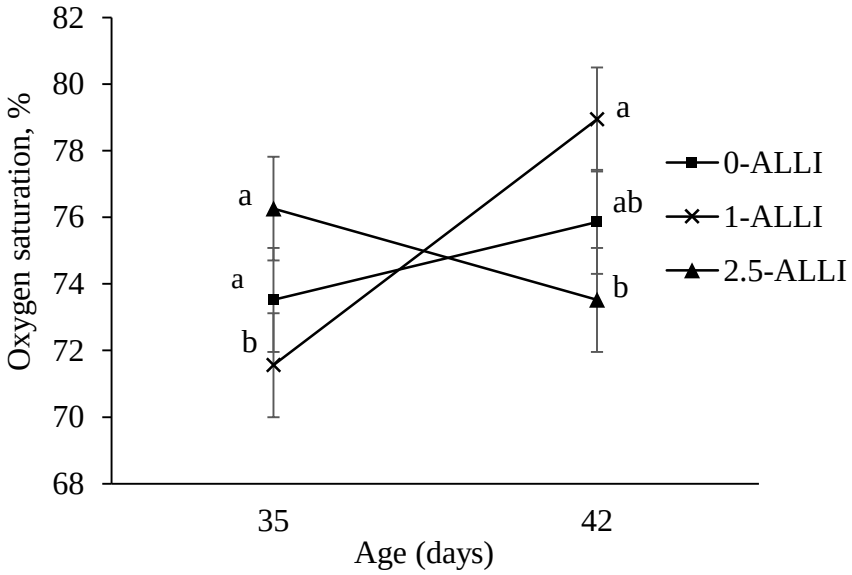

Figure 3. 1. Blood oxygen saturation in broilers treated with synthetic allicin. 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter in each age are significantly different ($P < 0.05$). Treatment = 0.8654, Age = 0.0061, Treatment × Age: 0.0001.

Table 3. 2. Breast skin pigmentation in broilers treated with synthetic allicin.

	Treatment				Age (days)			P-value		
Variable	0-ALLI	1-ALLI	2.5-ALLI	SEM	35	42	SEM	Treatment	Age	Treatment × Age
L*	62.88	63.72	63.19	0.49	62.82 b	63.70 a	0.26	0.2464	0.0010	0.8524
a*	3.38	3.85	3.72	0.29	4.11 a	3.19 b	0.13	0.2760	<0.0001	0.0230
b*	15.18	15.40	14.45	0.65	14.11 b	15.91 a	0.27	0.3378	<0.0001	0.8025

L*: lightness, a*: redness and b*: yellowness. 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter are significantly different ($P < 0.05$).

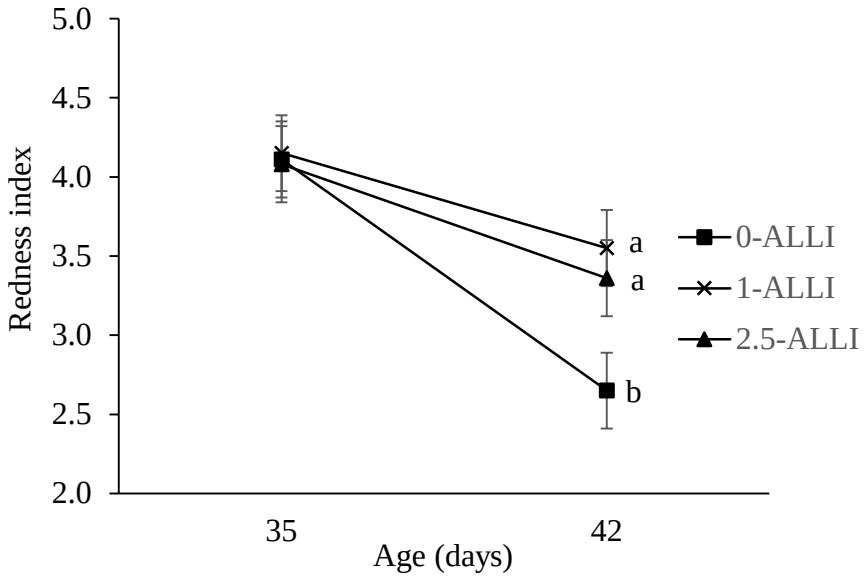

Figure 3. 2. Redness index in broilers treated with synthetic allicin. 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter in each age are significantly different ($P < 0.05$).

Hematocrit content

Hct% was no affected by treatment; however, it was observed that Hct% decreased significantly with age ($P < 0.05$; Figure 3. 3).

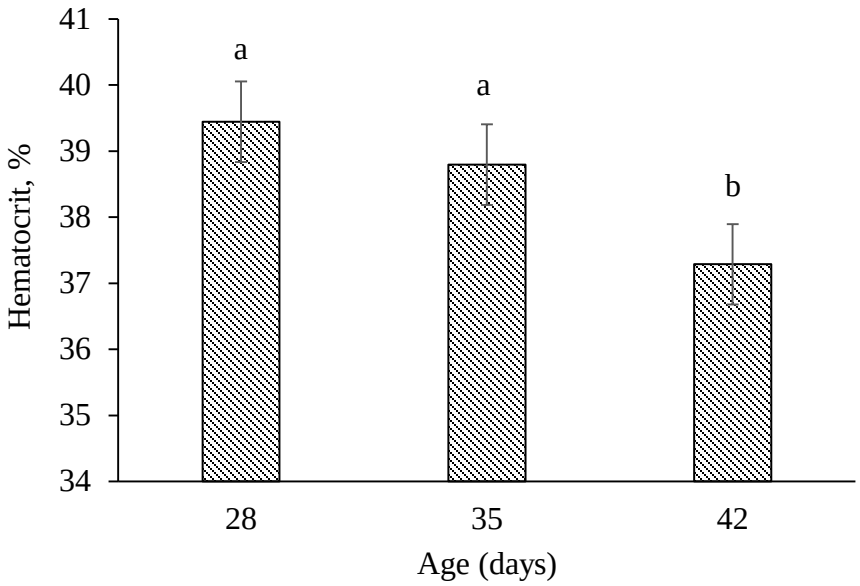

Figure 3. 3. Hematocrit content (%) of broilers treated with synthetic allicin. [a,b] Means with different letter are significantly different ($P < 0.05$). Treatment = 0.5184, Age = 0.0201, Treatment × Age 0.7201.

Right-to-total ventricular weight ratio and digestive organ variables

No differences among treatments were observed for RV:TV. It was found that RV:TV decreases as age increased (Figure 3. 4). Only liver weight was affected by treatment ($P < 0.05$); broilers in the 2.5-ALLI group showed the highest liver weight. The relative weight of spleen increases with age. In this study, age affected all variables of digestive organs (except pancreas weight); and it was found that the older broilers the lower empty relative weights and lengths of organs (Table 3. 3).

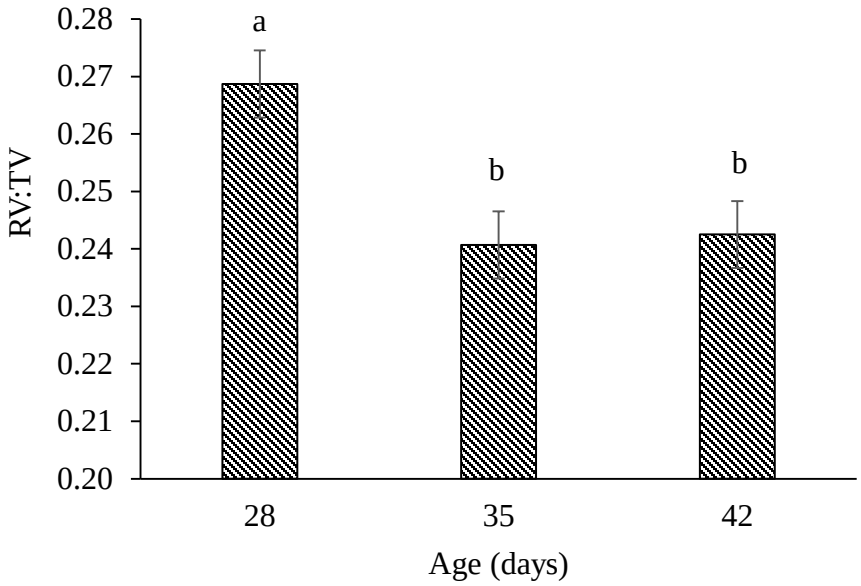

Figure 3. 4. Effect of synthetic allicin on right-to-total ventricular weight ratio (RV:TV) in broilers. [a,b] Means with different letter are significantly different ($P < 0.05$). Treatment = 0.2013, Age = 0.0032, Treatment × Age 0.7007.

Table 3. 3. Effect of synthetic allicin on size of digestive organ of broilers.

	Treatment				Age (days)				P-value		
	0-ALLI	1-ALLI	2.5-ALLI	SEM	28	35	42	SEM	Treatment	Age	Treatment × Age
Relative weight (g/kg live weight)											
Liver	21.80 b	21.66 b	22.85 a	0.49	23.72 a	21.95 b	20.64 c	0.51	0.0450	<.0001	0.3522
Spleen	1.12	1.12	1.13	0.06	0.86 b	1.21 a	1.31 a	0.05	0.9614	<.0001	0.8589
Pancreas	2.50	2.48	2.46	0.06	2.51	2.52	2.41	0.06	0.8711	0.2404	0.1896
Empty relative weight (g/kg live weight)											
Proventriculus	3.46	3.33	3.48	0.09	3.78 a	3.52 b	2.96 c	0.09	0.2730	<.0001	0.1980
Gizzard	12.96	12.21	12.78	0.35	13.76 a	12.80 b	11.39 c	0.39	0.1148	<.0001	0.4913
Small intestine	20.98	20.28	20.76	0.69	21.02 a	21.92 a	19.07 b	0.47	0.5918	<.0001	0.6524
Caeca	2.78	2.81	2.86	0.09	3.12 a	3.06 a	2.26 b	0.95	0.6939	<.0001	0.9263
Relative length (cm/kg live weight)											
Small intestine	99.87	100.63	100.34	1.29	130.12 a	93.26 b	77.46 c	1.43	0.8404	<.0001	0.5608
Caeca	10.24	10.40	10.37	0.12	12.92 a	9.70 b	8.39 c	0.18	0.3797	<.0001	0.4049

0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter either within treatment or age indicate significant differences ($P < 0.05$).

Angiotensin II type 1 receptor gene expression

The ATR1 mRNA expression in the heart of broilers from 1-ALLI group was lower than in the control group ($P < 0.05$; Figure 3. 5). However, no differences ($P > 0.05$) were found between treatments for mRNA expression of this gene in the lungs of broilers (Figure 3. 6).

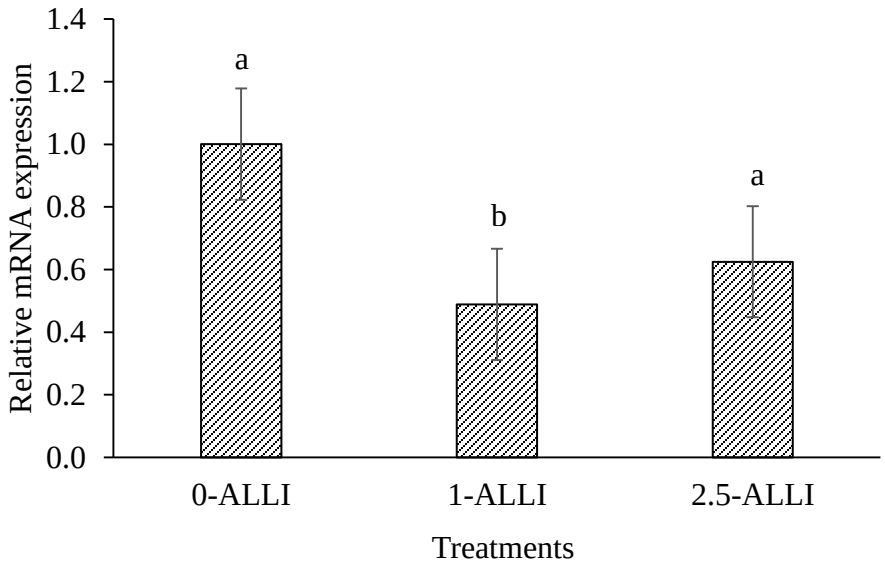

Figure 3. 5. Angiotensin II type 1 receptor mRNA expression in heart of broilers treated with synthetic allicin. 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter are significantly different ($P < 0.05$).

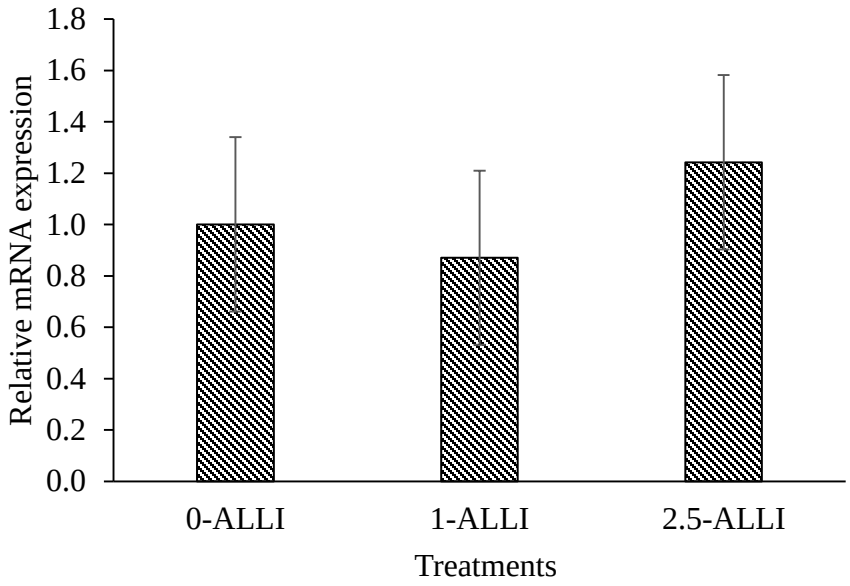

Figure 3. 6. Angiotensin II type 1 receptor mRNA expression in lungs of broilers treated with synthetic allicin. 0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW).

Ascites mortality and productive performance

Cumulative AS mortality was not affected (P = 0.1346) by treatment (0-ALLI = 9.66 ± 2.24%, 1-ALLI = 7.34 ± 2.24% and 2.5-ALLI = 12.00 ± 2.24%; data not shown in table). Feed intake, BW gain and feed conversion ratio were not affected by neither treatment nor age (P > 0.05); only feed intake was affected by treatment × age interaction (P > 0.05; Table 3. 4).

Table 3. 4. Effect of synthetic allicin on productive performance in broilers.

Age (days)	Treatment			SEM	P-value		
	0-ALLI	1-ALLI	2.5-ALLI		Treatment	Age	Treatment × Age
Feed intake (g/bird)							
21	595 a	575 ab	564 b	13	0.1371	<0.0001	0.0159
28	854 a	850 a	799 b	22			
35	1081 a	1065 a	1039 a	41			
42	1223 b	1315 a	1266 ab	29			
BW gain (g/bird)							
21	401	401	403	6	0.7231	<0.0001	0.7706
28	448	424	440	30			
35	592	625	616	27			
42	603	633	601	33			
Feed conversion ratio							
21	1.49	1.43	1.40	0.03	0.2166	<0.0001	0.7513
28	1.95	2.00	1.85	0.11			
35	1.85	1.71	1.70	0.12			
42	2.07	2.09	2.12	0.12			

0-ALLI or control [0 mg of allicin/kg of body weight (BW)], 1-ALLI (1 mg of allicin/kg of BW) and 2.5-ALLI (2.5 mg of allicin/kg of BW). [a,b] Means with different letter are significantly different ($P < 0.05$).

DISCUSSION

In the current study, mixed broilers Ross 308 strain were used, but a greater proportion of females than males were observed in the flock. AS was induced by exposing broilers to environmental hypoxia and low ambient temperature (18.0-19.0 °C), while they were fed *ad libitum* with a mash-form diet. It has been suggested that high growth rate of modern broilers result in an increased oxygen requirement which might be the reason for development of AS (Closter *et al*., 2012). Besides, broilers exposed to high altitude, show an increase in pulmonary blood vessels constriction and pulmonary vascular resistance, which in turn cause right ventricular hypertrophy

(Baghbanzadeh and Decuypere, 2008). In this regard, O_2 requirement is the most critical trigger of the AS in broilers (Julian, 2000), so that SaO_2 may serve as an indicator of AS susceptibility (Druyan *et al*., 2007; Aviagen, 2009b). Results of this study showed that at 35 days of age, birds in the 2.5-ALLI group showed higher value of SaO_2 than in broilers in the 1-ALLI group; however, at 42 days of age the opposite was observed; which suggests that allicin could promote arterial vasodilation (Ried and Fakler, 2014). Rahman (2007) mentioned that allicin showed vasodilatory activity in the pulmonary vascular bed of cat and rat; in this way, allicin reduces pulmonary arterial pressure (Kaye *et al*., 2000) allowing the blood to carry more O_2 to the tissues of broilers.

The cause of right ventricular failure in fast-growing chickens is the high O_2 requirement needed to sustain metabolism (Julian, 2000). Blood within the systemic arteries that is under-saturated with O_2 does stimulate erythropoiesis, which increases the Hct% (Wideman *et al*., 2013); so, the higher the Hct%, the more viscid the blood (Julian, 2000). Tekeli (2014) reported that in broilers with SA, the heart tries to pump more blood through the lungs to meet the O_2 requirements and right ventricle enlarges in response to increased workload of heart. In the current study, a reduction of Hct% and RV:TV was expected in broilers in the 1-ALLI or 2.5-ALLI groups, as it was observed in our previous studies. However, results of this study showed that Hct% and RV:TV were no affected by synthetic allicin. Besides we found that broilers sampled at 28, 35 and 42 days of age had a RV:TV below 0.27 (Balog *et al*., 2003) which indicate that the broilers were clinically healthy. Wideman (2001) reported that clinically healthy broilers have RV:TV ranging from 0.15 to 0.27, whereas broilers with pulmonary hypertension have RV:TV equal or above 0.28. It is important to note that in this study broilers of both sexes were used, and there was a greater number of females than males. Then, results obtained in this study may be attributed to the fact that female broilers have a low BW, and for this reason are less prone to developing AS than males (Decuypere

et al., 2000; Julian, 2000). Therefore, in the current study no differences on AS mortality were observed among treatments. In accordance with our results, Closter *et al*. (2012) mentioned that male broilers are more affected by AS than female broilers, and that the development of AS leads to an increased mortality.

On the other hand, higher values of redness were observed in breast skin of broilers in the 1-ALLI and 2.5-ALLI groups than in broilers from the control group. Our findings suggest that the highest values of SaO_2 observed in broilers that received 1-ALLI and 2.5-ALLI treatments, may be attributed to the fact that allicin could promote O_2 supply to the tissues. However, Closter *et al*. (2012) mentioned that AS leads to reduced meat quality, such as reddish color of the breast. Similarly, Hoving-Bolink *et al*. (2000) reported that AS is associated with a reduced numbers of capillaries per fibre in the pectoral muscle, so a reduction of O_2 supply to the muscle may have negative effects on meat quality. On the other hand, Castañeda *et al*. (2005) reported that redness (a*) value is not a good skin colour parameter in live birds because redness from blood vessels interfere with the reading.

Regarding to digestive organ variables, it was found that broilers in the 2.5-ALLI group showed higher liver weight than broilers in the others groups. Similarly, Brzóska *et al*. (2015) found that chickens receiving 1.50 mL of liquid garlic extract/kg of diet showed the highest percentage of liver weight as a percentage of carcass. El-Katcha *et al*. (2016) reported that supplementation with garlic extract (0.10 mg/kg diet) increased liver weight and relative weight compared with the control treatment; however, they found that liver weight and relative weight decreased with increased supplementation of garlic. Conversely, Issa and Omar (2012) mentioned that relative liver weight was not affected by garlic powder in broilers. Nevertheless, it has been suggested that high doses of garlic induce liver damage (Rana *et al*., 2006) and that differences in digestive organs

weight like liver, arise because of increased metabolic rate of the organs in attempt to metabolize the compounds of garlic.

Feed intake, BW gain and feed conversion ratio were no affected by treatment. However, a reduction in feed intake was observed with increasing doses of synthetic allicin (at day 21 and 28 days of age) due to treatment × age interaction. Varmaghany *et al*. (2015) reported that the inclusion of different concentrations levels of dietary garlic bulb (0, 5, 10 and 15 g/kg of diet) did not affect feed intake, BW gain and feed conversion from 1 to 42 days of age when broilers were raised under cold temperature conditions. On the contrary, Islam *et al*. (2017) found that garlic supplementation (1.0 %) in broiler diet increased BW gain (from 7 to 35 days of age) and improve feed conversion; whereas Brzóska *et al*. (2015) reported an increase in BW at 42 days with incorporation of liquid garlic extract at concentrations of 1.00, 1.50 and 2.25 mL/kg of diet; however, they did not find differences in feed conversion ratio. This discrepancy found in the literature may be attributed to the differences of the composition and amount of active compounds in garlic preparations and the responses that they may induce (Qidwai and Ashfaq, 2013). Also, the decreased feed intake recorded in this study may be explained due to intense flavor of the allicin (Adjei *et al*., 2015).

On the other hand, it has been reported that the renin angiotensin system is the main regulator of the cardiovascular system, and alterations in its function may lead to cardiac hypertrophy. Nevertheless, cardiac hypertrophy can be inhibited by the inhibition of the ATR1 (Youtz *et al*., 2014). Hassanpour *et al*. (2016) reported that ATR1 mRNA expression in the right ventricle of broiler with pulmonary hypertension induced by T_3 (3,5,3′-l-triiodothyronine) was increased compared to control chickens. Our results showed that ATR1 was downregulated in the heart of broilers from 1-ALLI group compared with the control group. Similarly, García-Trejo *et al*. (2016)

reported that allicin downregulates ATR1 as cardioprotective mechanism; also, these authors mentioned that the beneficial effects of allicin may be mediated by an improved endothelial function, vasodilator effects, downregulation of AT1R receptor, and hence reduction in ATR1 activity. Thus, there is a possibility that allicin may act through mechanisms that inhibit the renin-angiotensin system which plays a significant role in pulmonary hypertension

In conclusion, synthetic allicin improves SaO_2 in broilers and downregulates ATR1 in heart, which demonstrates the blood pressure lowering property and vasodilator activity of allicin; however, it reduces feed intake and increases relative liver weight. In further studies, administration of synthetic allicin using male broilers reared under ascites-inducing conditions in recommended because males are more prone to develop ascites syndrome.

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GENERAL CONCLUSIONS

The use of synthetic allicin in dietary interventions:

In male broilers:

- Reduces hematocrit content and the right-to-total ventricular weight ratio without compromising productive performance.
- Improves blood oxygen saturation and upregulates/downregulates ATR1 in heart.

In mixed sex broilers:

- Reduces feed intake.

Therefore, allicin represents an alternative to delay the progression of ascites syndrome in male broilers by improving some ascites related traits.