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Investigation of the Effect of Vitamin C In The Prevention of Transport-Induced Stress in Ring-Necked Pheasant (*Phasianus colchicus*)

Halka Boyunlu Sülünlerde (*Phasianus colchicus*) Nakil Kaynaklı Stresin Önlenmesinde C Vitamininin Etkinliğinin Araştırılması

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ÖZET

Amaç: Bu çalışmada, halka boyunlu sülünlerde (*Phasianus colchicus*) nakil kaynaklı stresin önlenmesi veya azaltılmasında C vitamininin etkinliği araştırıldı.
Materyal ve Metot: Çalışmada, 8-12 aylık yaşta, canlı ağırlıkları 1140-1525 g aralığında değişen toplamda 30 sülünden (15 erkek ve 15 dişi) oluşan bir kontrol ve iki deneme grubu oluşturuldu (n=10/grup). Deneme gruplarına sırasıyla 200 (AA1) ve 400 (AA2) mg/kg dozunda C vitamini günlük olarak içme suları içerisinde verildi. Bir haftalık uygulama sonrasında sülünler iki saat boyunca nakil stresine maruz bırakıldı. Plazma kortikosteron (CS) düzeyleri ile heterofil/lenfosit (H/L) oranları taşıma öncesi ve sonrası belirlendi.
Bulgular: Nakil öncesi ve sonrası elde edilen değerler karşılaştırtıldığında, kontrol grubunda CS değerlerinde ve H/L oranlarında nakil sonrası anlamlı artış belirlenirken (P<0,001), AA1 ve AA2 gruplarında sadece plazma CS düzeyinde anlamlı artış saptandı (Sırasıyla, P<0,05 ve P<0,001). Her üç grupta ölçülen parametrelerde nakil öncesinde istatistiksel fark yoktu. Nakil sonrası ise plazma CS düzeyleri ve H/L oranları deney gruplarında benzerdi, fakat deney gruplarına ait değerler kontrol grubundan anlamlı şekilde düşüktü (Sırasıyla, P<0,001 ve P<0,01).
Sonuç: Sülünlere nakil öncesi bir hafta süreyle oral C vitamini verilmesi, nakil kaynaklı stres yanıtında azalma sağlamaktadır.

Anahtar Kelimeler: C Vitamini, Heterofil/Lenfosit oranı, Kortikosteron, Nakil stresi, Sülün.

ABSTRACT

Objective: In this study, the efficiency of vitamin C in the prevention or reduction of transport induced stress in ring-necked pheasants was investigated.
Material and Method: In the study, a control and two experimental groups consisting of a total of 30 pheasants (15 males and 15 females) at the age of 8-12 months, with body weight of 1140-1525 g, were formed (n=10/group). The experimental groups were given daily doses of 200 (AA1) and 400 (AA2) mg/kg of vitamin C in drinking water, respectively. After one week of treatment, pheasants were exposed to transport stress for two hours. Plasma corticosterone (CS) levels and heterophile/lymphocyte (H/L) ratios were determined before and after transport.
Results: When the values obtained before and after transportation were compared, a significant increase was found in CS values and H/L ratios in the control group after transportation (P<0.001), while a significant increase was found only in plasma CS levels in the AA1 and AA2 groups (P<0.05 and P< 0.001, respectively). There was no statistical difference in the parameters measured in all three groups before transportation. After transportation, plasma CS levels and H/L ratios were similar in the experimental groups, but the values of the experimental groups were significantly lower than the control group (P<0.001 and P<0.01, respectively).
Conclusion: Oral administration of vitamin C to pheasants for one week before transportation provides a decrease in the stress response due to transportation.

Keywords: Corticosterone, Heterophil/Lymphocyte ratio, Pheasant, Transport stress, Vitamin C

INTRODUCTION

Transporting ring-necked pheasants (*Phasianus colchicus*) from rearing facilities to nature is a common practice in many European countries (Draycott et al., 2002; Draycott et al., 2005) and the USA (Schwartzkopf-Genswein et al., 2012), as it is in our country (Annual report, 2020), to protect the natural life and increase the number of birds suitable for hunting. The transportation process increases the stress load in birds for many reasons, such as catching, caged, sudden change in microclimate conditions, water and feed deprivation, sudden accelerations and decelerations, vibration, unfamiliar voice, restricted floor space, and social perturbation due to high animal intensity. The consequences of the stress during transport of birds were reviewed in detail for poultry especially broilers because different transport technics may cause an increase in mortality, decrease in poultry welfare due to stressful transport, and meat quality (Schwartzkopf-Genswein et al., 2012; Qi et al., 2017; Zaboli et al., 2019).

The effects of stress, at the molecular/cellular or physiological level, are mostly related to oxidative stress and damage to important biological molecules. Therefore, a number of feed-derived and supplemented antioxidants such as vitamins, minerals, amino acids, and phytogenic feed additives are added to minimize these unwanted effects of stress (Saeed et al., 2019).

Vitamin C (chemical names: ascorbate and ascorbic acid [AA]) is a 6-carbon lactone that is synthesized from glucose by poultry and many other animals in kidneys in birds and reptiles, and in the liver in some mammals (Ahmadu et al.,2016). Ascorbic acid is one of the most important antioxidants that can neutralize oxidative stress through an electron donation/transfer process and is involved in the biosynthesis of corticosterone, a hormone that increases energy supply during stress. Birds need more AA during stress because endogenous AA is insufficient to meet the bird's requirements, so adding AA to drinking water or feed helps lower corticosterone levels during stress (McKee and Harrison, 1995). It has been reported that, under a variety of stressful conditions, improved performance resulting from AA use is associated with suppressed stress responses demonstrated by a reduction in plasma adrenocorticotropic hormone (Sahin et al., 2003) and corticosterone (McKee and Harrison, 1995; Mahmoud et al., 2004) levels. Positive effects of ascorbic acid on immune response, feed consumption, live weight gain, rectal and body temperature, fertility, hatchability of fertile eggs, oxidative stress, carcass grade, carcass yield and weight gain have been reported in birds. (Saeed et al., 2019). Therefore, it has been reported that AA can be supplemented to reduce the negative effects of stress due to heat, transportation, and other environmental factors in poultry (Ahmadu et al., 2016).

In poultry, two of the most common physiological parameters of stress are circulating concentrations of corticosterone and the heterophil/lymphocyte (H/L) ratio. Transport stress can activate the hypothalamic-pituitary-adrenal axis and thereafter plasma corticosterone is elevated following a road journey (Kannan et al., 1997). This apparent activation of the hypothalamic-pituitary-adrenal axis is consistent with the observation of post-transport increases in the H/L ratio (Gross and Siegel, 1983; Maxwell, 1993; Mitchell and Kettlewell, 1998; Erisir et al., 2008).

While there are many studies that have revealed that vitamin C can moderate the effect of different stress factors on poultry (Zulkifli et al., 2000; Sahin et al., 2002; Sahin et al., 2003; Mahmoud et al., 2004; Minka and Ayo, 2011; Ghazi et al., 2015; Ahmadu et al., 2016) there are few studies investigating the effects of stress due to handling (Chloupek et al., 2009), transport (Voslarova et al., 2006; Suchy et al., 2007; Voslarova et al., 2012), translocation (Volfova et al., 2022) and other factors (Voslarova et al., 2013) in pheasants. To our knowledge, there is only one study in pheasants where Nowaczewski (2006) showed that supplementation with vitamin C reduced the tonic immobility reaction and CS level in stressed pheasants. Therefore, this study was undertaken to determine the stress-ameliorating effects of vitamin C supplementation in drinking water in response to transportation in pheasants.

MATERIALS and METHODS

Animals and experimental protocol

In total, 30 adults (8-12-months-old) healthy ring-necked pheasants (*Phasianus colchicus*; hereinafter, pheasant) of both sexes were used. The sample size of this study was determined using G-power software (G* Power 3.1.9.2, Heinrich–Heine–Universität, Düsseldorf, Germany). Based on the result of G*Power, the number of samples was at least eight pheasants for each group. However, 10 pheasants were used per group taking into account the possible losses. Pheasants were obtained from the Gelemen Pheasant Production Station, Samsun, and were brought from the station to the pen (41° 22′ N, 36° 12′ E) within plastic transport cages. This experiment was carried out with an average of 19±1° C outside temperature, 21±1° C inside temperature and 71±7% relative humidity. The birds were kept at a fixed light cycle of 15L:9D to mimic the early June conditions outside during the experiment. This study was approved by the Animal Experimentation Ethics Committee of Ondokuz Mayis University (Approval no: 2016/04).

The pheasants were divided into three groups considering their body weight (BW), with five males and five females in each group. The BW of the birds in the control group (C) and treatment groups (AA1 and AA2) were 1330±89 and (1350±129 and 1349±112 g), respectively. There was no statistical difference among the three groups for BW. All the groups were fed commercial feed obtained from the station to avoid unwanted effects of diet change. All birds were subjected to the adaptation period (a week) before the experiment. During this period, the daily average water consumption of each group was also determined.

Daily drinking water was supplied to the C group without AA supplementation while groups AA1 and AA2 were supplemented with 200 and 400 mg of AA/kg BW, respectively. Ascorbic acid was obtained by DSM Besin Maddeleri Ltd., Istanbul, Turkey. This supplementation was continued for seven days. A dose of AA, recalculated for the total BW of pheasants in each group, was dissolved in a predetermined volume of water in dark coloured bottles.

Pheasants were transported at the end of the supplementation period (day 8) to three consecutive parties to avoid exceeding the processing capacity. At each transport party, an equal number of birds from each group were loaded onto the truck with a crate (W=96 x L=57 x H=27 cm3) at the density of approx. 400 cm2kg-1. The crate was placed in such a way to prevent movement during transit and transported for two hours with an average speed of 60 km/hour. All three journeys were made on the same route.

Blood samples (1 ml) were obtained within 3 minutes to avoid change in monitored stress parameters due to sampling such as catching, handling, bleeding (Owen, 2011). Blood was taken from the brachial vein to tubes containing anticoagulant (EDTA) twice immediately before and after transportation to determine the level of plasma corticosterone and H/L ratio.

The samples were sent to the laboratory from the sampling room (within 30 min after blood collection) in a small styrofoam cooler. After their transfer to the laboratory, the blood smears were prepared using the May-Grunwald-Giemsa stain. The H/L ratios were determined one day later. Blood smears were examined under a microscope at a magnification of 100x with oil immersion and in each smear, heterophils and lymphocytes were counted until the cumulative total reached 100 cells, and then the H/L ratio was calculated.

The remaining blood samples were centrifuged at 10000 rpm for 10 min, plasma was decanted and then stored at -20 °C until corticosterone analyses. Plasma corticosterone levels were determined in duplicate by immunoassay according to guidelines provided by the manufacturer (Enzo Life Sciences, Corticosterone ELISA Kit, ADI-900-097, Lausen, Switzerland). Standards used in the current study were between 32-20000 pg/ml. The intensity of colour was measured at 405 nm with an 8-channel microtitration plate spectrophotometer (DAS, Digital and Analogue Systems, A3, Italy). The results were calculated by using the 4 parameters logistic curve-fit.

Statistical analysis

Statistical analysis was carried out using the SPSS statistical package (IBM SPSS Statistics, Ver. 21, USA). After the Shapiro-Wilk normality test and homogeneity of variance test, data with homogeneous variances (H/L ratio) were subjected to a one-way ANOVA to assess statistical significance, followed by a Tukey-HSD test for multiple comparisons. Due to the standard error of means exceed to their mean values, data with heterogeneous variances (corticosterone) were executed to GENMOD analysis with linked time function. Differences in corticosterone mean changed over time were analysed with the contrast structure (linear form) of the GENMOD procedure. Data are presented as mean±SD for H/L, and mean, median and their deviation for corticosterone.

RESULTS

Plasma corticosterone concentration was statistically higher after transportation in the control, AA1 and AA2 groups (P<0.001, P<0.05 and P<0.001, respectively) while the H/L ratio was higher only in the control group (P<0,001). When compared groups, plasma corticosterone level and H/L ratio were similar in all three groups before transportation. However, after transportation, both CS and H/L ratio were higher in the control group than the AA administrated groups (P<0.001 and P<0.01, respectively). Experimental groups also were similar to each other after transportation (Table 1).

Table 1. The stress parameters values obtained before and after transport in pheasants

		Groups									
		Control			**AA1**			**AA2**			
		Mean	**Median**	**Deviance**	**Mean**	**Median**	**Deviance**	**Mean**	**Median**	**Deviance**	**P value**
Corticosterone[1] (ng/ml)	BT	21.66	12.94	0.5646	24.06	10.60	0.2815	22.79	9.59	0.5864	>0.05
	AT	89.35^{a}	96.70		50.20^{b}	43.84		52.24^{b}	43.79		<0.001
	P value	<0.001			<0.05			<0.001			
		Mean±SD			**Mean±SD**			**Mean±SD**			**P value**
H/L ratio	BT	0.42±0.13			0.40±0.15			0.45±0.18			>0.05
	AT	0.76±0.21^{a}			0.52±0.09^{b}			0.58±0.10^{b}			<0.01
	P value	<0.001			>0.05			>0.05			

DISCUSSION

Road transport represents a critical stage in poultry production, and it is considered one of the main causes of stress. These stress factors include human-induced handling and crating, journey-induced noise, vibration, density, starvation, emotional factors such as unfamiliar sound and environment, and climatic factors such as extreme heat and humidity. The consequences of stress during transport of birds and coping with transport induced-stress were studied mainly in poultry by many researchers and reviewed by many authors (Kannan et al.,1997; Mitchell and Kettlewell, 1998; Zulkifli et al., 2001; Zhang et al., 2009; Vosmerova et al., 2010; Minka and Ayo, 2011; Schwartzkopf-Genswein et al., 2012; Qi et al., 2017). Although there are studies investigating the effects of transportation, there is no publication about alleviating transport-induced stress in pheasants. Therefore, we investigated whether AA is effective in alleviating stress in pheasants as in other poultry. Our results demonstrate that transportation is a stressful procedure in pheasants, as shown by an immediate increase in monitored parameters in the control group after transportation (Table 1). Plasma corticosterone concentration, which is considered the main indicator of stress in birds, was significantly higher after the travel than before transport in all groups. Although there was a numerical increase in the H/L ratio in all groups after transportation, the increase was statistically significant only in the control group. Considering increases in corticosterone and H/L ratio after transportation, we interpreted these results that transport-induced stress has occurred in all groups at different levels. Our results agreed with early researchers who reported that corticosterone (Suchy et al., 2007; Voslarova et al., 2012) or H/L ratio (Voslarova et al., 2006) was higher in transported pheasants in comparison with the non-transported control pheasants. Voslarova et al. (2006) and Suchy et al. (2007) investigated the effects of transport stress and reduction of floor area (290 and 195 cm^2/kg) on pheasants at 9 weeks of age, transported at different densities for 4 hours, and described changes in some biochemical and hematological parameters. Similarly, stress effects resulting from transport in common pheasant were examined by Voslarova et al. (2012), who observed an increase in the corticosterone concentration.

We compared parameters measured before and after transportation among groups to evaluate the effectiveness of AA. There was no statistical difference before the journey for all the parameters measured. After the journey, all parameters were similar for each experimental group, and they were lower than the control group. According to these results, it can be postulated that AA had a positive effect on reducing stress responses with the indication of decreased plasma corticosterone level and H/L ratio, and this agrees with the conclusions reached by many researchers (Kutlu and Forbes, 1993; McKee and Harrison, 1995; Zulkifli et al., 2001; Sahin et al., 2002; Sahin et al., 2003; Mahmoud et al., 2004; Minka and Ayo, 2011; Ghazi et al., 2015). These researchers used broilers or quails to determine the effectiveness of AA in dealing with stress depending on transportation or high temperature and used plasma corticosterone level, H/L ratio, or other biochemical parameters as stress markers.

The lower values of corticosterone level and H/L ratio recorded in pheasants administered AA following transportation suggested that the AA reduced or eliminated the adverse effects of road transportation stress in pheasants. Kutlu and Forbes (1993) reported that AA reduces the synthesis of corticosteroid hormones in birds. Similarly, Sahin et al. (2003) reported low concentrations of ACTH in quails reared at high temperature and fed a diet supplemented with AA. Ascorbic acid plays an important role in the biosynthesis of corticosterone, a hormone that increases energy supply during stress. AA alleviates the negative effects of stress by reducing the synthesis and secretion of corticosteroids (McKee and Harrison, 1995; Sahin et al., 2003; Mahmoud et al., 2004).

In conclusion, doses of 200 and 400 mg of AA exerted a positive influence on alleviating transport-induced stress in pheasants. It appears advisable to use a dose of 200 mg of AA per kg BW in water for one week before transportation of pheasants.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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