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RESEARCH ARTICLE

Effect of Free Choice Feeding as to Protein Levels on Oxidative Status in the Broilers Exposed to Heat Stress

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Summary

This study was conducted to evaluate the effects of free choice feeding on oxidative stress in broilers exposed to high temperature. At 21 days of age, 24 male Ross 308 strain chicks were divided into four groups of six with respect to feed composition and temperature regimen. Broilers were evenly divided into two environmental rooms with temperatures set at 22°C (group 1 and 2) and 30°C (group 3 and 4). The experiment consisted of 2 dietary treatments per temperature. Half of the birds in each room were feed according to traditional nutrition procedure (group 1 and 3) and the other half were feed according to free choice feeding system (group 2 and 4). For the free choice feeding systems, birds were allowed to choose between diets containing 18% or 23% crude protein. At the end of the 7th week, some oxidant (lipid hydroperoxide, total oxidant status and oxidative stress index) and antioxidant (total antioxidant capacity, total free sulfhydryl groups and ceruloplasmin) in the plasma and performance (feed consumption, body weight gain, feed convertion ratio and average daily protein intake) parameters were measured. High temperature treatment disrupted performance and oxidative status by elevated lipid hydroperoxide level, total oxidant status and oxidative stress index. On the other hand, antioxidant parameters such as total antioxidant capacity and free sulfhydryl level did not decrease, moreover, ceruloplasmin levels increased in response to elevated oxidative stress in broilers exposed to heat stress. As for free choice feeding, it did not affect oxidative status and performance parameters in any of the groups. These results suggest that high temperature treatment increased the oxidative stress in both feeding treatment groups. Free choice feeding failed to ameliorate oxidative stress caused by high temperatures in broilers.

Keywords: *Free choice feeding, Protein; Heat stress, Oxidative stress, Broiler*

Sıcaklık Stresine Maruz Broylerlerde Protein Yönünden Seçmeli Yemleme Uygulamasının Oksidatif Strese Etkisi

Özet

Çalışmada yüksek sıcaklık altında yetiştirilen broyler piliçlerde serbest seçmeli yemlemenin oksidatif stres üzerine etkisi araştırıldı. 21 günlük 24 adet Ross 308 ırkı broyler piliçler sıcaklık ve beslenme rejimi yönünden farklı ve her birinde 6 adet olmak üzere 4 eşit gruba ayrıldı. Broylerlerin ilk iki grubu 22°C'de (1 ve 2. grup), son iki grubu da 30°C (3 ve 4. grup) olmak üzere 2 farklı sıcaklık grubuna ayrıldı. Her sıcaklık grubundaki piliçlere beslenme yönünden geleneksel (1 ve 3. grup) ve serbest seçmeli yemleme (2 ve 4. grup) uygulandı. Serbest seçmeli yemleme uygulanan piliçlerin %18 ve %23 ham protein içeren yemleri seçmelerine izin verildi. Yedinci haftanın sonunda plazmada bazı oksidan (lipit hidroperoksit, total oksidan kapasite ve oksidatif stres indeksi), antioksidan (total antioksidan kapasite, total serbest sülfhidril grubu, seruloplazmin) parametreler ile performans parametreleri (yem tüketimi, canlı ağırlık artışı, yemden yararlanma oranı, ortalama günlük protein alımı) ölçüldü. Yüksek sıcaklık uygulamasının lipit hidroperoksit seviyesi, total oksidan kapasite ve oksidatif stres indeksini artırarak oksidatif strese neden olduğu ve performansı düşürdüğü görüldü. Diğer taraftan sıcaklık stresinin total antioksidan kapasite ve total serbest sülfidril seviyesini azaltmadığı, hatta artan oksidatif strese karşın serüloplazmin seviyesini artırdığı gözlendi. Serbest seçmeli yemleme uygulaması ise hiçbir grupta ne performansı ne de oksidatif ve antioksidatif parametreleri etkilemedi. Sonuç olarak; yüksek sıcaklık stresinin her iki beslenme uygulamasında oksidatif stresi artırdığı, serbest seçmeli yem uygulamasının ise oksidatif stresi azaltan herhangi bir etkisinin olmadığı gözlenmiştir.

Anahtar sözcükler: *Serbest seçmeli yemleme, Protein, Sıcaklık stresi, Oksidatif stres, Broyler*

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INTRODUCTION

Environmental stress is of major concern for poultry, especially in the hot regions of the world because of the resulting poor growth performance, nutrient utilization, immunosuppression and high mortality rate [1]. Stress has some commonality in the capacity to alter animal metabolism. A common denominator of the responses to stress is the redox homeostasis. The redox homeostasis is maintained by the balance between the production of reactive oxygen species (ROS) and antioxidant defense system. The production of ROS in low concentrations is absolutely necessary for some physiological processes such as cell differentiation and proliferation, apoptosis, and cell-mediated immunity. Moreover, many endogenous and exogenous factors such as eicosanoid and corticosteroid synthesis, ischemia-reperfusion and the metabolism of some drugs may cause an increase in ROS in the form of hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and superoxide anion. At the same time, enzymatic (superoxide dismutase, catalase, glutathione peroxidase, ect.) and non-enzymatic (uric acid, vitamin E, A, C, sulfhydryl groups, ect.) antioxidant defenses maintain ROS concentrations in the physiological range. When the balance between the antioxidant defense system and the production of ROS is disturbed, the process is defined as oxidative stress. Oxidative stress is associated with damage to a wide range of molecules species including lipids, proteins, and nucleic acids. Such oxidative damage might also lead to cellular dysfunction, and contribute to the development of a wide variety of diseases including inflammatory conditions, ischaemia-reperfusion injury, ageing process [2,3] and ascites in poultry [4]. Heat stress is reported to disturb the balance between the production of ROS and the antioxidant defense systems in broilers [5] and laying hens [6].

The improvement of rearing conditions during heat stress can be achieved by modifying the environment, building, breeding, and nutritional practices (protein, energy, water and electrolytes). Dietary medications are among the most preferred and practical ways to alleviate the effect of high environmental temperature in poultry [7]. In most of the experiments reported there is considerable variation in diet selection between individuals that might simply be a reflection of different requirements of the individuals for protein and energy [8]. Free choice feeding (FCF) may be suggested to overcome the negative effect of heat stress in broilers. Free choice feeding allows the animal to formulate its own diet by selecting the feeds according to the particular physiological requirements for maintenance and production. Thus, the performance of free choice-fed broilers was significantly better than that of the same birds fed the most sophisticated complete diets in hot environments [8,9].

In chicken exposed to high temperature, dietary protein level has received considerable attention because its metabolism is associated with higher heat production when compared with that of fats and carbohydrates. Consequently, diet-induced thermogenesis associated with protein raises concerns in hyperthermic broilers [10,11]. During many years, diets with low protein level have been recommended in order to reduce heat production in broilers under heat stress [10]. However, recent findings have demonstrated that low protein diets have negative effects on broiler performance when ambient temperature is high, because decreased feed intake along with a low level of protein induces amino acid deficiencies [12]. On the other hand dietary protein may play a role in the antioxidative defense mechanism by influencing the concentration of glutathione, which acts as a cellular antioxidant [13]. Moreover, Nieto et al.[14] reported that feeding broilers with low-protein diet from 10th to 24th day of age resulted in an increase in the requirement of metabolizable energy for maintenance and heat production.

Given the information mentioned above, FCF system with protein concentrate may have a potential for poultry production in reducing heat stress under hot climates. Offering free choice diets allows individuals to select the foods according to their need for maintenance and production and probably for a better adaptation to high environmental temperatures [9].

Although there are many studies concerning amelioration of the negative effects of heat stress, no studies were found in regard to the effect of FCF on oxidative status in broilers under heat stress. The present study was carried out to evaluatethe effect of FCF and traditional feeding systems on oxidative status in broilers exposed to heat stress from 21 to 42 days of age.

MATERIAL and METHODS

Animals and Experimental Desing

The study was carried out on 24 healthy male Ross 308 strain chicks. In the pre-experimental period (1 to 21 days of age), chicks were fed the same diet according to the requirements of NRC [15] *(Table 1)* and were kept in environmentally controlled rooms under conventional thermoneutral conditions. The birds were kept in a windowless house and given conventional artificial light (23 h light and 1 h dark). At 21 days of age, broilers were divided into four groups with respect to feed composition and temperature regimen. Broilers were evenly divided into two environmental rooms with temperatures set at 22°C (group 1 and 2) and 30°C (group 3 and 4). The experiment consisted of 2 dietary treatments per temperature. Half of the birds in each room were fed according to NRC [15] (group 1 and 3) and the other half were fed according to FCF system (group 2 and 4). For the FCF systems, birds were allowed to choose between the diets containing crude protein of 18% or 23%. Chicks had free access to feed and water.

Blood Samples

At the end of the 7th week, broilers were sacrificed by

Table 1. *Ingredients and chemical composition of broiler diet*
Tablo 1. *Broyler yemi içeriği ve kimyasal kompozisyonu*

Ingredient Composition	Diets Compositions (g/kg)		
	0-3 week	3-6 week	6-8 week
Maize	462.4	574.4	645.6
Soybean Meal	418.5	336.8	282.5
Limestone	16.0	14.0	13.1
Maize Oil	80.9	56.8	44.2
Dicalcium Phosphate	13.4	11.5	9.3
NaCl	4.6	3.3	2.6
Vit.-Min. Premix [1]	2.5	2.5	2.5
DL-Methionine	1.7	0.7	0.3
Calculated Nutrient Composition [2]			
Crude Protein	23	20	18
Calcium	1.00	0.90	0.80
Total Phosphorus	0.45	0.35	0.30
Sodium	0.20	0.15	0.12
Lysine	1.32	1.11	0.96
Methionine	0.52	0.38	0.32
Methionine+Cystine	0.90	0.72	0.63
Threonine	0.90	0.78	0.69
Tryptophan	0.28	0.24	0.20
Apparent Metabolisable Energy (Kcal/kg)	3.200	3.200	3.200

[1] The composition of vitamins and minerals per kilogram: Vit A, 15.000 IU; Vit D_3, 2.500 IU; Vit E, 40 mg; Vit K_3, 5 mg; Vit B_1, 3 mg, Vit B_2, 7 mg; Vit B_6, 5 mg; Vit B_{12}, 0.02 mg; Pantotenic acid, 10 mg; Niasin, 40 mg;Folic acid, 1 mg; Biotin, 0.07 mg; Mn, 80 mg; Fe, 60 mg; Zn, 60 mg; Cu, 5 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg; Cholin Cloride, 300 mg, [2] Calculated nutrient composition was based on NRC values

cervical dislocation. Immediately after blood collection, plasma was separated for assays. Oxidative and antioxidative parameters were measured in the plasma samples by using the Aeroset automated analyzer (Abbott) and spectrophotometer (Cecil 3000).

Measurement of Lipid Hydroperoxide

Lipid hydroperoxide (LOOH) levels were measured with the ferrous ion oxidation-xylenol orange assay. The principle of the assay is oxidation of ferrous ion to ferric ion via various oxidants and the produced ferric ion is measured with xylenol orange. LOOHs are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between with and without TPP pretreatment gives LOOH levels [16].

Measurement of Total Oxidant Status

The total oxidant status (TOS) was measured using an automated colorimetric measurement method developed by Erel [17]. In this method, oxidants in the plasma sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity, which is related to the total amount of oxidant molecules present in the sample, is measured spectrophotometrically. The assay is calibrated with ($HO^{\cdot}$) and the results are expressed in terms of µmol H_2O_2 Eq/L.

Measurement of Total Antioxidant Capacity

The total antioxidant capacity (TAC) was measured using an automated colorimetric method developed by Erel [18]. In this method, $HO^{\cdot}$ radical is produced by the Fenton reaction, and reacts with O-dianisidine to produce the dianisyl radical, which is yellowish-brown in color. Upon the addition of a plasma sample, the oxidative reactions initiated by the $HO^{\cdot}$ radicals are suppressed by the antioxidant components of the plasma, preventing the color change. The results are expressed as mmol Trolox Eq/L. The precision of this assay is lower than 3%. The major advantage of this test is that it measures the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound.

Calculation of Oxidative Stress Index

Oxidative stress index (OSI) is an indicator of the degree of oxidative stress and defines the ratio of TOS to TAC. To perform the calculation, the result unit of TAS (mmol Trolox Eq/L) was converted to µmol Eq/L and the OSI value was calculated by using following formula: OSI=[(TOS, µmol/L)/(TAS, (mmolTrolox Eq/L) x 100].

Measurement of Total Free Sulfhydryl Groups

Total free sulfhydryl groups (SH) of plasma were assayed according to the method of Hu et al.[19]. In brief, 1 mL of buffer containing 0.1M Tris, and 50 µL plasma was added to a spectrophotometer cuvette followed by the addition of 50 µL 10mM DTNB in methanol. Samples without DTNB were run for each sample as blanks. Following incubation for 15 min, absorbance was red at 412 nm on a spectrophotometer with a temperature controlled cuvette holder. The concentration of SH groups was calculated using reduced glutathione as free SH group standard.

Measurement of Ceruloplasmin

Ceruloplasmin (CP) levels were measured according to the method of Erel [20]. This is an automated colorimetric method based on the enzymatic oxidation of ferrous ion to ferric ion. The precision of this assay is lower than 3%.

Statistical Analysis

Statistical analysis was carried out with SPSS software 10.0 (SPSS Inc., Chicago, IL). Data were analyzed using General Linear Model procedure to study the effects of temperature (22°C and 30°C), dietary crude protein (18% and 23%) and the interaction between them. Significant differences were tested further using Duncan multiple range test to determine the differences among treatments. Values were given as means and standard deviation. The differences were considered to be significant when $P<0.05$.

RESULTS

High temperature treatment increased oxidative stress by elevated LOOH and TOS levels and OSI value ($P<0.05$) in both feeding systems *(Table 2)*. On the other hand, antioxidative parameters such as TAC and SH levels did not change ($P>0.05$), while CP levels increased ($P<0.05$) in the broilers exposed to heat stress *(Table 2)*. In addition, heat stres decreased performance (feed consumption, body weight gain, average daily protein intake) of broilers *(Table 3)*. As for FCFsystem, it affected neither oxidative status nor performance ($P>0.05$) compared with traditional feeding system in all groups *(Table 2,3)*. Thus, FCF did not improve oxidative stress at either normal or high temperature compared with traditional feeding system. The temperature and dietary treatment interactions were not significant ($P>0.05$) for all the parameters analyzed *(Table 2)*.

DISCUSSION

The birds are very susceptible to stress in comparison to other livestock animals. High ambient temperature is considered as a potent climatic stress causing an increase in

Table 2. Effects of high temperature and free choice feeding on oxidative stress in broilers. (mean±SD)
Tablo 2. Broylerlerde yüksek sıcaklık ve serbest seçmeli yemlemenin oksitatif strese etkisi (ortalama±SD)

Parameters	22°C		30°C		Statistical Analysis (P values)		
	1 NRC (n=6)	2 FCF (n=6)	3 NRC (n=6)	4 FCF (n=6)	Temperature	Diet	Interaction
LOOH (mmol H_2O_2 Eq/L)	3.93±0.23^a	4.16±0.59^a	5.24±0.80^b	5.24±0.73^b	0.000	0.064	0.673
TOS (mmol H_2O_2 Eq/L)	23.51±0.49^a	23.72±0.84^a	24.99±0.86^b	24.75±1.24^b	0.003	0.965	0.545
OSI (arbitrary unit)	1.33±0.05^a	1.32±0.06^a	1.43±0.06^b	1.37±0.08^{ab}	0.015	0.216	0.323
TAC (mmol Trolox Eq/L)	1.77±0.07	1.79±0.05	1.74±0.06	1.80±0.06	0.804	0.132	0.498
SH (mmol/L)	0.080±0.001	0.081±0.001	0.078±0.001	0.083±0.001	0.967	0.437	0.651
CP (mg/L)	270.90±22.50^a	270.75±7.26^a	304.32±42.70^b	301.77±9.76^b	0.006	0.819	0.831

a, b: *Means with different superscripts within a row are significantly different ($P<0.05$),* ***NRC:*** *National Research Council,* ***FCF:*** *Free choice feeding; LOOH: Lipid hydroperoxide,* ***TOS:*** *Total oxidant status,* ***OSI:*** *Oxidative stress index,* ***TAC:*** *Total antioxidant capacity,* ***SH:*** *Total free sulfhydryl groups,* ***CP:****Ceruloplasmin*

Table 3. *Effects of high temperature and free choice feeding on feed consumption, body weight gain, feed conversion ratio and average protein intake in broilers (mean±SD)*
Tablo 3. *Broylerlerde yüksek sıcaklık ve serbest seçmeli yemlemenin yem tüketimi, canlı ağırlık artışı, yemden yararlanma oranı ve günlük ortalama protein alımına etkisi (ortalama±SD)*

Parameters	22°C		30°C		Statistical Analysis (P values)		
	1 NRC (n=6)	2 FCF (n=6)	3 NRC (n=6)	4 FCF (n=6)	Temperature	Diet	Interaction
Feed Consumption (g)	4232.02±490.33^a	4187.02±146.25^a	3457.21±95.97^b	3656.23±277.48^b	0.001	0.530	0.323
Body Weight Gain (21-49 days)	2146.73±153.76^a	2261.23±138.58^a	1931.31±56.67^b	1945.72±257.14^b	0.001	0.357	0.472
Feed Convertion Ratio	1.97±0.09	1.86±0.16	1.79±0.05	1.90±0.26	0.322	0.980	0.115
Average Daily Protein Intake (21-49 days)	29.57±3.42^a	29.98±1.22^a	24.16±0.66^b	26.82±2.15^b	0.001	0.095	0.211

a, b: *Means with different superscripts within a row are significantly different ($P<0.05$),* ***NRC:*** *National Research Council,* ***FCF:*** *Free choice feeding*

the load of the physiological system. When body temperature is over 30°C, broilers are assumed to be under heat stress [21]. It has been well documented that high ambient temperature impaires oxidant/antioxidant status in broilers [5] and laying hens [6]. Plasma concentrations of oxidants can be measured separately. However, we also measured TOS values because it accurately reflects the oxidative status of plasma due to the additive effects of the oxidant components in plasma [17]. As expected, high environmental temperatures negatively affected the oxidant/antioxidant status of the broilers. LOOH, TOS levels and OSI values, indicators of the degree of oxidative stress, were significantly elevated ($P<0.05$) in both traditional and FCF feeding system at 30°C temperature compared to 22°C *(Table 2)*. The results can be explained as follows: heat stress increases ROS by disturbing the electron transport in mitochondria. Mujahid et al.[22] reported that mitochondrial superoxide anion production in heat-stressed broilers was significantly increased compared to that of control broilers. Moreover, stress impairs the absorption of antioxidant vitamins such as A and E, and may lead to endogenous deficiency of these vitamins by decreasing food intake [23].

The free sulphydryl groups act as important cellular scavengers of peroxides and so help to protect cells from damage by these molecules [2]. However, FCF treatment or heat stress did not change plasma SH levels in the current study ($P>0.05$). The gradual oxidation of the SH groups may be related to initial oxidative consumption of the more sensitive plasma chain-breaking antioxidants e.g. vitamin C, uric acid and bilirubin. *In vitro* and clinical studies have shown that the levels of these antioxidants fall more rapidly than SH levels [24].

Ceruloplasmin is a copper-containing plasma acute phase protein that has extracellular antioxidant properties. It acts as an antioxidant by several mechanisms: inhibiting iron-dependent lipid peroxidation and ($HO^{\cdot}$) formation from H_2O_2 by its ferroxidase activity, reacting with and scavenging H_2O_2 and superoxide anion, and inhibiting copper-induced LOOH by binding Cu^{+2} ions [25]. In the present study, despite oxidative stress parameters increased in both FCF and traditional feed broilers exposed to high temperature, the increase in CP levels ($P<0.05$) was an unexpected observation. Similarly, several studies reported that plasma CP levels elevated in spite of increased oxidative stress parameters in some human disorders [25,26] and during hyperthermia in rat [27]. The reason for the high temperature induced increase in the concentration of CP may be an adaptive response to increased ROS production [26].

Measuring the activity of individual antioxidants would give an indication of the activity of only one antioxidant, whereas TAC may represent the total antioxidant characteristics of all antioxidants in the plasma [18]. Therefore, we used TAC to assess the impact of high temperature and FCF administration on the redox state of plasma since this is a sensitive indicator for the efficacy of a wide range of antioxidants (e.g., bilirubin, uric acid, vitamin C, polyphenols, and some proteins) [2]. We expected a decrease in TAC values due to increased oxidative stress at the high temperature. Nevertheless, no significant difference in TAC values among temperature treatments was observed ($P>0.05$). If we had measured different individual antioxidant components, we would have shown possibly a difference in these antioxidant parameters as expected. The result might originate from an adaptive response of enzymatic and non-enzymatic antioxidants to oxidative status as did elevated CP and unchanged SH levels opposite to high oxidative status. On the other hand, this result may be explained by the level of uric acid (a strong antioxidant molecule) elevated by high temperatures in broilers [28]. In contrast to other plasma antioxidants, uric acid is more rapidly oxidized and so other antioxidants are protected from oxidation [24].

Considering that proteins have the highest heat increment among nutrients it has been suggested that protein levels should be reduced in diets of heat-stressed broilers [29]. However, recent findings have demonstrated that diets with reduced crude protein content decrease the performance of broilers reared under heat stress [12]. The dietary protein intake of animals changes with environmental temperature. When broilers are allowed to select freely from feed of suitable protein content, they appear able to match their dietary energy and protein requirement under different environmental temperatures [30]. Thus, the performance of free choice-fed broilers was significantly better than that of the same birds fed the most sophisticated complete diets in hot environments [8]. In the present study, we offered the broilers FCF treatment including crude protein in 18% and 23%, at both temperatures. No significant interaction between temperature and diet, and no significant differences ($P>0.05$) for all parameters among the groups were found *(Table 2,3)*. Unfortunately, FCF were not sufficient to overcome the detrimental effects of the high temperatures on the oxidant/antioxidant status. These results may be originated that average daily protein intake and feed consumption were not different in both feding system in the present study *(Table 3)*. Similary, Rose and Kyriazakis [31] reported that chickens are able to select a diet that provided a nutrient intake similar to the requirements published by NRC [15].

In conclusion, although high temperature treatment damaged oxidative status and performance, antioxidative defense mechanisms showed adaptive response to oxidative stress by means of increased CP level and unchanged TAC and SH levels in heat exposure broilers. On the other hand, FCF failed to ameliorate oxidative stres and performance in the broilers at both temperatures.

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