Effects of dietary sodium bicarbonate and ascorbic acid on hepatocytes antioxidant profile in broiler chicks raised under chronic heat stress

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ABSTRACT:

Broilers raised in heat stress conditions produce more free radicals, which cause oxidative stress. An experiment was conducted to study some strategies to ameliorate the effect of chronic heat stress on broiler chicks. A total number of 300 male broiler chicks (ROSS^{308}) 14-day-old were used with three treatments: CONT (control-without any supplementation), SBC (sodium bicarbonate 1g/kg diet) and AA (ascorbic acid 1g/kg diet) supplemented for 21 days starting from 15-day-old chicks. All treatments were kept under 32±1°C throughout the trial period. Liver samples were collected at 5 weeks of age and grow out performance measurements were calculated over the 21-day study. Results of liver total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and cytochrome c oxidase (CCOX) activities insignificantly decreased in SBC and AA, respectively, and glutathione peroxidase (GSH-PX), insignificantly increased in SBC and AA, respectively compared to CONT. SBC and AA increased significantly (P≤0.05) body weight (BWT), daily weight gain (DWG), and daily feed intake (DFI) at 3 and 4 weeks of age, while significantly increased (P≤0.05) body weight and increased daily feed intake at 5 weeks of age. These results indicate that SBC and AA may enhance the antioxidant capacity as a beneficial effect on broiler performance under chronic heat stress.

Keywords: Broilers; Antioxidants; Heat stress; Sodium bicarbonate; Ascorbic acid.

INTRODUCTION

Global warming caused by rising industrialization and environmental degradation has resulted in a constant increase in ambient temperature, and the harmful impacts of heat stress will become more obvious in the future. Economic losses in broiler production could result from climatic variables. Heat waves are to blame for a lot of these losses (Vale *et al.,* 2010). Heat stress is a very crucial factor in broiler production since it affects production and growth. Moreover, its impact can negatively affect the production and physiological performance of broiler chickens and causes more damage at the cellular level through oxidative stress (Akbarian *et al.,* 2016, Luo *et al.,* 2018).

Oxidative stress is referred to as the existence of reactive species (RS) exceeding the available antioxidant capacity of animal cells. Numerous radicals and metabolic byproducts are classified as "reactive oxygen/nitrogen/chlorine species" and are described as potentially toxic (Pisoschi and Pop 2015, Zhang *et al.,* 2016). Antioxidants are molecules that prevent or delay cellular damage by inhibiting or quenching free radical reactions (El-Bahr 2013). To minimize oxidative stress, some strategies such as feeding on sodium bicarbonate (SBC) and ascorbic acid (AA) have been employed.

In tissues, oxidative metabolism produces carbon dioxide, which the enzyme carbonic anhydrase converts to carbonic acid. The carbonic acid is a component of the bicarbonate/carbonic acid buffer system in which the bicarbonate ion performs its major function. The respiratory and renal systems work together with this, the most significant buffering mechanism of plasma, to control the acid-base balance of blood plasma (Balnave and Gorman 1993). Due to panting, which increases carbon dioxide loss and lowers the ideal water intake, birds under heat stress have an altered electrolyte balance. Supplementing the diet with 0.5 or 1 % SBC helps restore the acid-base balance lost in alkalosis resulting from panting in birds under heat stress (Majekodunmi *et al.,* 2013, Fisinin and Kavtarashvili 2015) and stimulates feed and water intake at high environmental temperature (Bhadauria *et al.,* 2017).

Ascorbic acid is a water-soluble antioxidant that is normally produced in adequate amount by metabolism in chickens when not stressed (Shoukry *et al.,* 2011). According to Mahmoud *et al.,* (2004); Kumar *et al.,* (2017) and Horváth and Babinszky (2019), the addition of AA (200 mg/kg feed) resulted in significant changes in plasma AA levels in broilers subjected to heat stress. Sahin *et al.,* (2002); Farooqi *et al.,* (2005) and Attia *et al.,* (2011) illustrated the role of free radicals of scavenging under different high temperature conditions with a

supplement of AA (150 to 500 mg/kg diet) and can also be demonstrated with the function of free radicals of scavenging. The study will be conducted to evaluate the effects of dietary SBC and AA on ameliorating the effect of chronic heat stress on male broiler chicks through their effects on hepatocyte antioxidant profile.

MATERIALS AND METHODS

Experimental Birds and Management:

The experiment was conducted at Poultry Research Station, Animal Production Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

In an open housing system, 300-one day-old, broiler chicks (Ross^{308}) were kept in floor pens and reared on a chopped mixture of wheat straw from one day old to 35 days of age with initial body weight of 50.00±0.65 g, chicks were brooded together and fed a basal diet with feed and water *ad libitum*. Chicks were subjected to standard lighting regimes during growth, light period was 24 hours/day throughout the first week of brooding and 22 hours/days from period of 7-35 days. All birds were healthy and clinically monitored and vaccinated according to a standard vaccination schedule. All birds were reared under similar management and hygienic conditions. At two weeks of age, chicks were weighed and grouped into three treatments distributed randomly in two blocks each block was halved into two plots. Each plot served as replicate. Chicks in each plot (25 chicks) has floor space (calculated based on 8 chicks/m^2) and kept under 32±1°C throughout the trial period.

Experimental Design and Feeding Program:

The experimental design was as follows: Control (CONT) was without any supplementation; SBC was supplied with sodium bicarbonate (1g/kg diet); AA was supplied with ascorbic acid (1g/kg diet) for 21 day starting form 15 day-old. Broiler chicks were fed on starter and grower diets days according to guidelines of (Ross^{308} broiler performance and nutrition supplement 2019) and (N.R.C. 1994). The trial period was classified into two feeding periods: starter period from 1 to 21 days of age and grower period from 22 to 35 days of age. Ingredients of diets of experiment and their nutrient composition are presented in Table (1).

Samples Collection and Tissue Preparation:

At 5 weeks of age, 5 birds from each treatment were used to measure total antioxidant capacity, glutathione peroxidase, superoxide dismutase activity, and cytochrome c oxidase activity in hepatocytes.

Perfusion Procedure: Chicken livers were perfused by a modification of the method of Bickerstaffe *et al.,* (1970). The chickens were anesthetized by right jugular vein injection (15 mg/kg body wt.) of sodium thiopental and the abdomen was opened through a transverse incision made just below the point of the sternum. The incision was extended to the right and left of the midline to reach the caudal angles of the sternum.

The left ventricle cannulated with a polyethylene cannula *in situ*, and the liver was perfused with ice-cold sodium phosphate buffer (0.05M, pH=7.4) as described below. The perfusate was pumped by peristaltic pump (Heidolph, Germany) through the heart at a constant rate of 35 ml/min. The right ventricle was incised immediately after cannulation and pumping the buffer. The liver was then perfused, and effluent fluid was removed from the cannulated right ventricle. The liver was sampled by excised the apex of right lobe then transferred to a Teflon pestle glass homogenizer. The liver samples were homogenized with the ice-cold perfusate buffer. The homogenate was centrifuged at 1000 × g for 15 minutes at 4°C to remove cell debris and nuclei, then the supernatant was harvested for the subsequent analyses.

Measurements:

Cytochrome C Oxidase Activity Determination (CCOX):

The supernatants as a crude mitochondrial suspension, were subjected to determination of CCOX activity using spectrophotometric methods according to (Mills 1970). All steps of preparing the supernatant were in contact with crushed ice.

Antioxidant Enzymes Activities Determination:

All steps were conducted in contact with ice. The supernatant after determination of CCOX activity were then frozen in liquid nitrogen vapor and stored at –80°C until total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) activities analyses. The concentrations of T-AOC, SOD, and GSH-PX activities were analyzed using the commercial

assay kits from Bio-diagnostic company (diagnostic and research reagents) using spectrophotometric methods according to the instructions of the manufacturer.

Tissue Protein Determination:

The protein content in supernatant was measured by using method of Lowry *et al.*, (1951). The results of CCOX, GSH-PX, T-AOC, and SOD activities were expressed as units per milligram of protein (U/mg protein).

Productive performance:

Through the experiment, grow-out performance was evaluated by recording body weight, body weight gain, and feed intake. Body weights of the chicks were recorded individually at the beginning of the experiment (14-day-old chicks) and in weekly basis subsequently. Feed intake for each plot was weekly recorded and divided by the number of chicks to obtain the estimated bird feed intake.

Statistical Analysis:

Complete randomized block design with replicates was done to test the differences among treatments according to Winer (1971). Least squares means was used for means separation in analysis of variance. All statistical analyses were done by Proc GLM of SAS software (SAS, 2015).

Complete randomized block design with replicates model:

$$\gamma_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

γ_{ijk} = Individual observation, μ = Population means, α_i = Effect of block, β_j= Effect of treatments,

$(\alpha\beta)_{ij}$ interaction between block and treatment, ε_{ijk} = Experimental error, **k**=number of replicates.

RESULTS AND DISCUSSION

The Liver Cytochrome C Oxidase and Antioxidant Enzymes Activities:

Table (2) shows that no significant effects of SBC and AA treatments were found in liver cytochrome C oxidase, total antioxidant capacity, superoxide dismutase, and glutathione peroxidase enzymes. However, there are numerical differences among the treatments. The lack of significance may be due to the small sample size.

Figure (1) shows activities of SBC and AA in cytochrome C oxidase level in liver. Treatments with SBC insignificantly decreased hepatic CCOX activity as compared to the control. Meanwhile, activity of CCOX in AA treatment were insignificantly increased as compared to the control. The percent changes of SBC and AA were 16.00 and 26.13%, respectively.

The complex cytochrome C oxidase is necessary for practically all energy production in cells (Babcock and Wikström 1992). Cytochrome C oxidase in the final step in the electron transport chain, receives electrons from cytochrome C and irreversibly transforms oxygen to water. The enhanced activity of CCOX results in improved mitochondrial function, as measured by higher mitochondrial respiration, membrane potential, and ATP levels (Lee 2021). The total oxygen used during electron transport is not converted to water by CCOX in the event of electron leakage, but rather to superoxide in respiratory chain complexes I and III (Akbarian *et al.,* 2016).

In present study, the elevated CCOX activity in AA treatment, indicated a higher mitochondrial respiration with more electron leakages during chronic heat stress (Venditti *et al.,* 2014). However, Gomez-Cabrera *et al.,* (2008) concluded that cytochrome C concentration as a substrate of CCOX in rats treated with ascorbic acid, did not result any significant change in the concentration of cytochrome C, which may indicate unaffected CCOX activity. In addition, Paulsen *et al.,* (2014) found that supplementing with ascorbic acid for 4-11 weeks increases in CCOX and GSH-Px levels in humans. Meanwhile, the decrease in CCOX activity in SBC treatment, which does not act as an antioxidant, may indicate that there are a reduced electron leakages during chronic heat stress. Florence (1985) established that excessive H_2O_2 degrades cytochrome c *in vitro*.

Figure (2) shows activities of SBC and AA in liver total antioxidant capacity in (A), superoxide dismutase in (B), and glutathione peroxidase in (C). Figure (2, A) shows activities of total antioxidant capacity levels in liver. Treatments with SBC and AA insignificantly decreased hepatic T-AOC activity as compared to the control. The percent changes of SBC and AA were 36.42 and 6.80 %, respectively.

Figure (2, B) shows activities of superoxide dismutase levels in liver. Treatments with SBC and AA insignificantly decreased hepatic SOD activity as compared to the control. The percent changes of SBC and AA were 33.81

and 25.43 %, respectively. In addition, Figure (2, C) shows activities of glutathione peroxidase levels in liver. Treatment with SBC insignificantly decreased and increased in AA hepatic GSH-Px as compared to the control. The percent changes of SBC and AA were 37.91 and 36.56 %, respectively.

In the current study, the various oxidative stress variables were examined in broiler chickens, Crapo and Tierney (1973) established that enzymatic antioxidant defense systems, which include superoxide dismutase and glutathione peroxidase, are natural lipid peroxidation defenders. In a cellular antioxidant reaction, SOD first converts superoxide anion to hydrogen peroxide. Following that, GSH-Px detoxifies the hydrogen peroxide produced (Jaeschke 1995). In the scope of this information several earlier studies have demonstrated that SBC and AA can successfully lower various forms of oxidative stress in chickens by enhancing antioxidant enzyme activities to protect cell membranes from oxygen and free radicals formation (Zhang *et al.,* 2009, Srilatha *et al.,* 2010, Li *et al.,* 2014, Yang *et al.,* 2014, Sohaib *et al.,* 2018). Ascorbic acid breaks the cycle of lipid peroxidation in cell membranes, which reduces the biochemical signals of stress in the antioxidant defense system (Nimse and Pal 2015). Thus, ascorbic acid can neutralize ROS and reduce oxidative stress.

The present study shows that the decreases in T-AOC and SOD of mitochondrial content in hepatocytes in SBC and AA groups and increases in GSH-Px activity in AA group, suggest improved mitochondrial function and fewer electron leaks after prolonged heat stress at 32 ºC.

According to Yin *et al.,* (2018), SOD activity in ascorbic acid supplemented groups was significantly (P≤0.05) reduced whencompared with the control group, because ascorbic acid administration significantly enhanced exogenous antioxidants, which reduced the demand for endogenous antioxidants. Also, ascorbic acid supplementation reduced overall of T-AOC and SOD activities (Cumming *et al.,* 2014). In comparison to chickens treated with 0.5 % SBC, Peng *et al.,* (2013) found that a decrease in GSH-Px activity may deteriorate oxidative lesion in chickens treated with 2.0 and 4.0 % SBC. It seems that broilers' redox equilibrium would be disturbed by SBC supplementation at levels more than 1.0 % SBC. Improved mitochondrial performance may also lower hydrogen peroxide (H_2O_2) in the cytosol (Venditti *et al.,* 2014), contributing to the detected increases in GSH-Px activity in the AA group, suggest improved mitochondrial function with fewer electron leaks during prolonged heat stress.

Productive Performance:

Figure (3) shows that the main effects of SBC and AA at 3 and 4 weeks of age on body weight in (A), daily weight gain in (B), and daily feed intake in (C) were significant (P≤0.05). Meanwhile, the main effects of SBC and AA on BWT were significant (P≤0.05), where they were not significant for DWG and DFI at 5 weeks of age.

The final body weights were less than the (2.1 to 3.7 kg) target weight range for (Ross^{308}) at day 35, demonstrating the detrimental effects of heat stress. In the present study, birds treated with SBC and AA gave improve (P≤0.05) body weight (Fig. 3A) and daily weight gains (Fig. 3B). When body weight was analyzed on a weekly basis the data clearly indicated that at 21 and 28 days of age the lowest (P≤0.05) body weight and daily weight gains were in birds fed the control diet compared to birds fed on diet containing SBC or AA. On the other hand, birds treated with SBC at 35-day had better (P≤0.05) body weights than birds fed the control diet with no supplement. Meanwhile, no significant differences were observed in daily weight gains at 35 days of age among treatments.

The daily feed intake showed that all birds treated with SBC and AA had higher (P≤0.05) daily feed intake than birds in the control group (Fig. 3C). At 21 and 28 days of age, birds treated with SBC and AA compared to birds in control group showed a significant increase (P≤0.05) in daily feed intake. However, at 35 days of age, no significant differences were detected in daily feed intake among treatments.

The body weights of the chickens in the current study were lower than those predicted for 35-day-old chicks, showing the negative effects of the birds exposure to high ambient temperatures paired with high humidity (32±1°C and 55±5%RH) during the trial. The low performance of the birds, particularly those in control group, was consistent with previous observations (Balnave and Gorman 1993, Hayat *et al.,* 1999, Khattak *et al.,* 2012).

Siegel (1995), Celik and Ozturkcan (2003) and Celik and Ozturkcan (2003) showed that when the ambient temperature rise exceeded the thermoneutral zone (above 32 ºC), chicken growth performance was reduced. Indeed,

Wallis and Balnave (1984) and Zuprizal *et al.,* (1993) observed that high ambient temperatures impacted broilers by reducing protein and amino acid digestibility, which, when exposed to heat stress, may limit the activity of the protein-digesting enzymes trypsin, chymotrypsin, and amylase. In the present study, the final BWT and DWG were lower in the birds fed a ration devoid of any antioxidants than in the birds treated with SBC and AA.

Our results in harmony with many previous results, Roussan *et al.,* (2008) and Sahin *et al.,* (2003) reported that the broilers in the heat stress supplemented with ascorbic acid and SBC groups had significantly ($P<0.05$) better live BWT and gain than birds in the heat stress non-supplemented groups as a dietary supplement, sodium bicarbonate can aid to maintain acid-base and electrolyte balance while also alleviating respiratory alkalosis caused by heat exposure (Mujahid 2011).

Although chickens have the potential to synthesis ascorbic acid, it is insufficient when subjected to stresses such as high ambient temperatures, high humidity, and a high production rate (Whitehead and Keller 2003, Khan *et al.,* 2012). Environmental stresses, according to Pardue and Thaxton (1986), can alter ascorbic acid utilization or synthesis in chickens. Actually, heat stress has a detrimental influence on broiler chicken growth performance, and ascorbic acid included into the ration enhances male body weight gain, which is consistent with previous literature reports (Mahmoud *et al.,* 2004, Lin *et al.,* 2006, Attia *et al.,* 2009). Yoo *et al.,* (2016) proposed that antioxidants may improve digestibility by enhancing DWG and BWT, however no significant improvement in DFI was detected. According to their findings, it is required to supplement of SBC and AA in heat-stressed chicks.

CONCLUSION:

In conclusion, SBC and AA treated, partially restored the chronic heat stress induced impairment in hepatic tissue and growth performance. Our findings further suggest that AA may be more effective than SBC treatment in enhancing stress tolerance and antioxidative capacity in heat-stressed broilers. Furthermore, it appears that SBC and AA supplement employed in this study provide antioxidant protection by activating antioxidant enzymes and scavenging ROS. This impact may operate as a backup strategy for living cells when they are challenging with chronic heat stress, as is the case in the current investigation.

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Table 1: Ingredient and nutrient composition of rations on dry basis:

Ingredient (%)	Starter (1-21 days of age)	Grower (22-35 days of age)
Yellow corn	48.45	52.30
Soybean meal	37.00	31.70
Corn gluten meal	7.00	8.30
Sunflower oil	3.20	3.70
Monocalcium phosphate	1.60	1.40
Sodium chloride	0.30	0.30
Limestone	1.70	1.60
DL-methionine	0.20	0.18
Lysine	0.25	0.22
Vitamin-mineral premix[1]	0.30	0.30
Total (Kg)	100	100
Calculated chemical composition[2]		
Crude protein %	23.00	21.50
Metabolizable energy (Ka/kg feed)	3000	3100
Calcium %	0.96	0.87
Available phosphorus %	0.48	0.44
DL-methionine	0.56	0.51
Lysine	1.29	1.29
Methionine+ Cystine %	1.08	0.99

[1]Provided per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 4000 IU; vitamin E, 30 IU; vitamin K, 2.3 mg; thiamine, 2.2 mg; riboflavin, 8 mg; pantothenic acid, 24.3 mg; niacin, 65 mg; pyridoxine, 4 mg; folic acid, 1.2 mg; biotin, 0.25 mg; vitamin B12, 3 mg; choline, 600 mg; iron from ferrous sulfate, 60 mg; copper from copper sulfate, 7.5 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 110 mg; iodine from ethylene diamine dihydroidide, 1.8 mg; selenium from sodium selenite, 0.35.
[2]Calculated chemical composition values according (N.R.C., 1994).

Table 2: The effect of sodium bicarbonate (SBC) and ascorbic acid (AA) on hepatocytes cytochrome C oxidase, total antioxidant capacity, superoxide dismutase glutathione peroxidase of broiler chicks raised under chronic heat stress.

Variables	Dietary Treatments			
	CONT	SBC	AA	P[2]
Cytochrome C oxidase (U/min/mg protein)	0.3061 ±0.08	0.257 ±0.08	0.386 ±0.08	0.544
Total antioxidant capacity (mM/mg protein)	36.98 ±11.49	23.45 ±6.58	34.47 ±8.73	0.556
Superoxide dismutase (U/mg protein)	209.00 ±59.00	138.00 ±46.00	156.00 ±51.00	0.617
Glutathione peroxidase (U/mg protein)	1095.00 ±307.00	680.00 ±190.00	1496.00 ±400.00	0.270

[1]Least square means ± pooled Standard Error [2]Probabilities = (P≤0.05)

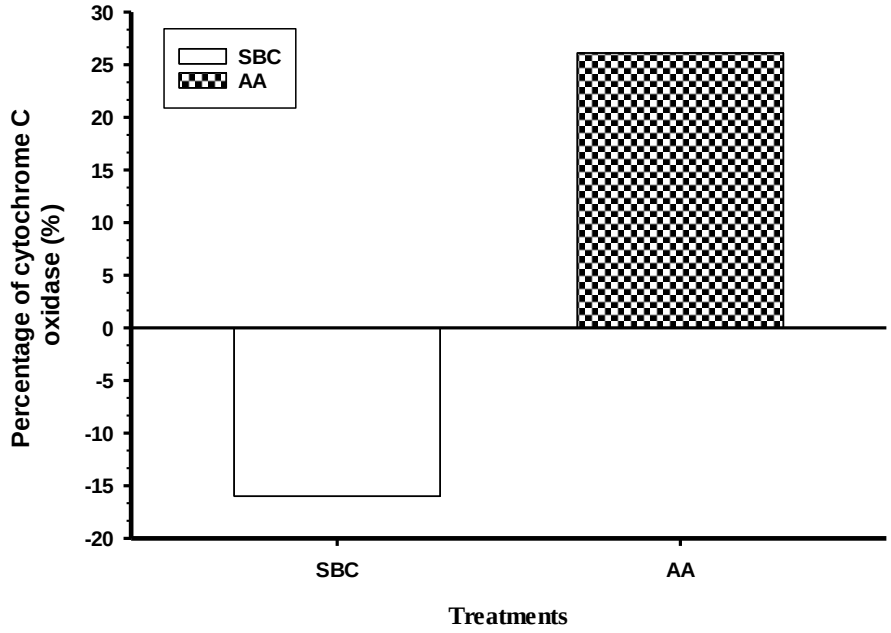


Figure 1: The effect of dietary sodium bicarbonate (SBC) and ascorbic acid (AA) on hepatocytes cytochrome C oxidase activity as a percent of the control group of broiler chicks raised under chronic heat stress at 5 weeks of age.

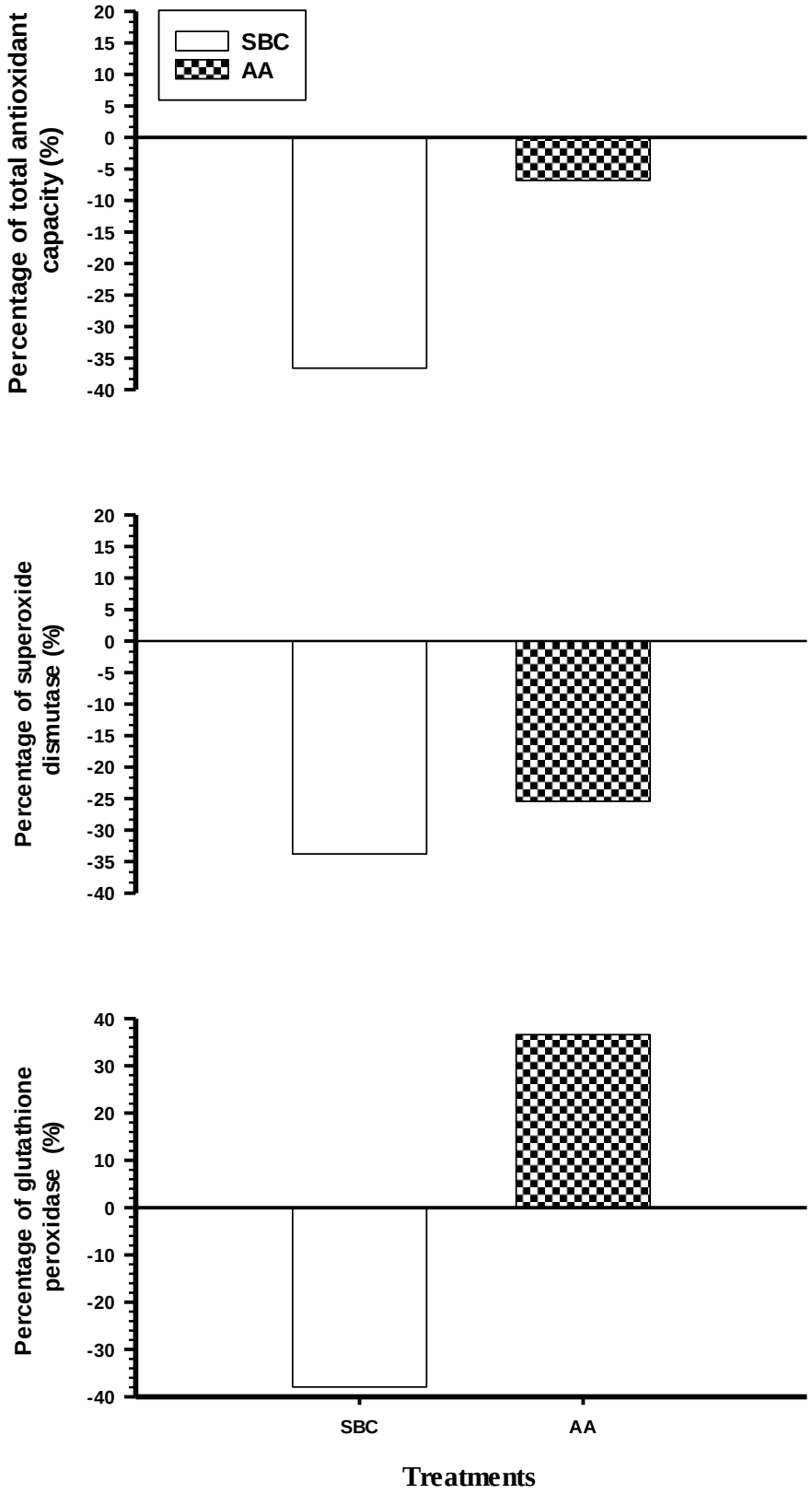
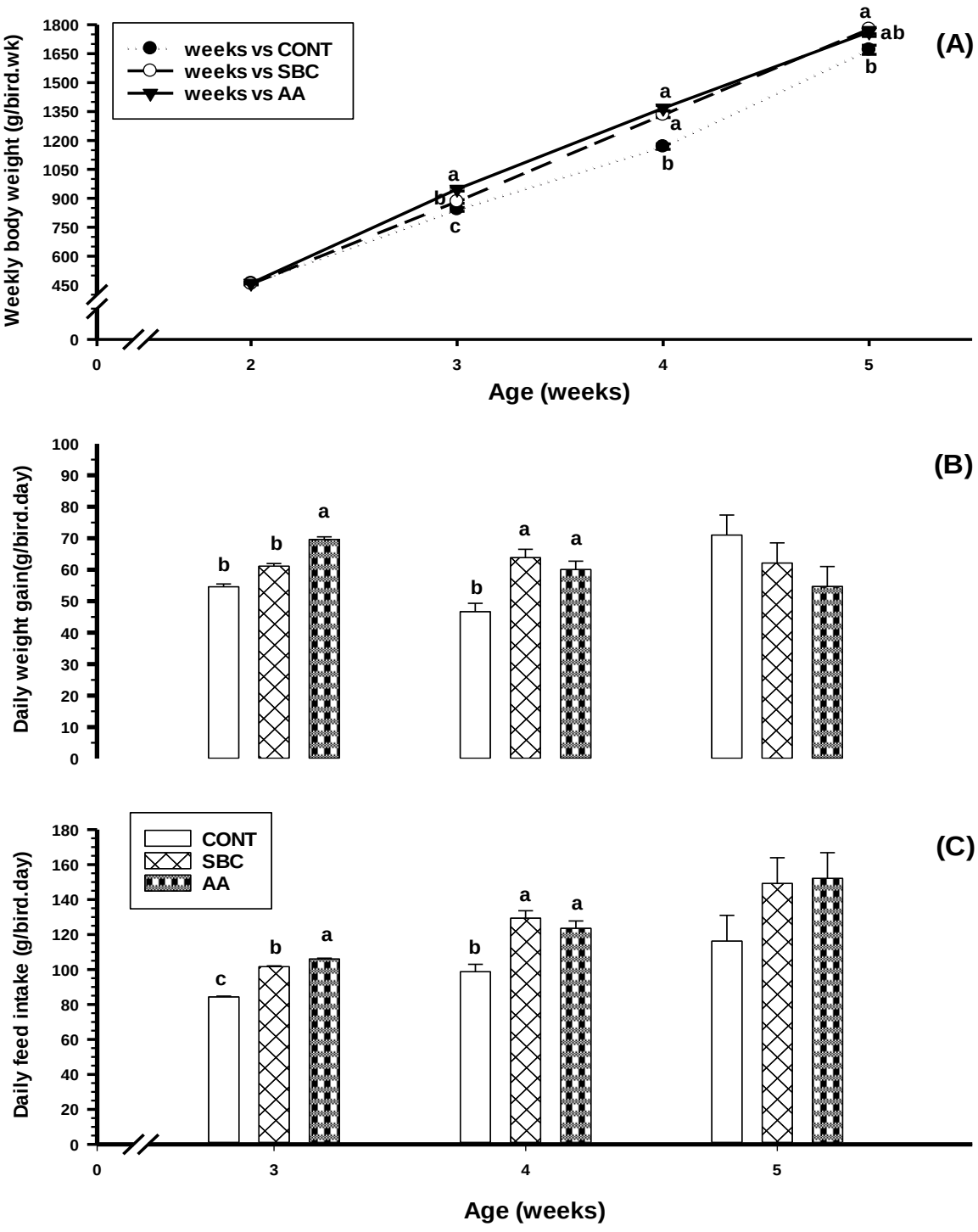


Figure 2: The effect of sodium bicarbonate (SBC) and ascorbic acid (AA) on hepatocytes total antioxidant capacity in (A), superoxide dismutase in (B), and glutathione peroxidase in (C) as percent of the control group of broiler chicks raised under chronic heat stress at 5 weeks of age.



[1]Least square means ± Standard error.
[a,b,c] Means having different letter exponents are significantly different (p ≤ 0.05) within age.

Figure 3: The effect of dietary sodium bicarbonate (SBC) and ascorbic acid (AA) on body weight (A), daily weight gain in (B) and daily feed intake in (C) of broiler chicks raised under chronic heat stress.

تأثير إضافة بيكربونات الصوديوم وحمض الأسكوربيك في العليقة على خصائص مضادات الأكسدة في خلايا الكبد فى كتاكيت التسمين المربى تحت الإجهاد الحراري المزمن

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الملخص العربى:

دجاج التسمين المربى تحت الإجهاد الحراري المزمن ينتج المزيد من الجذور الحرة التي تسبب الإجهاد التأكسدي. أجريت تجربة لدراسة بعض الإستراتيجيات للتخفيف من تأثير الإجهاد الحراري المزمن على كتاكيت التسمين. تم استخدام عدد 300 كتكوت تسمين من سلالة الروس308 عمر 14 يوم قسمت على ثلاث معاملات: المعاملة الأولي: الكنترول (CONT) تغذت فيها الطيور بدون أي إضافة، المعاملة الثانية: وفيها تم إضافة بيكربونات الصوديوم بمعدل 1جم/ كجم عليقة (SBC) والمعاملة الثالثة: تم إضافة حمض الإسكوربيك بمعدل 1جم / كجم عليقة (AA)، واستمرت التجربة لمدة 21 يومًا بدءًا من عمر 15 يومًا. تم الاحتفاظ بالطيور في جميع المعاملات تحت 32 ± 1 درجة مئوية خلال فترة التجربة. تم جمع عينات الكبد في عمر 5 أسابيع وحساب قياسات الأداء الإنتاجي على مدار 21 يومً. أظهرت النتائج انخفاض نشاط مضادات الأكسدة الكلية (T-AOC)، فوق أكسيد الديسموتيز (SOD) ونشاط السيتوكروم سي أوكسيديز (CCOX) بصورة غير معنوية في معاملة الـ SBC ومعاملة AA على التوالي، كما زاد نشاط انزيم الجلوتاثيون بيروكسيديز (GSH-PX) بصورة غير معنوية في الـ SBC وAA، على التوالي مقارنة بمعاملة الكنترول (CONT). أوضحت النتائج زيادة معنوية في وزن الجسم، ومقدار زيادة الوزن اليومي، واستهلاك العلف اليومي في عمر 3 و4 أسابيع لمعاملات الـ SBC وAA مقارنة بمعاملة الكنترول، بينما في عمر 5 أسابيع زاد بصورة معنوية كلا من وزن الجسم واستهلاك العلف اليومي فقط للطيور المعاملة بالـ SBC وAA. تشير هذه النتائج إلى أن SBC وAA قد يعززان من قدرة مضادات الأكسدة ولها تأثير مفيد على أداء دجاج التسمين تحت الإجهاد الحراري المزمن.

الكلمات الإسترشادية: دجاج التسمين، مضادات الأكسدة، الإجهاد الحراري، بيكربونات الصوديوم، حمض الإسكوربيك.