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Journal

Journal of Animal Physiology and Animal Nutrition, 101(5)

ISSN

0931-2439

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Publication Date

2017-10-01

DOI

10.1111/jpn.12591

Peer reviewed

Journal of Animal Physiology and Animal Nutrition

DOI: 10.1111/jpn.12591

ORIGINAL ARTICLE

Liver mitochondrial oxygen consumption and proton leak kinetics in broilers supplemented with dietary copper or zinc following coccidiosis challenge

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Summary

Mitochondrial respiration was assessed in sixteen 7-day-old broilers as a subset of a larger study assessing the effects of Cu and Zn supplementation above requirements with a coccidiosis challenge on gain/feed ratio. The birds were selected from four treatments (four birds/treatment): a control diet (Cu 15 mg/kg and Zn 60 mg/kg) + coccidiosis challenge (CC), a Cu diet with 245 mg/kg Cu from tribasic copper chloride (TBCC) + CC, a negative control diet (Cu 15 mg/kg and Zn 60 mg/kg) - CC and a Zn diet with 2000 mg/kg Zn from ZnO. The diets were composed of 49% corn, 40% soybean meal, 6.2% vegetable oil (diet dry matter = 90.62%, crude protein = 21.37%, fat = 7.7%, metabolizable energy = 12.1 MJ/day) and were fed for 14 days. Birds were dissected, and approximately one gram of liver tissue was used for mitochondrial oxygen consumption and proton leak kinetics assays. Respiratory control ratio and mitochondrial proton leak assessed by calculating rates of oxygen consumption at 175mV membrane potential were greater for the negative control group, but there were no differences in average gain/feed among treatments. In summary, broilers that did not undergo coccidiosis challenge had lower proton leak and higher respiratory control ratio. However, the impact of supplementation of Cu and Zn above requirements did not appear to prevent changes in respiratory control ratio and proton leak kinetics with coccidiosis challenge.

Keywords broilers, minerals, mitochondria, feed efficiency, proton leak

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Received: 4 September 2014; accepted: 26 July 2016

Introduction

Avian coccidiosis, an inflammation of the small intestine caused by protozoa of the genus Eimeria, is considered to be one of the most important diseases of domestic poultry with significant decreases in poultry performance (Stanley et al., 2004). Deficits in immunological functions have been observed in Zn-deficient poultry; therefore, avian coccidiosis may be prevented or treated by supplementing broilers with increased levels of the antioxidant minerals Cu and Zn. Increased Cu in plasma is associated with increased ceruloplasmin which can protect cells by increasing antioxidant defence. Chicks challenged with lipopolysaccharide have increased requirements for Cu (Koh et al., 1996). Zinc is essential to mount an immune response, and supplementation above requirements may increase antibody production (Bartlett and Smith, 2003). For both poultry and humans, Zn deprivation has been associated with decreased number of T lymphocytes, T-cell mitogen response and diminished T-helper and NK-cell cytotoxic function (Fraker et al., 1986; Hambidge, 1989). Cu and Zn also have antioxidant properties in their catalytic function in copper- zinc superoxide dismutase (Cu-Zn SOD) (Halliwell and Gutteridge, 1989). Cu-Zn SOD has been found in both cytosol and extracellular environments, and its activity decreases with diets low in Cu (Paynter et al., 1979; Klevay, 1990). The main role of Cu-Zn SOD is to catalyse the dismutation of superoxides into oxygen and hydrogen peroxide. Thus, supplemental Cu and Zn above recommended requirements may help protect liver cells and mitochondria from oxidative stress.

Mitochondria are the site for oxidative phosphorylation to convert energy from NADH and FADH to ATP. Inefficiency in this process due to protons leaking across the inner mitochondrial membrane may

account for approximately 20–30% of resting energy requirements (Brand and Divakaruni, 2011). Furthermore, when reactive oxygen species production exceeds antioxidant capacity, mitochondria are exposed to oxidative damage which may decrease their ability to synthesize ATP. Therefore, the objective of this study was to evaluate the supplementation of high levels of Cu or Zn on feed efficiency, mitochondrial oxygen consumption and proton leak of 21-day-old broilers challenged with coccidiosis to determine whether supplementation of Cu or Zn above requirements can overcome negative effects of coccidiosis on mitochondrial respiration.

Materials and methods

Broilers and management

Animals

The experiment and all procedures were approved by the University of California, Davis Animal Care and Use Committee.

Newly hatched broiler chicks were obtained from Ellenwood Hatchery (Cobb x Cobb, Foster Farms, Waterford, CA, USA) and were housed in one Petersime battery. At 3 days of age, broilers were individually weighed and assigned to treatments. Chicks received *ad libitum* access to diets and water.

Experimental design

Mitochondrial respiration measurements were performed on 16 broiler chicks at 21 days of age. The chicks were from a larger study examining effects of copper or zinc supplementation above requirements on gain/feed ratio in birds with coccidiosis challenge (CC) (Kurzbard, 2011). The larger study included 224 birds divided among seven treatments with 32 birds per treatment. Each treatment had eight pens with four birds per pen. Pens were randomly distributed across three Petersime batteries.

Chicks used for mitochondrial respiration measurements represented four of the treatments with two pens per treatment and two chicks per pen for a total of 16 chicks. Prior to mitochondrial respiration measurements, chicks were fed a basal diet that met all dietary broiler chick requirements (NRC, 1994). Treatments included two basal diet treatments (Table 1): a positive control with chicks infected at 8 days of age and a negative control with chicks not exposed to Eimeria. For the Cu and Zn diets, chicks had their infected basal diets supplemented with 245 mg/kg Cu from TBCC or 2000 mg/kg Zn from ZnO, respectively (Micronutrients, Indianapolis, IN). Broilers from Cu or Zn treatments were infected at 8 days of age. BW and FI were measured weekly. Feed efficiency was expressed as gain-to-feed ratio and was calculated from cumulative FI divided by final BW.

Table 1 Diet composition

Ingredients, as-fed basis	%
Corn	49
Soybean meal	42
Vegetable Oil	6.2
Ca Carbonate	2.0
Limestone	1.4
Salt	0.50
DL Methionine	0.36
Vitamin mix*	0.25
Mineral mix†	0.25
L-Lysine HCl	0.12
Choline chloride	0.080
L-Threonine	0.060
Ferrous Sulphate	0.050

*Ingredients supplied per kilogram of diet: vitamin A, 1500 IU, 6 mg; thiamine HCl, 1.8 mg; pyridoxine HCl, 3.0 mg; folic acid, 0.55 mg; biotin, 0.15 mg; vitamin D_3, 400 IU; vitamin E, 10 IU; vitamin K, 0.55 mg; riboflavin, 3.6 mg; pantothenic acid, 10 mg; niacin, 25 mg; vitamin B12, 0.01 mg; ethoxyquin, 125 mg;
†Cu, 6 mg; Zn, 36 mg; Mn, 60 mg; Se, .2 mg.

Infection

Two ml of Advent® coccidiosis control vaccine (Novus, St. Louis, MO, USA) was slowly mixed into 48 ml of phosphate-buffered saline (PBS) in a 50-ml conical tube. The tube was capped and then shaken vigorously for 30 s. Eight ml of the PBS + Advent® mixture was dispensed into six 10-ml syringes. Each syringe held 8–10x doses of Advent® to be distributed among the eight replications. One syringe was filled with 8 ml PBS for negative control birds. At 8 days of age, all groups were dosed with their respective syringes into the feed. To determine the amount of feed to use for infection, 24-h FI was measured from five randomly selected pens 1 day prior to infection. Individual treatment feeds were added to a professional mixer (Hobart, Troy, OH, USA) at levels 120% of average 24-h FI to account for daily increases in FI. Syringe contents of PBS and Advent® were slowly added to the feed mixer on low speed. Mixer speed was increased to high for 30 s. Feed was removed and added to designated feeders. The mixer was thoroughly cleaned before the next diet was mixed. To assure that broilers were indeed infected with coccidiosis, records of weight gain, oocysts content in excreta and intestinal histology samples were obtained.

Mitochondria assays

After 21 days of feeding, four birds per treatment were randomly selected from two pens, weighed and then killed by asphyxiation using CO_2. A gram of liver per bird was excised and used for mitochondria isolation using methods outlined by Cawthon et al. (1999, 2001).

Mitochondrial isolation

Liver tissues were minced in isolation media (220 mM mannitol, 70 mM sucrose, 20 mM Tris, 1 mM EDTA and 0.1% (w/v) BSA, pH 7.4 at 4 °C). The minced tissue was homogenized in a Potter-Elvehjem vessel with a Teflon pestle of 0.16 mm clearance maintained on ice. The homogenate was centrifuged at 1800 × *g* for 10 min, and the resulting supernatant was centrifuged at 8100 × *g* for 10 min to obtain the mitochondrial pellet. Fatty acid-free BSA was used in the isolation of mitochondria to sequester free fatty acids (that can induce or cause proton leak in the inner mitochondrial membrane). The pellet was resuspended and washed twice in 10 ml isolation solution with and without BSA at 8100 × *g* for 10 min each. The resulting mitochondrial pellet was suspended in 200 μl of isolation medium and placed on ice for oxygen consumption and proton leak kinetics assays as described below. Protein concentration was determined using the Bradford protein assay with BSA as the standard.

Measurement of mitochondrial oxygen consumption (nmol O/mg mitochondrial protein/min)

Mitochondrial oxygen consumption was measured using a Hansatech Clark-type oxygen electrode (Norfolk, UK; Eastbrook, 1967; Ramsey et al., 2004). Mitochondria (0.35 mg protein/ml final concentration) were incubated in 1 ml of oxygen consumption medium (120 mM KCl, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM Hepes and 1 mM EGTA) in a magnetically stirred incubation chamber maintained at 30 °C. Rotenone (5 μM) was used to block the electron transport chain at Complex I, and state 4 respiration (non-phosphorylating respiration) was determined in mitochondria following the addition of 5 mM succinate. State 3 respiration was measured in mitochondria incubated in the presence of 5 mM succinate and 100 μM ADP. RCR was determined by dividing state 3 by state 4 (Bottje et al., 2002).

Measurement of mitochondrial membrane potential (mV)

Δp was assessed using a $TPMP^+$-sensitive electrode (Ramsey et al., 2004). All measurements were completed in duplicate and simultaneous to determinations of mitochondrial oxygen consumption. Rotenone (5 μM) and oligomycin (8 μg/mg protein) were used to block electron transport chain at Complex I and ATP synthase. Nigericin (0.4 μg/ml) was added to convert the pH component of Δp to membrane potential units (mV), allowing Δp to be measured in mV units (Ramsey et al., 2004). Data from the two electrodes (oxygen and $TPMP^+$) were collected by data acquisition software (Hansatech Oxygraph System, Norfolk, UK) allowing real-time simultaneous measurements of mitochondrial oxygen consumption and Δp. MMP in millivolts was calculated based on the Nernst equation as:

MMP = 61.5 log ([TPMP] added−external [TPMP]) × TPMP binding correction/(0.001 × mg of protein/ml × [TPMP]), where the TPMP binding correction was 0.4 $(\mu l/mg \text{ of mitochondrial protein})^{-1}$ (Rolfe and Brown, 1997).

Mitochondrial proton leak kinetics

The kinetic responses of proton leak to MMP were determined by titrating the electron transport chain with malonate (0.1–2.5 mM), an inhibitor of succinate dehydrogenase, in the presence of oligomycin (8 μg/mg mitochondrial protein). Proton leak kinetics were visualized by plotting MMP against H^+ proton leak across the inner membrane. Proton leak was compared among treatments by calculating H^+ proton leak across inner membrane at a common membrane potential (175 mV).

Statistical analyses

All statistical analyses were performed using R Project for Statistical Computing (version 2.15.1) using Mixed model ANOVA with fixed effect of treatments and random effect of pen. Data are presented as least square means, and mean comparisons were performed using *t*-tests. For liver mitochondrial respiration, initial and final BW and feed-to-gain ratio were tested as covariates and if significant were included in following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$$

where Y_{ij} = nmols of oxygen consumed/mg mitochondrial protein min^{-1}, μ is the overall mean, α_i is treatment group (i = 1,2,3,4), β_j is the effect of pen (j = 1,2) and ε_{ij} are the residuals which follow a normal distribution N(0, σ^2). A probability level of $p \leq 0.05$ was considered statistically significant. For the analysis of proton leak kinetics, curves were transformed to be linear using the log function of Excel (Microsoft, 2007) and rates of oxygen consumption at

a membrane potential of 150 mV were compared for the four treatment groups using analysis of variance.

Results and discussion

Infected broilers had increased lymphocytes in intestinal histology samples and an average of 1541 oocysts/g of excreta compared to non-infected broilers (data not shown). From results of the larger study (Kurzbard, 2011), there were no differences in BW, FI and gain-to-feed ratio (Table 2. $p = 0.41$; $p = 0.85$; $p = 0.83$, respectively) with coccidiosis challenge or Cu supplementation. However, coccidiosis challenge did result in lower FI which increased to control levels with the Zn supplementation treatments. But BW gain and gain/feed were not different (Table 3. $p > 0.05$, $p = 0.90$ respectively). Broilers in the negative control and Cu diet groups had the greatest BW and FI for the overall trial period compared to other treatments, but they were not statistically significant. Proton leak (state 4 respiratory rate) was lower in negative control broilers and resulted in lower RCR rates for the negative control treatment (Table 4). No differences in mitochondrial state 3 respiratory rates of isolated liver mitochondria were observed among treatments. While supplementation of Cu did not alter whole animal response, state 3 respiration was lower, so the increase in proton leak relative to control may be a result of CC. There was no difference between Cu-supplemented and positive control RCR, but Cu supplementation may decrease oxygen consumption due to CC. The lowest average state 4 respiration values were observed in the negative control group (14.26 nmols of O min^{-1} mg protein $^{-1}$), and the highest average values were observed in birds that were infected regardless of Cu or Zn supplementation. There were also differences in RCR (state 3/state 4) that corresponded to the differences in state 4. Therefore, differences in RCR respiration were due to large differences in state 4 respiration. Unlike Zn supplementation increasing FI in CC broilers, Cu and Zn supplementation did not appear to overcome effects of CC with increasing proton leak. An increase in proton leak has previously been linked with increasing FI in beef steers (Acetoze et al., 2015), and Bottje et al. (2002) did not find a difference in mitochondrial oxygen consumption or RCR in livers from broilers with high and low feed efficiency. But in this study, there were no changes at the whole animal level which could be reflecting (1) that the infection was not of sufficient magnitude or duration to change feed efficiency, (2) although RCR decreased significantly with CC, numbers of broilers per treatment were not high enough to detect a response to Cu or Zn supplementation, (3) high levels of Cu supplementation may also interfere with Zn absorption and metabolism leading to a marginal Zn deficiency in the Cu-supplemented treatment and therefore no response by the mitochondria or (4) Cu or Zn may be used for other metabolic functions related to the immune response but not mitochondrial function for instance antioxidant defence by copper-bound ceruloplasmin and the sequestration of Fe and Zn functions in the citric acid cycle. Little is known about the effects of supplementing high levels of Cu or Zn on animal response, mitochondrial respiration or the ability of an animal to cope with an immune challenge. Therefore, explanations 1, 2 and 4 for the results in Tables 2–4 are difficult to cite conclusively. However, the number of birds enrolled in the mitochondrial respiration study may have been too small to conclusively state that Cu and Zn supplementation does not affect mitochondrial

Table 2 Performance of 21-days broilers with and without a coccidiosis challenge and Cu supplementation

	Negative control	Positive control	245 mg/kg Cu	SEM
Body weight gain, kg	1.17	1.21	1.22	0.024
DMI*, kg	1.42	1.44	1.45	0.031
Gain/Feed	0.822	0.845	0.844	0.025

*DMI: dry matter intake.

Table 3 Performance of 21-days broilers with and without a coccidiosis challenge and Zn supplementation

	Negative control	Positive control	2000 mg/kg Zn	SEM
Body weight gain, kg	1.13	1.02	1.13	0.033
DMI*, kg	1.33^{a}	1.17^{b}	1.30^{ab}	0.034
Gain/Feed	0.846	0.861	0.872	0.017

Rows not followed by the same superscript letter are significantly different ($p < 0.05$).

*DMI: dry matter intake.

Table 4 Least square mean liver mitochondrial respiration from 21-day broilers under a coccidiosis challenge that were fed Cu or Zn* (nmol O_2/mg mitochondrial protein/min)

$n = 16$	Negative control	Positive control	245 mg/kg Cu	2000 mg/kg Zn	SEM
State 3	27.3	28.1	22.6	27.1	2.5
State 4	7.10^{b}	10.9^{a}	10.0^{a}	10.5^{a}	0.91
RCR*	4.25^{b}	2.60^{a}	2.31^{a}	2.46^{a}	0.37

Means in a row not followed by the same superscript letter are significantly different ($p < 0.05$).

*RCR = respiratory control ratio (state 3/state 4).

respiration. Data presented in Tables 2 and 3 were a summary of many more birds. Mitochondrial respiration measurements must be performed on fresh tissue; therefore, the number of birds used was limited by the amount of time available to make the mitochondrial measurements. Previous studies have shown results using five to eight birds per treatment. But, variability between birds was extremely high for state 3 respiration (Table 4) so more birds per treatment may have increased the significance of RCR and state 3 respiration for Cu supplementation.

Mitochondrial proton leak accounts for approximately 20% of the total resting energy expenditure based on estimates of oxygen consumption in liver and muscle associated with proton leak (Ku et al., 1993; Rolfe and Brown, 1997). Therefore, it is an important contributor to net energy for maintenance. Mitochondrial proton leak is a process that allows dissipation of Δp which uncouples oxidative phosphorylation from ADP decreasing the efficiency of ATP synthesis (Ramsey et al., 2004). Values of membrane potential (mV) and H^+ proton leak across inner membrane (nmol/mg mitochondrial protein/min) were recorded simultaneously and plotted to create a kinetics curve (Fig. 1). There were differences ($p = 0.02$) in mitochondrial proton leak isolated from liver of infected broilers supplemented with Cu or Zn when comparing rates of H^+ proton leak across inner membrane to the same membrane potential (175 mV). Therefore, proton leak kinetics between the negative control group and all the other infected groups differed. Broilers from the negative control group had a greater increase in membrane potential for a given increase in respiration and had lower proton leak at higher oxygen consumption rates than the infected broilers. The same curve shows that maximum leak-dependent respiration (represented by the points on the *far right* of each curve) was different among treatment groups. Averages of H^+ proton leak across the inner membrane for a fixed mitochondrial membrane potential of 175 mV were 126.72, 79.08, 64.24 and 94.68 nmols mg mitochondrial protein^{-1} min^{-1} for the negative control, positive control and Cu or Zn diets respectively. Broilers that were in the negative control group had lower maximum leak-dependent respiration and higher membrane potential, which implies a lower proton leak. However, broilers that were immune-challenged regardless of Cu or Zn supplementation had the highest rates of oxygen consumption to maintain 175 mV of membrane potential.

The difference in proton leak kinetics between the negative control and immune-challenged groups could reflect differences in liver oxidative damage between groups. Both copper and zinc are part of the Cu-Zn SOD complex and play a role in managing oxidative stress by mitigating the detrimental effects of cytosolic ROS. ROS can cause mitochondrial membrane damage and lead to increased proton leak (Brand and Divakaruni, 2011).

The immune challenge compromised FI in the positive control broilers, and Zn supplementation increased FI almost back to original (but not significant) levels (Table 3). However, the immune challenge did not alter FI, gain or gain–to-feed ratio in CC broilers (Table 2). It is unknown why those CC broilers did not respond to the infection and performed as well or better than negative controls both with and without Cu supplementation. Therefore, Zn

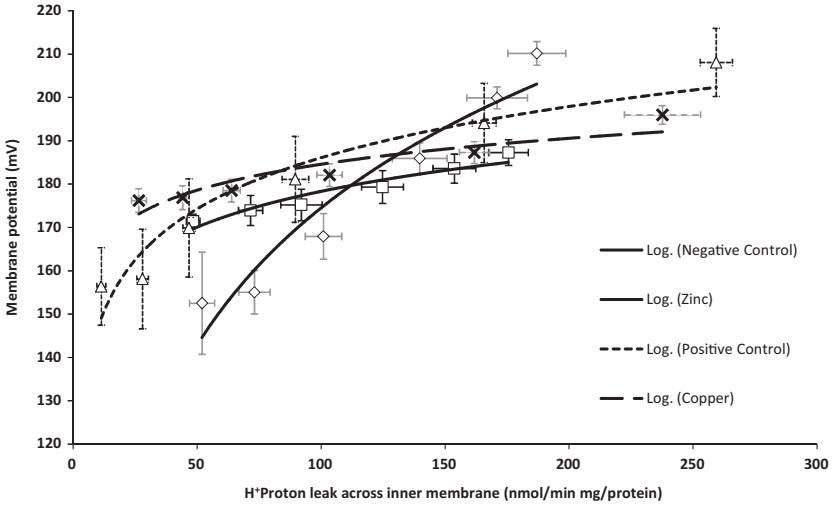

Fig. 1 Liver mitochondrial proton leak kinetics in broilers following coccidiosis challenge with or without supplemental Cu or Zn ($n = 16$).

supplementation above requirements could have value in a production environment to maintain FI and potentially maintain BW gain.

Conclusion

This study shows that a coccidiosis immune challenge altered mitochondrial proton leak kinetics and RCR in broilers. Supplementation of Zn above requirement levels did not appear to mitigate associated mitochondrial changes. It is possible that dietary supplementation with Zn for longer periods of time prior to infection or increased numbers of broilers per treatment may be required to observe liver mitochondrial changes in coccidiosis-infected broilers.

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