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PROTOZOOLOGY - ORIGINAL PAPER

Intestinal injury caused by *Eimeria* spp. impairs the phosphotransfer network and gain weight in experimentally infected chicken chicks

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Abstract

Parasitic infections caused by protozoan belonging to genus *Eimeria* are considered important for the poultry industry, due to their severe intestinal lesions and high mortality rates, causing significant economic losses. Although several mechanisms of coccidiosis pathogenesis are known, the effects of this infection on intestinal enzymes linked to adenosine triphosphate (ATP) metabolism, as creatine kinase (CK), adenylate kinase (AK), and pyruvate kinase (PK), remain unknown. Thus, the aim of this study was to evaluate whether coccidiosis impairs enzymes linked ATP metabolism in the intestine of chicken chicks. For this, 42 animals that were 2 days old were divided into two groups: uninfected (the negative control group) and experimentally infected on second day of life (the positive control group). On days 5, 10, and 15 post-infection (PI), fecal samples were collected for oocyst counts; intestinal tissue was collected in order to evaluate CK, AK, and PK activities, as well as parameters of the oxidative stress and histopathology. On days 10 and 15 PI, infected animals showed high counts of oocysts in fecal samples and intestinal lesions compared to the control group. Cytosolic CK activity was higher in infected animals on days 10 and 15 PI compared to the control group, while mitochondrial CK activity was lower on days 5, 10, and 15 PI. Also, AK activity was lower in infected animals on days 10 and 15 PI compared to control group, while no differences were observed between groups regarding PK activity. In relation to parameters of oxidative stress, intestinal lipid peroxidation and reactive oxygen species levels were higher in infected animals on days 10 and 15 PI compared to the control group, while non-protein thiol levels were lower on day 10 PI. On the 15th day, infected animals had lower body weight ($P < 0.05$). Based on this evidence, inhibition of mitochondrial CK activity causes an impairment of intestinal energetic homeostasis possibly through depletion on ATP levels, although the cytosolic CK activity acted as an attempt to restore the mitochondrial ATP levels through a feedback mechanism. Moreover, the impairment on energy metabolism appears to be mediated by excessive production of intestinal ROS, as well as oxidation of lipids and thiol groups.

Keywords Poultry farm · Coccidiosis · Oxidative stress · Energetic metabolism

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Introduction

Avian coccidiosis is a disease caused by protozoan parasites of the genus *Eimeria*, which is of great importance worldwide due to causing elevated mortality and morbidity rates, causing economic losses that exceed 3 billion dollars a year (Dalloul and Lillehoj 2006). This parasite affects the intestinal tract of chick, causing lower performance and poor feed conversion, as well as reduction on body weight gain (Ritzi et al. 2016). Alterations caused to the intestinal mucous of birds can be verified between 4 and 7 days post-infection (PI), i.e., in a period that parasites multiply in the enterocytes, eliciting modifications on the structure and morphology of the intestinal villi, as well as their destruction. The infection impairs the

turnover of intestinal crypts causing fluid loss, hemorrhage, and invasion of necrotizing bacteria in the mucosa, favoring the appearance of enteritis (Kitessa et al. 2014). The effects of a parasitic infection in the intestinal tissue and its effect on adenosine triphosphate (ATP) remain poorly understood. Thus, more studies are needed to understand the mechanisms involved on disease pathophysiology, such as the involvement of enzymes belonging to phosphotransfer network: creatine kinase (CK), adenylate kinase (AK), and pyruvate kinase (PK).

The phosphotransfer network is a pathway that exerts an essential role on cellular homeostasis by facilitating the equilibrium between sites of ATP generation and utilization, since an efficient use of cellular energy is required for suitable cell functions (Carrasco et al. 2001). CK is considered a central controller of energy metabolism by catalyzing reversible transfer of phosphoryl group from ATP to adenosine diphosphate (ADP) and creatine to produce phosphocreatine (PCr), acting as an energy source, buffer, and energy transporter, shuttling energy from subcellular sites (mitochondria) to sites of energy consumption (cytosol) (Schlattner et al. 2006). AK catalyzes the reversible transfer of the γ-phosphate group from a phosphate donor to adenosine monophosphate (AMP), releasing two molecules of ADP, which double the energetic potential of ATP and contribute to the efficient communication between cytosolic and mitochondrial ATP levels (Dzeja and Terzic 2003). Finally, PK is a key enzyme of the glycolytic pathway that catalyzes the irreversible transfer of phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP (Wang et al. 2002), the principal route of energy provision for suitable cellular and tissue functions. Recently, a study conducted by Freitas et al. (2008) demonstrated that *E. acervulina* inhibits ATP synthesis in experimentally infected broilers (*Gallus gallus domesticus*), but the pathways involved in this process remain unclear. Thus, our hypothesis is that the impairment of intestinal enzymes of the phosphotransfer network may be a pathway involved in the inhibition of ATP synthesis during coccidiosis.

Enzymes belonging to the phosphotransfer network are highly susceptible to lipid oxidative damage, as well as by impairment of the antioxidant system (Glaser et al. 2010). Also, a study conducted by Venkataraman et al. (2009) demonstrated that CK, PK, and AK enzymes might be one of the targets for reactive oxygen species (ROS), contributing to its inactivation. Oxidative stress is considered an imbalance between antioxidant/oxidant status, which occurrs when the production of free radicals is faster than they are scavenged by the antioxidant system (Xing et al. 2012), which contributes to lesions of biological macromolecules such as lipids and proteins, with consequent tissue damage (Winterbourne 2015), leading to the initiation and progression of infectious diseases. In this sense, a study conducted by Koinarski et al. (2005) demonstrated that *E. acervulina* causes lipid and protein peroxidation, as well as impairment of antioxidant enzymes (catalase and superoxide dismutase), contributing to disease pathogenesis. Thus, our hypothesis is that oxidative damage can contribute to alterations to the phosphotransfer network of chicken chicks with coccidiosis. Therefore, the aim of this study was to evaluate whether coccidiosis experimental *Eimeria* sp. infection may impair enzymes of the ATP metabolism in the intestines of chicken chicks.

Material and methods

Animals, housing, and feed

Forty-two chicks (1 day old; Cobb 500® lineage; 46 g body weight) were acquired in a commercial incubator in the city of Chapecó (Southern, Brazil). The animals were randomly allocated into two pens with wood shavings bedding in a completely randomized experimental design. Room temperature was maintained using incandescent bulbs. The same diet was provided for both groups, composed of corn and soybean meal providing all nutritional requirements for chicks (Rostagno et al. 2011). Water and feed were provided ad libitum.

Study design

The animals were divided into two groups of 21 chicks each, as follows: uninfected and orally infected (two drops = 30 μL) on the second day of life with 35,000 oocysts of *Eimeria* spp. (*E. mitis* = 10,000 oocysts, *E. acervulina* = 8000 oocysts, *E. praecox* = 8000 oocysts, *E. tenella* = 5000 oocysts, and *E. maxima* = 4000 oocysts). The inoculum (not attenuated) used in this experiment was donated by Biovet Laboratory (Vargem Grande Paulista, SP, Brazil).

Sampling

On days 5, 10, and 15 PI, seven animals from each group were anesthetized with halothane in a gas chamber followed by cervical dislocation. After euthanasia, fecal samples were collected from the cloaca for parasitological examination. Jejunum was collected and stored at − 20 °C in order to measure the activities of enzymes belonging to the phosphotransfer network and oxidative stress parameters. Also, intestinal fragments (duodenum, jejunum, and cecum) were collected for histopathological analyses.

Parasitological analyses

The centrifugal flotation technique was used to quantify the number of oocysts per gram of feces, as recommended by Monteiro (2010). The fecal sample (1 g) was dissolved in

15 mL of sucrose solution and centrifuged during 5 min at 2000 rpm. Fecal examination was performed using a light microscope (× 100).

Tissue preparation

The intestine was washed in SET buffer (0.32 M of sucrose, 1 mM of EGTA, 10 mM of Tris–HCl, pH 7.4) and homogenized (1:10 *w*/*v*) in the same SET buffer with a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 800×*g* for 10 min at 4 °C. Part of this supernatant was used to evaluate AK activity and parameters of oxidative stress; the pellet was discarded and the supernatant was once again centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant containing cell cytosol was collected for the determination of PK and CK activities. The pellet, containing mitochondria, was washed twice with the same SET buffer and then resuspended in 100 mM of Trizma and 15 mM of $MgSO_4$ buffer (pH 7.5) to evaluate mitochondrial CK activity.

CK, PK, and AK activities

CK activity was assayed in the reaction mixture containing the following final concentrations: 65 mM of Tris–HCl buffer, pH 7.5, 7 mM of PCr, 9 mM of $MgSO_4$, and 5 μg of protein in a final volume of 150 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP and stopped after 10 min by the addition of 1 μmol of ρ-hydroxymercuribenzoic acid. The creatine level was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL of 2% α-naphthol and 0.1 mL of 0.05% diacetyl in a final volume of 1 mL and read at 540 nm after 20 min. Results were expressed as nmol of creatine formed per min per mg of protein.

AK activity was measured with a coupled enzymatic assay using hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al. (1999). The reaction mixture contained 100 mM of KCl, 20 mM of HEPES, 20 mM of glucose, 4 mM of $MgCl_2$, 2 mM of $NADP^+$, 1 mM of EDTA, 4.5 U mL^{-1} of HK, 2 U mL^{-1} of G6PD, and 20 μL of the homogenate. The reaction was initiated by the addition of 2 mM ADP, and the reduction of $NADP^+$ was evaluated at 340 nm for 3 min in a spectrophotometer. ADP, $NADP^+$, G6PD, and HK were dissolved in Milli-Q water. The concentration of the reagents and the assay time (3 min) were used to assure linearity of the reaction. The results were expressed as μmol of ATP formed per min per mg of protein.

PK activity was assayed as described by Leong et al. (1981). The incubation medium consisted of 0.1 M of Tris/HCl buffer, pH 7.5, 10 mM of $MgCl_2$, 0.16 mM of NADH, 75 mM of KCl, 5.0 mM of ADP, 7 U L-lactate dehydrogenase, 0.1% (*v*/*v*) Triton X-100, and 20 μL of the mitochondria-free supernatant in a final volume of 500 μL. After 10 min of pre-incubation at 37 °C, the reaction was started with the addition of 1 mM PEP. All assays were performed in duplicates at 25 °C. Results were expressed as μmol of pyruvate formed per min per mg of protein.

ROS levels

Intestinal ROS levels were determined by the dichlorodihydro-fluorescein (DCFH) oxidation method described by LeBel et al. (1992), recently published in details by Biazus et al. (2017), using excitation and emission wavelengths of 485 and 538 nm, respectively, and results were expressed as units per DCF per mg of protein.

TBARS

As an index of lipid peroxidation, thiobarbituric acid reactive substance (TBARS) formation during an acid-heating reaction was determined as previously described by Ohkawa et al. (1979). Malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by the absorbance at 532 nm and were expressed as MDA equivalent (nmol MDA/mg of protein).

NPSH levels

Intestinal non-protein thiol (NPSH) levels were determined colorimetrically at 412 nm as previously described by Ellman (1959) and published in details by Baldissera et al. (2014). A cysteine solution was used as reference standard. Non-protein thiols were expressed as micromoles of SH per gram of tissue.

Protein determination

Protein content in the intestinal homogenate was determined by the method of Coomassie blue G dye (Read and Northcote 1981), using serum bovine albumin as the standard.

Histopathology

Intestinal samples (duodenum, jejunum, and cecum) were collected and conserved in 10% formaldehyde solution. Intestinal fragments (2 to 5 cm length and 0.5 to 1 cm thickness) were placed in paraffin blocks, stained by the hematoxylin–eosin (HE) method and analyzed under light microscope by two pathologists.

Statistical analysis

The data were submitted to normality test (Shapiro–Wilk test), and the data regarding the number of oocysts did not show normal distribution, and thus, a non-parametric test (Mann–

Whitney *U* test) was used to evaluate the difference between groups for $P < 0.05$. The other data set showed normal distribution, and a parametric test (Student's *t* test) was used to evaluate the difference between groups using $P < 0.05$. All results were presented as mean ± standard deviation.

Results

Parasitic burden, clinical signs, and histopathology

On day 5 PI, no excretion of *Eimeria* spp. oocyst was observed in both groups, while the presence of oocysts was observed on days 10 and 15 PI in the infected group (Fig. 1). No clinical signs were observed in both groups on days 5 and 10 PI, while apathy, feathers ruffled, and tremors were observed in infected group on day 15 PI. The body weight of the animals on day 15 experiment was 412 ± 10.7 g and 247 ± 13.8 g for control and infected, respectively.

No intestinal lesions were observed in both groups on day 5 PI and remained without lesions in the control group during all evaluated experimental periods. Chicks experimentally infected by *Eimeria* spp. showed mild to moderate (day 10 PI) and moderate to high (day 15 PI) quantities of degenerated oocysts in the small intestinal villi, as well as microgametes, macrogametes, and oocysts in high quantities (Fig. 2).

Enzymes of the phosphotransfer network

There were no differences between groups regarding intestinal cytosolic CK activity on day 5 PI; however, on days 10 and 15 PI, this activity was higher in infected animals compared to control animals. On the other hand, mitochondrial CK activity was lower in infected animals compared to the control group on days 5, 10, and 15 PI. AK activity was lower in infected animals compared to the control group on days 10 and 15 PI (Table 1). No significant difference was observed between groups regarding intestinal PK activity in all evaluated periods (Table 1).

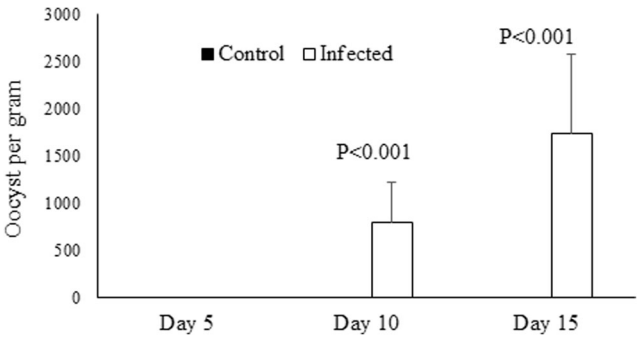

Fig. 1 Oocyst count per gram of feces on days 5, 10, and 15 post-infection (PI) of chicks experimentally infected by *Eimeria* spp.

Oxidative stress parameters

There were no significant differences between groups regarding intestinal ROS, TBARS, and NPSH levels on day 5 PI. On the other hand, intestinal ROS and TBARS levels were higher in infected animals compared to the control group on days 10 and 15 PI, while NPSH levels were lower on day 10 PI (Table 2).

Discussion

In this study, we observed, for the first time, that coccidiosis causes an impairment of the intestinal phosphotransfer network, eliciting an imbalance of bioenergetics between ATP production and ATP synthesis. Also, our data revealed that intestinal oxidative damage can be associated with impairment of enzymes belonging to the phosphotransfer network.

The suitable integration of energetic network ensures the energy homeostasis during stressor conditions, such as those caused by parasitic diseases (Baldissera et al. 2018a). Thus, the measurement of the phosphoryltransfer network provides new perspectives to understand alterations in bioenergetics caused by parasitic diseases. We observed that mitochondrial CK activity was inhibited during coccidiosis, indicating an impairment in the phosphocreatine (PCr) production as an attempt to restore ATP levels, similarly as observed by Baldissera et al. (2018b) in the spleen of silver catfish (*Rhamdia quelen*) naturally infected by *Ichthyophthirius multifiliis*. According to these authors, the inhibition of intestinal mitochondrial CK activity can impair the communication between sites of energy production and energy utilization, causing a severe impairment of energy supply in a tissue with high-energy demand, as the intestines. It is important to emphasize that the existence of microcompartments of CK isoenzymes (cytosolic and mitochondrial) is considered key to maintaining cellular energetic homeostasis (Alekssev et al. 2012). In this sense, we observed that intestinal cytosolic CK activity was stimulated on days 10 and 15 PI, which can be considered a situation known as energetic compensation, as observed by Baldissera et al. (2017a, b) in the kidneys of silver catfish experimentally infected by *Aeromonas caviae.* It is important to highlight that there exists a mutual compensatory relationship between CK isoenzymes to safeguard cellular energy economy, which contributes to efficient intracellular energetic communication to maintain the balance between cellular ATP consumption and production in an attempt to preserve the bioenergetics balance (Janssen et al. 2000). In this sense, the communication of compartment-specific isoenzymes of CK (cytosolic and mitochondrial) exerts multiple roles in cellular energy balance through two pathways: a first pathway is associated to the build-up of a global cellular

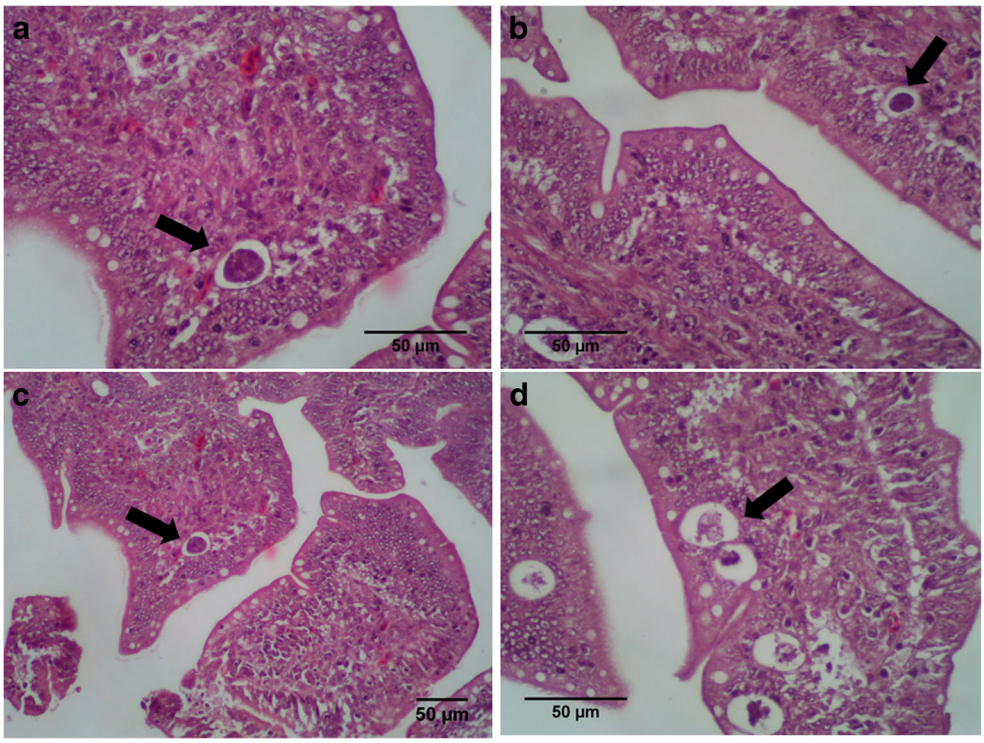

Fig. 2 Small intestine of chicks experimentally infected by *Eimeria* spp. showed degenerated oocysts (downward arrow) on villi to mild to moderate quantity (day 10 PI) (**a**–**c**) and moderate to severe quantity (day 15 PI) (**d**) (H&E)

energy buffer in the form of PCr that can be used to regenerate ATP during a temporal mismatch between ATP generation and ATP consumption, while the second pathway is linked to PCr formation and consequently facilitation of CK/PCr shuttle in order to correct a spatial mismatch between ATP generation and ATP consumption within a cell (Schlattner et al. 2006). Thus, coccidiosis inhibits mitochondrial CK activity, leading to an imbalance of energy homeostasis, which can be compensated by the stimulation of cytosolic CK activity.

In this work, intestinal AK activity was stimulated by coccidiosis, which can be considered an attempt to improve intestinal bioenergetics imbalance, as observed in the spleens of silver catfish naturally infected by *I. multifiliis* (Baldissera et al. 2018b). It is important to highlight that there is a compensatory relationship between AK and CK in order to prevent or reduce the impairment of cellular energy homeostasis and consequently depletion of ATP (Dzeja and Terzic 2009). In this sense, a reduction of intestinal mitochondrial CK activity may promote a high-energy phosphoryl transfer by the

Table 1 Intestinal cytosolic creatine kinase (CK-CYT), mitochondrial creatine kinase (CK-MIT), adenylate kinase (AK), and pyruvate kinase (PK) activities of chicks experimentally infected by *Eimeria* spp.

Variable	Day	Control	Infected	*P* value
CK-CYT (nmol of creatine formed/min/mg of protein)	5	1.20 (0.10)	1.37 (0.20)	0.145
	10	1.02 (0.11)	1.61 (0.29)	0.001**
	15	1.05 (0.07)	1.72 (0.24)	0.001**
CK-MIT (nmol of creatine formed/min/mg of protein)	5	1.39 (0.14)	1.06 (0.05)	0.001**
	10	1.49 (0.16)	1.08 (0.09)	0.001**
	15	1.39 (0.04)	1.07 (0.10)	0.001**
AK (pmol ATP formed/min/mg protein)	5	1.13 (0.07)	1.05 (0.05)	0.358
	10	1.25 (0.06)	0.90 (0.05)	0.001**
	15	1.25 (0.05)	0.97 (0.07)	0.024*
PK (μmol pyruvate formed/min/mg protein)	5	3.68 (0.61)	3.67 (0.41)	0.885
	10	3.87 (0.33)	3.75 (0.65)	0.805
	15	3.75 (0.30)	3.88 (0.47)	0.824

*$P < 0.05$; **$P < 0.001$ shows difference between groups

Table 2 Non-protein thiols (NPSH), thiobarbituric acid reactive substances (TBARS), and reactive oxygen species (ROS) levels of chicks experimentally infected by *Eimeria* spp.

Variable	Day	Control	Infected	*P* value
NPSH (μmol SH/g of tissue)	5	0.90 (0.17)	1.01 (0.15)	0.541
	10	0.88 (0.15)	0.39 (0.04)	0.001**
	15	0.81 (0.15)	0.61 (0.09)	0.124
TBARS (nmol MDA/mg of tissue)	5	22.6 (3.1)	21.6 (4.3)	0.824
	10	23.0 (3.7)	34.0 (4.4)	0.001**
	15	23.6 (4.0)	29.4 (3.0)	0.025*
ROS (U DCF/mg of protein)	5	255.9 (46.4)	299.9 (47.3)	0.175
	10	291.0 (35.5)	402.4 (31.4)	0.001**
	15	232.4 (17.3)	273.7 (26.3)	0.036*

*$P < 0.05$; **$P < 0.001$ shows difference between groups

AK system, since AK facilitates the transfer and utilization of gamma and beta-phosphoryls in ATP molecule, which doubles ATP energetic potential, contributing to the efficient communication between nucleus and cytosol ATP levels (Dzeja and Terzic 2003).

To identity a possible pathway involved in the inhibition of the phosphotransfer network, we evaluated parameters linked to oxidative damage, since CK, AK, and PK are susceptible to free radical (especially ROS) (Glaser et al. 2010). We observed an increase of intestinal ROS production with concomitantly lipid peroxidation after 10 and 15 days of infection, as observed in plasma of chicken chicks infected by *E. acervulina* 8 days PI (Koinarski et al. 2006). Excessive free radical production and damage to lipids may explain the impairment of the intestinal phosphotransfer network, as observed in this present study. Glaser et al. (2010) demonstrated that oxidative damage to lipids is associated with the inhibition of cerebral CK activity during exposure to methylmercury, in agreement with our findings. Also, we observed a reduction on thiol content in intestinal samples of infected animals on day 10 PI, which also contributes to the inhibition of intestinal mitochondrial CK activity. There should be corroboration since thiol group is present in the CK enzymatic structure, exerting a putative role in the protection against inactivation by ROS, developing an important role for suitable enzymatic CK activity (Koufen and Stark 2000). Thus, the intestinal excessive ROS production and lipid oxidative damage, as well as the decrease of the thiol content, can be a pathway involved on the impairment of phosphotransfer network.

Based on this evidence, inhibition of mitochondrial CK activity caused an impairment of the intestinal energetic homeostasis possibly through depletion of ATP levels, although the cytosolic CK activity acted as an attempt to restore the mitochondrial ATP levels through a feedback mechanism. Moreover, the impairment on energy metabolism appears to be mediated by the intestinal excessive production of ROS, as well oxidation of lipids and thiol groups. In summary, coccidiosis causes an impairment of the intestinal bioenergetics between ATP synthesis and utilization, besides damaging the weight gain.

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Compliance with ethical standards

Commission of ethics All procedures were approved by the Ethical and Animal Welfare Committee (CEUA) from the Universidade do Estado de Santa Catarina (UDESC), under protocol number 3096260917.

Conflict of interest The authors declare that there is no conflict of interest.

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