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Ectonucleotidases and adenosine deaminase activity in laying hens naturally infected by *Salmonella* Gallinarum and their effects on the pathogenesis of the disease

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ABSTRACT

Salmonella Gallinarum is the etiologic agent of fowl typhoid that affects chickens and turkeys causing egg production drops, infertility, lower hatchability, high mortality, and as a consequence severe economic losses to the poultry industry. The alterations in NTPDase and adenosine deaminase (ADA) activities have been demonstrated in several inflammatory conditions; however, there are no data in the literature associated with this infection. Thus, the aim of this study was to evaluate the activities of NTPDase, 5′nucleotidase, and ADA in serum and hepatic tissue of laying hens naturally infected by *Salmonella* Gallinarum. Liver and serum samples were collected of 27 laying hens (20 *S.* Gallinarum infected and 7 uninfected). NTPDase and 5′-nucleotidase activities in serum were increased ($P < 0.001$) in infected animals to hydrolysis of substrate ATP, ADP and AMP. In addition, it was observed decreased ($P < 0.001$) in ADA activity in serum of laying hens naturally infected by *S.* Gallinarum; as well as increased ($P < 0.001$) ADA activity in liver tissue of infected laying hens. Histopathological analyses revealed that *S.* Gallinarum caused fibrinoid necrosis in liver and spleen associated with infiltrates of heterophils, macrophages, lymphocytes, and plasma cells. Considering that NTPDase and ADA are involved in the cell-mediated immunity, this study suggests that activities of these enzymes could be important biomarkers to determine the severity of inflammatory and immune responses in salmonellosis, contributing to clarify the pathogenesis of the disease.

1. Introduction

Several bacterial pathogens are involved in poultry diseases. Economic losses triggered by these pathogens represent 10–20% of the gross value of production in poultry industries. Among them, *Salmonella* Gallinarum is an important problem for conventional poultry in developed countries, such as North America, and also poultry industries in underdeveloped countries [1]. *S.* Gallinarum is an invasive pathogen and cause systemic diseases of domestic poultry [2], and the clinical signs included anorexia, diarrhea and dehydration. As a consequence, decreased egg production, infertility, lower hatchability, and increased mortality are frequently observed [3], causing important economic losses.

Ectonucleotidases are capable of hydrolyzing ATP and ADP, such as NTPDase that hydrolyze ATP (adenosine triphosphate) into ADP (adenosine diphosphate) and AMP (adenosine monophosphate); degradation continues with the 5′-nucleotidase activity that hydrolyze AMP in adenosine. Complete ATP hydrolysis by NTPDase

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and 5′-nucleotidase may be relevant to general pathogenesis of diseases due to increase nucleotides concentrations [4]. Alterations in the ATP, ADP and AMP hydrolysis were observed in humans infected by bacterial meningitis, and the increase of these nucleotides may be an important protective mechanism in order to increase adenosine production [5]. Similarly, to what may occur in salmonellosis, as we point out that ATP is an important inflammatory mediator, unlike adenosine which has anti-inflammatory action. Adenosine deaminase (ADA) is an enzyme that catalyzes the conversion of adenosine to inosine, and closely regulates extracellular adenosine and inosine concentrations in mammals. This enzyme also acts as an endogenous regulator of the innate immune, playing an important role on T lymphocytes proliferation and differentiation [6]. Furthermore, adenosine regulates cell metabolism and triggers a variety of physiological effects in cell proliferation [7]. Changes in ADA activity in serum are observed in others important bacterial pathogens, such as *Mycobacterium tuberculosis* [8], as well as changes in the ADA activity and histopathological findings, which are essential for rapidly diagnosis.

Considering the importance of adenine nucleotides hydrolysis in the response against bacterial pathogens, the lack of participation and involvement of ectonucleotidases and ADA on chickens infected by salmonella, basic studies are needed for comprehension of the pathogenesis of the disease. The aim of this study was to evaluate the activities of NTPDase, 5′-nucleotidase and ADA in serum as well as ADA in hepatic tissue of laying hens naturally infected by *S*. Gallinarum.

2. Materials and methods

2.1. Laying hens

For this study, 20 liver samples from hens with 27 weeks of life were collected. These animals came from a farm known to be infected by *S*. Gallinarum according to the Official Sanitary Inspection Agency of Santa Catarina State (*CIDASC)* in Chapecó, Brazil. According to the National Plan for Poultry Health (Brazilian Agriculture Ministry) all hens from positive farms must be sacrificed. These animals were Isa Brown breed and showed clinical signs of the disease (ruffled feather and apathy). For comparison purposes, liver samples were collected from seven hens know to be negative for *S*. Gallinarum with the same breed and age of other property, but even producer. Importantly, that all laying hens included in this study received the same diet (commercial ration, 16% crude protein). Liver samples from all 27 laying hens (20 infected and 7 uninfected) were divided into three fragments: the right lobe was frozen for analysis of ADA, and the left lobe was used to histopathology. The present study was approved by the Ethics Committee for Use of Animals (CEUA) of Universidade do Estado de Santa Catarina, under number protocol 1.38.15.

2.1.1. Histopathology

At the necropsy, the left hepatic lobes and spleens were fixed in 10% buffered formalin, processed for routine histopathological analysis and stained by hematoxylin-eosin (H&E).

2.2. Collection and sample preparation

For sampling laying hens were manually contained. Whole blood (4 mL) was collected from the brachial vein using a 5.0 mL syringe and needle 25 × 7. These samples were stored into tubes without anticoagulant; serum was obtained after centrifugation at 3500 g for 10 min, and stored at −20 °C for the NTPDase, 5′-nucleotidase and ADA assays. A fragment of liver was removed, weighed and homogenized with sodium phosphate buffer 50 mM (pH 6.5). Each homogenate was centrifuged for 30 min at 14,000 g at 4 °C. The supernatant was then collected to analyze ADA activity. The serum and liver tissue were stored at 20 °C during 7 days. After this period, the analyzes were performed.

2.3. NTPDase and 5′-nucleotidase seric activity

NTPDase and 5′nucleotidase activity in serum were determined as previously described by Oses et al. [9]. The reaction mixture for the NTPDase activity containing 3 mM of ATP or ADP as substrate and 112.5 mM Tris–HCl (pH 8.0), already used for the activity of 5′-nucleotidase the reaction mixture containing 3 mM of AMP as substrate and 100 mM Tris–HCl (pH 7.5). The reaction mixtures were incubated with approximately 1.0 mg of homogenized protein at 37 °C for 40 min in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000× g for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method described by Chan et al. [10]. In order to correct non-enzymatic hydrolysis, we performed controls by adding the homogenized after the reaction was stopped with TCA. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein (nmol of Pi/min/mg protein).

2.4. ADA in serum and liver

ADA activity in serum and liver was measured according to Giusti and Gakis [11]. Reaction was initialized by the addition of substrate (adenosine). ADA activity in liver was quantified by the method of Giusti [12], which is based on the direct measurement of the formation of ammonia produced when the enzyme acts on adenosine. The volume of 25 μL of liver homogenate was used. The enzymatic reaction was started by addition of 500 μL of 21 mM adenosine as substrate. The reaction was stopped by adding 1.5 mL of 106/0.16 mM phenol–sodium nitroprusside to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline-hypochlorite (sodium hypochlorite). Released ammonia reacted with alkaline-hypochlorite and phenol in the presence of the catalyst-sodium nitroprusside to produce indophenol (a blue color). The concentration of ammonia was directly proportional to the absorbance of indophenol, read at 620 nm. Ammonium sulfate 75 μM was used as standard. All measures were carried out in triplicate and ADA activity in liver homogenates was expressed as U/mg of protein. Protein content in liver homogenates was determined by the method of Bradford [13] using bovine albumin serum as the standard.

2.5. Statistical analysis

After subjected to normality test (Shapiro), and found that the data have normal distribution, this were analyzed by the Student's *t*-test ($P < 0.05$), and summarized as means and standard deviations.

3. Results

3.1. Histopathological lesions

In 8/20 *S*. Gallinarum positive laying hens, it was observed mild (4/20), moderate (3/20) and severe (1/20) multifocal fibrinoid necrosis associated with moderate or severe infiltrates of macrophages, heterophils, lymphocytes and plasma cells (Fig. 1 A–B) in the liver. One bird showed severe diffuse fibrinoid perihepatitits