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NTPDase and 5′-nucleotidase as inflammatory markers in cattle naturally infected by *Eurytrema coelomaticum*

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ABSTRACT

The aim of this study was to evaluate seric NTPDase and 5′nucleotidase activities of cattle naturally infected by *Eurytrema coelomanticum*, as well as to correlate them to histopathological lesions in the pancreas and the degree of parasitism. Blood samples and pancreas of 51 bovines were collected on a slaughterhouse in Southern Brazil: 33 from cattle naturally infected by *E. coelomanticum* (the Group A), and 18 from uninfected animals (the Group B). Infected animals showed an average of 532 parasites per pancreas. In the pancreatic histology, ducts displayed hyperplasia, stenosis, proliferation of fibrous tissue, and interstitial inflammatory infiltration of lymphocytes. The serum from infected animals showed an increase in NTPDase activity when ATP was used as substrate ($P < 0.001$). For the ADP substrate, there was no difference between groups regarding NTPDase activity ($P = 0.37$), as well as 5′-nucleotidase activity ($P = 0.27$). Correlating NTPDase activity (ATP substrate) with the degree of histopathological lesions (rho = 0.66, $P < 0.001$) and the parasitic load on the pancreas (rho = 0.65, $P < 0.001$), a positive correlation was observed. Similar results were found between the degree of histopathological lesions and NTPDase activity (ADP substrate; rho = 0.29, $P = 0.03$), and 5′nucleotidase activity (rho = 0.35, $P = 0.01$). Based on the results of NTPDase and 5′nucleotidase enzymes in cattle naturally infected by *E. coleomanticum*, it is possible to suggest that these enzymes are involved in the modulation of inflammation, and they can act as markers of inflammatory response.

1. Introduction

Eurytrematosis is a disease caused by *Eurytrema coelomaticum*, a trematode that has cattle as definitive host [1,2]. This parasite is an incidental finding, usually a post-mortem finding at the slaughterhouse or during necropsies [2,3]. This parasite is usually located in pancreatic ducts but it can be found sporadically in the bile ducts and small intestine of parasitized individuals [1–3]. Infection of the definitive host occurs by ingestion of locust containing metacercariae of *E. coelomaticum*, which migrates from the small intestine to other organs of the host. This fact is related to the onset of chronic pancreatitis with severe interstitial and periductal fibrosis, compromising food digestion by inhibiting pancreatic secretion of enzymes [1–3]. Affected animals may display production losses, and emaciation, but in most cases they show asymptomatic infections, and generally the diagnosis is made only post-mortem [3]. However till date, the participation and influence of eurytrematosis on enzymes of the purinergic system involved in diverse physiological functions of mammals, is unknown.

Ectonucleotidases regulate the purinergic signaling, enzymes that hydrolyze nucleotides, and thus, regulate the concentration of these molecules in the extracellular space. NTPDase (EC 3.6.1.5) is responsible for the hydrolysis of the ATP into ADP and AMP con-

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secutively, while the 5′-nucleotidase (EC 3.1.3.5) hydrolyzes AMP to adenosine, an important endogenous anti-inflammatory molecule [4]. These nucleotides have participation in the immune response and inflammatory process, and they are object of extensive research [5,6].

Adenosine triphosphate (ATP) and diphosphate (ADP) have innumerous intracellular metabolic functions, while in the extracellular environment they act as signaling molecules that interact with purinergic receptors (P1 and P2) founded in the plasma membrane of leukocytes, platelets and other cells [7]. These nucleotides are in the micromoles range in the blood of healthy animals, but in pathological cases of cell lysis and tissue damage, these nucleotides have their concentration increased, and consecutively the interaction of these nucleotides with their receptors increase as well [8]. Possible functions performed by purinergic signaling are vasodilation, platelet aggregation, leukocyte activation, and immune modulation [7,9].

The ratio of adenine nucleotides and ectonucleotidases activity has an important role in modulating the inflammatory response against the etiological agent. In this context, the aim of study is to evaluate the seric activities of NTPDase and 5′nucleotidase in cattle naturally infected by *E. coelomaticum*, and to relate these results with histopathological findings and parasitic load on the pancreas.

2. Materials and methods

2.1. Animals and experimental design

For this study, 51 samples were collected from cattle slaughtered in an abattoir located in Concórdia, Santa Catarina, Southern Brazil in February 2015. Animals infected by *E. coelomaticum* (n = 33) formed the Group A and uninfected animals (n = 18) composed the Group B, according previous describe by Schwertz et al. [10]. It is noteworthy that the animals were apparently healthy.

From each animal, pancreas and blood samples were collected. Blood samples were collected after the desensitization of cattle during bleeding, stored in tubes without anticoagulant, centrifugated at 3500 rpm for 10 min, and stored at −20 °C to measure NTPDase and 5′-nucleotidase activities.

Each pancreas was divided into three fragments; two laterally and a fragment from the center of the right lobe to histopathology. The samples were fixed in buffered formalin 10% and routinely processed for hematoxylin and eosin technique (H&E). In addition, the number of parasites upon opening this organ was determined for each animal.

2.2. NTPDase and 5′-nucleotidase activities

NTPDase and 5′-nucleotidase seric activities were determined as previously described by Oses et al. [11]. The reaction mixture for the NTPDase activity contained 3 mM of ATP or ADP as substrate, and 112.5 mM Tris HCl (pH 8.0) and for 5′ nucleotidase 3 mM of AMP as substrate, and 100 mM Tris-HCl (pH 7.5) were used. The mixtures were incubated with approximately 1.0 mg of homogenized protein at 37 °C for 40 min on 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 released inorganic phosphate (Pi) was measured by the method of Chan et al. [12]. In order to correct non-enzymatic hydrolysis, control samples were used.

Protein concentration in sera samples was determined by the Coomassie blue method according to Bradford [13], using bovine serum albumin as the standard. Therefore, the results of enzymatic activities were expressed as nanomoles of Pi released per min per milligram of protein (nmol of Pi/min/mg protein).

2.3. Statistical analysis

The data of infected and uninfected cattle were first analyzed descriptively; measures of central tendency (median) and data dispersion (max and min) were computed for NTPDase (substrate ADP), NTPDase (substrate ATP), 5′-nucleotidadse (substrate AMP), and finally the number of parasites in pancreas. Further, all variables were submitted to Shapiro Wilk's W-test for normally distribution verification. Since most of the variables did not meet the assumption of parametric testing, a nonparametric test for two independence groups using the Mann-Whitney *U* test was performed. In addition, Spearman rank correlation was performed between NTPDase (ADP substrate); NTPDase (ATP substrate), 5′-nucleotidase with the number of parasites in the pancreas and histopathology results. It was considered statistically different when *P*-value < 0.05. The whole statistical process was carried out with R-language, v.2.15.2 (R Development Core Team, 2012).

3. Results

Each pancreas from the Group A had an average of 532 *E. coelomaticum* ranging between 12 and 2578 (Fig. 1). Histopathology revealed epithelial hyperplasia of pancreatic ducts, sometimes causing stenosis, fibrous connective tissue proliferation in the vicinity of ducts and interstitial inflammatory infiltrate, predominantly lymphocytic, as well as, parasites in the light of hyperplastic ducts of pancreas from infected animals (Fig. 1).

Comparison between groups (infected and uninfected) was performed by descriptive statistics and differences were accessed by a non-parametric test (Mann-Whitney U). A significant difference between groups for NTPDase activity was observed when the substrate ATP was used (Mann-Whitney 22.15, P < 0.001), i.e. occurred a seric increase in NTPDase activity in infected cattle (Fig. 2). For NTPDase activity (substrate ADP) no significant difference between groups were found (P = 0.37) (Fig. 2).

A positive correlation between NTPDase (substrate ATP) and the number of parasites was found (rho = 0.65, P < 0.001), as well as, with the degree of histopathological lesions in the pancreas (rho = 0.66, P < 0.001). There was no significant correlation between NTPDase activity (substrate ADP) with the number of parasites, however a significant correlation between this enzyme (substrate ADP) with the degree of histopathological lesions in the pancreas was observed (rho = 0.29, P = 0.03) (Fig. 3).

For 5′-nucleotidase activity when comparing groups, it was found no significant difference (P = 0.27). The correlation between 5′nucleotidase and the number of parasites in the pancreas was not significant, however a significant positive correlation with the degree of histopathological lesions in the pancreas was observed (rho = 0.35, P = 0.01; Fig. 4).

4. Discussion

Cattle are massively infected by *Eurytrema coelomanticum* in some parts of southern Brazil, and high-grade pancreatic lesions and poor body condition characterizes the severe cases [14]. The presence of the parasite in pancreatic ducts affects normal organ function, leading to loss of glandular tissue by fibrous tissue proliferation, as well as chronic pancreatitis seen in the histopathological analysis [2]. The infection generally remains asymptomatic, despite pancreas damage observed by histopathological analysis, which stimulate pancreatic cells to release a number of pro-inflammatory mediators in the blood stream [2,6].