Eurythematosis as a developmental model of the Diabetes Mellitus type 1 pathological condition: pathophysiological parameters and oxidative stress

Eurytrematose como modelo de desenvolvimento da patologia da Diabetes Mellitus tipo 1: parâmetros fisiopatológicos e stress oxidativo

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ABSTRACT
Eurytrematosis is a helminthic disease caused by trematodes belonging to the genus Eurytrema spp. that parasitize the pancreas of many animals and humans. This parasitosis causes chronic fibrosing pancreatitis, fat infiltration in the pancreatic parenchyma, besides damaging the exocrine pancreas, which is similar to that found in patients with Diabetes Mellitus type 1 (DM1). The current work aimed to evaluate the use of bovine pancreas infected with E. coelomaticum as a model to study DM1 pathophysiology. It was carried out macroscopic analyses, parasite identification, total pancreatic lipid determination and oxidative damage biomarkers levels of pancreas naturally infected with E. coelomaticum. Macroscopically, we observed that the infected pancreas had duct obstruction, organ stiffness due to the visible presence of fibrosis, increased adipose tissue deposition, increased protein and lipid damage, as well as increased antioxidant biomarkers (GSH, CAT and VIT C). Thus, it is possible to show that DM1 may have pancreatic parasitism as a possible primary origin. However, more studies are needed to better investigate this possible primary origin; the results obtained here suggest that the use of pancreas parasitized by E. coelomaticum could be a model to investigate DM1 pathophysiology.

Keywords: Eurytrema coelomaticum, E. pancreaticum, Oxidative stress, Lipid, Diabetes Mellitus

INTRODUCTION
Eurytrematosis is a globally known zoonotic disease caused by trematodes belonging to the genus Eurytrema spp (Bassani et al., 2007; 2006; Ilha et al., 2005; Ma et al., 2014; Ogawa et
al., 2019; Surian et al., 2022). The Eurytrema spp cycle requires two intermediate hosts: I) the snail of the genus Bradybaena and; II) the grasshopper of the genus Conocephalus (Bassani et al., 2007). Cattle, goats and sheep can become accidental hosts through the ingestion of contaminated grasshoppers present in the pasture (Ma et al., 2014; Mohanta et al., 2015; Rojo-Vazquez et al., 2012). Humans can also be infected by Eurytrema due to poor food hygiene habits and food culture, considering countries where insect consumption is common (Grist, 2008; Ishii et al., 1983; Tessele et al., 2013). Once infected by Eurytrema spp, humans and animals have their pancreatic and biliary ducts infected by this trematode (Li & Lu, 2011; Ma et al., 2014; Mohanta et al., 2015; Rojo-Vazquez et al., 2012; Schwertz et al., 2016), causing the clinical signs observed in infected subjects.

Considering human beings, the species E. pancreaticum can cause Eurytrema that is commonly associated to a clinical and pathological condition of chronic interstitial pancreatitis. Analyzing the necropsy of reported cases of human Eurytrematosis, dilation and fibrosis of the pancreatic duct were observed, besides fat infiltration in the parenchyma (Ishii et al., 1983; Takaoka et al., 1983). In animals, the species E. coelomaticum is the most found (Li & Lu, 2011; Ma et al., 2014; Mohanta et al., 2015; Rojo-Vazquez et al., 2012).

Due to the E. coelomaticum zoonotic aspects, it is suggested that clinical and pathological changes, as well as endocrine and exocrine pancreatic lesions are similar between humans and animals (Belém et al., 1994). Animals may show high plasma concentrations of amylase and glucose, in addition to ketonuria (Grosskopf et al. 2016; Harada et al., 1980; Headley et al., 2009; Ilha et al. 2005; Quevedo et al., 2013; Sakamoto et al., 1980). Besides having other clinical signs in common, we can cite: cachexy (Belém et al., 1986; Mattos et al., 1987) and glycosuria (Belém et al., 1986; Harada et al., 1980).

Organic changes of parasitic origin found in humans and animals suggest the development of some pathological conditions, such as Diabetes Mellitus Type 1 (DM1) (Gepts & Lecompte, 1981; Capen, 2001). DM1 is a chronic disease characterized by progressive, immune-mediated destruction of pancreatic β cells islets with consequent insufficient insulin secretion (Atkinson & Eisenbarth, 2001; Dantas et al., 2009; Sousa et al., 2016). However, the occurrence of pathological conditions such as DM1 originated from parasitic infection is based on the literature only in the description of clinical, biochemical and pathological conditions. Additional studies are needed to prove that parasitism can be one of this disease’s primary origin.

DM1 pathophysiology has been extensively studied through several models. Chemical induction of diabetes has already been performed in rats (Dordevic et al., 2017; Eleazu et al., 2017; Gite et al., 2017; Nazratun et al., 2017; Pipkin et al., 2017), mice (Chen et al., 2017),
Zebrafish (Leontovich et al., 2016), monkeys (Park et al., 2017) and pigs (Hiridis, 2016; Pepper, 2013).

Regarding the Eurytrematosis relevance in human health and particularly in animal health, the present study aims to evaluate the use of bovine pancreas naturally infected with E. coelomaticum as a model to study the DM1 physiopathogenesis.

2 MATERIAL AND METHODS

2.1 ETHICAL ASPECTS

The research was approved by the Ethics Committee on the Use of Animals of the Universidade Federal da Fronteira Sul (Protocol CEUA/UFFS: 23205.005061/2014-20).

2.2 EXPERIMENTAL DEVELOPMENT

The experiment was carried out by evaluating the pancreas of adult male Nellore (Bos indicus) cattle collected in a slaughterhouse located in the city of Capitão Leônidas Marques - Paraná, Brazil. After slaughter, the pancreas were removed and placed on inspection trays for parasitism macroscopic confirmation and then separated in two experimental groups: naturally infected with E. coelomaticum (n=12) and non-infected, for control purposes (n=3). Later, part of the pancreas fragments were sent, under refrigeration, to the Laboratório de Saúde Única da Universidade Federal da Fronteira Sul, Realeza-PR. The fragments were submitted to macroscopic evaluation, microscopic confirmation of the parasite species and total lipid dosage. Besides this, part of the pancreatic samples were sent to the Laboratory of Pharmacology and Toxicology (Farmatox) of the Federal University of Santa Maria (Santa Maria – RS) for oxidative stress markers biochemical determination.

2.3 TREMATODE IDENTIFICATION

The pancreas were macroscopically analyzed and images were collected to confirm the parasite contamination. The collected parasites were fixed in Railliet and Henry's solution (930 mL of distilled water, 6 g of sodium chloride, 50 mL of formalin and 20 mL of glacial acetic acid) (Fernandez et al., 2006). Subsequently, the trematodes were cleared with 80% acetic acid for the species’ microscopic confirmation. Images were recorded using a digital eyepiece, Dino-Lite brand coupled to an optical microscope.
2.4 TOTAL PANCREATIC LIPID DETERMINATION

The total pancreatic lipid determination was performed according to Bligh and Dyer (1959) method, which measures the tissue fat through the extraction performed with an organic solvent and measuring the difference between the weights of the degreased sample. A 0.5 g sample of pancreatic tissue was homogenized with the aid of a Sonifier (Hielscheer UP4005 40 MHz) with a mix of chloroform/methanol (10:1 – 1 g of tissue for each 10 mL). After this process, the tissue was transferred to a graduated flask and the volume (5 mL) measured with a chloroform/methanol mixture. The system was filtered and its volume measured.

This way, for every 5 mL of the filtrate, 1 mL of deionized water was added. Then, the system was subjected to agitation until obtaining a single phase. The resulting suspensions were centrifuged for 5 minutes at 1500 rpm, for phase separation. With the aid of a pasteur pipette, the organic phase (chloroform, where the lipids are found) was separated and transferred to a previously weighed petri dish. For total solvent evaporation, the plate was kept in an oven at 100 ºC for 1 hour, followed by cooling in a desiccator, and then, it was weighed again. The calculation of total lipids determination considers the extracted phase as corresponding to 60% of total volume.

2.5 TISSUE PREPARATION FOR BIOCHEMICAL ANALYSIS

After collecting the pancreas at the slaughterhouse, a tissue sample was taken from each pancreas to perform the biochemical assays of oxidative status. The tissue was prepared according to the following method: each sample was homogenized, in a 1:10 ratio (weight/volume), in TrisHCl buffer (10 mM and pH 7.4) and centrifuged at 3000 rpm for 10 min. The supernatant was collected and used for oxidative status biomarker analyses.

2.6 OXIDATIVE STRESS BIOMARKER ANALYSIS

The protein carbonyl (PC) levels were quantified by the method of Yan; Traber e Packer (1995), with some modifications. Soluble protein was incubated with 2,4-dinitrophenylhydrazine (DNPH; 10 mM in 2M HCl) or 2M HCl at room temperature for 1 hour. In sequence, denaturation buffer (150 mM sodium phosphate buffer pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99,8%) and hexane (99,5%) were added, mixed by shaking and centrifuged. Protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturation buffer. Each DNPH sample was quantified at 370 nm in a spectrophotometer against the corresponding HCl sample (blank). Results were expressed as nmol protein carbonyl/g tissue.
Lipid peroxidation was determined through the thiobarbituric acid reactive substances (TBARS) levels in the tissues, a method that consists of heating the biological material with thiobarbituric acid (TBA) in an acidic medium and measuring the formation of a pink colored product, spectrophotometrically (535 nm), according to Ohkawa; Ohishi e Yagi (1979). Results were expressed as nmol malondialdehyde (MDA)/g tissue.

Tissue reduced glutathione (GSH) levels were determined after the reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The yellow color formed was measured in a spectrophotometer at 412 nm, according to Boyne e Ellman (1972) with modifications (Jacques-Silva et al., 2001). A standard curve using cysteine was used to calculate the GSH content in the samples. Results were expressed as µmol GSH/g tissue.

Catalase activity (CAT) was spectrophotometrically quantified using the method of Aebi (1984), which involves monitoring the H₂O₂ disappearance in the presence of cell homogenate (pH 7 at 25 °C) at 240 nm for 120 s. Enzyme activity was expressed in mmol tissue H₂O₂/min/g tissue.

Vitamin C (VIT C) was estimated as described by Galley et al. (1996). This method consists of the production of an orange chromogen by reaction with dinitrophenylhydrazine at 37 °C. The product is spectrophotometrically measured at 520 nm. A standard curve using ascorbic acid was used to calculate the VIT C content and expressed in mg VIT C/g tissue.

2.7 STATISTICAL ANALYSIS

The results’ comparison of the analyses among control and infected groups was performed using Student's T test. Values of p < 0.05 were considered statistically significant for all comparisons. The results are expressed as mean ± standard deviation of the mean. The GraphPad® Prism version 7.01 program (GraphPad Software - San Diego, CA, USA) was used to analyze data and design the figures.

3 RESULTS

3.1 MACROSCOPIC ANALYSIS OF BOVINE PANCREAS

Macroscopic analysis of pancreas from control bovine and naturally infected pancreas is shown in Figure 1.

The bovine pancreas’ anatomopathology is shown in Fig. 1A. On macroscopic observation, the control pancreas from non-parasitized animals did not reveal any morphological changes (Fig. 1B-a). However, in pancreas of naturally infected animals by E. coelomaticum it was observed dilation and thickening of the pancreatic duct wall, duct lumen filled with
specimens of parasites, whitish areas caused by the parenchyma (fibrosis) (Fig. 1B-b) and a firmer organ. More detailed views of collagen deposition and ductal obstruction by the parasites were also observed (Fig. 1B-c-d).

3.2 E. COELOMATICUM PARASITES MORPHOLOGICAL AND BIOMETRIC CHARACTERIZATION

Morphological and biometric analyses indicated that the parasites have a typical trematode morphology, characterized by a foliaceous body with an oral and a ventral cup, an ovary, branched uterus and two testicles (Fig. 2A). The parasites’ eggs are elliptical in shape, naturally brown in color and have an operculum at one end (Fig. 2B).

3.3 TOTAL LIPIDS DETERMINATION IN BOVINE PANCREAS

The total lipids determination was performed in control pancreas and pancreas infected with E. coelomaticum. After applying the Student's T test, it was possible to observe that the parasitized pancreas had a higher fat percentage when compared to non-parasitized pancreas (p < 0.05), showing that infected animals have greater adipose tissue deposition in the pancreatic parenchyma (Fig. 3).

3.4 INFLUENCE OF PANCREATIC INFECTION CAUSED BY E. COELOMATICUM ON OXIDATIVE STRESS MARKERS

Statistical analysis by Student's T test indicated that the E. coelomaticum infection caused protein and lipid damage in the pancreatic tissue when compared, respectively, with PC and TBARS levels in the control group (p < 0.05). However, to compensate these damages, the antioxidant defenses of the infected group were increased, as GSH and VIT C levels, and CAT activity were higher than in the control group (p < 0.05) (Table 1).

4 DISCUSSION

Humans and animals can undergo similar biological changes from the same origin or caused by a different agent, such as those caused by parasites (Grist, 2008; Tessele et al., 2013). Regarding this, Eurytrematosis is a parasitosis caused by the infection of Eurytrema spp., considered endemic in several regions of Brazil, and it triggers the development of a pathological condition similar to DM1 (Azevedo et al. 2004; Bassani et al., 2006; Gepts & Lecompte, 1981).

Brazil ranks 5th in the world in terms of diabetes incidence with a significant number of 16,8 million people. Approximately 10% of this number represents those affected by DM1 (IDF,
DM1 is characterized by the destruction of β cells in the pancreatic islets, causing insulin secretion insufficiency. Insulin levels deficit causes hyperglycemia, which will cause problems in heart, kidney, neural problems, and etc. (Atkinson & Eisenbarth, 2001; Dantas et al., 2009; Sousa et al., 2016). Therefore, there is an urgent call for the study of DM1 pathophysiological mechanisms to mitigate its consequences.

This way, the suggestion of using bovine pancreas naturally infected with E. Coelomaticum to study the physiopathogenesis and oxidative stress biochemical patterns in Eurytrematosis in the development of a similar condition to DM1 becomes even more relevant. Our study observed: I) histopathological changes in the pancreas of the infected group, such as duct obstructions and fibrosis; II) the E. coelomaticum presence in the infected pancreas; III) an increase in lipid tissue percentage in infected pancreas and IV) increased protein and lipid damage, besides compensatory increase in antioxidant defenses, which were assessed by GSH, VIT C and CAT activity in the infected group.

Post-mortem inspection is usually performed through a macroscopic examination, which may favor a suspicion for the diagnosis of pathologies developed throughout life (Lima et al., 2007; Tessele et al., 2013). The macroscopic analysis of the pancreas in this study identified the presence of countless adult parasites in the infected group. The parasites were responsible for the pancreatic ducts obstruction, in addition to the increase in connective tissue deposition, factors that favor this tissue’s stiffening. The findings are in accordance with Hossa et al., (2013) and Graydon et al., (1992) where macroscopic analyses revealed, together with parasitic structures, firm multifocal areas and paleness and decreased organ size. Furthermore, the pathological changes found in the present study are similar to those found in humans with Eurytrematosis (Ishii et al., 1983; Takaoka et al., 1983).

Literature has already shown that, in humans, pancreatic ducts obstruction can lead to a high and progressive loss of pancreatic acinar cells and consequently to exocrine pancreatic insufficiency (Nousia-Arvanitakis, 1999). Other pancreatic conditions may be related to this pathological condition, such as pancreatic acinar atrophy; chronic pancreatitis; fibrosing interstitial pancreatitis; severe protein malnutrition; pancreatic hypoplasia and DM1 (Westermarck; Wiberg, 2012).

Multiple illnesses can have the same macroscopic features, which can increase the misdiagnosis probability occurring at the time of macroscopic inspection. Thus, complementary tests can be performed, aiming at confirming the presumptive diagnosis (Mendes et al., 2013). At first, the classification of trematodes occurred according to their host, erratic location in the organs or even the position of the parasite in the pancreas (Yamamura, 1989). Subsequently,
identification was carried out through phenotypic characteristics, morphology of organ structures and cytological analysis (Bassani et al. 2006; Bassani et al. 2007; Mohanta et al. 2015; Zheng et al. 2007).

The morphological and biometric characterization of E. coelomaticum parasites isolated from the pancreatic lesions of naturally infected bovine was performed in this study. The parasites collected showed a typical trematode morphology characterized by a foliaceous body with an oral and a ventral suction cup, an ovary, a branched uterus and two testicles. The parasites’ eggs had an elliptical shape, a naturally brown color and an operculum at one end. These observed characteristics are in accordance with what has already been presented and proposed in previous studies (Mohanta et al., 2015; Wiroreno et al., 1987; Yamamura, 1989).

When pancreatic ducts are obstructed, there is a tendency to replace the glandular tissue (pancreatic acinar cells) by fibrous and adipose tissue with varying patterns of change, such as partial or complete replacement of the pancreas by adipose tissue (Nousia-Arvanitakis, 1999). The total lipid tissue percentage analysis in the pancreas revealed that the infected group had a higher fat percentage when compared to the non-parasitized pancreas. This fact shows that infected animals have greater adipose tissue deposition in the pancreatic parenchyma. These changes that occur in Eurytrematosis can lead to an impairment of the pancreatic endocrine function (Belém et al., 1986; 1997).

Studies have already shown that the occurrence of chronic interstitial fibrosing pancreatitis in bovine pancreas parasitized by E. coelomaticum, with exuberant fibroplasia, promotes the replacement of extensive areas of the pancreatic parenchyma. In addition, the accumulation of parasite eggs and involvement of the endocrine pancreas have been observed with a reduction in numbers and dimensions of the islets of Langerhans (Oliveira & Bechera, 1988). Thus, this endoparasitosis can cause several disorders in the secretory functions of the pancreas and digestive and metabolic processes dependent on the pancreatic function can present changes such as decreased digestibility and, consequently, reduced food assimilation (Yamamura, 1989). It is known that the islets of Langerhans constitute the endocrine portion of the pancreas, and are formed by β and α cells, which produce, respectively, insulin and glucagon, hormones that play fundamental roles in regulating the metabolism of glucose, lipids and proteins (Westermarck & Wiberg, 2012). The destruction of β cells in the pancreas results in absolute insulin deficiency and, consequently, hyperglycemia, a DM1 characteristic condition (Lehninger et al., 2014).

Tissue damage, which can be measured through oxidative stress biomarkers, reflects the emergence of chronic diseases, as in the case of DM (Rocha et al., 2006). In this sense, studies
have already shown that the presence of free radicals can contribute to the destruction of pancreatic β cells (Elgawish et al., 1996; Hunt et al., 1990; Zyzak et al., 1995). Oxidative status biomarkers are present in all cellular tissues, such as pancreatic tissues, and through them, it is possible to measure levels and assess tissue oxidative damage (Liu et al., 2010; Yang & Lee, 2015).

The balance disruption between pro-oxidant phenomena and cellular antioxidant defenses is called oxidative stress. Once the production of reactive species or pro-oxidant phenomena overlaps the body's antioxidant capacity, there will be associated oxidative damage (Brum et al., 2020; Schimites et al., 2020).

In this context, the oxidative status assessment in the pancreatic tissue of the infected group evidenced the presence of protein and lipid damage (through an increase in PC and TBARS, respectively). Additionally, a compensatory increase in the enzymatic (CAT) and non-enzymatic (GSH and VIT C) antioxidant system was observed in the infected group compared to the control one. This oxidative stress evidence may be associated with several complications that induce cell membrane damage (Brum et al., 2020; Frey et al., 2006; Lazarotto et al., 2020). Based on our findings, we can consider that the lipoprotein damage observed in the group of infected pancreas may happen because of the destruction of pancreatic cells caused by endoparasitosis. Among pancreatic cells, β cells stand out, which are closely related to DM1 (Atkinson & Eisenbarth, 2001; Dantas et al., 2009; Sousa et al., 2016) and this pathological condition has already been related to physiological changes of parasitic origin (Gepts & Lecompte, 1981). The increase in antioxidant defenses observed in this study is triggered because of the body's natural balance to maintain the physiological homeostasis.

The physiological changes caused by changes in the pancreatic tissue of those affected by DM1 are similar to those found in pancreas parasitized by E. coelomaticum in humans and animals. We believe that this proposed model could become an important tool in the search for better treatments for the disease. The design of new studies using naturally infected bovine pancreas would enable new alternatives to reverse cell damage and/or improve the insulin synthesis mechanism in DM1 conditions.

5 CONCLUSION

The pathological similarity of pancreatic parasitism between animals (E. coelomaticum) and humans (E. pancreaticum) can be explored to clarify some diseases pathophysiology, such as DM1. The results of this study suggest that the pathophysiological and biochemical alterations observed in the analysis of the pancreas of bovine parasitized by E. coelomaticum were similar
to those found in DM1 cases. This similarity could enable the use of this model to study DM1 physiopathogenesis. Molecular studies related to the proposed model are of great importance and would reinforce the evidence presented here.

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