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Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with *Enteromyxum scophthalmi*

A.P. Losada [a], R. Bermúdez [b], L.D. Faílde [a], M.I. Quiroga [a,*]

[a] Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[b] Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain

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ABSTRACT

Enteromyxum scophthalmi is the causative agent of turbot enteromyxosis, an intestinal parasitisation that produces severe desquamative enteritis leading to a cachectic syndrome and eventually the death. It is well known the importance of the innate immune response against parasites in fish, with the release of antimicrobial substances such as reactive oxygen and nitrogen species, produced by the inducible nitric oxide synthase (iNOS). This enzyme is mainly found in phagocytes, but also in structural cells from the intestinal mucosa. The aim of this study was to characterize iNOS in intestine and lymphohaematopoietic organs (spleen and anterior kidney) of turbot by means of immunohistochemistry in order to assess the possible changes of this enzyme through the infection. The presence of the enzyme was evaluated in control and *E. scophthalmi*-infected turbot. The results showed immunoreactivity in the apical border of enterocytes and mild staining of goblet cells in both control and infected turbot although it was more evident and widespread in infected turbot compared to control. Moderate numbers of iNOS+ cells were present in the lamina propria-submucosa of fish which presented moderate and severe inflammatory infiltrates at this level. In spleen and kidney, iNOS+ cells were scattered through the parenchyma and, in severely infected fish, tended to be allocated near the vascular structures and melano-macrophage centres. The number of positive cells at the lymphohaematopoietic organs was significantly higher in infected turbot and increased as infection progressed. The increase in the expression of iNOS in the tissues of *E. scophthalmi*-infected turbot was more evident in individuals with severer lesions. The measurement of the levels of iNOS during turbot enteromyxosis reveals a possibly delayed response that would not able to eliminate the parasites but would exacerbate mucosal injury.

1. Introduction

Enteromyxosis is an important disease in turbot culture, with high prevalence and mortality rates [1,2] caused by *Enteromyxum scophthalmi*. This myxozoan parasite colonizes the intestinal epithelium of cultured turbot [3] and provokes epithelial damage, desquamation of the lining epithelium and mucosal inflammation that result in nutrient malabsorption, diarrhoea and eventually death after a cachectic syndrome [1,4]. The agent is responsible for heavy economic losses, due to the great number of turbot affected via cohabitation and contamination of marine water supplies [4] and nowadays there is not effective treatment against this parasitisation [5].

The innate immune response against parasites is of great importance in fish. Several humoral and cellular factors are activated in case of infection. Cytotoxic effector molecules, such as reactive nitrogen species, are mainly produced by activated phagocytes [6], and have been demonstrated in infections caused by other myxosporeans, as in *Enteromyxum leei*-infected sharp snout sea bream [7].

In mammals, during intestinal inflammation, synthesis of nitric oxide (NO) is consistently elevated in the intestinal mucosa as well as in the lamina propria-submucosa (LP) [8–10]. This molecule is produced by the enzyme NO synthase (NOS). There are three types of NOS: neuronal (nNOS) and endothelial (eNOS) Ca^{2+}-dependent, and inducible (iNOS) Ca^{2+}-independent, whose expression rises during inflammatory events and lasts longer than nNOS and eNOS activity [11,12]. This inducible form appears in almost all cell types,

Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IT, infected turbot; LP, lamina propria-submucosa; MMC(s), melano-macrophage centre(s); NIT, non-infected turbot; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PBS, phosphate saline buffer; PE, post exposure; RT, room temperature; SEM, standard error of the mean.

* Corresponding author. Tel.: +34 982 822 304; fax: +34 982 822 001.
E-mail address: misabel.quiroga@usc.es (M.I. Quiroga).

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and in large amount in phagocytes [13,14]. It is known that the expression of iNOS in granulocytes and macrophages rises during stressing and inflammatory events [15], participates in the macrophage respiratory burst [13,16], produces a cytotoxic environment and supports proinflammatory events [17]. The presence of iNOS in the gastrointestinal mucosa mainly occurs in the apical pole of the enterocytes [18] and its expression is significantly increased in mammals and teleosts during bacterial and protozoan infections, comprising an important tool against pathogens [9,19–22].

Several studies evaluated the innate immune response of fish by means of different techniques, most of them measuring gene expression and serum levels of different mediators [2,6,23–25], but none of them have used immunohistochemistry to characterize the distribution of iNOS and the localization of iNOS+ cells in turbot. For this reason, the main aim of our study is the detection of iNOS by means of immunohistochemistry, in order to establish the changes in the expression of this enzyme in cells of the immune system and mucosa throughout the response against *E. scophthalmi*.

2. Materials and methods

2.1. Fish

All experimental fish came from a farm allocated in North-western Spain. *E. scophthalmi*-infected turbot were employed as donor fish. A total of 160 uninfected turbot were checked for the absence of the parasite and 80 individuals used as control and the other 80 as recipient fish. Fish were kept in 500 L tanks and water temperature throughout the experiment was 14.3 ± 1.1 °C.

2.2. Infection

The experimental infection was performed by effluent transmission between stocks suffering serious mortality caused by *E. scophthalmi* and recipient fish in order to better recreate the natural infection. The uninfected recipient fish were placed in a tank which received the effluent water from a tank containing donor fish.

2.3. Sampling procedure

Five recipient and five control fish were sacrificed by chilling on ice and severing the spinal cord at days 20, 40, 56, and 78 post exposure (PE). At 107 days PE, only three recipient fish were sampled, since they were the only survivors. Samples of spleen, kidney and intestine (pyloric caeca, anterior, mid and hindgut) were taken.

2.4. Light microscopy

Tissues were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 3 μm thick were stained with haematoxylin–eosin and toluidine blue and examined to detect the presence of myxosporean stages and to evaluate the histological changes. Fish were considered infected when the parasite was detected on histological sections from any part of the intestine, the target organ. Infection intensity and the presence and severity of typical lesions of the disease (intestinal inflammation and desquamation, spleen and kidney leukocyte depletion) were semi-quantitatively classified from no infection or absence of lesions (0) to severe infection or lesions (3+), according to Bermúdez et al. [2]. Briefly, the slight degree consisted of low intensity parasitisation, absence of intestinal epithelial desquamation and absence or scarce inflammation; in the moderate degree the parasite burden was higher (from 30 to 80% of intestinal folds affected) and there was mild to moderate epithelial desquamation and moderate inflammatory response in the connective tissue of the mucosa; the severe degree was characterized by evident gross lesions (mainly cachexia, ascitis and intestinal haemorrhages), high amount of developmental stages of *E. scophthalmi* in the lining epithelium, even with the presence of scarce spores, severe epithelial desquamation and high number of inflammatory cells in the lamina propria-submucosa (LP). The entire histological damage represents the total sum of the histological lesions observed in the three studied organs for each fish.

2.5. Immunohistochemistry

Tissues fixed in Bouin's fluid and embedded in paraffin were cut to 3 μm thickness. The slides were routinely dewaxed and rehydrated. All incubations were performed at room temperature (RT) in a humid chamber and slides were washed with 10 mM phosphate-buffered saline (PBS) 0.5% Tween 20, pH 7.4, in three successive immersions of 5 min. Endogenous peroxidase was blocked by Peroxidase Blocking Solution (Dako, Denmark) for 1 h. No antigen retrieval was required. Primary rabbit polyclonal antibody NOS-2 (RB-1605, Thermo Fisher Scientific, UK) was incubated for 2 h. Immunohistochemical staining was performed using the horseradish peroxidase (HRP) anti-rabbit EnVision (Dako). Diaminobenzidine (Dako) was used as chromogen. Mammal tissues were used as positive controls. Negative controls were included by replacement of the primary antibody by PBS.

2.6. Quantitative analysis of iNOS+ cells

In order to later on analyze the quantity and distribution of iNOS+ cells in kidney, spleen and the sections of intestine, four groups of turbot were classified according to their health status: absence of lesions, slight, moderate and severe lesions. To count the mean number of immunostained cells, five digitalized images of each studied tissue were randomly taken at 200× magnification using an Olympus DP72 camera connected to a photomicroscope Olympus BX51. All the image parameters were set manually at the same levels to standardize the results. The iNOS-positive cells were counted using Image Pro-Plus 4 (Media Cybernetics Inc., Bethesda, MD).

2.7. Statistics

Data were analyzed using PASW Statistics 18 (SPSS Inc., Chicago, IL). Records of each organ were studied separately. Results were reported as the mean ± SEM, and they were obtained for each individual and each organ from the summation of counts of positive cells in the five images. A Kruskal–Wallis One Way Analysis of Variance on Ranks followed by paired comparisons was employed to seek differences amongst the groups. Results were considered significant at $P < 0.05$.

3. Results

3.1. Light microscopy and mortality

The histological study showed developmental stages of *E. scophthalmi* in the intestinal epithelium from the day 20 PE. The distribution of the parasitic forms started in the pyloric caeca and anterior gut, and spread to the rest of the digestive tract. As the disease progressed, a higher number of developmental stages of *E. scophthalmi* were found in the intestine and an increase in the severity of lesions was observed, with moderate epithelial desquamation and inflammatory infiltrates in the LP. The parasite