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Genes related to cell-mediated cytotoxicity and interferon response are induced in the retina of European sea bass upon intravitreal infection with nodavirus

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ABSTRACT

Viral diseases are responsible for high rates of mortality and subsequent economic losses in modern aquaculture. The nervous necrosis virus (NNV) produces viral encephalopathy and retinopathy (VER), which affects the central nervous system, is considered one of the most serious viral diseases in marine aquaculture. Although some studies have localized NNV in the retina cells, none has dealt with immunity in the retina. Thus, for the first time, we intravitreally infected healthy specimens of European sea bass (*Dicentrarchus labrax*) with NNV with the aim of characterizing the immune response in the retina. Ultrastructural analysis detected important retinal injuries and structure degradation, including pycnosis, hydropic degeneration and vacuolization in some cell layers as well as myelin sheaths in the optic nerve fibres. Immunohistochemistry demonstrated that NNV replicated in the eyes. Regarding retinal immunity, NNV infection elicited the transcription of genes encoding proteins involved in the interferon (IFN) and cell-mediated cytotoxicity (CMC) responses as well as B and T cell markers, demonstrating that viral replication influences innate and adaptive responses. Further studies are needed to understand the retina immunity and whether the main retinal function, vision, is affected by nodavirus.

1. Introduction

Nodavirus (NNV) has become one of the most devastating marine fish viruses worldwide, and it represents a serious economic threat to aquaculture [1], affecting both marine and freshwater fish species [1–3]. The virus causes viral encephalopathy and retinopathy (VER), which is characterized by symptoms of neurological damage and high mortality rates of up to 100%, especially in larvae and juvenile European sea bass (*Dicentrarchus labrax*), one of most susceptible species to the virus [4,5], which is extremely important in Mediterranean aquaculture. NNV is a bipartite, naked, icosahedral virus of 25–30 nm, composed of 2 positive single-stranded RNA fragments, RNA1 and RNA2, which are capped but not polyadenylated. The capsid protein (CP) is encoded by the RNA2 and is involved in host specificity whilst RNA1 codes for the non-structural protein RNA-dependent RNA polymerase (RdRp) [6,7]. In addition, a sub-genomic RNA1 transcript, RNA3, encodes the protein B2 which seems to be pivotal for virus accumulation since it antagonizes hosts siRNA and is only expressed in newly infected fish cells [8–10].

NNV infects cells from the brain, spinal cord and retina, causing extensive tissue degradation and altering their functioning [1]. The first evidence of retina damage was described several decades ago, when extensive vacuolation, especially in cells from the bipolar and ganglionic layers, was observed in a variety of fish species [11–14]. This injury to the retina structure is associated with the presence of NNV in the tissue and represents one of the key histopathological features of VER disease. For example, viral particles have been detected in the nuclear and ganglion cell layers and adjacent to the circumferential

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germinal zone at the ciliary margin close to the iris of Atlantic halibut (*Hippoglossus hippoglossus*) [15,16]. As in other fish species, retina cell damage in European sea bass was described in most layers from the outer limiting membrane to the ganglion layer, with viral particles observed in the nuclear region concomitantly with cell damage [17–19]. As an immune privileged site, the retina's regeneration process after impairment is difficult [20] and so any immune response after infection or injury might be regulated in a particular manner. Despite this, several studies have focused on the retinal detection of NNV and, although severe brain cell damage results in massive mortalities [1], no studies have been carried out to analyse the loss of eye function including the immune response.

This study is the first to address NNV colonization in the retina and the immune responses developed by the tissue, in order to elucidate whether the damage and immune responses might impair fish vision. Intravitreal infection of the right eye of juveniles of European sea bass triggered the massive infection in both retinas at 6 days. Cell damage was extensive in the retinal layers, optical centre and the medullary region of the retina enhancing the expression of genes related with interferon (IFN) and cell-mediated cytotoxicity (CMC) responses, as well as B and T lymphocytes probably leading to retina disfunction. However, further studies are needed to clarify whether vision is affected.

2. Materials and methods

2.1. Animals

Healthy juvenile specimens of European sea bass (*Dicentrarchus labrax* L.) were bred and kept at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia). The fish were kept in 14 m^3 tanks with the natural sea water temperature, a flow-through circuit, a suitable system for aeration and filtration and a natural photoperiod. Fish were fed daily with 1% of their biomass with commercial pellets (Skretting). The environmental parameters and food intake were recorded daily. Fish with a mean body weight (bw) of 12.3 ± 1.4 g were transported to the University of Murcia (Spain) aquaria to perform *in vivo* infections (see below). Fish were randomly divided into two tanks, kept in 450–500 l running seawater (28‰ salinity) aquaria at 25 °C with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the onset of the experiments. Before sampling, all specimens were anesthetized with 40 μl/l of clove oil, completely bled and immediately decapitated and weighed. The experiments described comply with the Guidelines of the European Union Council (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments of the IEO (Permit Number: 2010/02) and of the University of Murcia (Permit Number: A13150104).

2.2. Nodavirus stock

NNV (strain It/411/96, genotype RGNNV) was propagated in the SSN-1 cells [21]. The SSN-1 cells were grown at 25 °C in Leibovitz's L15-medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 50 μg/ml gentamicin (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25 °C until the cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following the methodology described previously [22], before being used in the experiments.

2.3. Intravitreal infection

Sedated fish with 20 μl/l of clove oil were infected by a single injection of 2 μl of SSN-1 culture medium (mock-infected) or with culture medium containing a total dose of 10^4 $TCID_{50}$/fish of NNV in the right eye vitreous body, leaving the left eye free of injection. Fish (n = 6 fish/group and time) were sampled at 1, 4, 24, 72 (3 days) and 144 (6 days) h after viral injection. Retinas were individually sampled and immediately frozen in TRIzol Reagent (Life Technologies) for later RNA isolation or placed into fixative solutions for microscopic examination.

2.4. Immunohistochemistry

Six days (144 h) post-injection, retinas (n = 4 fish/group) from both eyes were fixed in Bouin's solution for 16 h at 4 °C, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 3 μm. After dewaxing and rehydration, the sections were subjected to an indirect immmunohistochemical (IHC) process using two antibodies specific to: (i) the NNV capsid protein (anti-CP, Ø233 antibody) or (ii) the NNV B2 protein (anti-B2, Ø6073 antibody) at the optimal dilution of 1:500, as previously described [10]. In brief, the sections were incubated at 60 °C for 30 min, dewaxed in xylene, rehydrated in a series of ethanol baths and washed in running water. Prior to staining the tissue sections were autoclaved for 15 min in 0.01 mM citric acid (pH 6.0) for antigen retrieval. To prevent non-specific antibody binding, sections were blocked by using 5% bovine serum albumin (BSA; Sigma) in Tris buffered saline (TBS, 0.05 M, pH 7.6; Merck) for 20 min. The primary anti-B2 (Ø6073 antibody) or anti-CP (Ø233 antibody) sera were diluted in TBS containing 2.5% BSA and incubated for 30 min at 37 °C, and then washed for 5 min with TBS. The Vectostain® universal ABC-AP kit (Vector Laboratories), which provides both the secondary antibody (biotinylated anti-mouse/rabbit immunoglobulin) and avidin-biotin alkaline phosphatase (ABC-AP) was used. After TBS washes, the sections were incubated for 5 min with DAKO Fuchsin Substrate- and Cromogen system (Dako), followed by washing in running tap water before counterstaining with Shandon's haematoxylin and mounting in aqueous mounting medium (Aquatex, BDH laboratory). The specificity of the reaction was determined by using sections of tissue from control fish and by omitting the primary antibody on sections of tissue from infected fish.

2.5. Transmission electron microscopy

For transmission electron microscopy (TEM), the right eye retina was removed from the specimens and immersed in the fixative (1% paraformaldehyde, 1.26% glutaraldehyde and 0.02% $CaCl_2$ in 0.1 M phosphate buffer, pH 7.4) at 4 °C overnight. After fixation, the retinas were post-fixed with 2% OsO_4 for 1 h, dehydrated in ethanol, and embedded in Epon-812 (Polysciences) for TEM study. Semithin sections from each retina were observed and photographed with an Optic Microscope Leica DMRB. Thin sections of 70 nm were contrasted with uranyl acetate and lead citrate and observed in a Zeiss EM10C/CR electron microscope.

2.6. Gene expression by real-time qPCR

For the qPCR study, we only used the left eye retina in order to determine the effect of NNV and replication and avoid the local inflammation or leucocyte infiltration produced by the injection procedure in the right eye.

Total RNA was independently extracted from the left eye retina (n = 6 independent fish/group and time) with TRIzol Reagent (Life Technologies) following the manufacturer's instructions, and quantified with a spectrophotometer (Cecil Instruments Ltd). Isolated RNA was DNase I treated (amplification grade, 1 unit/μg RNA, Life Technologies) and the SuperScript III RNase H^- Reverse Transcriptase (Life Technologies) was used to synthesize the first strand cDNA with 1 μl of random hexamers (0.25 μg/μl; Life Technologies) from 1 μg of total