Interaction of the attenuated recombinant rIHNV-Gvhsv GFP virus with macrophages from rainbow trout (*Oncorhynchus mykiss*)

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Abstract

One of the most important threats to the salmonid aquaculture industry is infection caused by novirhabdoviruses such as infectious haematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV). Using reverse genetics, an avirulent recombinant rIHNV-Gvhsv GFP strain was generated, which was able to replicate as effectively as wild type IHNV in a fish cell line and in macrophages. Although this recombinant virus induced protective responses against IHNV and VHSV, the response did not involve the production of antibodies or modulate the expression of some antiviral genes. To determine the immune mechanisms underlying the protection conferred by the rIHNV-Gvhsv GFP virus, different immune parameters (NO production, respiratory burst activity and the induction of apoptosis) were assessed in the macrophage population. The results obtained in the present work may indicate that the Nv protein could be important in the modulation of NO and ROS production. rIHNV-Gvhsv GFP did not appear to have a clear effect on nitric oxide production or apoptosis. However, an increased respiratory burst activity (with levels induced by the recombinant virus significantly higher than the levels induced by the wild type virus), suggests a stimulation of the macrophage population, which could be related to the protection against virulent viruses.

Keywords: **rIHNV-Gvhsv GFP; IHNV; Rhabdovirus; Macrophages; TEM; ROS; Apoptosis; Trout**

1. Introduction

Vaccination has an important role in large-scale commercial fish farming and has been a key reason for the success of salmonid fish cultivation. This culture has been highly affected by infections caused by the infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV), the two major causes of mass mortality (Bootland and Leong, 1999; Smail, 1999). Although a lot of research is being done for the control of these diseases, only one live attenuated vaccine for VHSV is available in Germany (Enzmann, Tübingen), and Novartis Animal Health (Switzerland) commercialised a DNA vaccine against IHNV (Apex®-IHNV) for use in Canada.

Although alternative methods to the traditional formulations such as live virus vaccines (Sommerset et al., 2005), recombinant DNA vaccines (Lorenzen et al., 2002) or live recombinant virus (Biacchesi et al., 2002; Romero et al., 2008) have been tried to generate vaccines, their development is limited by safety concerns for the consumer and for the environment. Using reverse genetics methodology, a new line of live recombinant IHNV strains were developed by Biacchesi et al. (Biacchesi et al., 2000a; Biacchesi et al., 2000b; Biacchesi et al., 2002) and tested in vaccination trials (Romero et al., 2005; Novoa et al., 2006; Romero et al., 2008). Two of the six IHNV genes were modified in the recombinant virus used in the present study (rIHNV-Gvhsv GFP). One of them was the G glycoprotein gene which encodes the G protein, involved in viral pathogenicity and capable of eliciting protective antibody production against various IHNV strains (Engelking and Leong 1989). It was replaced with the gene from VHSV. The amino acid

homology between the G protein of IHNV and VHSV indicates a high degree of structural and functional similarity between the two fish rhabdovirus glycoproteins (Lorenzen et al., 1993). Although the G-VHSV protein is able to induce nonspecific protection against IHNV in experimental challenges in fish (Lorenzen et al., 1998; Kim et al., 2000) the production of specific antibodies is restricted by the epitope structure of VHSV (Engelking and Leong, 1989; Lorenzen et al., 1990). The other gene modified was the small non-virion protein Nv, which has been proven to be nonessential for recombinant IHNV, although its deletion affects replication in cell culture (Thoulouze et al., 2004). Therefore, the Nv gene can be used as a site of insertion for foreign genes and can serve as vector for expressing additional antigens. This non-structural Nv gene was replaced with the green fluorescent protein (GFP). We have previously demonstrated that this recombinant virus was apathogenic for zebrafish and rainbow trout. Moreover, vaccination trials showed that it was able to induce protective responses against experimental infections with IHNV or VHSV in both species (Novoa et al., 2006; Romero et al., 2008). However, we observed that the non-specific protective response analyzed by measuring the gene expression level of some antiviral genes, and the specific immune response evaluated through the antibodies production, did not appear to be involved in this protection, and it was suggested that other immune mechanisms could be responsible for the protection conferred by the rIHNV-Gvhsv GFP virus (Novoa et al., 2006; Romero et al., 2008).

Leukocytes are target cells for the replication of IHNV (Chilmonczyk and Winton, 1994). The viral replication occurs fast in cell culture being detected the first ultrastructural changes of the cytoplasm as early as 24-36 h post-infection by electron microscopy (Björklund et al., 1997; Kazachka et al., 2007). Moreover, the viral titre of IHNV peaked at 2 days post-infection in rainbow trout leucocytes (Chilmonczyk and Winton, 1994). The cycle of infection occurs by series of well described events in the following order: adsorption, penetration and uncoating, transcription, translation, replication, assembly and budding (Bootland and Leong, 1999). The morphogenesis and replication cycle of the recombinant virus rIHNV-Gvhsv GFP was analysed using transmission electron microscopy (TEM) and compared with wild type IHNV to assess if the changes introduced in the viral genome modified the efficacy of viral replication.

Leukocytes constitute an important part of the cellular defence against bacterial and viral infections in fish (Secombes, 1994) by secreting reactive oxygen and nitrogen intermediates and by their phagocytic capacity (Marletta et al., 1988; Nathan and Hibbs, 1991; Secombes and Fletcher, 1992). Taking this into account, we analysed in primary cell cultures enriched with kidney macrophages different immune mechanisms triggered by the viral infection (IHNV or rIHNV Gvhvs GFP), such as NO production, respiratory burst activity and also the induction of apoptosis, to better understand the mechanisms underlying the protection induced by the rIHNV-Gvhsv GFP virus.

2. Materials and Methods

2.1. Virus titration

IHNV (French isolates 32/87) (Laurencin, 1987) and the recombinant viruses rIHNV-Gvhsv GFP, rIHNV GFP (Novoa et al., 2006) and rGvhsv (Romero et al., 2005) were propagated in the fish epithelial cell line EPC, which is derived from common carp (*Cyprinus carpio*) (Tomasec and Fijan, 1971). EPC cells were cultured in Eagle's minimum essential medium (MEM Invitrogen, GIBCO) supplemented with 10% foetal bovine serum (FBS Invitrogen, GIBCO), penicillin (100 IU/mL) (Invitrogen, GIBCO), and streptomycin (100 µg/mL) (Invitrogen, GIBCO), and buffered with 7.5% sodium bicarbonate (Invitrogen, GIBCO), and were incubated at 20 ºC. The viruses were inoculated on EPC cells grown in MEM with antibiotics and 2% FBS at 15 ºC. When the cytopathic effect (CPE) was complete, the supernatants were harvested and centrifuged to eliminate cell debris. Viruses were then titrated according to Reed and Muench (1938).

2.2. Primary cell cultures enriched with kidney macrophages

Primary cell cultures enriched with macrophages from rainbow trout (mean weight 22 g) were obtained (Secombes, 1990). Briefly, the anterior kidney was removed aseptically and passed through a 100-µm nylon mesh using Leibovitz medium L-15 (Invitrogen, GIBCO) supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), heparin (10 U/mL) (Invitrogen, GIBCO) and 2% FBS. The resulting cell suspension was placed on a 34%-51% Percoll density gradient (GE Healthcare) and centrifuged at 500 g for 30 min at 4 ºC. The interface cells

were collected and washed twice in L-15 containing 0.1% FBS, spinning at 500 g for 5 min. The viable cell concentration was determined by trypan blue exclusion. Cells were resuspended in L-15 with 0.1% FBS and dispensed into 24-well plates at a concentration of 10^6 cells/mL. Adherent cells were attached to the bottom of the wells by incubating 3h at 18 ºC. After this period supernatants and non-adherent cells were removed. All the animal experiments were reviewed and approved by the CSIC National Committee on Bioethics.

2.3. Cell infections

Primary cell cultures enriched with kidney macrophages were infected with the rIHNV-Gvhsv GFP virus or wild type IHNV at a multiplicity of infection of 1 (MOI 1). After 30 min of adsorption, cells were washed and incubated at 15 ºC in L-15 medium supplemented with 2% FBS. Confluent EPC cell cultures were also infected with IHNV or rIHNV-Gvhsv GFP virus as positive control groups. Infected cell cultures were sampled at 24, 48 and 72 h post-infection (p.i.). Samples were frozen and thawed twice to release the viral particles inside the cells. After centrifugation at 12000 g for 5 min, the supernatants were stored at -80 ºC until use. Titration of supernatants was measured in triplicate according to the protocol described by Reed and Muench (1938). Results were expressed as the mean ± standard deviation (SD) and were compared using a t test ($p<0.05$).

In addition, infected cultures were counterstained with DAPI (Sigma Aldrich) according to the manufacturer's instructions to observe the CPE and the

morphological changes with high-resolution spectral confocal microscopy (Leica TCS SPE).

2.4. Viral morphogenesis

The study of the viral morphogenesis by electron microscopy was conducted in EPC cells 1, 2, 3, 5 and 6 days post-infection. Cells were fixed for 1 h in 2% glutaraldehyde in 0.1M cacodylate buffer. After two washes, cells were fixed in 2% osmium tetroxide (Invitrogen) and included in 0.25% technical agar (Cultimed). Small squares of agar were processed for Poly/Bed 812 and Araldite grade 502 (Polysciences, Inc.) resin blocking. Thin sections (1 µm) stained with 0.5% toluidine blue were analysed by light microscopy. Ultrathin sections were then stained with uranyl-acetate (FLUKA) and lead citrate (FLUKA) and observed with a Philips CM 100 transmission electron microscope (TEM).

2.5. Analysis of innate immune cell parameters

The different immune responses induced by macrophages against experimental infections with recombinant rIHNV-Gvhsv GFP virus were analysed and compared with the responses induced by wild type IHNV.

Primary cultures enriched with head kidney macrophages were obtained from eight adult fish (mean weight 22 g), placed in 96-well plates (10^6 cells/mL) and infected with rIHNV-Gvhsv GFP virus, rIHNV GFP virus or rGvhsv at different MOI (1 and 0.01) in L-15 with 0.1% FBS at 15 ºC. Primary cultures were also infected with wild type IHNV or treated with culture medium as positive and

182 negative control groups, respectively. At 24, 48 and 72 h p.i., 50 µL of the
183 supernatants were removed from the wells, and a nitrite assay was conducted. The
184 remaining cells were grouped in three pools, and the respiratory burst activity was
185 measured by the chemiluminescence (CL) method.
186
187 ***2.5.1. Nitrite assay***
188 Nitric oxide (NO) production of head kidney macrophages was assayed
189 using the method described by Neumann et al. (1995). Briefly, after incubation of
190 macrophages at 15 ºC for 24, 48 and 72 h, 50 µL of the supernatants were
191 removed and placed in a separate 96-well plate. One-hundred microlitres of 1%
192 sulphanilamide (Sigma) was added to each well followed by 100 µL of 0.1% N-
193 naphthyl-ethylene-diamine (Sigma). Optical density (O.D.) was measured at 540
194 nm, and the molar concentration of nitrite was determined from standard curves
195 generated using known concentrations of sodium nitrite. All the treatments were
196 assayed in triplicate for each fish. Data were compared using a t test ($p<0.05$).
197 Results are expressed as the mean ± SD.
198
199 ***2.5.2. Chemiluminescence assay***
200 The reactive oxygen species (ROS) production was measured 24 h, 48 h
201 and 72 h p.i. in infected macrophages (MOI 1 and 0.01) as the emission of
202 Relative Luminescence Units (RLUs) after cell membrane stimulation with
203 phorbol myristate acetate (PMA, Sigma) and amplification by 5-amino-2,3-
204 dihydro-1,4-phthalazinedione (Luminol, Sigma) according to the protocol

205 described by Rodriguez et al. (2008). Triplicate wells were used in all
206 experiments. To determine if the viral infection could trigger macrophage
207 respiratory burst activity, cells were infected as described above, and the O.D. at
208 550 nm was measured after 30 min without PMA stimulation. Data were
209 compared using a t test ($p<0.05$). ROS production index were calculated by
210 dividing the values obtained in infected cells by the values obtained in controls
211 Results are expressed as the mean ± SD.
212
213 ***2.5.3. Apoptosis assay***
214 Primary cultures enriched with head kidney macrophages from four rainbow
215 trout (22 g mean weight) were obtained by the method described above. The cell
216 concentration was adjusted to 10^6 cells/mL, and cells were placed on 24-well
217 tissue culture plates (Falcon). Cells were infected with IHNV or rIHNV-Gvhsv
218 GFP at a MOI 1. A negative control group was treated with culture medium, and a
219 positive control group was treated with UV light for 30 min. Samples were taken
220 at 24 and 72 h p.i., and Annexin V-PE (BD Biosciences) and 7AAD (BD
221 Biosciences) staining were measured by flow cytometry as described by Romero
222 et al. (2008). Data obtained from the four replicates were compared using a t test
223 ($p<0.05$).
224
225 **3. Results**
226
227 ***3.1. Viral replication***

228 The recombinant rIHNV-Gvhsv GFP virus replicated as effectively as wild
229 type IHNV in both the macrophage cell culture and the EPC cell line (Fig. 1). The
230 viral titre increased along with time, changing from $3x10^4$ $TCID_{50}$/mL to $2x10^5$
231 $TCID_{50}$/mL in cell cultures enriched with kidney macrophages. Similar increases
232 were recorded for IHNV, the titres of which reached $5x10^5$ $TCID_{50}$/mL in
233 macrophages 72 h p.i. The viral titre also increased in EPC cells infected with
234 IHNV and rIHNV-Gvhsv GFP, reaching $5x10^5$ $TCID_{50}$/mL at 72 h p.i. No
235 significant differences were observed between wild type and recombinant virus-
236 infected samples in all cell types and time-points examined.

237 Infected EPC cells and infected macrophages showed a typical “bunch of
238 grapes” appearance upon IHNV infection (Fig. 2A and 2B). Although association
239 between damaged cells and GFP fluorescence was observed, no morphological
240 changes were observed in fluorescent virus-infected cells either in the cytoplasm
241 or in the nucleus until 48 h p.i. Confocal microscopy revealed that only a small
242 percentage of the cell population was actively infected by the rIHNV-Gvhsv GFP
243 virus (Fig. 2C and 2D). The green fluorescence emitted by the viral GFP protein
244 was focused in the cytoplasm. Green cytoplasmic extensions were also observed
245 (Fig. 2C and 2D). Virus-induced CPE was first observed in infected cells 24 h p.i.
246 using light microscopy on semi-thin sections (Fig. 3). The earliest ultrastructural
247 changes in virus-infected cultures involved a reduction of cell volume. This stage
248 was rapidly followed by a condensation of chromatin around the nuclear
249 membrane. Vacuolization of the cytoplasm and condensation of the chromatin
250 into one or several dense bodies was evident. Total CPE was observed at day 6

p.i., when the cytoplasm was completely vacuolated and dead cells and cellular debris were observed.

3.2. Viral morphogenesis

The replication cycle of the rIHNV-Gvhsv GFP virus was described by electron microscopy observations. The virions of the recombinant virus were typically bullet-shaped, with approximate measurements of 110 nm X 70 nm (Fig. 4A). At 24h p.i., viral particles were attached to the cell surface (Fig. 4B). Subsequent internalization by endocytosis of the viral particles into the cytoplasm was observed (Fig. 4C). Endocytic compartments with an electron-dense granular material inside were frequently observed at 24 h (Fig. 4D). At the same time (24 h p. i.) cells with destructive changes in the cytoplasm were observed. Cytoplasm inclusion bodies were observed as the first sign of virus replication (Fig. 4E and 4F). Chains of ribosome-bound mRNAs were detected in the cytoplasm (Fig. 4G). Different stages of maturation were detectable at cellular membrane (Fig. 4H). The next visible form of the virus replication (also at 24h p.i.) was the presence of high amount of viruses into cytoplasmatic vacuoles (Fig. 4I). They induced the disintegration of the cell structure and caused the release of the viral particles. Also individual viruses were budded from the cell surface (Fig. 4J). At 48-72 h after viral infection we observed many cells collapsed into apoptotic bodies with different sizes. After 72 h p. i. almost all cells of the monolayer were completely destroyed by the infection.

3.3. Analysis of innate immune cell parameters

3.3.1. NO production

The levels of nitrite measured in kidney macrophages infected with the rIHNV-Gvhsv GFP virus or rIHNV GFP were significantly different from the levels measured for cells infected with IHNV or rGvhsv, regardless of the MOI used (Fig. 5). In both experimental conditions (MOI 1 and 0.01), the levels of nitrite induced by the recombinant viruses rIHNV-Gvhsv GFP and rIHNV GFP were similar to the levels recorded in the control group. However, infection with wild type IHNV and rGvhsv always induced values of nitrite lower than the GFP recombinant viruses. Statistically significant differences were obtained under all of the experimental conditions except for the sample taken at 24 h p.i. at MOI 1

3.3.2. ROS production

Infection of macrophages with the recombinant viruses rIHNV-Gvhsv GFP, rIHNV GFP, rGvhsv and the IHNV wild type virus at MOI 1induced significant increases in respiratory burst activity with respect to the controls at 24 h, 48 h and 72 h p.i. (Figure 6). However, at MOI 0.01 only infected cells with the recombinant viruses rIHNV-Gvhsv GFP and rIHNV GFP induced a significant increase with respect to controls during all the experiment. The ROS levels induced by the recombinant virus rIHNV-Gvhsv GFP was always significantly higher than the levels induced by the IHNV wild type at all samples and time-points (Fig. 6). IHNV and rIHNV-Gvhsv GFP could not trigger respiratory burst

296 activity in infected macrophages 30 min after infection in the absence of PMA
297 (data not shown).
298
299 ***3.3.3. Apoptosis assay***
300 The most important morphological changes in the population structure were
301 observed in cells treated with UV light at 72 h (Fig. 7A). The recombinant
302 rIHNV-Gvhsv GFP virus induced similar apoptotic and necrotic levels in infected
303 kidney macrophages as the wild type IHNV at 24 and 72 h p.i. (Fig. 7B). At 72 h,
304 the percentage of apoptotic macrophages (stained with Annexin V-PE) induced by
305 IHNV was the same as that induced by rIHNV-Gvhsv GFP virus (23%). The
306 percentage of necrotic cells (stained with 7AAD) was always lower than 10%. UV
307 light treatment induced the highest amount of cellular damage (apoptosis and
308 necrosis) in both primary cultures. An increase in the number of apoptotic
309 macrophages was observed from 24 to 72 h, changing from less than 10% at 24 h
310 p.i. up to 80% at 72 h p.i. (Fig. 7B). Although the percentage of macrophages
311 infected by the recombinant virus (as measured by GFP using the FL-1 channel,
312 530 nm) increased from 24 to 72 h p.i., less than 13% of the cell population
313 supported the viral replication (data not shown).
314
315 **4. Discussion**
316 IHNV is able to replicate in a variety of established cell lines (Ristow and
317 DeAvila, 1994; Bootland and Leong, 1999; Lorenzen et al., 1999) as well as in
318 many fish tissues (Wolf, 1988; Yamamoto et al., 1990), resulting in the presence

319 of virus in body fluids and mucus (Mulcahy et al., 1982; LaPatra et al., 1989).
320 Haematopoietic tissues are particularly susceptible to IHNV infection (Yasutake
321 and Amend, 1972; Estepa and Coll, 1994), and peripheral blood and kidney
322 leukocytes are involved in the pathogenesis of IHNV (Chilmonczyk and Winton,
323 1994). Our results showed that the recombinant rIHNV-Gvhsv GFP virus
324 replicated in leukocyte populations derived from the kidney of rainbow trout as
325 effectively as the wild type IHNV. Under all conditions studied, the viral titre
326 increased along with time and followed the same kinetics as IHNV, reaching
327 similar values at 72 h p.i. These data revealed that macrophage populations could
328 serve as target cells in the initial phase of rIHNV-Gvhsv GFP infection. Although
329 Novoa et al. (2006) and Romero et al. (2008) suggested that the changes
330 introduced in the genome decreased virulence in experimental infections *in vivo*,
331 no changes in replication efficiency were observed in infected cell cultures.
332 However, only a small percentage of the macrophage population (less than 13%)
333 supported viral replication *in vitro* as observed by confocal microscopy and flow
334 cytometry. Similar results were reported by Tafalla et al. (1998) using
335 immunofluorescence assays, in which only 8% of trout monolayer macrophages
336 expressed VHSV-specific fluorescence three days p.i. Estepa et al. (1992)
337 obtained a variable percentage of positive cells, ranging between 20-50%, five
338 days p.i. by flow cytometry analysis.

339 The recombinant rIHNV-Gvhsv GFP virus did not produce morphological
340 changes in the cytoplasm or in the nucleus until 48 h p.i., which coincides with
341 observations in VHSV-infected macrophages (Estepa and Coll, 1991). This virus-

induced CPE appeared as focal areas resembling those induced by wild type IHNV, and cells had the morphology of apoptotic cells. Similar changes were also described by Björklund et al. (1997) in an EPC cell line infected with the spring viraemia of carp virus (SVCV), suggesting that apoptosis could be a generalised cell killing mechanism after viral infections. It is interesting to note that although the recombinant rIHNV-Gvhsv GFP virus was able to induce CPE in macrophage populations, no histological lesions were observed in kidney, liver, spleen or brain (Novoa et al., 2006; Romero et al., 2008).

The rIHNV-Gvhsv GFP virus initiated the replication cycle by attaching to the cell surface, most probably using different cellular receptors, such as fibronectin-like protein complex, phosphatidylserine, sialic acid, and other cell adhesion molecules (Schlegel et al., 1983; Haywood, 1994; Broughan and Wunner, 1995; Bearzotti et al., 1999), and its viral glycoprotein (G) spikes (Wagner, 1987). Although the rIHNV-Gvhsv GFP virus had the G protein replaced by the G protein from VHSV, no changes were observed in its ability to infect cultured cells, and viral particles attached to the surface were frequently observed in TEM. Also, many cytoplasmic vesicles with viral particles inside were observed, suggesting that the virus entered the cell by endocytosis as has been described with other enveloped viruses (Lenard and Miller, 1982; Matlin et al., 1982; Marsh, 1993). Finally, the final assembly of rIHNV-Gvhsv GFP virus occurred predominantly at the cell membrane and sometimes at the membranes of the Golgi cisternae. After assembly, new virions were found adsorbed to neighbouring cell membranes. Taking the data from the experimental titrations together with the

365 microscopy observations, we suggest that neither viral morphogenesis nor the
366 replication ability of the recombinant virus were affected by the genome
367 modification.

368 With regard to the immune cell parameters, it is well known that macrophages
369 are key cells in the first stages of a viral infection. Positive feedback mechanisms
370 and synergistic interactions intensify the immune response and give rise to potent
371 bactericidal and antiviral mechanisms, such as NO and ROS production (Reiss
372 and Komatsu, 1998; Ellermann-Eriksen, 2005). NO is usually produced by
373 macrophages in response to proinflammatory cytokines, bacterial
374 lipopolysaccharide (LPS), parasites or viruses (Marletta et al., 1988; Nathan and
375 Hibbs, 1991; Tafalla et al., 1999; Tafalla et al., 2000; Tafalla et al., 2001).
376 However, NO production has been shown to be ineffective for protecting against
377 viral infection in human cell lines (López-Guerrero and Carrasco, 1998). We
378 observed that IHNV and the pathogenic recombinant virus rGvhsv were able to
379 significantly inhibit NO production, probably as a viral mechanism to overcome
380 the host immune defences. This has also been described for VHSV (Tafalla et al.,
381 2001), as the virus was able to suppress NO production in infected macrophages
382 from turbot. In contrast, the recombinant virus rIHNV-Gvhsv GFP and rIHNV
383 GFP were not able to inhibit this production, and the nitrite values recorded were
384 similar to the levels registered in the control group. This NO concentration might
385 inhibit the earliest stages of viral replication and thus prevent viral spread,
386 promoting viral clearance and recovery of the fish. These results may indicate that
387 the Nv protein could be important in the inhibition of NO production.

Viral infections may also trigger the production of ROS (Akaike et al., 1998). The role of ROS in apoptosis induction during viral infections has been reported in mammals (Staal et al., 1990; Roederer et al., 1992; Kohno et al., 1996; Skulachev, 1997; Skulachev, 1998; Kulms et al., 2000). Moreover, it has been described that high levels of NO and ROS can modulate and activate apoptotic cell death (Brüne et al., 1998; Tripathi and Hildeman, 2004). However, the results obtained in fish so far have been contradictory. We previously observed (Tafalla et al., 1998) no changes in ROS production in macrophages from rainbow trout and turbot infected *in vitro* with VHSV. This lack of respiratory burst activity could be correlated with the small number of macrophages that were actively infected. Similar results were later observed by Chilmonczyk and Monge (1999) and Tafalla and Novoa (2001). However, Stohlman et al. (1982) described that in some cases, ROS production mediates the antiviral activity of macrophages, and Siwicki et al. (2003) reported a significant reduction in the response of macrophages isolated from rainbow trout and infected with VHSV. The decrease in ROS production could be a mechanism induced by the virus to evade the host immune response. Moreover, it could prevent the activation of other pathways to eliminate infected cells, such as apoptosis (Skulachev, 1998). In contrast to these previous results, we reported here a significant increase in the respiratory burst activity of macrophages infected with rIHNV-Gvhsv GFP. Moreover, the respiratory burst levels induced by this recombinant virus were significantly higher than the levels induced by the wild type virus. Although the level of cell infection described in this work was the same as the percentage observed by

411 Tafalla et al. (1998), macrophages infected with IHNV and rIHNV-Gvhsv GFP
412 were able to induce a high respiratory burst activity. However, the recombinant
413 virus was not able to trigger the response by itself, in agreement with the results
414 obtained by Tafalla et al. (1998) with VHSV. In any case, the high levels of
415 respiratory burst activity observed here reflected the activation of the macrophage
416 population against the viral infection.

417 The effects of rIHNV-Gvhsv GFP on the induction of apoptosis are of
418 particular interest because it is well known that this virus is able to replicate in
419 macrophages. Apoptosis has an important role in many viral infections
420 (Thoulouze et al., 1997; Gadaleta et al., 2002; Blaho, 2003; Blaho, 2004) and has
421 been shown to be involved in the cell death caused by rhabdovirus in cell lines
422 and tissues (Björklund et al., 1997; Chiou et al., 2000; Eléouët et al., 2001; Du et
423 al., 2004). Romero et al. (2008) described that rIHNV-Gvhsv GFP induced higher
424 apoptosis levels than wild type IHNV in the EPC cell line. However, in the
425 present work, a low level of apoptosis induction was recorded in primary cell
426 cultures infected with the recombinant or the wild type virus. To a certain extent,
427 this result was expected, as less than the 13% of the population supported the viral
428 replication. Our results are in accordance with previous results obtained by
429 Thoulouze et al. (1997), who analysed apoptosis induction by an attenuated rabies
430 virus strain in mouse and human lymphocytes and obtained low levels of
431 apoptosis.

432 The rIHNV-Gvhsv GFP virus did not appear to have a clear effect on NO
433 production or apoptosis in macrophages. However, the macrophage population

seemed to be stimulated by the recombinant virus, given the observed respiratory burst activation. In a previous study, MacKenzie et al. (2008) analysed the response in trout head kidney after intraperitoneal challenge with the recombinant rIHNV-Gvhsv GFP virus, IHNV and LPS. The results showed that infection with the recombinant virus induced a similar gene expression pattern to infection with the native IHNV at 24 h p.i. However, a divergence in the viral response was observed after 72 h, with the recombinant virus showing a recovery response more similar to that observed for the LPS treatment, suggesting that different mechanisms of activation were induced.

The results obtained in the present work may indicate that the Nv protein could be important in the modulation of NO and ROS production on infected cells. Moreover, the good levels of protection against experimental infection with IHNV and VHSV conferred by the recombinant virus rIHNV-Gvhsv GFP could be the result of stimulation or activation of the cellular innate immune system, although more efforts should be made to further clarify the interaction between the virus and the fish immune system.

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672 **Figure legends**

673

674 **Fig. 1.** Time course of the viral titre in head kidney macrophages infected with
675 rIHNV-Gvhsv GFP or IHNV. Infected EPC cell lines were the positive control.
676 Values represent the mean and SD from three different experiments. Numbers on
677 the x axis indicate hours post-infection.

678

679 **Fig. 2.** Morphological changes induced by the rIHNV-Gvhsv GFP virus in the
680 EPC cell line and the macrophage cell culture (A and B, respectively) at 48 h p.i.
681 under confocal microscopy (MOI 1). A correlation between damaged cells and
682 GFP fluorescence was observed in the EPC cell line and the macrophage cell
683 culture (C and D, respectively). Green: recombinant virus on infected cells. Blue:
684 nuclear DAPI staining. Scale bar 250 μm.

685

686 **Fig. 3.** Morphological changes observed under light microscopy in semi-thin
687 sections of EPC cell line and macrophages infected with rIHNV-Gvhsv GFP
688 virus. The virus-induced CPE was first apparent at 24 h p.i. Vacuolization of the
689 cytoplasm and condensation of the chromatin into one or several dense bodies was
690 observed at 48 h p.i. After six days, the cytoplasm was completely vacuolated,
691 and dead cells and cellular debris were observed.

692

693 **Fig. 4.** Viral morphogenesis and replication in the EPC cell line under electron
694 microscopy. (A and B) Infection by rIHNV-Gvhsv GFP and IHNV was initiated

by the attachment of the viruses to the cell surface. CS: cell surface (scale bar 100 nm). (C) Internalization of the recombinant virus (arrow head) by endocytosis (scale bar 200 nm). (D) The appearance of endocytic compartments with the virus inside was frequent (scale bar 200 nm). (E and F) Viral cores released into the cytoplasm (arrows) (scale bar 200 nm). (G) Chains of ribosome-bound mRNAs of the recombinant virus in the cytoplasm (arrows) (scale bar 200 nm). (H) Newly synthesized viral proteins associated in the cytoplasm with *de novo* replicated genomic RNA to form ribonucleoprotein cores (arrows) (scale bar 200 nm). (I) Release of the new viral progeny (rIHNV-Gvhsv GFP) by cytoplasmic vesicles (arrows) and disintegration of the cell structure (scale bar 200 nm). (J) The budding of individual viruses from the cell surface was also observed (arrows) (scale bar 100 nm).

Fig. 5. Production of nitrite radicals in kidney macrophages infected with rIHNV-Gvhsv GFP, rIHNV GFP, rGvhsv or IHNV at different MOI (1 and 0.01) at 24, 48 and 72 h p.i. Results represent the mean ± SD of three replicates. (*) Significant differences regarding to controls ($p<0.05$).

Fig. 6. Respiratory burst activity induced by the recombinant rIHNV-Gvhsv GFP virus, rIHNV GFP, rGvhsv and IHNV (MOI 1 and 0.01) in primary cultures enriched with macrophages at 24 h, 48 h and 72 h p.i. Results represent the mean ± SD of three replicates. (a and b) Significant differences regarding to values obtained in control group and in infected cells with IHNV, respectively ($p<0.05$).

718

719 **Fig. 7.** (A) FSC/SSC density plots of the cell population at 72 h post-treatment.
720 (B) Percentage of apoptotic and necrotic kidney macrophages infected with
721 rIHNV-Gvhsv GFP or IHNV or treated with UV light as a positive control 24 and
722 72 h p.i. The apoptotic and necrotic cells were stained with Annexin V-PE and 7-
723 AAD, respectively. Results represent the mean ± SD of four replicates. (*)
724 Significant differences ($p<0.05$).

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726

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Figure 1

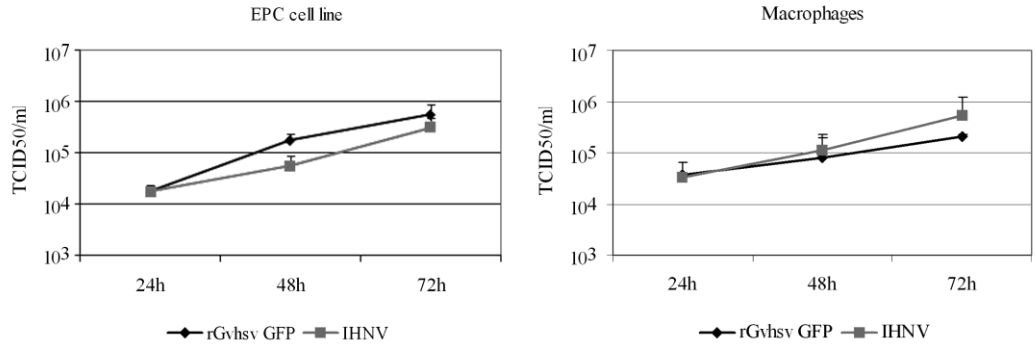

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Figure 2

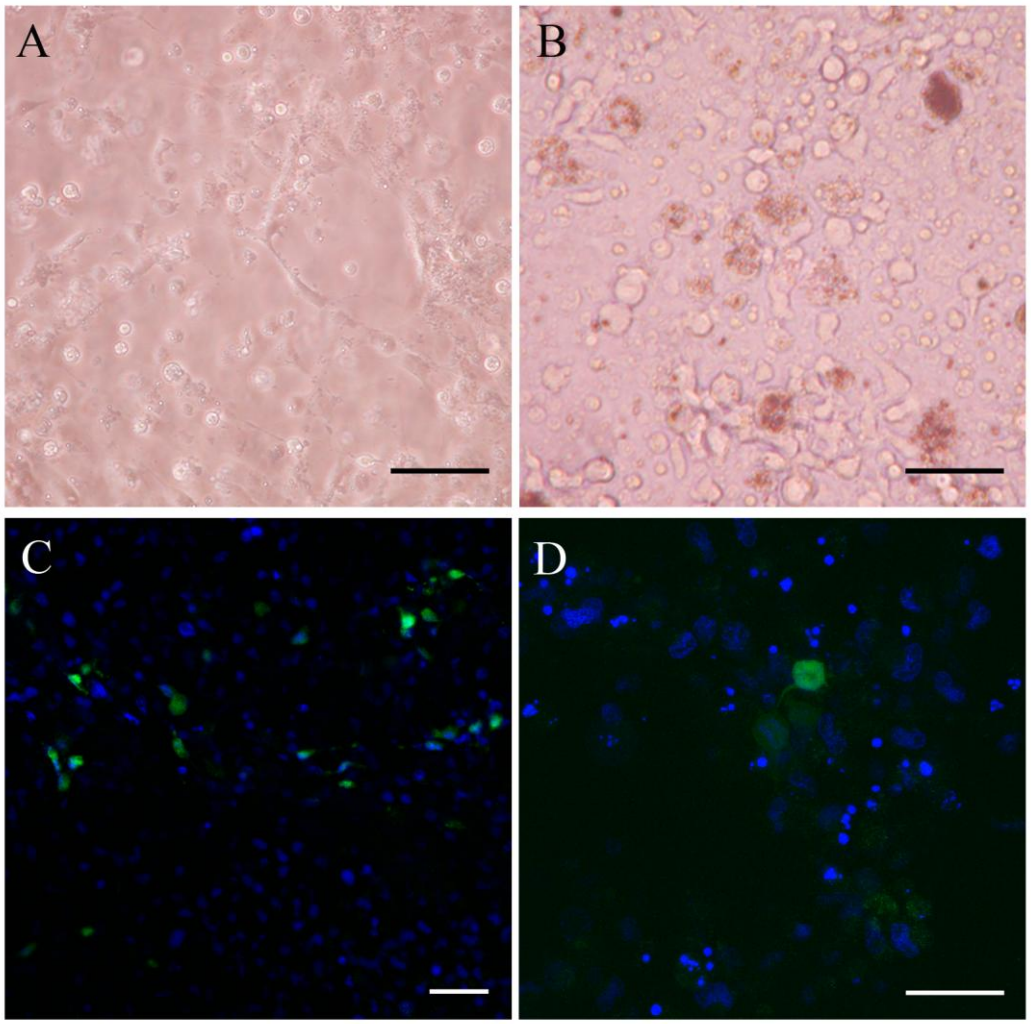

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Figure 3

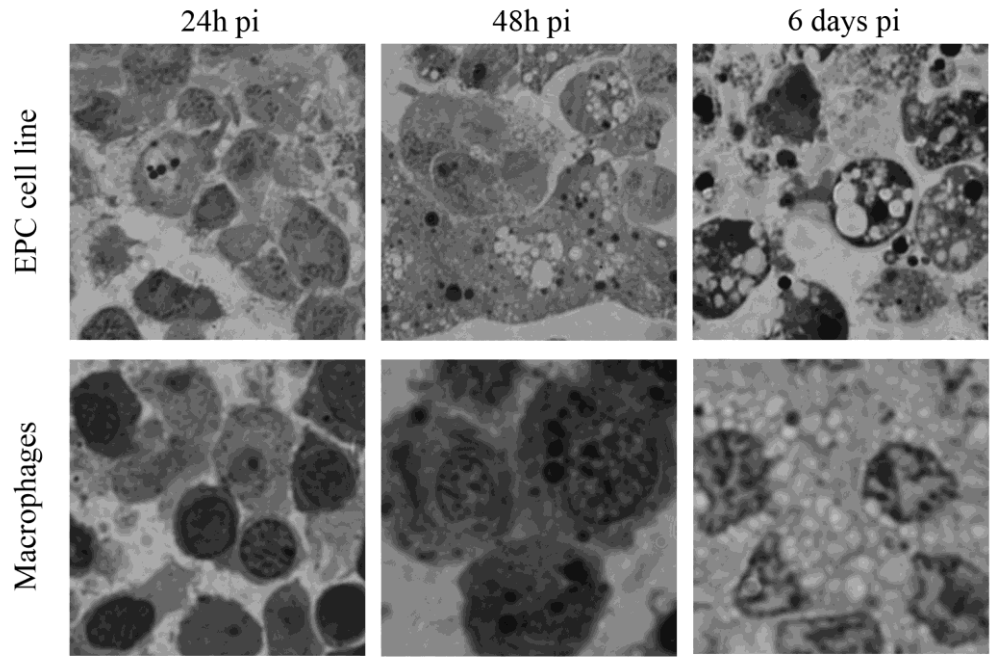

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Figure 4

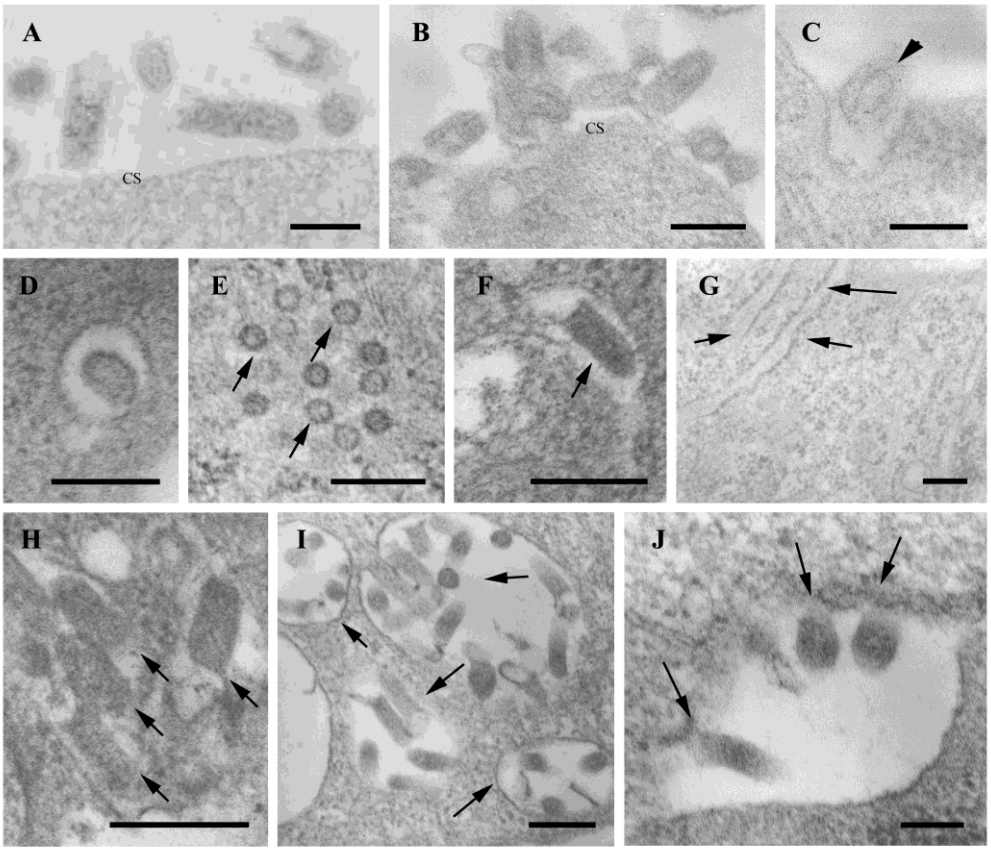

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Figure 5

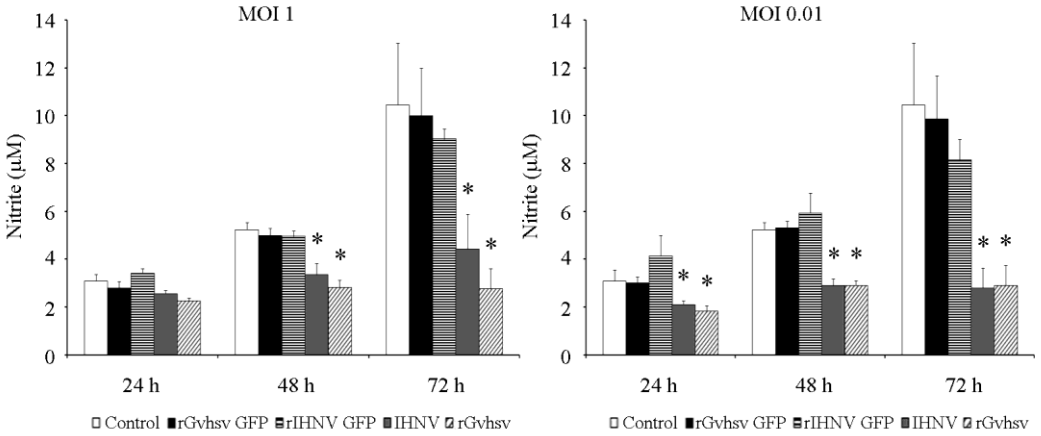

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Figure 6

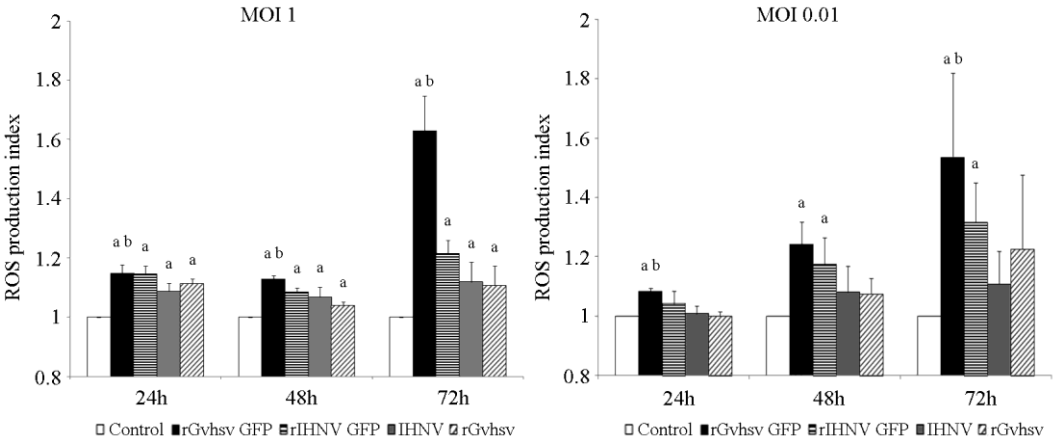

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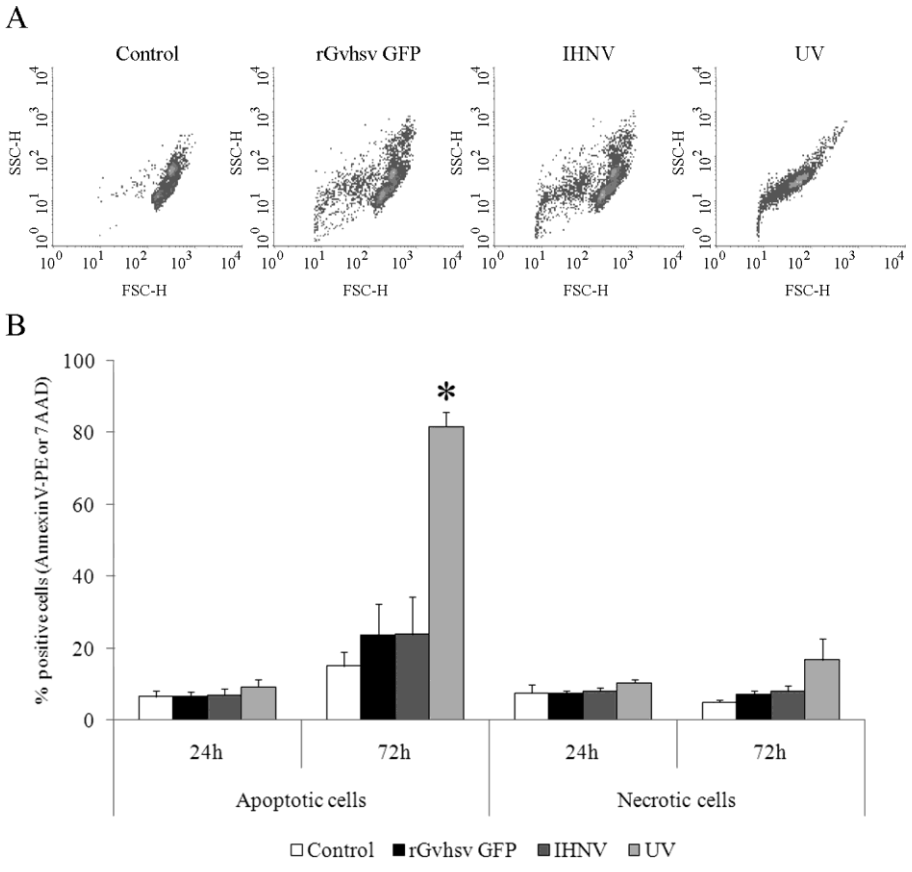
Figure 7



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