Virus Research 263 (2019) 73–79

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Evaluation on the antiviral activity of ribavirin against infectious hematopoietic necrosis virus in epithelioma papulosum cyprini cells

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ARTICLE INFO

Keywords:
Ribavirin
Antiviral activity
IHNV

ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) causes high mortality in several economically important salmonid fishes, but there is no approved therapy up to now. To address the urgent need for therapeutics to combat IHNV infection, we investigate the anti-IHNV activities of 14 common antiviral agents using epithelioma papulosum cyprini (EPC) cells in this study. Among these agents, ribavirin shows the highest inhibition on IHNV replication, with an inhibitory percentage of 99.88%. And the 72 h half maximal inhibitory concentrations (IC_{50}) of ribavirin on IHNV glycoprotein is 0.40 mg/L. In addition, ribavirin significantly inhibits apoptosis and cellular morphological damage induced by IHNV. Mechanistically, ribavirin could damage the viral particle of IHNV. Moreover, ribavirin could be used for prevention of IHNV infection. Therefore, ribavirin is considered to develop as a promising agent to treat IHNV infection.

1. Introduction

Infectious hematopoietic necrosis virus (IHNV) is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Ammayappan et al., 2010). The virus is first described in Sockeye salmon (*Oncorhynchus nerka*) fry hatcheries in western North America in the early 1950s (Rucker et al., 1953), from where it also quickly spread to Europe and Asia causing high epizootics in trout and salmon species worldwide (Winton, 1991). IHNV has strong pathogenicity and extensive transmissibility, which has brought huge economic losses in the aquaculture industry of the world. Outbreaks of infectious hematopoietic necrosis (IHN) result in losses approaching 100%, depending on the species and size of the fish, the virus strain and environmental conditions (Rachel et al., 2013). Moreover, no effective methods are available for the control of the disease currently in the aquaculture industry. One of the reasons is lacking effective drugs for use and the pharmacological mechanism (Zhao et al., 2017). Besides, it is still difficult to control IHNV with the vaccine, due to the delivery method of vaccine showing labor intensive and impractical for large-scale administration in the freshwater commercial aquaculture industry. The vaccines are mainly administered by intraperitoneal injection or intramuscular injection (Plant and Lapatra, 2011). Overall, it is important to develop antiviral agents that can be used for prophylaxis or as antiviral agents against IHNV infection.

Ribavirin is a synthetic nucleoside analog used as an inhibitor of a large number of viruses in mammals and other vertebrates (Beaucourt and Vignuzzi, 2014). In fish, it has been reported that some viral infections are inhibited by ribavirin. In particular, infections with viral hemorrhagic septicemia virus (VHSV) (Marroquí et al., 2007), chum salmon reovirus (CSV) (Dewitte-Orr and Bols, 2007), infectious pancreatic necrosis virus (IPNV) (Jashés et al., 1996; Migus and Dobos, 1980), and infectious salmon anemia virus (ISAV) (Rivas-Aravena et al., 2011) have been shown to be dramatically reduced by ribavirin. In addition, ribavirin as an antiviral agent has great antiviral activity in the treatment of IHNV (Hudson et al., 1988). Although ribavirin exhibit excellent disease-resistant potency for targeting IHNV, little information exists on its efficacy and the mechanisms used by the compound on aquatic viruses.

Diverse pathological changes usually appear in different species or cells after virus infection such as cytopathic effect (CPE). The same phenomenon is also observed in the IHNV-infected epithelioma papulosum cyprini (EPC) cells under optics microscope. The cell apoptosis, ultrastructure of the cell surface, viral replication, and nucleus are likely to be accompanied by a series of subtle changes and these problems need further study. Therefore, further studies will be needed to close these knowledge gaps.

In this study, the anti-IHNV activities of 14 common antiviral agents were investigated using EPC cells. Ribavirin, the active compound, was

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https://doi.org/10.1016/j.virusres.2019.01.008
Received 17 November 2018; Received in revised form 12 January 2019; Accepted 12 January 2019
Available online 14 January 2019
0168-1702/

chosen for further potential mechanism study. It was further validated in secondary assays, including virus titration assay, CPE reduction analysis, nucleus damage observation, microtubule structure, and ultracentrifugation. The results set the foundation for the development of anti-IHNV agents and the further functional mechanism exploration of the antiviral agents.

2. Materials and methods

2.1. Cell lines, virus and compounds

The EPC cell line was kindly provided by Prof. Ling-Bing Zeng (Yangtze River Fisheries Research Institute, Wuhan, Hubei, China). Cells were maintained at 25 °C in 5% CO_2 atmosphere in Medium 199 (Hyclone, USA) cell culture containing 10% fetal bovine serum (FBS) (ZETA LIFE, USA), streptomycin 100 μg/mL and penicillin 100 U/mL. The IHNV (strain Sn-1203, isolated from infected rainbow trout in China, kindly provided by Prof. Tong-yan Lu, Heilongjiang River Fishery Research Institute Chinese Academy of Fishery Sciences, Harbin China) was propagated in EPC cells at 15 °C as previously described (Zhao et al., 2017). Ribavirin and thirteen common antiviral agents were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China).

2.2. Anti-IHNV activity assay of 14 compounds

For the screening assay, 14 compounds were diluted to the highest concentrations which cell viability was > 80% based on trypan blue exclusion dye staining test and compound solubility. To detect IHNV by qPCR, EPC cells were cultured in 12-well plates to a monolayer and infected with IHNV (1×10^3 50% tissue culture infective dose ($TCID_{50}$)) for 2 h at 15 °C. After that, the medium was replaced with cell maintenance medium containing 14 compounds and incubated at 15 ± 0.5 °C for 72 h. Afterwards, the media was removed, RNA extracted, cDNA obtained and qPCR carried out as below indicated.

2.3. RNA isolation, cDNA synthesis, and qPCR assays

Total mRNA from each sample was extracted using Trizol (TaKaRa, Japan) according to the manufacturer's protocols. RNA was reverse transcribed using HiScript Q Select RT SuperMix for qPCR (+gDNA wiper) (TaKaRa, Japan). Quantitative PCR of G gene was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using AceQ® qPCR SYBR® Green Master Mix (TaKaRa, Japan) with the following parameters: 95 °C for 30 s and then 40 cycles at 95 °C denaturation for 5 s, followed by at 60 °C annealing for 35 s. The glycoprotein (G) primers were designed with Primer Premier 5 software and β-actin was used as the internal reference and listed in Table 1 (Shao et al., 2016). Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method with the formula (Livak and Schmittgen, 2001).

2.4. Dose effect of ribavirin on IHNV replication

Ribavirin was diluted to six concentrations (3.16, 1.58, 0.79, 0.40, 0.2 and 0.10 mg/L) using cell maintenance medium. DMSO at 0.06‰ was served as vehicle control. Virus infection and ribavirin treatment were mentioned above. The expression of IHNV G related to the β-actin gene was measured.

Table 1
Sequences of primer pairs used for the analysis of gene expression by real-time PCR.

Genes		Primer sequences (from 5′ to 3′)
β-actin	Forward	GCTATGTGGCTCTTGACTTCGA
	Reverse	CCGTCAGGCAGCTCATAGCT
IHNV glycoprotein (G)	Forward	GCACAAAGGCTCCATCTATC
	Reverse	TGTACTGGGCGACGTATT

2.5. Cytotoxicity assay of ribavirin

For cytotoxicity assay, EPC cells with a density of 1×10^4 per well were seeded into 96-well plates containing 100 μL growth medium and incubated for reaching approximately 80–90% confluence. Subsequently, the cells were exposed to cell maintenance medium (medium 199 supplemented with 5% FBS) containing ribavirin at six concentrations (100.00, 50.11, 25.11, 12.58, 6.30, and 3.16 mg/L) for 72 h. The cells without the treatment of compounds were used as the control. After incubation, the viability of cells was examined with cell counting kit-8 assay (CCK-8, Beyotime, China) according to the manufacturer's protocol. The viability was determined by measuring the optical density at 450 nm using a microplate reader (M200, Tecan, Mannedorf, Switzerland).

2.6. CPE and virus titration reduction assays

Virus multiplication and titration assays were performed as described in a previous study (Shen et al., 2018). EPC cells were cultured in 96-well plates (1×10^4 cells/well) for 24 h. Then, the medium was replaced with 100 μL cell maintenance medium containing 1×10^3 $TCID_{50}$ IHNV. After 2 h of infection, the medium was replaced again with maintenance medium containing 3.20 mg/L ribavirin. Each sample was directly observed and photographed under an inverted microscope.

2.7. Fluorescence observation for nucleus damages and microtubule structure

Cells were incubated with 1×10^3 $TCID_{50}$ IHNV and ribavirin-virus mixture for 72 h at 15 °C. Then samples were collected and washed with 0.1 M phosphate buffer saline (PBS) three times. Subsequently, cells were dyed with 1 mg/L DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) and 5 mg/mL DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) for 20 min (Beyotime, China). The cover glass grown cells were mounted on a glass slide and fluorescence was observed with an upright fluorescence microscopy (LeicaDM5000, Germany).

2.8. Ultrastructural analysis on cell membrane surface

The IHNV-infected and compound ribavirin (3.20 mg/L)-treated EPC cells were collected at 72 h post infection (p.i.), and fixed with 2.5% glutaldehyde at 4 °C overnight. Afterwards, each sample was washed with 0.1 M PBS three times, dehydrated in a sequence of ethanol solutions (30%, 50%, 70%, 80%, 90%, 95% and 100%), passaged twice through acetone and replaced and stored with isoamylacetate. After fully dried with a critical point dryer, cells were stuck to the glass slide (5 mm × 5 mm) with polylysine and sputter-coated with gold-palladium. According to the study of Liu et al. (2015), EPC cells were observed by using a field emission scanning electron microscopy (FE-SEM, S-4800, Hitachi, Japan) at 10 kV (Liu et al., 2015).

2.9. Ultracentrifugation assay

Before incubating IHNV with ribavirin *in vitro* and recovering the virion particles by ultracentrifugation, IHNV (1×10^3 $TCID_{50}$) was treated with ribavirin (3.20 mg/L) for 1, 2 and 4 h at 25 °C. After that, each sample was ultracentrifuged at 33,000*g* for 1.5 h at 4 °C and the pellet was resuspended with 5% FBS M199 medium. EPC cells were infected with the above-treated virus, and the related viral protein mRNA were detected after 72 h incubation.

Table 2
List of all the compounds used in the study, their CAS numbers, purities, concentrations and effects on the expression of IHNV glycoprotein. Each value represents mean ± standard error of mean.

Compound	CAS number	Purity (%)	Concentration (mg/L)	Expression of IHNV glycoprotein (%)
1-Adamantanamine	768-94-5	98	25	67.76 ± 1.78
Ribavirin	36791-04-5	98	10	0.12 ± 0.13
Moroxydine hydrochloride	3160-91-6	99	100	109.43 ± 6.24
Acyclovir	59277-89-3	99	50	126.65 ± 1.26
Ganciclovir	82410-32-0	98	25	125.83 ± 2.88
1-Adamantanecarboxylic acid chloride	2094-72-6	98	10	122.25 ± 2.61
Vidarabine	24356-66-9	99	10	73.11 ± 1.45
Guanine	73-40-5	98	25	313.06 ± 11.50
Guanosine hydrate	118-00-3	98	25	43.71 ± 1.41
Cytarabine	147-94-4	98	50	195.11 ± 2.97
Spongouridine	3083-77-0	98	25	79.46 ± 2.92
Oseltamivir phosphate	204255-11-8	98	25	222.26 ± 10.90
Isoprinosine	36703-88-5	98	25	99.05 ± 3.74

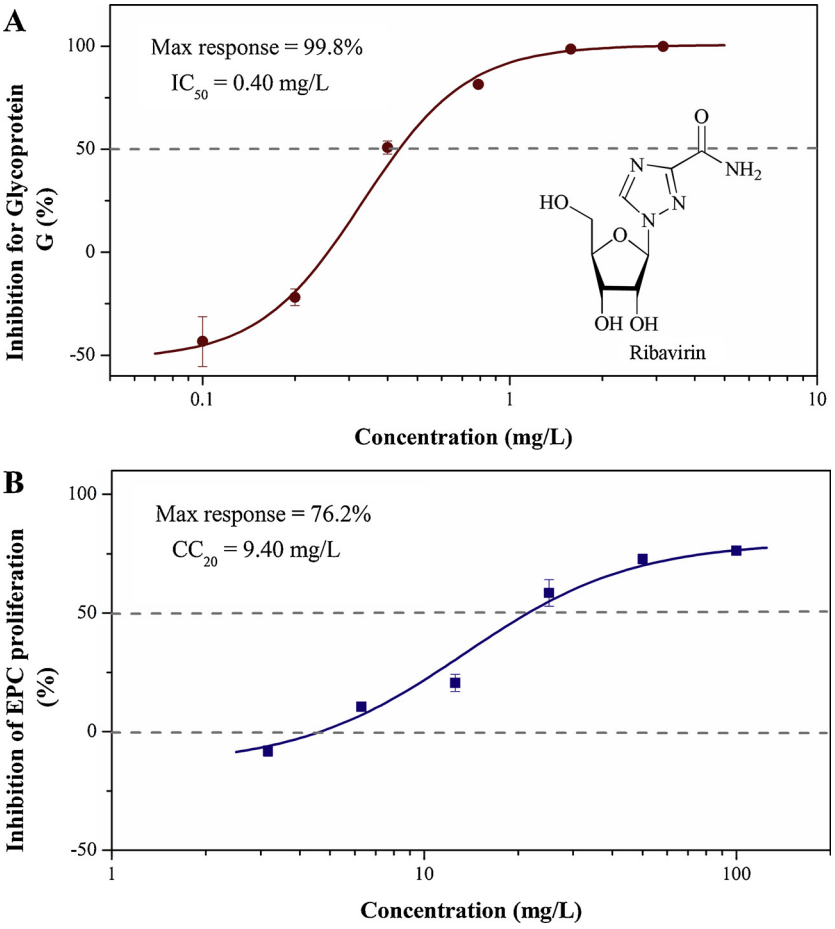

Fig. 1. Confirmatory six-point dose-response curves for compound ribavirin in EPC cells. The percent inhibition of the compound in the IHNV assay is shown in red (A), and the percent cytotoxicity of the compounds on the host cell is shown in blue (B). The maximum percent inhibition observed (Max response) of IHNV and EPC were indicated. Error bar indicates the SD.

2.10. Statistical analysis

Drug response curves were represented by a logistic sigmoidal function with a maximal effect level (Amax) and a Hill coefficient represented the sigmoidal transition, which was performed with Origin 8.1. The data were analyzed by probit analysis which was used for calculating the IC_{50} and CC_{20} of the compound at the 95% confidence interval by using the SPSS 18.0 for Windows (SPSS Inc. an IBM Company). Values were expressed as the mean ± standard deviation (SD) or the mean ± standard error (SEM) and statistical analysis was performed with SPSS 18.0 software (SPSS Inc., USA), using one-way ANOVA after normalization to determine significance. *P* values less than 0.05 were considered statistically significant, **, $p < 0.01$; *, $p < 0.05$.

3. Results and discussion

3.1. Anti-IHNV activities of the 14 compounds

The anti-IHNV activities of the 14 common antiviral agents were presented in Table 2, respectively. Compounds were considered active if the expression of IHNV G was lower than 50%. Surprisingly, among the 14 common antiviral agents tested, ribavirin with an inhibitory percentage of > 90% proved to be the most effective. Regrettably, there was no effective anti-IHNV agent among the other 13 agents.

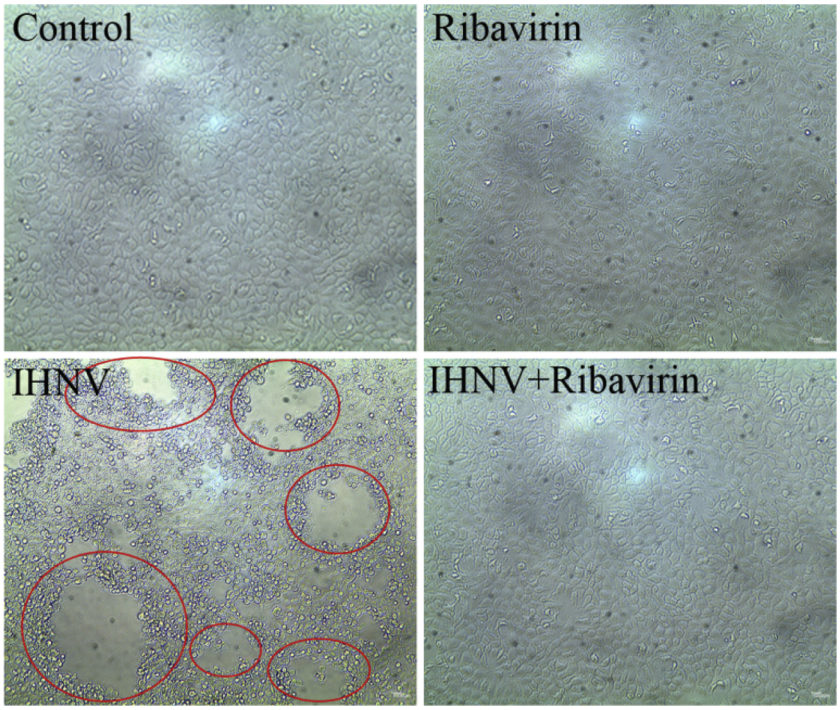

Fig. 2. Morphological protective effect of compound ribavirin against IHNV in EPC cells. CPE was shown in red ovals.

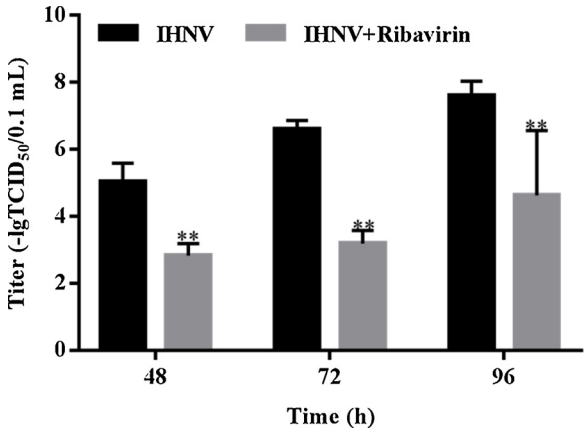

Fig. 3. Compound ribavirin reduced the titers of IHNV in EPC cells. Data were shown as mean ± SD.

3.2. Antiviral activity of ribavirin against IHNV

Due to the high antiviral activity of ribavirin against IHNV, the dose effect of ribavirin on IHNV replication was investigated. As shown in Fig. 1, ribavirin had a concentration-dependent inhibition on the expressions of IHNV G. The 72 h IC_{50} of ribavirin on IHNV G was 0.40 mg/L (Fig. 1A). Additionally, the maximum responses of the inhibition were 99.8% for G under the condition of 3.16 mg/L ribavirin. It should be noted that 3.16 mg/L ribavirin had no significant influence on the viability of EPC cells. In EPC cells, the safe concentration of ribavirin was calculated as 9.40 mg/L (Fig. 1B).

In accordance with gene expressions, CPE of IHNV-infected cells in presence of ribavirin was decreased significantly at 72 h (Fig. 2). Specially, CPE appeared in EPC cells at 72 h post infection with IHNV. The virus-free cells were maintaining the state of normal growth without any CPE. Of particular concern was the finding that ribavirin could block the virus-induced CPE. Similarly, significant inhibition of IHNV was shown in ribavirin-treated EPC cells in the measurement of the viral titer (Fig. 3). IHNV titers were $10^{5.03}$ (48 h p.i.), $10^{6.60}$(72 h p.i.) and $10^{7.60}$ (96 h p.i.) $TCID_{50}$/0.1 mL); whereas IHNV titers were $10^{2.83}$ (48 h p.i.), $10^{3.19}$ (72 h p.i.) and $10^{4.63}$ (96 h p.i.) $TCID_{50}$/0.1 mL in the ribavirin-treated group. The results above indicated that ribavirin could significantly inhibit IHNV replication in EPC cells.

3.3. Effects of ribavirin on IHNV-induced apoptosis

Based on the observation that typical apoptotic features including cellular morphology disappeared, nuclear fragmentation and cytoplasmic degradation in viral-infected cells (Fig. 4), we found the apoptotic features weakened in drug-treated cells where the nucleus remained a normal spindle shape and the quantity of apoptosis body was reduced sharply. These results suggested that treatment with ribavirin can block the occurrence of apoptosis in IHNV-infected cells to some extent.

3.4. Ribavirin had morphologically protective effect on EPC cells

The result of scanning electron demonstrated that the morphology of normal cells was intact, round and plump, and retained spherical shape with a clear edge. Inversely, cells infected with IHNV appeared typical apoptotic features including cell shrinkage, volume reduction and cell blebbing after 72 h infection. By direct inhibitory effect of ribavirin on virions replication, the IHNV-induced morphological change in EPC cells were dramatically blocked, contributing to maintain the normal growth situation and intrinsic morphology (Fig. 5).

3.5. Ribavirin could damage the viral particle of IHNV

Analysis of the above results, we hypothesized that ribavirin might have an effect on the function of IHNV G protein, to against IHNV infection in EPC cells. Thus, we tested whether ribavirin destroyed the infectivity of IHNV virion particles. By incubating IHNV with ribavirin *in vitro* and recovering the virion particles by ultracentrifugation before inoculation of cultures, we clearly demonstrated that incubation of the virus with ribavirin significantly decreased IHNV infectivity in EPC cells (Fig. 6). The results presented in this section suggested that incubation of the virus with ribavirin could damage the viral particle.

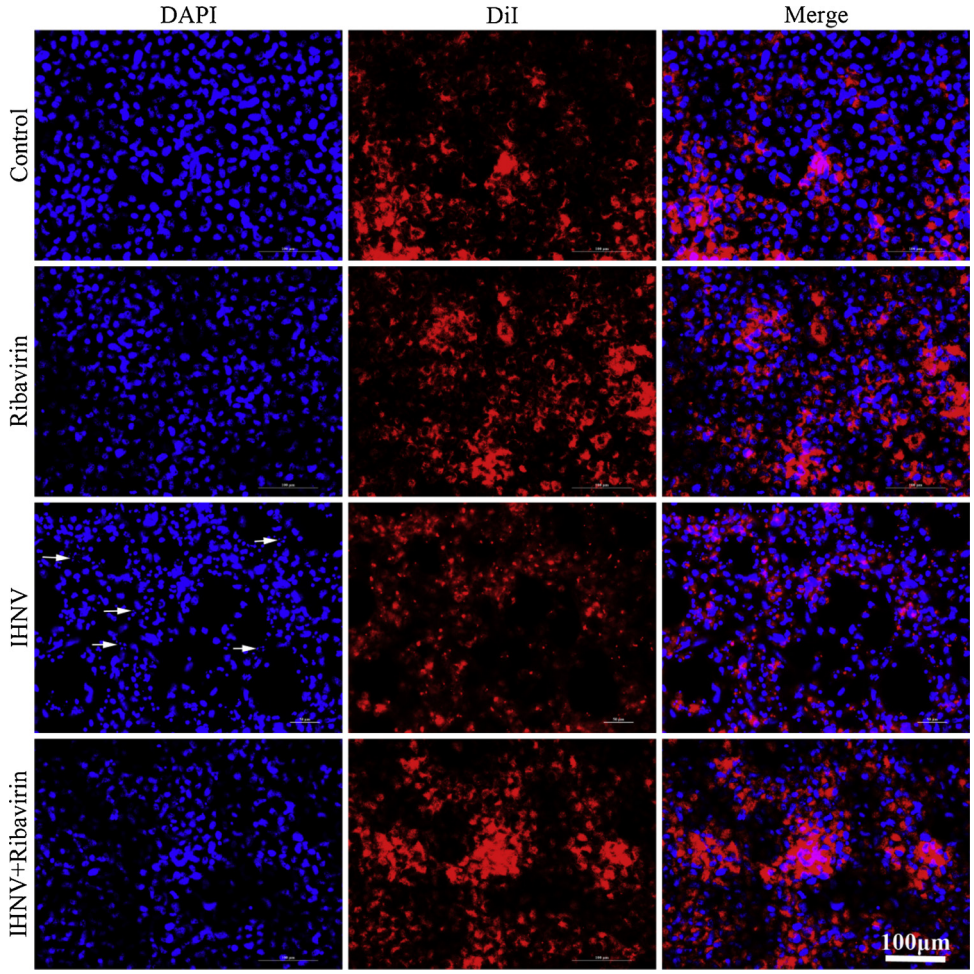

Fig. 4. Fluorescence microscopy images of nucleus damage in EPC cells. The cells were dyed after 72 h p.i. and apoptosis body was detected as arrows indicating.

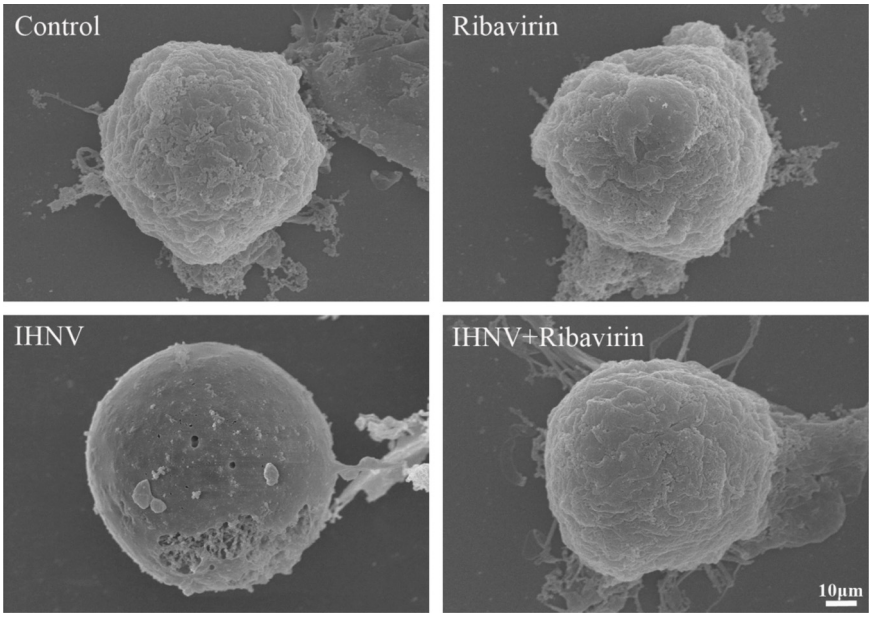

Fig. 5. Scanning electron microscopy images showed the morphology of EPC cells in normal, ribavirin-treated, the morphological changes of cells after IHNV infection, and protective effect of ribavirin in cell morphology after 72 h treatment.

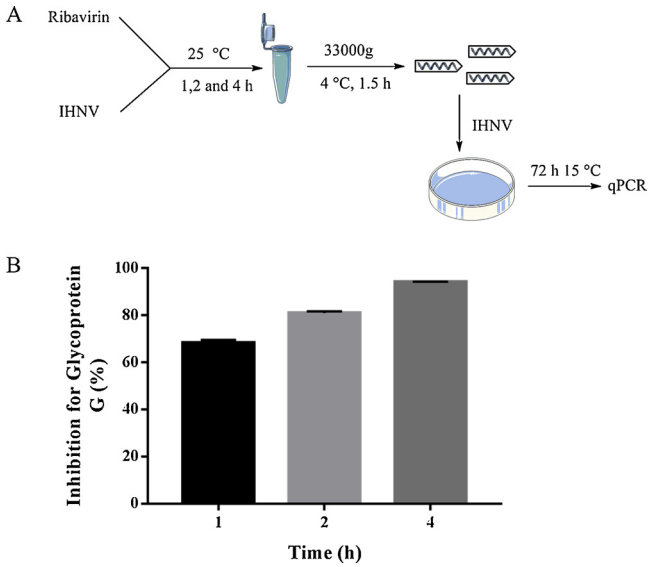

Fig. 6. The infectivity of IHNV was affected by ribavirin. (A) Workflow of the experimental design followed in B. (B) qRT-PCR analysis of G encoding gene. Error bars indicate the SEM.

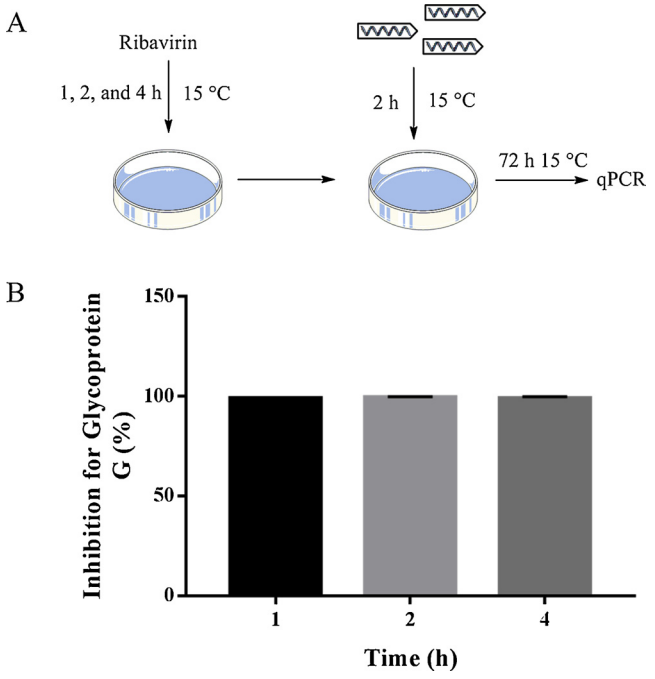

Fig. 7. Ribavirin can be used for prevention of IHNV infection. (A) Workflow of the experimental design followed in B. (B) qRT-PCR analysis of the G protein-encoding gene. Error bars indicate the SD.

Therefore, ribavirin could be a virucidal agent.

3.6. Ribavirin could be used for prevention

To investigate whether ribavirin can be used for prevention of IHNV, we pretreated EPC cells with ribavirin for 1, 2 and 4 h. After treatment with ribavirin, EPC cells were washed with PBS three times and then infected with IHNV (1×10^3 $TCID_{50}$) for 2 h (Fig. 7A). After incubation for 72 h, the G protein mRNA was detected by qRT-PCR. As shown in Fig. 7, pretreatment with ribavirin could inhibit IHNV replication, which suggested that ribavirin can be used for prevention.

Aquatic virus infections directly influence the aquaculture industry and have attracted much attention in recent years because these pathogens cause severe disease in aquatic animals. Consequently, controlling rhabdovirus has become more important in aquaculture, and some studies have made advances in viral characterization (Xu et al., 2014; Ji et al., 2017; Wang et al., 2016; Wu et al., 2017; Ahmadivand et al., 2017; Dixon et al., 2016). However, the IHNV is still difficult to control at present because it is not enough to reduce the high mortality by immunoregulation and vaccine administration when the virus causes severe disease (Ren et al., 2018). Moreover, there was no effective drug that can be used in IHNV controlling currently. The present study for the first time explored the associated molecular mechanisms underlying how ribavirin inhibit IHNV.

Ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carboxamide) is a broad-spectrum antiviral agent with *in vitro* and *in vivo* inhibitory activity against DNA and RNA viruses (Rivas-Aravena et al., 2011). But there is still a gap between the pharmacological function and mechanism of ribavirin on controlling various viruses. The results in this study indicated that ribavirin could effectively inhibit the viral proliferation in host cells at a dose-response manner. Besides, ribavirin could block IHNV induced apoptosis and maintain the normal cell ultrastructure. Mechanistically, ribavirin can affect IHNV infectivity directly and can be used for prevention of IHNV infection. Taken together, the antiviral activity provided the basis for the development and utilization of ribavirin as an anti-IHNV agent in aquaculture.

Notes

The authors declare no competing financial interest.

Acknowledgments

The authors thank Prof. Ling-bing Zeng in Yangtze River Fisheries Research Institute for providing EPC cells and Professor Tong-yan Lu in Heilongjiang River Fishery Research Institute Chinese Academy of Fishery Sciences for providing IHNV strains. This work was supported by the National Natural Science Foundation of China (No. 31772873), the Excellent Young Talents Program of Northwest A&F University (No. 2452018029), and Key Technology Research and Development Program of Shaanxi Province (NO. 2016NY-082).

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