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Carbon nanotube-based nanocarrier loaded with ribavirin against grass carp reovirus

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ABSTRACT

Infectious diseases of viral origin cause major aquatic production losses in different parts of the world. Because of formidable barriers for gastrointestinal tract, skin and cell, large amounts of antiviral drugs have limited therapeutic effect. In the current study, functionalized single-walled carbon nanotubes (SWCNTs) were selected as a drug carrier to carry antiviral drug for the treatment of viral diseases on fish. The results show that increasing antiviral drug (ribavirin) intake was observed by SWCNTs carrier and therapeutic dosage to kill grass carp reovirus is significantly reduced. At 12 d post infection, survival rate and infection rate were 29.7% and 50.4% for naked ribavirin treatment group exposed to the highest concentration (20 mg/L); however, survival rate of 96.6% and infection rate of 9.4% were observed in 5 mg/L ribavirin-SWCNTs treatment group. In addition, the drug detention time in different organs and tissues (blood, gill, liver, muscle, kidney and intestine) was also significantly extended (about 72 h) compared with the same dosage in naked ribavirin treatment group. Moreover, the toxicity of functionalized SWCNTs in grass carp can be minimal, and physiological markers (some antioxidant enzymes activities, apoptotic factors activities and their corresponding genes expression) remained within normal ranges following treatment. Our results indicated that drug delivery with functionalized SWCNTs can improve the antiviral effect on grass carp and has a potential application value to control fish viral diseases in aquaculture.

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1. Introduction

Grass carp reovirus (GCRV), as a large family of double-stranded RNA (dsRNA) viruses, can cause severe hemorrhagic disease in fingerling and yearling populations of grass carp (*Ctenopharyngodon idella*), and result in a mortality rate of up to 85% during an outbreak (Cheng et al., 2008; Xue et al., 2013). To prevent the occurrence of virus disease and reduce the economic losses, lots of measures have been adopted, such as the use of vaccines (Fang et al., 2007; He et al., 2011; Xue et al., 2013), immunostimulants (Li et al., 2013a) and antiviral drugs (Rodríguez Saint-Jean et al., 2013). An inactivated vaccine was applied as the main method to prevent GCRV, but this kind of vaccine has subtype specificity, which limits its application (Xu et al., 1994). In addition, aquatic animal inoculation needs lots of human, material, time and financial resources (Shekhar and Lu, 2009). Large amounts of immunostimulants and antiviral drugs also have limited efficacy to prevent the transmission of virus in the fish farming industry (Ferrara and Reddy, 2006). However, in many cases they had no effect because of several formidable barriers (gastrointestinal tract, skin and cell) (Patton and Platz, 1992). Currently, the emerging field of nanotechnology may play an important role in overcoming these barriers by pharmacological advantages at the nano-scale. Carbon nanotubes (CNTs) as a novel nanomaterial have been used in biomedical research for drug delivery (Endo et al., 2008; Sun et al., 2010) and cancer therapy (Meng et al., 2010; Wu et al., 2012). For cancer therapy, *in vitro* study showed CNTs encapsulation permitted the drugs to be used at a 100 folds lower concentration compared to exogenous treatment yet achieve a comparable 70% cancer kill rate (Wu et al., 2012). Thus, CNTs as drug carrier could be used to break through the biological barriers, and improve drug delivery and therapeutic efficacy.

Ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carbaxaide) is a broad-spectrum antiviral agent with *in vitro* and *in vivo* inhibitory activity against DNA and RNA viruses (Rivas-Aravena et al., 2011). The mechanisms to explain ribavirin antiviral activity have been proposed: direct inhibition of the viral RNA polymerase (Toltzis et al., 1988), inhibition of inosine monophosphate dehydrogenase

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(Parker, 2005), inhibition of the "capping" of the 5′ end of viral mRNAs (Marroquí et al., 2008) and induction of error catastrophe as a result of accumulation of mutations (Graci and Cameron, 2002). Particularly for viral pathogens of fish, ribavirin dramatically affects the infective cycle of some viruses, such as infectious pancreatic necrosis virus (Jashés et al., 1996), viral hemorrhagic septicemia virus (Marroquí et al., 2007), chum salmon reovirus (DeWitte-Orr and Bols, 2007) and infectious hematopoietic necrosis virus (Hudson et al., 1988).

In this study, ribavirin was selected as an antiviral agent to evaluate the antiviral effects on GCRV infection. Grass carp is employed as a model for viral therapy studies because it is susceptible to GCRV (Fang et al., 2007). Using the chemical modification methods, the ribavirin was link to the surface of CNTs and further we investigated the *in vivo* pharmacological of naked ribavirin and ribavirin-CNTs from basic mechanisms to experimental therapeutics. In addition, the acute toxicity of CNTs on grass carp was also examined. Our data provide evidence that ribavirin-CNTs cause a dramatic decrease in GCRV accumulation, making it a plausible candidate for preventing this disease among farmed grass carp.

2. Materials and methods

2.1. Reagents and materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2-(*N*-morpholino) ethanesulfonic acid (MES), *N*-hydro-xysuccinimide (NHS), *N*,*N*-dimethylformamide (DMF), succinic anhydride, *N*,*N*-diisopropylcarbodiimide (DIC) and dimethyl sulfoxide (DMSO) were obtained from Sinopharm Chemical Reagent Co., Ltd. with a purity ⩾99%; bovine serum albumin (BSA) and ribavirin (⩾98%) were purchased from Sigma–Aldrich (Shanghai) Trading Co., Ltd (Shanghai, China). The raw single-walled carbon nanotubes (SWCNTs) were provided by Chengdu Organic Chemicals Co., Ltd. Chinese Academy of Sciences (Chengdu, China). All other chemicals obtained from Sinopharm Chemical Reagent Co., Ltd. with a purity ⩾99%.

2.2. Functionalization of SWCNTs and ribavirin

As show in Fig. 1, the raw SWCNTs were treated by the mixture of concentrated HNO_3/H_2SO_4 (1:3 v/v) at 120 °C for 30 min under reflux with stirring to produce the o-SWCNTs (Pan et al., 2005). The BSA-SWCNTs were prepared by covalently bonding the BSA to o-SWCNTs through the diimide-activated amidation process as previously reported (Li et al., 2010). Briefly, o-SWCNTs samples (0.2 g) were added in 100 mL MES buffer solution (0.1 M, pH = 5.6). After sonicated for 30 min, EDAC (0.6 g) and NHS (0.4 g) were added into the solution and sonicated for 2 h. Then the mixture were centrifuged at 9,000 g for 10 min to remove supernatant liquid, the sediment was washed with 0.1 M MES buffer solution (50 mL × 3 times). After that, the sediments were dispersed in 50 mL PBS solution (phosphate buffered saline, pH = 7.4), and BSA (1.0 g) was added into the solution; then the mixture was sonicated for 2 h and stirred for other 48 h. The resulting mixture was transferred to a cellulose ester dialysis membrane tubing (cut-off molecular weight 100,000) for dialysis against fresh water. Subsequently, the solution was centrifuged (9,000 g, 10 min); the BSA-SWCNTs sample (dark-colored sediment) was obtained. Ribavirin active ester was prepared starting from ribavirin according to reported procedures (Colla et al., 1983). To a solution of the ribavirin (1.7 g) in DMF (10 mL) was added succinic anhydride (1.4 g) and the reaction mixture stirred at 70 °C for 24 h. The solvent was removed under reduced pressure to give the succinyl ribavirin ester. A solution of succinyl ribavirin ester (1.0 g), NHS (0.5 g) and DIC (0.5 mL) in DMF (10 mL) were stirred mechanically at room temperature until esterification complete. The solvent was removed under reduced pressure to obtain the ribavirin succinimide active ester. To a solution of ribavirin succinimide active ester (0.5 g) in DMF (5 mL) was added in 40 mL BSA-SWCNTs borate buffer (5 mg/mL, pH = 8.7). The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was dialyzed against fresh deionized water for 3 d and dried in vacuum at 60 °C to obtain ribavirin-BSA- SWCNTs (R-SWCNTs).

2.3. Experimental fish and virus

Grass carps were kindly provided by a fish farm in Heyang (Shannxi, China) and acclimatized in the laboratory for two weeks before experimental manipulation. Fish were maintained at 28 °C in aerated water and fed daily with commercial dry feed pellets (Hello Fish Dry Pellets; Beijing, China). Possible virus contamination in fish and feed was evaluated by reverse transcription quantitative real-time PCR (RT-qPCR) to confirm they were free from GCRV (Zhang et al., 2010). Grass carp without any physical deformities, swimming behavior abnormities or clinical signs were used in the present study. The GCRV strain used as a challenge

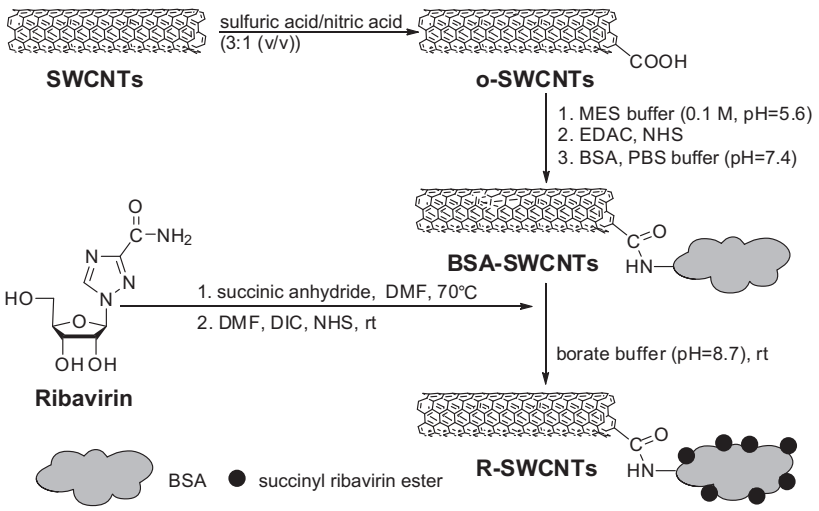

Fig. 1. The schematic procedure of the functionalization of SWCNTs and ribavirin.

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