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The protective immunity against grass carp reovirus in grass carp induced by a DNA vaccination using single-walled carbon nanotubes as delivery vehicles

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ABSTRACT

To reduce the lethal hemorrhagic disease caused by grass carp reovirus (GCRV) and improve the production of grass carp, efficient and economic prophylactic measure against GCRV is the most pressing desired for the grass carp farming industry. In this work, a novel SWCNTs-pEGFP-*vp5* DNA vaccine linked *vp5* recombinant in the form of plasmid pEGFP-*vp5* and ammonium-functionalized SWCNTs by a chemical modification method was prepared to enhance the efficacy of a *vp5* DNA vaccine against GCRV in juvenile grass carp. After intramuscular injection (1, 2.5 and 5 μg) and bath administration (1, 10, and 20 mg/L), the ability of the different immune treatments to induce transgene expression was analyzed. The results showed that higher levels of transcription and expression of *vp5* gene could be detected in muscle tissues of grass carp in SWCNTs-pEGFP-*vp5* treatment groups compare with naked pEGFP-*vp5* treatment groups. Moreover, antibody levels, immune-related genes, and relative percentage survival were significantly enhanced in fish immunized with SWCNTs-pEGFP-*vp5* vaccine. In addition, we found that a good immune protective effect was observed in bath immunization group; which at a concentration of 20 mg/L could reach the similar relative percentage survival (approximately 100%) in injection group at a dose of 5 μg. All these results indicated that ammonium-functionalized SWCNTs could provide extensive application prospect to aquatic vaccine and might be used to vaccinate fish by intramuscular injection or bath administration method.

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1. Introduction

Grass carp reovirus (GCRV) which causes lethal hemorrhagic disease with a mortality rate of 85% in fingerling populations of grass carp (*Ctenopharyngon idellus*) is presently one of the most serious pathogens harming the grass carp industry in China [1,2]. The other side, grass carp as an economically important freshwater fish is cultivated widely in more than 40 countries [3] and its global production is more than 4.3 million tonnes, where of China produces 4.2 million tonnes in 2012 [4].

To prevent the hemorrhagic disease caused by GCRV and improve the production of grass carp, an effective vaccine against GCRV is the most pressing desire for the grass carp farming industry. Until recently, an inactivated GCRV vaccine is the only commercial vaccine in Asia [5]. In China, traditional methods including attenuation of wild-type viruses to generate live vaccines and formalin-inactivation to produce dead vaccines are still put into use to develop effective preventive strategy against GCRV [6]. Because of the unpopular use of traditional vaccines at present, further improvement of vaccines in terms of efficacy, safety, manipulation method, and manufacturing cost is an urgently need for the prevention of grass carp hemorrhagic disease caused by GCRV.

The DNA vaccine (delivered intramuscularly) consisting of naked plasmid DNA that will result in expression of pathogenic proteins in the muscle tissue of the vaccinated fish is currently one of the most promising vaccine preparations against fish diseases [7]. The first demonstration of the efficacy of a DNA vaccine in fish was rainbow trout immunized against infectious hematopoietic

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necrosis virus [8]. GCRV differed from orthoreovirus in a number of characteristics such as absence of an antigenic relationship and unequal numbers of genome segments contains the core composed of five proteins: VP1, VP2, VP3, VP4, VP6 and the outer capsid composed of 200 trimers of VP5-VP7 heterodimers [9]. In previous reports, polyclonal antibodies against μ1 and σ3 were found to be able to neutralize Mammalian reovirus *in vitro* [10]. Moreover, VP7 only shows a very low sequence identity of 12% with its counterpart σ3 of Mammalian reovirus, while the sequence identity between VP5 and μ1 is 2% [11]. In addition, the viral VP5 protein was involved in viral infection and bacterially-expressed VP5 could be suitable for developing subunit vaccine for the control of GCRV infection [12]. Thus, VP5 was selected to test its DNA vaccine potential against GCRV in this work.

Until now, intramuscular injection is considered as a prophylactic manner for DNA vaccine implementing. However, intramuscular injection of naked DNA vaccine generally induces small and transient immune protection effect in fish [13]. What is more, there are some limitations in this method. Not only is the process of anesthetizing, handling and injecting stressful for the fish, but it is also labor intensive and costly for the farmer [14]. Importantly, it is not adaptive to inject small fish less than 20 g, which are often the most susceptible to disease, in large numbers [15]. Therefore, bath immunization becomes an attractive measure to prevent the disease, which provides the convenience for large-scale vaccination and the advantage of few stress caused by handling to fish [16]. However, the efficacy of bath immunization is not ideal for several barriers of piscine gill and skin epithelium [17]. In a word, it is necessary to develop an effective carrier and a novel measure for DNA vaccines.

Within the family of emerging nanomaterials, carbon nanotubes (CNTs) display very novel physicochemical characteristics and have been explored for many practical use such as biology, physics, chemistry and medicine [18]. CNTs are potentially useful in their exploitation as scaffolds for subunit vaccine compositions with these features including stability *in vivo*, low toxicity, lack of intrinsic immunogenicity and the ability to be appended with multiple copies of antigens [19]. In the field of medicine, functionalized SWCNTs support a lot of promise for transporting bioactive molecules such as genes, subunit vaccines, drugs and other therapeutic agents [20]. In previous study, ammonium-functionalized single-walled carbon nanotubes (SWCNT-NH_3^+):DNA complexes are capable of transfecting cells *in vitro* and the large surface area of the SWNT-NH_3^+ allow more DNA to tightly associate [21]. Similarly, a study using functionalized SWCNTs as delivery vectors showed that the expression of gene reached 10-fold higher levels than the plasmid DNA delivered alone [22]. Moreover, functionalized SWCNTs were a promising carrier to enhance the immune response of recombinant vaccines, and might be used to vaccinate juvenile fish by intramuscular measure or bath administration method [23,24]. Therefore, using the functionalized SWCNTs as vaccine delivery vehicle to pass through the cell barriers will greatly induce a better protective immune response in fish. The functionalized SWCNTs as a carrier for DNA vaccine can serve as an adjuvant and has a positive impact on the DNA vaccine, but, safety is first priority to be considered when preparing a subunit vaccine on grass carp. The potential health risks of SWCNTs and their impact on the aquatic environment should be evaluated. SWCNTs can pass through the membranes of the gills and/or intestine and enter the circulation. Then, nanoparticles shifted to the liver and intestine of larvae and were eliminated in the feces [25]. Previous studies have reported that the chorion of zebrafish embryo was an effective protective barrier to SWCNT, because the size of the pores on the embryo chorion was nanoscaled [26]. Moreover, in our previous study, functionalized SWCNTs have no toxicity on *G. rarus* embryos due to the protective effect of embryo chorion and have the potential to affect aquatic life when released into the aquatic environment and reached high concentration. SWCNTs able to enter and well dispersed in newly hatched larvae which are more sensitive than the embryo or adult stage, eventually cleared from the body. Newly hatched larvae exposed to functionalized SWCNTs yielded various morphological malformations, had decreased heart rate and body length, altered swimming speed and increased cellular death in a concentration-dependent pattern [27]. In addition, Templeton et al. [28] demonstrated that copepods ingesting purified SWCNTs showed no significant effects on mortality, development and reproduction across exposure. The authors suggested that the toxic effects on copepods were likely induced by the Co and Ni catalysts used in the production of SWCNTs.

In this study, a novel SWNTS-pEGFP-*vp5* DNA vaccine linked *vp5* recombinant in the form of plasmid pEGFP-*vp5* and ammonium-functionalized SWCNTs by a chemical modification method was prepared to enhance the efficacy of a *vp5* DNA vaccine against GCRV in grass carp. We evaluated the immune response elicited by different forms of the DNA vaccine and investigated the efficacy in vaccinated fish after intramuscular injection and bath implementation. This work will provide a good guide for the use of SWCNTS-DNA vaccine delivery systems in fish farming industry in the future.

2. Materials and methods

2.1. Experimental fish and virus

Healthy grass carps in developmental stage (3.5 ± 0.4 cm in body length and 1.1 ± 0.2 g in body weight) were purchased by a fish farm (hanzhong, China) and acclimatized in the laboratory for one month before experiment. They were maintained in a quarantine area by maintenance in 300 L aerated aquaria at 27 ± 1 °C and fed twice daily with a diet of commercial dry pellets (Wuhan Tianlong Feed Company, Wuhan, China) at 5% of their body weight per day. To confirm fish were free from GCRV, possible GCRV pollution in fish and feed was evaluated by reverse transcription quantitative real-time PCR (RT-qPCR) [29]. Prior to experiments, fish were narcotized with tricaine methanesulfonate (Sigma, USA) involving injection. The GCRV strain (097 strain) used as a challenge pathogen in this work was isolated from the infected grass carp in fish farm located in Rougu (Shaanxi, China) and stored in our laboratory [30]. The enrich method and the 50% tissue culture infective doses ($TCID_{50}$) of the virus were performed according to the previous report and established protocols [31,19]. Briefly, energetic grass carp (10 g in body weight) formerly free of GCRV were intraperitoneally injected with 100 μL of GCRV (3.63×10^7 $TCID_{50}$/mL), suspended in PBS, per gram body weight. All the fish were recorded activity and mortality every hour and the fish infected virus in 6 h–7 d post-challenge were collected until the termination of the experiment. The enrichment of GCRV was resort to homogenate of fish infected virus with 0.6% physiological saline, filtration and centrifugation, obtaining the GCRV supernate. Animal administration was in accordance with the guidelines of the Animal Experiment Committee, Northwest A&F University [24].

2.2. Construction of expression cassette

pEGFP-c1 (Clontech, USA) with cytomegalovirus (CMV) immediate early promoter driven the expression of the cloned gene was used as the original plasmid. The full-length *vp5* gene was amplified through PCR using primer pairs consisting of GCRV-F (5′-CGCGCTAGCATGTGGAACGTTCAAACCT-3′ the underline indicates *Nhe* I site) and GCRV-R (5-CCGCTCGAGTCACTTGCCGGGCCACAAGCTC-3′

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