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Short communication

Immersion vaccination of Mandarin fish *Siniperca chuatsi* against infectious spleen and kidney necrosis virus with a SWCNTs-based subunit vaccine

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ABSTRACT

Infectious spleen and kidney necrosis virus (ISKNV) cause a high mortality disease which lead to significant economic loss on mandarin fish in China. There is no effective drug or vaccine against this fatal disease at present. Meanwhile, many drugs and vaccines had no effect in many cases account of several impenetrable barriers (cell, skin and gastrointestinal tract). Here we reported an immersion subunit vaccine system (SWCNTs-MCP) encoding *MCP* gene of ISKNV based on single-walled carbon nanotubes (SWCNTs). To evaluate its efficacy against ISKNV, we found a stronger and longer duration immune response (serum antibody production, enzyme activities and immune-related genes expression) can be induced in fish vaccinated with SWCNTs-MCP in comparison with those vaccinated with MCP alone. Importantly, SWCNTs can increase the immune protective effect of naked subunit vaccine by ca. 23.8%. Thereby, this study demonstrates that SWCNTs as a promising carrier for subunit vaccine might be used to vaccinate large-scale juvenile mandarin fish by bath administration approach.

1. Introduction

As the commercially and common breeding industry that there is a large farming production in China, mandarin fish (*Siniperca chuatsi*) are kinds of delicious food [1]. However, with the expansion of mandarin fish farming industry, different pathogenic microorganisms have resulted in frequent outbreaks of disease, which bring substantial economic losses to the aquaculture industry [2]. Infectious spleen and kidney necrosis (ISKN) which caused by Infectious spleen and kidney necrosis virus (ISKNV) is an environmentally and economically important disease affecting more than 50 marine and freshwater fish species, primarily mandarin fish. Due to the highly infectious nature of ISKNV and its potential impact on susceptible fish populations globally, now ISKNV has been listed by the Office International Des Epizooties (OIE) [3–5].

Vaccine is widely accepted as an effective control against viral diseases [6]. Li et al. found that protection against ISKNV infection by vaccinated with vaccine encoding a kind of effective antigen (ORF093) have been verified, with the relative percentage survival range from 47% to 63%. Fu et al. prepared the MCP DNA vaccine to immune mandarin fish by injection in combination with QCDC adjuvant with the relative percentage survival (RPS) of 80%. Pan et al. prepared the cell inactivated vaccine to immune mandarin fish by injection with the RPS over 90% both in laboratory and field [7–10]. As the key structural component of the iridovirus particles comprising 40–45% of the total particle poly-peptide, the major capsid protein (MCP) has been used for vaccine construction against ISKNV, however, the protection induced by MCP-based vaccine is limited, with the highest protection rate of 64.3% at the injection dose of 50 mg/fish [8]. To achieve a relatively good immune effect through immersion immunization, an effective vaccine vector can be used as an effective way to enhance the effects of vaccine.

As a novel nanomaterials, carbon nanotubes (CNTs) possess mainly three features: (i) stability *in vivo* and low toxicity, (ii) lacking intrinsic immunogenicity, (iii) antigen load carrying capacity, which can be used as a candidate carrier of vaccine delivery [11,12]. Previous studies demonstrated that functionalized CNTs can cross cell membranes and delivery cargos such as vaccine into cell [13,14]. Currently studies have verified that CNTs as the vaccine carrier can elicited the immunoprotective effect in vaccinated fish, full protection were obtained by using single-walled carbon nanotubes as a vaccine carrier against grass carp reovirus [15–17], furthermore, single-walled carbon

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Table 1
Primers used for the analysis of mRNA expression by RT-qPCR.

Genes	Accession no.	Primer sequences (from 5′ to 3′)	Product size (bp)	
MCP-F/R	KC775382	Forward	CGGAATTCATGTCTGCAATCTCAGGT	1362
		Reverse	GACGGATCCTTACAGGATAGGGAAGCCTG	
β-actin	AY885683	Forward	ATTTCCGACACGGAGAGG	199
		Reverse	CATGGGTTTAGGATACGCTC	
IgM	AF327363.1	Forward	GGATTTTGTGGGAGGACGA	266
		Reverse	AAGTCAGGGTCACCGTTTCT	
IRF-1	JN859505.1	Forward	CAGAGCAGCAAGATTTCCCATT	167
		Reverse	CCCCGATCTCAACTGACAAAG	
Mx	AY392097	Forward	TGTTCCAGTGTCTGCTGATTGA	345
		Reverse	GACAGTCCAGGAAACGCATATA	
IL-1β	AY647430.1	Forward	GATGGTCCCGAAGCAACCTT	201
		Reverse	TCTCCAGGCACATTTCCACC	
IL-8	JQ513375.1	Forward	TGCGAGGAGACTGAGATCATTG	297
		Reverse	CAGCGTCTTTTGTTGGACATGA	
TNF-α	AJ311800.2	Forward	TGTGCCGCCGCTGTCTGCTTCACGCT	291
		Reverse	GATGAGGAAAGACACCTGGCTGTAGA	
ISKNV-MCP	HQ317465.1	Forward	TGCCCATTCCCCTCTTC	192
		Reverse	CGCTCCTCGCTTGTCAGTA	
MCP-F/R	HQ317465.1	Forward	CGGGATCCATGTCTGCAATCTCAGGT	1362
		Reverse	GACCTCGAGTTACAGGATAGGGAAGCCTG	
ORF086	NC_003494.1	Forward	CAAGCTGGCCGTCGAGTACA	115
		Reverse	CTGAGCCTTTATCACATCCCT	

The underlined nucleotides indicated cutting sites for restriction enzyme *BamH* I and *Xho* I.

nanotubes as delivery vehicles can enhance the immunoprotective effects of a recombinant vaccine against *Aeromonas hydrophila* with the highest relative percentage survival of 80% [18].

In this work, on the basis of hydroxyl and amino condensation reactions, functionalized single-walled CNTs (o-SWCNTs) as carriers were used to load immersion subunit vaccine encoding MCP protein of ISKNV. Mandarin fishes were immunized by MCP/SWCNTs-MCP subunit vaccine via bath administration. Furthermore, we evaluated the immune response elicited in vaccinated fish. The study reported herein provides a helpful reference for the use of SWCNTs-DNA vaccine delivery systems in fish farming industry.

2. Material and methods

2.1. Virus, cell line and experimental fish

Infectious spleen and kidney necrosis virus isolate (ISKNV-QY) and Chinese perch brain (CPB) cells were kindly provided by Professor Xiao-Zhe Fu (Pearl River Fisheries Research Institute, Guangdong, China). ISKNV-QY was propagated in CPB cells at 28 °C in medium 199 (Invitrogen, Carlsbad, CA), pH 7.0, plus 10% fetal calf serum. Virus titers for challenge were determined as the established protocols [8].

Juvenile mandarin fish (*S. chuatsi*) weighing 6.0 ± 0.5 g were purchased from a local farm in Guangzhou city. Fishes were maintained in tanks with sufficient aeration in flow-through system. Mud carps were used to feed mandarin fish twice daily. All of the experimental animals were handled according to the guidelines of the Animal Experiment Committee, Northwest A&F University.

2.2. Construction of recombinant pE-SUMO-MCP plasmid

The pE-SUMO (Clontech, USA) was used as the original plasmid. A primer pairs named MCP-F (the underline indicates *EcoRI* site) and MCP-R (the underline indicates *BamHI* site) were used to amplify ISKNV *MCP* gene, the optimum conditions for PCR were as follows: 94 °C for 30 min, 30 cycles at 94 °C for 30 s, 56 °C for 50 s 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The purified PCR product of *MCP* gene was digested with *EcoRI* and *BamHI* and inserted into pE-SUMO to generate recombinant pE-SUMO-MCP plasmid. PCR amplification, restriction enzyme digestion and DNA sequencing were used to verify the construction of pE-SUMO-MCP plasmid.

2.3. Expression and antiserum preparation of recombinant MCP proteins

Isopropyl-β-D-thiogalacto-pyranoside (IPTG, Sigma-Aldrich Trading Co., Ltd, Shanghai, China) was used to induce the expression of recombinant MCP protein. The empty pE-SUMO was used as the control fusion protein. The fusion protein was analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamid gel electrophoresis) following the previously method [17]. Then the recombinant proteins were assessed by western blotting analysis. The antibodies were used the diluted anti-His-tag monoclonal antibody (abcom, Cambridge, MA, USA) (1:1500) and HRP-conjugated goat anti-mouse IgG (1:1000) (Beijing CoWin Biotech Corp., Beijing, China). DAB horseradish peroxidase color development Kit (Qiagen, Hilden, Germany) were used to visualize the result. The recombinant MCP protein with the hexa-histiding tag at the N-terminus was purified by using Ni-NTA agarose (Qiagen, Shanghai, China) which we purified it according to the method provided by the manufacturer and then determined by Micro BCA Protein Assay Kit (Beijing CoWin Biotech Corp., Beijing, China).

2.4. Preparation of SWCNTs-MCP subunit vaccine

The raw SWCNTs (95% purity black powder, 1–2 nm in outside diameter, 0.4–3 μm in length) were purchased from Chengdu Organic Chemicals Co., Ltd. Chinese Academy of Science (Chengdu, China). The preparation of SWCNTs-MCP subunit vaccine was according to our previous study [17]. Briefly, the SWCNTs were dispersed in mixture of conc. H_2SO_4 and conc. HNO_3 (3:1 v/v) under reflux with stirring at room temperature for 36 h to generate the oxidized SWCNTs (o-SWCNTs). The MCP protein combined with the o-SWCNTs were carried out through the amidation reactions, briefly, 5 g o-SWCNTs, 1-Ethyl-3-(3-dimenthylaminopropyl) carbodiimide hydrochloride (9 g) and N-hydro-xysuccinimide (10.3 g) were dispersed in 2000 mL 2-(N-morpholino) ethanesulfonic acid buffer solution (0.1 M,pH = 5.6) for 3 h. Meanwhile, after the vacuum freeze-dried MCP protein (7.0 g) sonicated in the 500 mL PBS solution (phosphate buffered saline, pH = 7.4) for 3 h, the mixture were stirred for other 48 h. Then the treated o-SWCNTs and MCP protein were mixed and stirred for 36 h (in shade). Subsequently, after centrifugation and freeze-drying the SWCNTs-MCP powder was obtained.

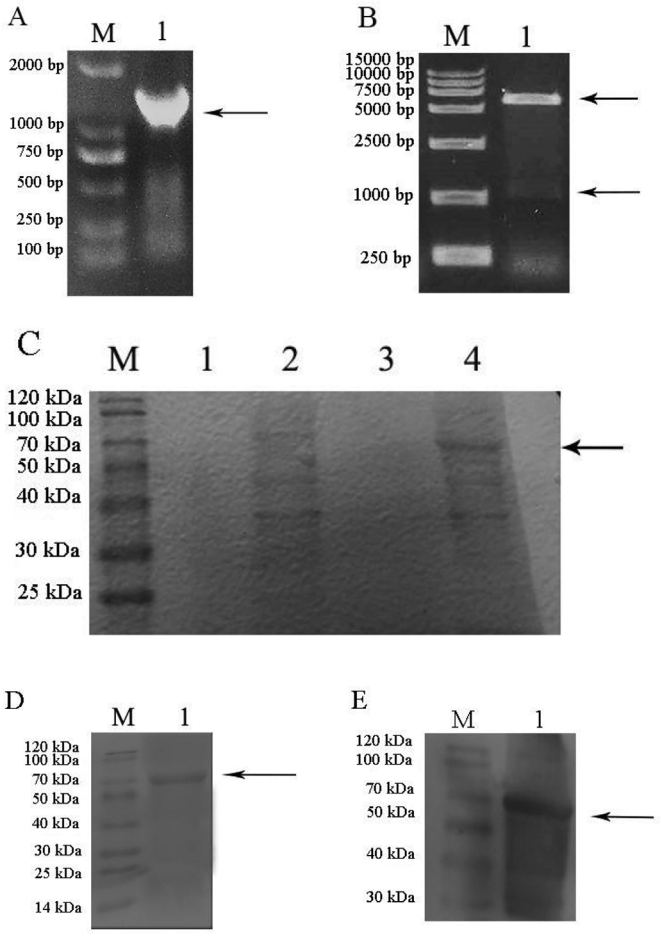

Fig. 1. Aanalysis of MCP expression. (A) RT-PCR amplification of MCP: lane M, DNA marker; lane 1, MCP. (B) Analysis of recombinant plasmid pE-MCP: lane M, DNA marker; lane 1, double enzymes digested pE-MCP with BamH I and EcoR I. (C) SDS-PAGE analysis of expressed pE-MCP: lane M, standard protein marker; lane 1, soluble lysates from E. coli BL21 strain transformed with pE-MCP un-induced; lane 2, insoluble lysates from E. coli BL21 strain transformed with pE-MCP un-induced; lane 3, soluble lysates from E. coli BL21 strain transformed with pE-MCP induced by IPTG; lane 4, insoluble lysates from E. coli BL21 strain transformed with pE-MCP induced by IPTG. (D) The purified recombinant MCP protein with SDS-PAGE analysis: lane M, standard protein marker; lane 1, the purified recombinant MCP protein. (E) Western blotting analysis of MCP protein with anti-his-tag monoclonal antibody: lane M, standard protein marker; lane 1, MCP protein.

2.5. Immunization and challenge experiment

During the vaccination, the rearing water temperature was kept at 25 ± 0.5 °C. The vaccinated fish were fed with mud carp daily. Before experiments, the kidney and spleen of fish were randomly sampled for the detection of the ISKNV by qPCR to make sure that they were free from ISKNV [4]. Disease-free mandarin fishes were randomly separated into eleven groups (50 fish per serving), including control group (PBS), SWCNTs group (20 mg/L SWCNTs alone) and ten vaccinate groups (MCP groups and SWCNTs-MCP groups containing five concentrations (1.25, 2.5, 5, 10 and 20 mg/L), respectively). All of the fish were vaccinated via bath administration for 10 h. Subsequently, the vaccinated fish were transferred to different tanks and monitored daily.

On the 28st day post-vaccination, fish in vaccinated, SWCNTs group and control groups (each group, n = 25) were transferred to new tanks and challenged by intraperitoneal injection with 50 μL 3.98 $\times 10^6$ $TCID_{50}$/mL of live ISKNV in saline buffer, and the relative percentage survival (RPS) were recorded daily for 14 days after viral challenge. Dead fish were collected and recorded daily. Moreover, PCR assay was used to confirm ISKNV infection in challenged fish, primers (ORF086) used for viral detection can be found in Table 1.

Relative percentage survival (RPS) = 1-[% mortality rate (vaccinated fish)/% mortality rate (control fish)] × 100.

2.6. DNA extraction, RNA isolation, cDNA synthesis and qPCR assays

DNA extraction from spleen (5 fish per group) was performed as described previously. Briefly, spleen tissues (0.5–1.5 cm^3) were taken from the mandarin fish at 14 days after ISKNV challenge, then samples were pulverized together to powder in liquid nitrogen, and dissolved in 3 mL genomic DNA isolation buffer (1.0% sodium dodecyl sulfate, 100 mM NaCl, 50 mM Tris HCl, 100 mM EDTA, pH 8.0, 20 μg/mL RNase). After incubated for 1 h at 37 °C, proteinase K was added to a concentration of 150 μg/mL and the samples were incubated at 60 °C overnight. DNA was extracted by conventional phenol-chloroform procedures.

To analyze the immune-related genes expression, total RNAs were obtained from the spleen tissues in each group (5 fish per group) at 1, 7, 14, 21 and 28 days after vaccination with TRIzol reagent. HiScript Q Select RT SuperMix for aPCR (+gDNA wiper) (Vazyme, China) was performed to reverse transcribed the purified RNA into cDNA.

Quantitative real-time PCR (qRT-PCR) was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) with the following procedure: 95 °C for 5 min and 40 cycles at 95 °C denaturation for 15 s, followed by 60 °C annealing for 60 s. The extracted DNA was used as template for RT-PCR amplification with specific primers SM-F/R. The *β-actin* was used as an internal control (Table 1). All qRT-PCR reactions were performed for three biological replicates and repeated with two independent samples. Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method with the formula, $F = 2^{-\triangle\triangle Ct}$, $\triangle\triangle C_t = (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}}) - (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}})_{\text{control}}$ [19].

2.7. Measurement of antibody level

The titers of the antibodies were measured by ELISA (Enzyme-linked immunosorbent assay) as described elsewhere [17–19]. For analyses of the presence of specific, neutralizing antibodies, vaccinated, SWCNTs and control fish (5 fish per group) were sampled weekly until 4 weeks for antibody determination. Serum samples preparation and determination were according to previous method [18]. Briefly, the blood collected from the caudal vein of mandarin fish was placed overnight at 4 °C and then centrifugated at 5000 × g for 15 min. The supernatant was collected and stored at −20 °C until use. Purified recombinant MCP protein was used as antigen. Fish serum samples was used as antibody. Anti-His-tag monoclonal antibody (abcom, Cambridge, MA, USA) was used as primary antibody, HRP-conjugated goat anti-rabbit IgG (Beijing CoWin Biotech Corp., Beijing, China) was used as secondary antibody. The primary and secondary antibodies were diluted at 1:1500 and 1:1000, respectively. Followed by color development using tetramethylbenzidine, TMB (Tiangen Biotech, Beijing, China) was used as colorimetric substrate. The plate was read at 450 nm by using a precision microplate reader (Molecular Devices Corp., Palo Alto, CA). The antibody response was expected in terms of O.D.

2.8. Statistical analysis

All data were analyzed using SPSS 22 Software (IBM, USA), one-way ANOVA were used to analyzed after normalization. For antibody titers, DNA detection, and immune-related genes expression were analyzed with two-tailed student's t-test; the relative percentage survival were transformed to square-root arcsine values before performing the differences test with SPSS statistical software. The differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$.

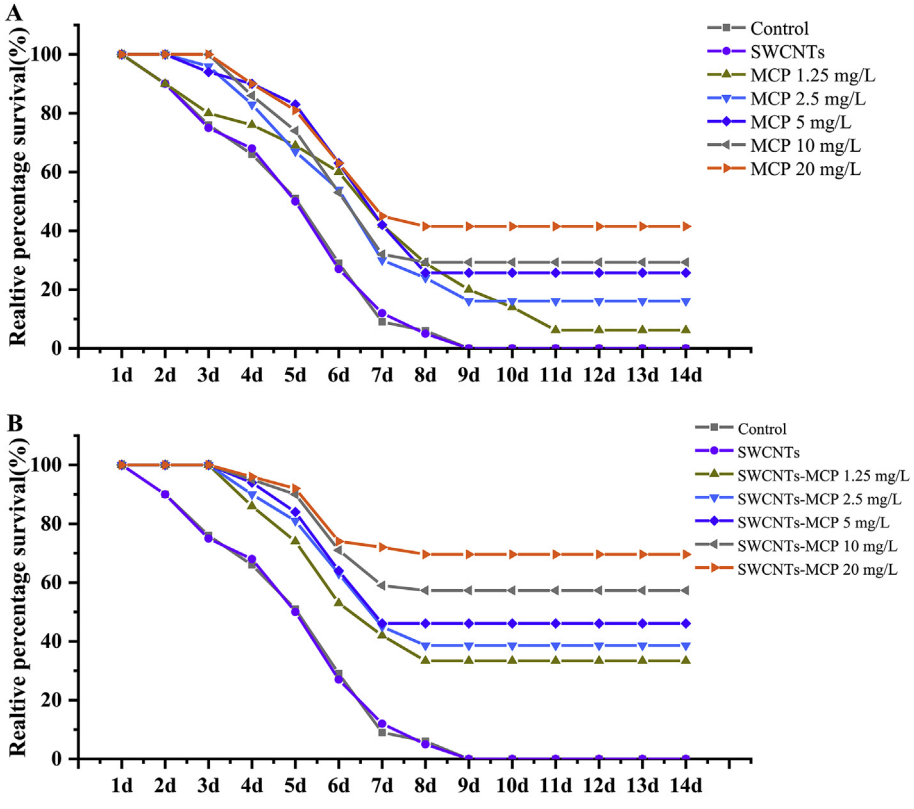

Fig.2

Fig. 2. The relative percentage survival of vaccinated fish. Mandarin fish were vaccinated with MCP, SWCNTs-MCP, and control group challenged with ISKNV. The relative percentage survivals were calculated at the end of the monitored period.

Table 2
Relative percentage survival (RPS) of fish challenged with ISKNV.

Fish injected	RPS
Control	0%
SWCNTs	0%
MCP 1.25 mg/L	6.2%
MCP 2.5 mg/L	16.1%
MCP 5 mg/L	25.7%
MCP 10 mg/L	29.3%
MCP 20 mg/L	41.5%
SWCNTs-MCP 1.25 mg/L	33.4%
SWCNTs-MCP 2.5 mg/L	38.6%
SWCNTs-MCP 5 mg/L	46.1%
SWCNTs-MCP 10 mg/L	57.3%
SWCNTs-MCP 20 mg/L	69.6%

3. Results

3.1. Expression and characterization of recombinant MCP

The *MCP* gene (size in 1362 bp) was gained by PCR amplification with primer pair as shown in Fig. 1A. Restriction enzyme digestion (Fig. 1B) and sequence analysis (date not shown) were used to confirm the construction of recombinant plasmid pE-SUMO-MCP. Fig. 1C showed that an appropriate-sized protein (about 70 KDa) was expressed in *E.coli* when induced by IPTG. The purified recombinant MCP protein was showed in Fig. 1D with SDS-PAGE. Furthermore, Western blot using monoclonal mouse anti-his-tag antiserum confirmed that the recombinant protein is a 70 KDa his-tag fusion protein, which was the molecular weight expected (Fig. 1E). After the recombinant MCP protein was purified, the recombinant MCP was scanned by gel image analysis system to calculate the percentage of MCP in total whole protein, and the recombinant MCP accounted for about 72% of all proteins in the solution.

3.2. Protection of vaccinated fish

During 14 days after challenge, the relative percentage survivals (RPS) were analyzed (Fig. 2). Vaccinated groups (MCP and SWCNTs-MCP groups) showed a significant improved survival compared with control groups. And the results showed that there was no significant difference in SWCNTs group compare with the control group. What's more, the RPS in SWCNTs-MCP groups via bath immunization were higher than that in MCP groups, the protective efficacy of SWCNTs-MCP vaccinated group (20 mg/L) was the highest, with the RPS value of 69.6% (Table 2). Furthermore, Table 2 showed that SWCNTs as a promising vehicle can enhance ca. 28.1% of the RPS in SWCNTs-MCP vaccinated fish compared with naked MCP immersed fish. RT-PCR was used to detect ISKNV infection in challenged fish with the primers of ORF086 (data not shown). Typical clinical symptoms of ISKNV infection can be observed in the dead fish, besides, no pathogen other than ISKNV was detected.

3.3. Virus loads reduction in vaccinated fish

QRT-PCR was used to analyze the viral clearance in vaccinated fish on days 14 after challenge, vaccinated fish after ISKNV challenge (5 fish per group) were randomly sampled to collect spleen. QRT-PCR assay was performed with primers of ISKNV *MCP* gene (ISKNV-MCP), the primers can be found in Table .1. Results (Fig. 3) showed that ISKNV could be effectively and gradually cleared from the spleen tissues of fish after challenge. No significant difference was observed between the SWCNTs group and the control group.

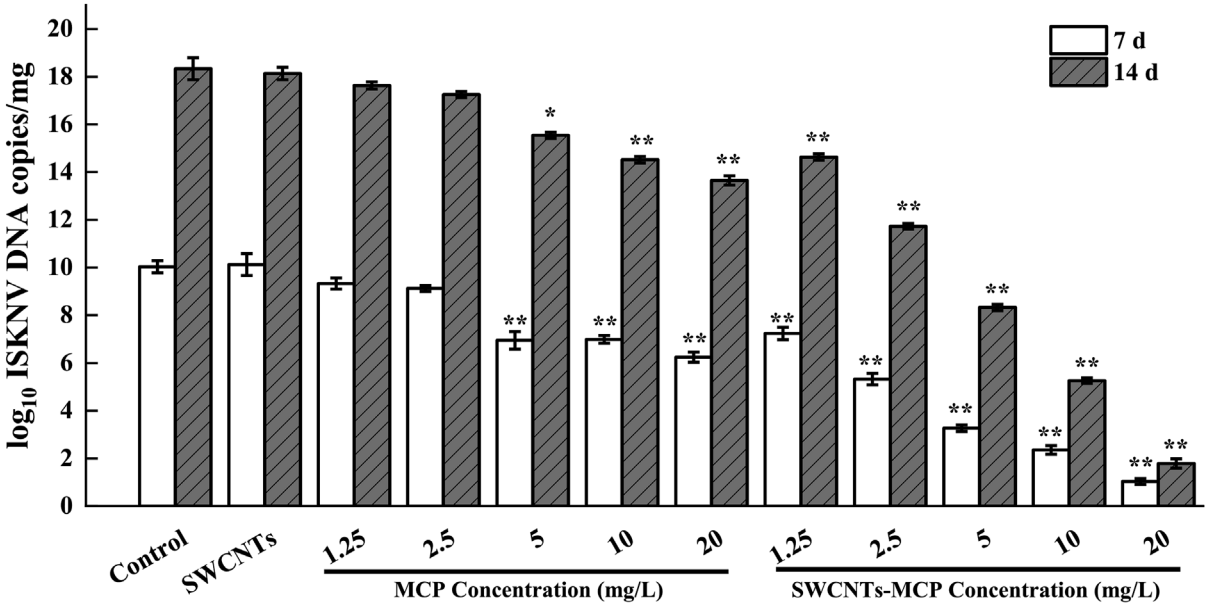

Fig.3

Fig. 3. Comparison of ISKNV copies among mandarin fish after challenged with ISKNV. To detect the ISKNV copy numbers, we construct a recombinant plasmid (pc-MCP) which was used as the template to generate a standard curve for the TaqMan real-time PCR by inserting the MCP gene sequence of ISKNV. Three replicates were set for the tests, with five fish per replicate. Data are means for three assays and presented as the means ± SE. Values that were significantly different from the control which are indicated by asterisks (one-way ANOVA, **P < 0.01; *P < 0.05).

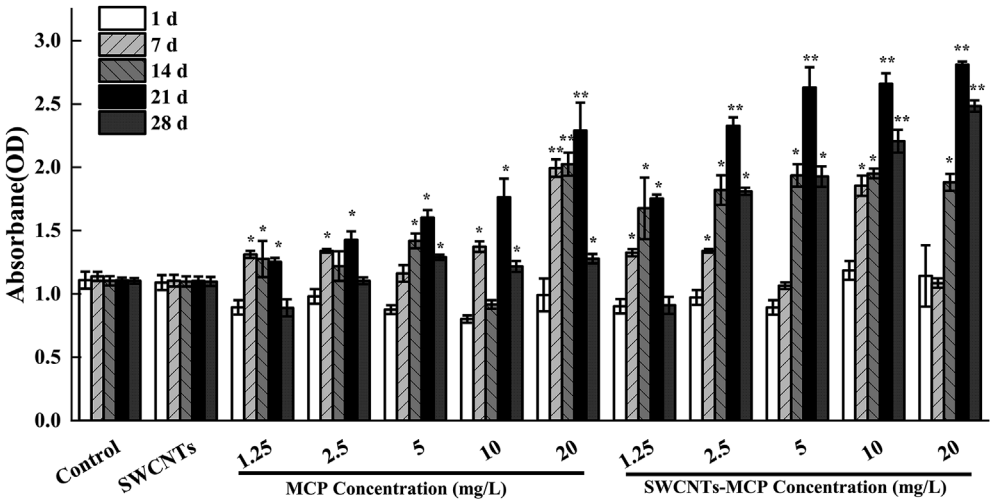

Fig. 4. Serum antibody production in vaccinated mandarin fish. Sera were collected from the fish at 1, 7, 14, 21 and 28 d post-vaccination, and serum antibodies against recombinant MCP were determined by ELISA. Three replicates were set for the tests, with five fish per replicate. Data are means for three assays and presented as the means ± SE. **P < 0.01; *P < 0.05.

3.4. Serum antibody production

The results of ELISA in Fig. 4 showed that the antibody levels increased as MCP concentration extending and reached a peak titer at 3-week post vaccination, and then they were gradually attenuated. Meanwhile, higher levels (about 1–2 times) of antibody can be found in fish vaccinated with SWCNTs-MCP, when compared with naked MCP at the same immunization concentration. However, no significant neutralizing activity was observed in control groups and the SWCNTs group.

3.5. Changes of non-specific immune parameters post-vaccination

As shown in Fig. 5, acid phosphatase assay (ACP), superoxide dismutase activity (SOD), alkaline phosphatase assay (AKP), and complement C3 activity in different treatment groups (MCP and SWCNTs-MCP groups) and control groups were recorded. The results showed that these four immune parameters in vaccinated groups were significantly higher than those in the control groups, furthermore, these effects were dose dependent (the higher dose group is higher than low dose group). There was no significant Changes of non-specific immune parameters in control groups and the SWCNTs group. In addition, these four immune-related activities in SWCNTs-MCP vaccinated groups were about 1.4–2 fold higher than those in MCP vaccinated groups.

3.6. Immune-related genes expression

The effect of vaccination on the expression of immune genes was examined by qRT-PCR analysis of the transcription of the genes encoding immunoglobulin M (*IgM*), interferon-regulatory factor 1 (*IRF-1*), Mx protein (*Mx*), interleukin 1 beta (*IL-1β*), interleukin 8 (*IL-8*) and tumor necrosis factor α (*TNF-α*). The results showed that all examined genes were significantly exhibited in MCP and SWCNTs-MCP vaccinated fish, when compared with the control groups (Fig. 6). And all

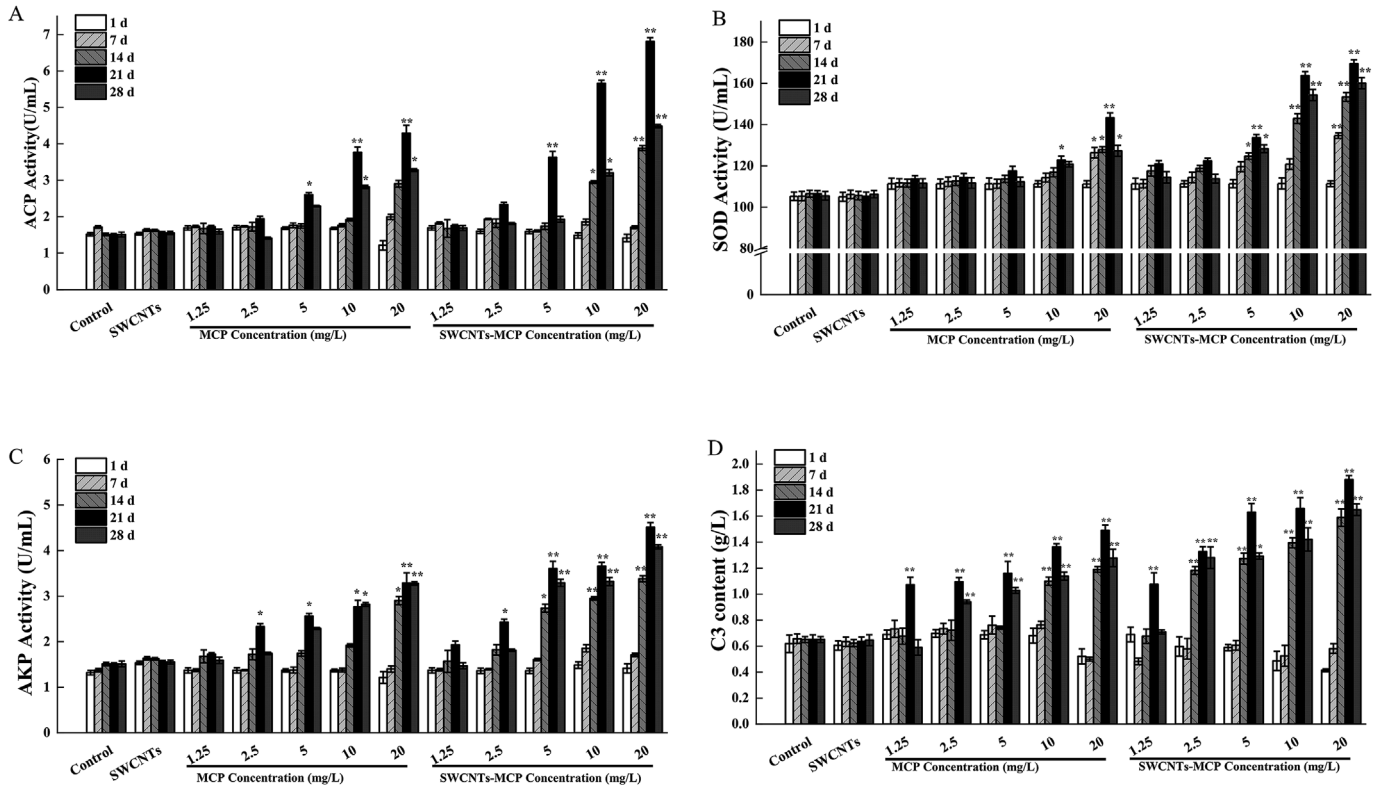

Fig. 5. Changes of immune parameters post-immunization by bath immunization: (A) ACP Activities; (B) SOD Activities; (C) AKP Activities and (D) C3 Content. Data are represented as mean ± SE. **P < 0.01; *P < 0.05. Three replicates were set for the tests, with five fish per replicate.

examined genes were not significantly difference in the SWCNTs group and the control group. In addition, the relative levels of immune genes in SWCNTs-MCP vaccinated groups were significantly higher than that in naked MCP vaccinated groups. Furthermore, these immune-related genes were significantly up-regulated (1.8–11.3 times) along with the increase in the vaccine concentration.

4. Discussion

Up to now, ISKNV has brought huge economic losses to many fish farms. However, there is no available commercial medicine or vaccine to cure or prevent ISKNV, moreover, the current ISKNV vaccine induced limited protection against ISKNV [20–22]. To enhance the efficacy of ISKNV vaccine, herein we reported a convenient immersion vaccine (SWCNTs-MCP) against ISKNV, results confirmed that SWCNTs-based subunit vaccine could induce protective immune responses in mandarin fish.

As many studies reported, vaccination via injection is by far the most effective method of combating disease. However, it is labor intensive, costly and not feasible to vaccinate large numbers of fish. Fry who is too small to be injected is usually vaccinated by immersion or by the oral route [23,24]. In this study, we vaccinated juvenile common carp via bath administration. Immersion is a convenient for large-scale immunization [25]. However, due to skin barrier and selective permeability of the cell membrane, it is not easy for most biological macromolecules including proteins and plasmid enter into fish body, which is also the obstacle for vaccine applications [26]. Hence, an efficient and economic strategy for the prevention of aquatic viruses infection becomes urgently needed.

Herein, we chose SWCNTs as vaccine carrier to break through the skin barriers and load more subunit vaccine into fish body. After 14 days ISKNV infection, the highest survival rate (69.6%) was observed in 20 mg/mL SWCNTs-MCP vaccinated group. Moreover, the highest levels of antibody level, enzyme activities were observed in 20 mg/mL SWCNTs-MCP vaccinated group. Lots of previous studies have confirmed that SWCNTs as a promise vaccine carrier can significantly enhance the efficacy of vaccine via bath administration. Our results also showed that there was no significant difference on the expression of immune-related genes, serum antibody production and the relative percentage survival after challenge et al. between the SWCNTs group and the control group, and there was no significant adverse effect on the mandarin fish. Zhu et al. reported that SWCNTs as the vaccine carriers could significantly enhance the efficacy of subunit vaccine and DNA vaccine against grass carp reovirus (GCRV) via bath administration, with the 100% survival rate [18]. Zhang et al. also found that SWCNTs as a promising vaccine carriers could enhance (1.3–2.4 times) the immune protective effect of vaccine against spring viremia of carp virus (SVCV) [27]. There has also been a report of SWCNTs being used as antigen carriers, whereby Wilms' tumor antigen was solubilized onto single-walled CNT scaffolds which were rapidly internalized into antigen-presenting cells for recognition by T cells [28]. There was also reported that immunized with peptide-functionalized carbon nanotubes enhances virus specific neutralizing antibody response [29]. All these results above were corresponding with our study.

To further investigate the immune response induce by SWCNTs-based vaccine against ISKNV, we further analyzed the immune-related genes (*TNF-α*, *Mx*, IRF-1, *IL-1β*, *IL-8*, *and IgM*) expression in vaccinated fish. All these genes were significantly up-regulated (1.8–11.3 times) in vaccinated carps, when compared with control groups, moreover, fish vaccinated with SWCNTs-MCP vaccine induced higher levels of immune-related genes expression (1.2–1.9 times) than fish immunized with naked MCP vaccine alone. The expression of *Mx* and *IRF-1*, were belong to *I-IFN* system associated genes. When a fish is infected with a virus, *I-IFN*, which plays a crucial role in non-specific immunity, can be activated rapidly [30]. In bony fish, the non-specific immune system usually play a key role in combating infection, with the I-IFN system representing the main antiviral mechanism [30]. Previous studies on the flounder revealed the role of *IRF-1* in regulating early immune

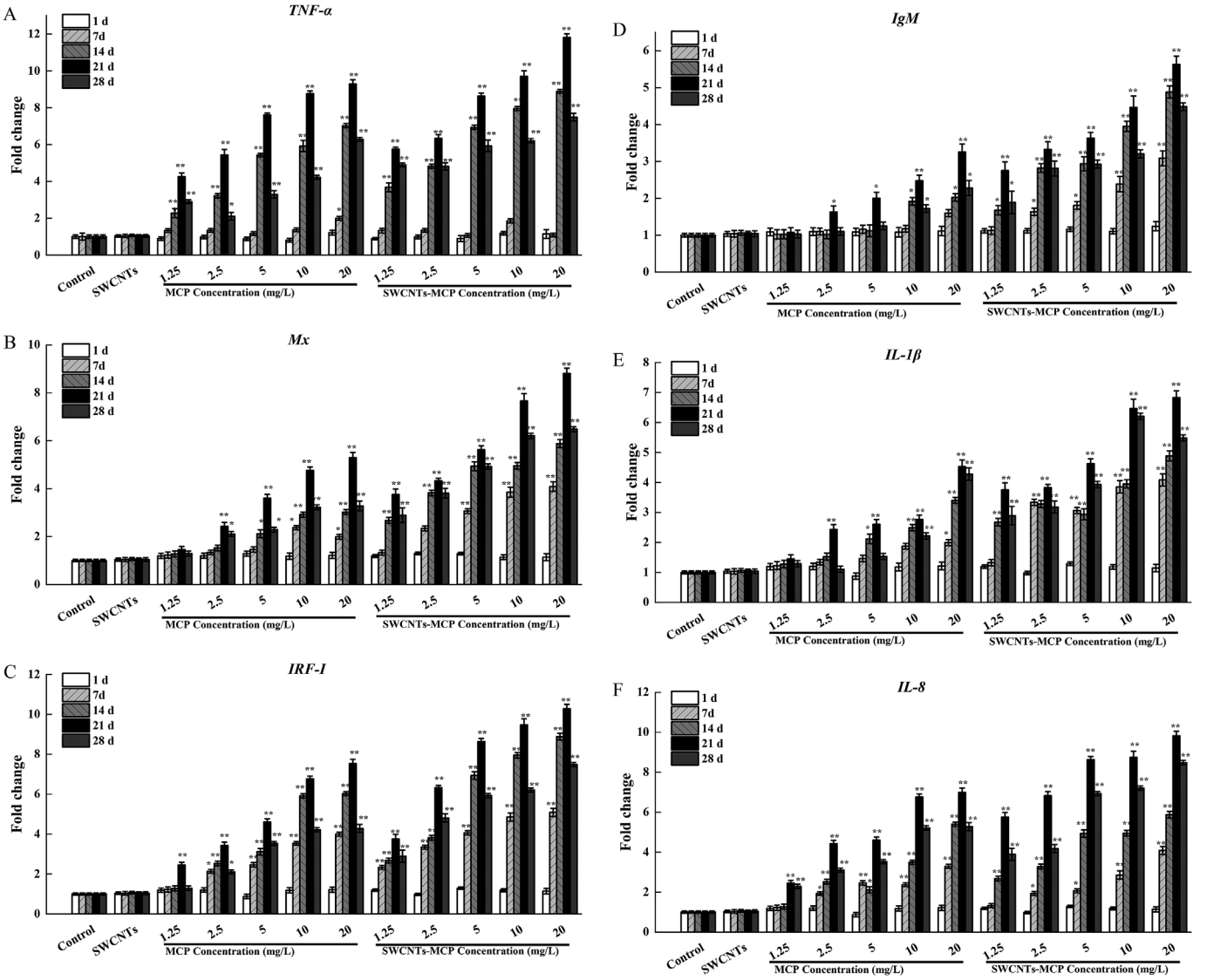

Fig. 6. qRT-PCR analysis of the expression of immune-related genes in mandarin fish vaccinated with different vaccine formulations. The changes of the expression in immune-related genes after bath immunization: (A) *TNF-α*; (B) *Mx*; (C) *IRF-1*; (D) *IgM*; (E) *IL-1β*; (F) *IL-8*. Samples were collected from the common carp at 1, 7, 14, 21 and 28 d post-vaccination. Data are means for three assays and presented as the means ± SE. Values that were significantly different from the control which are indicated by asterisks (one-way ANOVA, **P < 0.01; *P < 0.05).

responses in fish and provided a possible genetic adjuvant for fish vaccination [31]. Li et al. found that I-IFN can inhibit acute infection of ISKNV [5]. *TNF-α*, an important pro-inflammatory factor, produced by various cells, can modulates immune function and mediates inflammatory responses in mammals, which exhibit similar functions in fish [32,33]. Up-regulation of these genes indicates that the host may have induced humoral and cellular antiviral responses against ISKNV. Some researcher found that functionalized SWCNTs support a lot of promise for transporting bioactive molecules such as genes, subunit vaccines, drugs and other therapeutic agents [34]. Functionalized SWCNTs can interact with mammalian cells and enter cells via cytoplasmic translocation, and they therefore can deliver a range of therapeutic reagents into the cell [14,35]. In previous study, ammonium-functionalized single-walled carbon nanotubes (o-SWCNTs): DNA complexes are capable of transfecting cells in vitro and the large surface area of the o-SWCNTs allow more DNA to tightly associate [36]. 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 [37]. There's a study shows that grass carp immunized with DNA vaccine encoding the vp7 gene of grass carp reovirus using carbon nanotubes as a carrier molecule, showed that SWCNTs loaded with DNA vaccine stimulates a long lasting innate and adaptive immunity and gives protection (The highest relative percentage survival was 100%) from GCRV in grass carp as compared with conventional naked DNA vaccine [16]. Our results and previous studies demonstrated that functionalized SWCNTs as vaccine carrier can improve the content of the vaccine in fish and enhance the immune effect of the vaccine. Hence, all our results showed that SWCNTs-based immersion vaccine could induce strong immune protective response in mandarin fish against ISKNV.

5. Conclusion

In summary, our results showed that functionalized SWCNTs loaded with subunit vaccine encoding MCP protein gene conferred long duration and significant protection to juvenile mandarin fish against ISKNV after bath administration. This study presents key findings that demonstrate the efficacy and commercial potential for this immersion subunit vaccine.

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