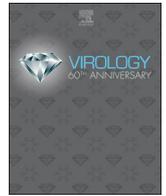




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Evaluation of immune response and protection against spring viremia of carp virus induced by a single-walled carbon nanotubes-based immersion DNA vaccine

Chen Zhang, Yu-Ying Zheng, Yu-Ming Gong, Zhao Zhao, Zi-Rao Guo, Yi-Jun Jia, Gao-Xue Wang, Bin Zhu*

College of Animal Science and Technology, Northwest A&F University, Yangling, 712100, China

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ABSTRACT

Spring viremia of carp virus (SVCV) has caused mass mortality in cyprinids, with case fatality rates of young fish up to 90%, resulting in enormous economic losses in the aquaculture industry. Immersion vaccination is considered as the most effective method for juvenile fish to combating disease, due to its convenience for mass vaccination and stress-free administration. However, immune responses following immersion vaccination are generally less robust and of shorter duration as those induced through intraperitoneal injection. Herein, to enhance the efficient of immersion vaccine, functionalized single-walled carbon nanotubes (SWCNTs) as carrier were used to manufacture immersion DNA vaccine system (SWCNTs-pEGFP-M) with chemical modification. Results showed that SWCNTs-pEGFP-M could enter into fish body via immersion administration and express antigen proteins in fish kidney and spleen. Moreover, stronger and longer duration immune responses (including serum antibody production and immune genes expression) can be induced in fish vaccinated with SWCNTs-pEGFP-M in comparison with those vaccinated with pEGFP-M alone. Notably, SWCNTs can increase the immune protective effect of naked DNA vaccine by ca. 23.8%. Altogether, this study demonstrates that SWCNTs as a promising DNA vaccine carrier might be used to vaccinate large-scale juvenile fish by bath administration approach, which can provide an outlook for future vaccination strategies against SVCV.

1. Introduction

As a cytopathic virus belonging to the genus *Sprrivivirus* of the family *Rhabdoviridae*, spring viremia of carp virus (SVCV) is widespread throughout the world with highly contagious and pathogenic to cyprinid fish (Ahne et al., 2002; Ashraf et al., 2016). The genome of SVCV is composed of a negative, single-stranded RNA that encodes five structural proteins, including glycoprotein (G), matrix protein (M), nucleoprotein (N), polymerase (L) and phosphoprotein (P) (Emmenegger and Kurath, 2008; Kanellos et al., 2006). G protein as well as M protein are two major outer membrane protein of SVCV (Teng et al., 2007). G protein is considered as the viral antigenic protein that determines the infectivity and serological properties of the virus (Shchelkunov and Shchelkunova, 1989; Teng et al., 2007). M protein provides the bullet-shaped structure of the virus and binds the nucleocapsid to the cytoplasmic domains of the G proteins embedded in the viral envelope, the matrix protein takes multi-function in the virus infection cycle, including participating in virus budding and assembly,

inhibiting protein nucleocytoplasmic transport, and regulating host gene transcription and including cell apoptosis. (Shao et al., 2016; Teng et al., 2007). Moreover, M protein is generally considered as a promising candidate antigen for recombinant influenza vaccine (Tompkins et al., 2007). Our previous study indicated that M protein could be used as an antigen for SVCV DNA vaccine construction (Zhang et al., 2018). Therefore, to further investigate the immunoprotective of M protein against SVCV, we selected M protein as the antigenic viral protein of our DNA vaccine.

There are three major administration routes for fish vaccination: intraperitoneal or intramuscular injection, oral administration and bath immunization (Jechlinger, 2014). Vaccination by intraperitoneal or intramuscular injection is by far the most effective method of combating disease. However, the obstacles of injection route focused on labor intensive, handling stress and other unexpected side effects on vaccinated fish (Plant and Lapatra, 2011; Sudheesh and Cain, 2016). Meanwhile, it is not practical to inject large amounts of fish under 10 g, while fish smaller this are often the most susceptible to disease. Oral

* Corresponding author. Northwest A&F University, Xinong Road 22nd, Yangling, Shaanxi, 712100, China.
E-mail addresses: wanggaoxue@126.com (G.-X. Wang), zhubin1227@126.com (B. Zhu).

vaccine is an effective way to arouse strong mucosal immunity and was considered as an important approach to induce antiviral activity, nevertheless, the stability of antigens was the hurdle when vaccine pass through the stomach and intestine which would largely diminished the immunity of oral vaccine (Caruffo et al., 2016; During et al., 2000; Ghosh et al., 2015). The immersion administration is gentle and safe. However, immune responses following immersion vaccination are generally less robust because of several formidable barriers including skin, cell and gastrointestinal tract (Hoare et al., 2017; Sudheesh and Cain, 2016). Therefore, efficient vectors could be a vital approach to delivery immersion vaccine into fish body and induce stronger immunoprotective against SVCV (Ding et al., 2017).

As a novel nanomaterial, SWCNTs possess 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 (De Volder et al., 2013; John et al., 2014). Specifically, SWCNTs are uniquely equipped to carry cargos (such as antigens and drugs) across biological membranes (Kostarelos et al., 2007; Porter et al., 2007). Their use for vaccination could allow effective utilization of antigens that have previously not been able to induce adequate or appropriate responses, as well as providing significant means of enhancing and modulating immune response (Gong et al., 2015; Liu et al., 2016; Wang et al., 2015; Zhu et al., 2015). Therefore, to further enhance the efficient of immersion vaccine, single-walled carbon nanotubes (SWCNTs) were used as the DNA vaccine carriers against SVCV in this study.

In this work, on the basis of hydroxyl and amino condensation reactions, functionalized single-walled CNTs (SWCNTs-NH₂) as carriers were used to load pEGFP-M DNA vaccine encoding matrix protein of SVCV. Common carps were immunized by pEGFP-M/SWCNTs-pEGFP-M DNA vaccine via bath administration. Furthermore, immune responses elicited in vaccinated fish were evaluated. The study reported herein provide a helpful reference for the use of immersion SWCNTs-DNA vaccine delivery systems in fish farming industry.

2. Material and methods

2.1. Experimental fish

Common carps (*C. carpio*) weighing 4.0 ± 0.5 g were purchased from a local SVCV-free farm in Yangling (shannxi, China). Carps were bred in laboratory for 28 days prior to vaccination. The water temperature for common carps were maintained at 20–23 °C. Commercial dry feed pellets (Hellow Fish Dry Pellets; CVM Products, Beijing, China) were used to fed carps twice daily. All of the experimental animals were handled according to the guidelines of the Animal Experiment Committee, Northwest A&F University.

2.2. Virus and cell lines

SVCV (strain 0504) kindly provided by Professor Qiang Li (Dalian Ocean University, Dalian, China), was propagated in epithelioma papulosum cyprini (EPC) cell line (Bioleaf, China) at 20 °C in minimum essential medium (MEM), pH 7.0 (Life Technologies, USA) plus 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Virus titers for challenge were determined as the established protocols (Emmenegger and Kurath, 2008), with a modified incubation temperature of 20 °C.

Epithelioma papulosum cyprinid (EPC) cells (kindly provided by Prof. Ling-bing Zeng in Yangtze River Fisheries Research Institute, Wuhan, Hubei, China) were cultured at 25 ± 0.5 °C in humidified atmosphere with 5% CO₂, and maintained in Medium 199 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; ZETA LIFE, USA).

2.3. Amino-single walled carbon nanotubes (SWCNTs-NH₂)

SWCNTs-NH₂ (95% purity black powder, 1–2 nm in outside

diameter, 5–30 μm in length, floating catalyst) were purchased from Chengdu Organic Chemicals Co., Ltd. Chinese Academy of Science (Chengdu, China).

2.4. Recombinant *M* plasmid constructions

The pEGFP-C1 (Clontech, USA) encoding a green fluorescent protein (GFP) which has brighter fluorescence and higher expression in mammalian cells, was used as the original plasmid. QIAGEN Viral RNA Mini Kit (Qiagen, Hiden, Germany) was used to extract the viral genomic RNA of SVCV. RNA PCR Kit (AMW) Ver.3.0 (Takara, Shiga, Japan) was used to converted the extracted RNA into cDNA. Specific primers used to clone SVCV *M* gene was designed based on the reference sequence published in GenBank which accession number is NC_002803. A primer pair consisting of SM-F (5'-CCCTCGAG ATGCTACTCTAAGAAAG CTC-3', the underline indicates (*Xho*I site) and SM-R (5'-CGGGATCCA TCTCCCATGAACAGGGAA-3', the underline shows *Bam*HI site) was utilized. The purified PCR product of *M* gene was digested with *Xho*I and *Bam*HI and inserted into pEGFP-C1 to generate recombinant pEGFP-M plasmid. PCR amplification, restriction enzyme digestion and DNA sequencing were used to verify the construction of pEGFP-M plasmid.

2.5. Preparation of SWCNTs-pEGFP-M DNA vaccine

SWCNTs-pEGFP-M was constructed refer to previous protocols (Zhu et al., 2015). Briefly, phosphate buffered saline (PBS, pH = 7.4) were used to dissolve SWCNTs-NH₂, then SWCNTs-NH₂ solution and pEGFP-M plasmid solution were mixed with the charge ratio (7:1 m/m), at last the mixture were wild agitated at 28 °C. The complexes were advised to use within 30 min after composed.

2.6. RNA isolation, cDNA synthesis and qPCR assays

For RNA isolation and cDNA synthesis cDNA synthesis, total RNAs were obtained from the kidney tissues in each group (3 fish per group) at 1, 3, 7, 14 and 21 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 were 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 $2^{-\Delta\Delta C_t}$ method with the formula, $F = 2^{-\Delta\Delta C_t}$, $\Delta\Delta C_t = (C_{t, \text{target gene}} - C_{t, \text{reference gene}}) - (C_{t, \text{target gene}} - C_{t, \text{reference gene}})_{\text{control}}$ (Livak and Schmittgen, 2001).

2.7. Expression of recombinant *M* plasmid in EPC cells

Immunofluorescence assay was performed on pEGFP-M infected EPC cells to further examine the native-form of endogenously synthesized matrix protein (*M*). The protocol was carried as previously described (Zhang et al., 2017), briefly, recombinant plasmid DNA was isolated with the Endofree Plasmid Mini Kit (Omega, USA) following the manufacturer's instructions and the concentration was measured by the NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA). Before transfection, EPC cells were cultured in 6 well plates (Corning, USA) with a density of 3×10^5 /well and grown at 25 °C for 70–80% confluence. The EPC cells were transfected with the indicated plasmids of pEGFP-M for 6 h using FuGENE® 6

Table 1
Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	Accession no.		Primer sequences (from 5' to 3')	Product size (bp)
<i>β-actin</i>	M24113	Forward	GCTATGTGGCTCTTGACTTCG	85
		Reverse	CCGTCAGGCAGCTCATAGCT	
SVCV-G	AY527273.1	Forward	GGAGACTGGGTAGAAA	374
		Reverse	CAAAATACTCTTGAGGCCG	
<i>TNF-α</i>	AJ311800.2	Forward	TGTGCCGCCGCTGTCTGCTTCACGCT	291
		Reverse	GATGAGGAAAGACACCTGGCTGTAGA	
<i>Cxcr 1</i>	AB010468.1	Forward	GCAAATTGGTTAGCCTGGTGA	144
		Reverse	AGGCGACTCCACTGCACAA	
<i>Cxca</i>	AJ421443	Forward	CTGGGATTCTGACCAITGGT	88
		Reverse	GTTGGCTCTCTGTTTCAATGCA	
<i>IL-10</i>	JX524550.1	Forward	GTCATCCTTTCTGCTCTGGTT	91
		Reverse	CCACAAATGAGCAACAGTCA	
<i>IFNγ2b</i>	JX181980.1	Forward	GCTCAAGAAGTATGCAGAAACTC	151
		Reverse	TCTGGCTTGTCTCTCCT	
<i>I-IFN</i>	AB376666.1	Forward	CAGAGTCAATGCTCCGCTT	297
		Reverse	CTCAGATGACTGCCGTTGC	
<i>IgM</i>	AB004105	Forward	CACAAGCGGGAAATGAAGA	145
		Reverse	CTGATAAAGCTTTGCACTTCAGCA	
<i>IgZ 1</i>	AB598367	Forward	AAACCAACCCTGAGTGTGGT	164
		Reverse	TAAAGACCTTCAGTATTCA	
<i>CD8β</i>	XM_019120131.1	Forward	AAAGTGCATATGTGCATCAG	168
		Reverse	TATCCTCTGAACAACAT	
<i>CD4</i>	DQ400124.1	Forward	AGTGGGATCAAAGGGCGAA	214
		Reverse	ATTCCAGAGACAGAGAGT	
<i>MHC-I</i>	XM_019103672.1	Forward	CAAGCAAAAAAGATTTC	177
		Reverse	ACAGCTGATCTGAAGCCC	
<i>MHC-II</i>	S62611.1	Forward	TGCAGTGCCTATGACTTC	191
		Reverse	GAGCTGGCGTGCTCCA	

Transfection Reagent (Promega, USA) in serum-free M199. Fluorescence was observed with an upright fluorescence microscopy (Leica-DM5000, Germany).

2.8. Detection of antigen protein in fish tissues

Healthy common carp (30 fish per group) were exposed to pEGFP-M (35 mg L^{-1}) and SWCNTs-pEGFP-M (35 mg L^{-1}) by immersion for 10 h and then transferred in standard dilution water, respectively. Kidneys and spleens were sampled and cleaned from each treatment and fixed in 4.0% paraformaldehyde. Frozen slicer (SAKURA, PORAR-DM, Japan) was used to cut kidneys into $5 \mu\text{m}$ -thick sections. Subsequently, the sections were stained with 1.0 mg L^{-1} DAPI (Beyotime, China), and then observed under a fluorescence stereomicroscope (Leica DM5000B, Germany).

2.9. Vaccination

The time schedule for vaccination was shown in Fig. 1. During the vaccination, the rearing water temperature were kept at $20 \pm 0.5^\circ\text{C}$. Disease-free common carps were randomly separated into nine groups (160 fish per serving), including three control groups (PBS group, pEGFP-C1 group, SWCNTs group) and six vaccinate groups (pEGFP-M groups and SWCNTs-pEGFP-M groups containing three concentrations (5 , 15 and 35 mg L^{-1}), 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. At 3 days post-immunization, the fish were boost immunized with the same regime as for the primary immunization protocol.

2.10. Analysis of specific IgM in serum (ELISA)

The titers of the antibodies were measured by ELISA (Enzyme-linked immunosorbent assay) as described elsewhere (Hao et al., 2017; Navot et al., 2004). For analyses of the presence of specific, neutralizing antibodies, vaccinated and control fish (3 fish of 3 independent experiments) were sampled weekly until 6 weeks for antibody determination. Serum samples preparation and determination were according to previous method (Zhang et al., 2017). Briefly, the blood collected from the caudal vein of common carp was placed overnight at 4°C and then centrifugated at $5000 \times g$ for 15 min. The supernatant was collected and stored at -20°C until use. Purified recombinant M protein was used as antigen. Anti-Common carp (*Cyprinus carpio carpio*)/Koi carp (*Cyprinus carpio koi*) IgM monoclonal antibody labeled with horseradish peroxidase (Aquatic Diagnostics Ltd., England) was diluted with PBS containing 3% skimmed milk at the ratio of 1:1000 before use, 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).

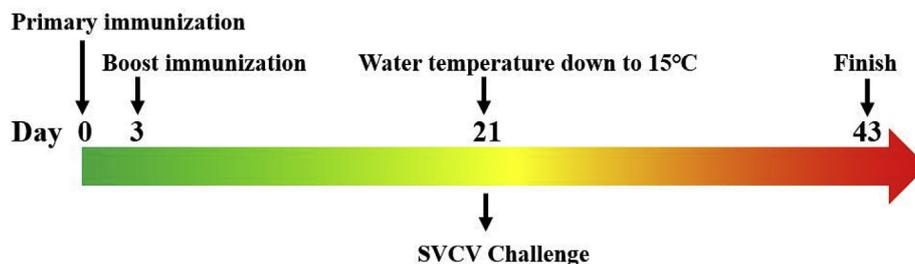


Fig. 1. Time schedule for immunization and challenge.

2.11. Virus challenge

Before virus challenge, the water temperature was gradually lowered from 20 °C to 15 °C at a rate of 1–2 °C/day. At the time point of 21 days post-vaccination, fish in vaccinated and control groups (each group, $n = 80$) were transferred to new tanks and challenged by intraperitoneal injection with 50 μL 6.0×10^4 TCID₅₀ mL⁻¹ of live SVCV in saline buffer, and the relative percentage survival (RPS) were recorded daily for 22 days after viral challenge. Dead fish were collected daily, recorded, and examined for clinical signs of SVCV. Moreover, PCR assay was used to confirm SVCV infection in challenged fish, primers (SVCV-G) used for viral detection can be found in Table 1.

Relative percentage survival (RPS) = $1 - [\% \text{ mortality rate (vaccinated fish)} / \% \text{ mortality rate (control fish)}] \times 100$

2.12. Statistical analysis

All data were analyzed using SPSS 22.0 Software (IBM, USA), one-way ANOVA were used to analyzed after normalization. Differences in antibody response and immune genes expression were analyzed with Duncan's test, values with different letters are significant ($P < 0.05$); The relative percentage survival was analyzed with Log-rank (Mantel-Cox) Text ($*P < 0.05$, $**P < 0.01$).

3. Results

3.1. Analysis of pEGFP-M plasmid expression in vitro

The expression of M protein *in vitro* was observed by immunofluorescence on the transfected EPC cells. As Fig. 2 reveals that pEGFP-M plasmid could expressed in EPC cell with bright green fluorescence.

3.2. Detection of SWCNTs-pEGFP-M DNA vaccine in immersion vaccinated fish

Tissue section observation was performed to check the internalization and expression of SWCNTs-DNA vaccines in fish kidney and spleen. As shown in Fig. 3, the nucleuses were stained with DAPI (blue fluorescence). The green fluorescence is corresponded to the antigen proteins. No obvious green fluorescence was observed in spleen or kidney of unhandled carps or pEGFP-M vaccinated carps, respectively. Notably, strong green fluorescence was identified in kidney and spleen of fish vaccinated with SWCNTs-pEGFP-M, which indicated that SWCNTs-pEGFP-M could be internalized *in vivo* and express antigen proteins in fish kidney and spleen.

3.3. Serum antibody production

The antibody levels of sera samples obtained from vaccinated fish during 1–6 weeks were evaluated. As shown in Fig. 4, there were significant enhancement of antibody levels in fish vaccinated with pEGFP-M and SWCNTs-pEGFP-M. Specifically, the antibody level of fish immersed with SWCNTs-pEGFP-M were significantly higher than that of fish vaccinated with pEGFP-M at the same vaccinate concentration ($P < 0.05$). In addition, the antibody level reached a peak titer at about 3-week post immunization and subsequently they were gradually declined. This result indicates that SWCNTs based immersion vaccine can induce high and long-term of specific antibodies level in vaccinated fish.

3.4. Immune genes expression

To further determine the immunoprotective effect of our SWCNTs-based immersion DNA vaccine. Immune genes including tumour necrosis factor α (*TNF- α*), CXC chemokine receptor 1 (*Cxcr 1*), CXC chemokine a (*Cxca*), interleukin 10 (*IL-10*), interferon gamma-2beta (*IFNg2b*), type I interferon (*I-IFN*), immunoglobulin (*IgM*), immunoglobulin Z1 (*IgZ 1*), T-cell surface glycoprotein CD8 beta (*CD8 β*), cluster of differentiation 4 (*CD4*), major histocompatibility complex class I (*MHC-I*) and major histocompatibility complex class II (*MHC-II*) were selected to reveal the immune response in vaccinated fish. As Fig. 5 shown, all above genes were significantly up-regulated (1.8–22.6 times) in vaccinated carps, when compared with control groups (PBS, pEGFP and SWCNTs). Moreover, carps vaccinated with SWCNTs-pEGFP-M induced higher levels of immune genes expression than those immunized with pEGFP-M ($P < 0.05$).

3.5. Protection of DNA vaccination

During 22 days after challenge, the relative percentage survival (RPS) were analyzed (Fig. 6). Vaccinated groups (pEGFP-M and SWCNTs-pEGFP-M groups) showed a significant improved survival compared with control groups (PBS, pEGFP and SWCNTs groups). What's more, the RPS in SWCNTs-pEGFP-M groups via bath immunization were higher than that in pEGFP-M groups ($P < 0.01$), the protective efficacy of SWCNTs-pEGFP-M vaccinated group (35 mg L⁻¹) was the highest, with the RPS value of 46.3% (Table 2). Furthermore, Table 2 showed that SWCNTs as a promising vehicle can enhance ca. 23.8% of the RPS in SWCNTs-pEGFP-M vaccinated fish compared with naked pEGFP-M immersed fish. Typical clinical symptoms of SVCV infection can be observed in the dead fish, besides, no pathogen other than SVCV was detected.

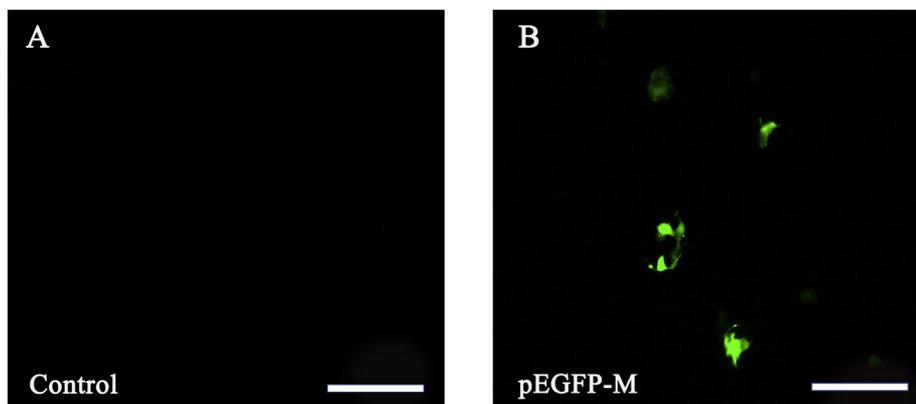


Fig. 2. Immunofluorescence analysis of EPC cells expressing the M protein after transfection with pEGFP-M. Cells were transfected with the recombinant pEGFP-M plasmid encoding a SVCV matrix protein and green fluorescent protein (GFP) fusion protein (green). Scale bars: 50 μm .

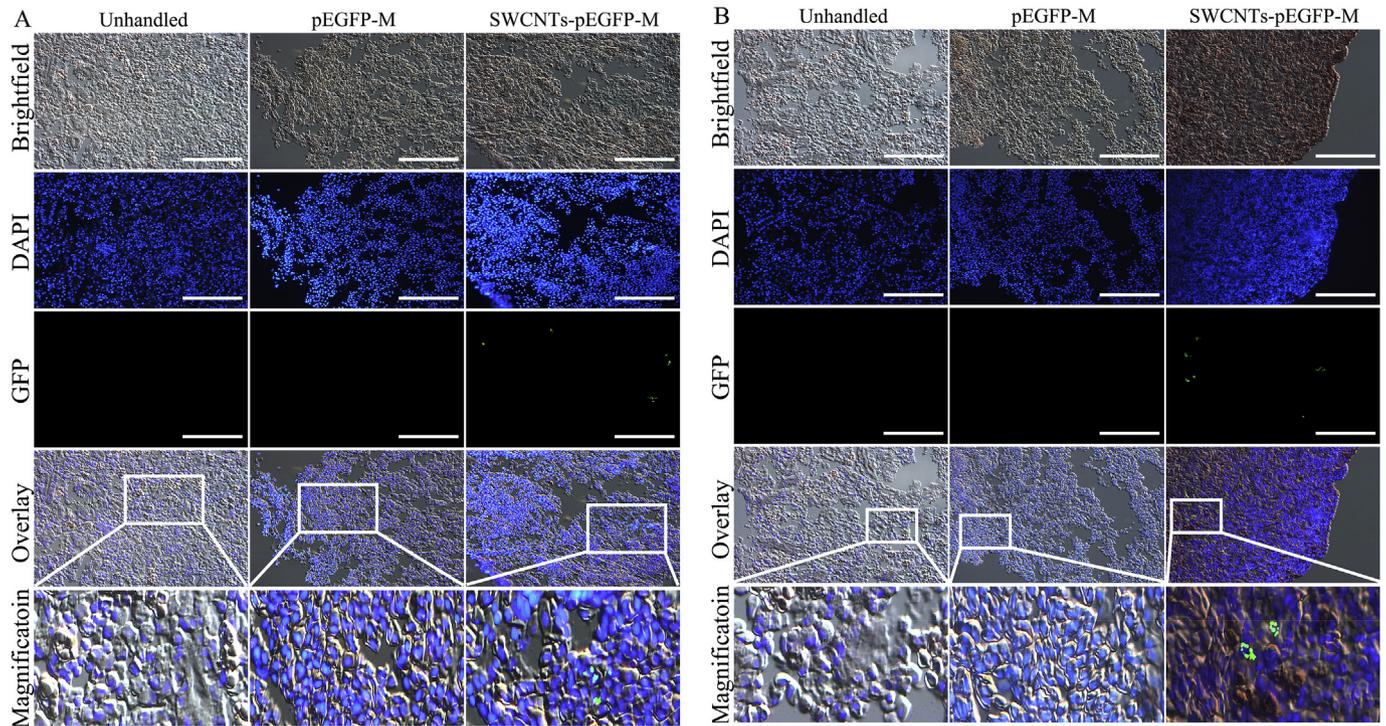


Fig. 3. Tissue cryosections of carp kidneys (A) and spleens (B) after immersed with vaccines. The expression of DNA vaccines was expressed with green fluorescence (green channel); The cell nucleus was labeled with DAPI (blue channel). Scale bars: 50 μm.

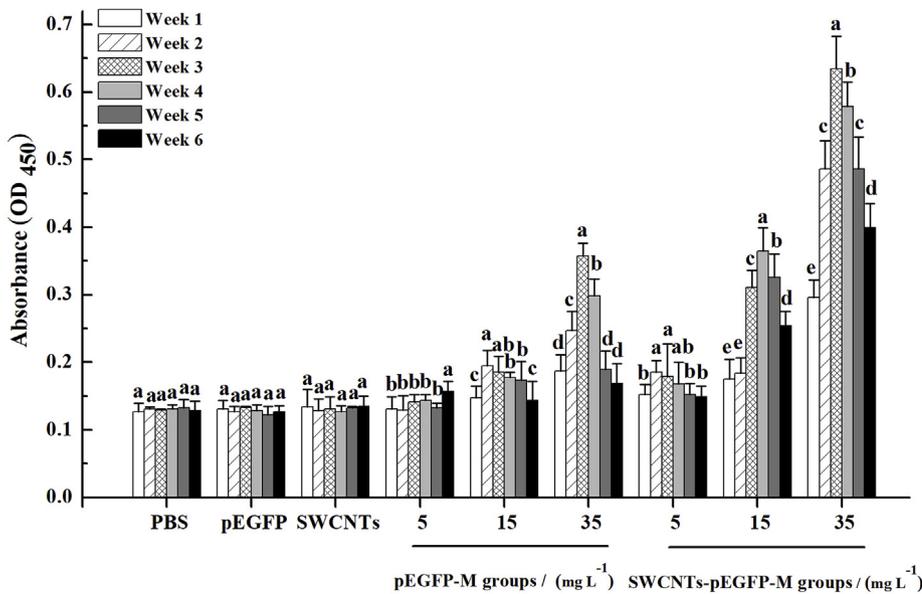


Fig. 4. Serum antibody production in vaccinated common carp. Sera were collected from the fish at 1–6 weeks post-vaccination, and serum antibodies against recombinant M were determined by ELISA. Data are means for three assays and represented as mean ± SD. Data at the same sampling time with different letters are significantly different ($P < 0.05$).

4. Discussion

As the causative agent of spring viremia of carp (SVC), SVCV still imperil the development of aquaculture, in particular cyprinid farming industry (Ashraf et al., 2016; Boonthai et al., 2017). As a promise prophylaxis, vaccination is commonly considered as a major improvement to induce immune protective effect against fish disease (Li et al., 2015; Wang et al., 2016). Up to now, the current SVCV DNA vaccine exert limited protection (Ashraf et al., 2016). In this study, modified SWCNTs were used to load our DNA vaccine. To our knowledge, this is the first report demonstrating the use of SWCNTs-based immersion DNA vaccine (SWCNTs-pEGFP-M) encoding full-length matrix protein of SVCV could protect juvenile common carp against SVCV. This study

confirmed the immunogenicity of SVCV matrix protein, which can be used for vaccine construction. Moreover, SWCNTs was proved as a potential vehicle for immersion DNA vaccine.

Fry who is too small to be injected is usually vaccinated by immersion or by the oral route (Cui et al., 2015; Valero et al., 2016). In this study, we vaccinated juvenile common carp via bath administration. Immersion is a convenient route for large-scale immunization (Zhu et al., 2017). 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 (Yang et al., 2016). In addition, the rapid tissue clearance and on-site degradation of DNA are both obstacles for DNA vaccination (Kayansamruaj et al., 2017). Therefore, it is

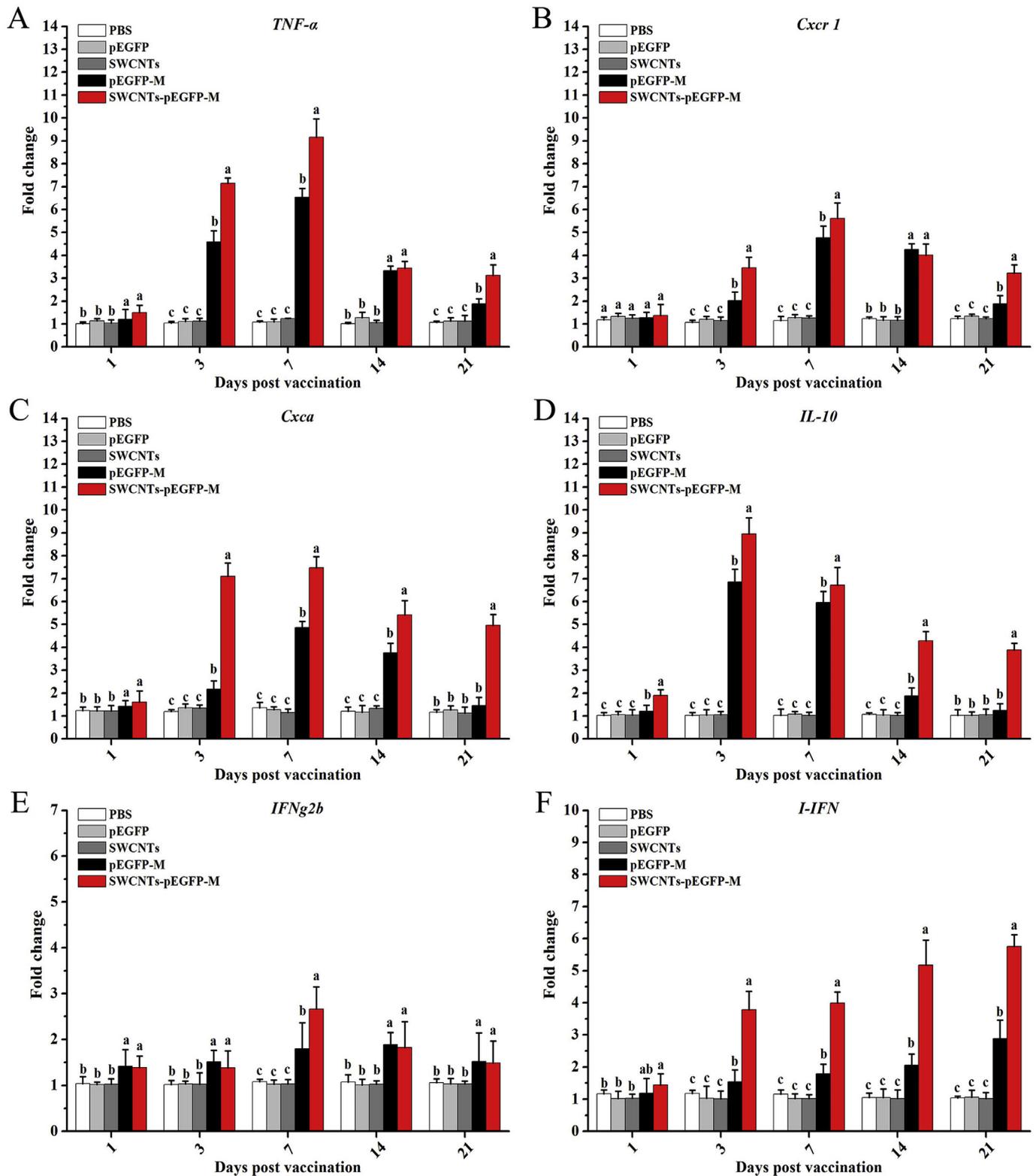


Fig. 5. Time course analysis of immune genes expression in kidney of fish immersed with PBS, pEGFP, SWCNTs, pEGFP-M and SWCNTs-pEGFP-M at 1, 3, 7, 14 and 21 days after vaccination. Common carp (3 fish/group) were sampled and the kidney were pooled and processed to determine the expression of *TNF-α* (A), *Cxcr 1* (B), *Cxca* (C), *IL-10* (D), *IFNγ2b* (E), *I-IFN* (F), *IgM* (G), *IgZ 1* (H), *CD8β* (I), *CD4* (J), *MHC-I* (K), *MHC-II* (L) genes by qRT-PCR. The β -actin was used as an internal reference. Data are means for three assays and represented as mean \pm SD. Data at the same sampling time with different letters are significantly different ($P < 0.05$).

important to develop efficient delivery technologies to settle these limitations. (Hao et al., 2017; Wang et al., 2016). In this study, the functionalized SWCNTs were used as DNA vaccine carrier. The fluorescence images in Fig. 3 revealed SWCNTs could delivery our DNA vaccine

enter kidney and spleen after 10 h immersion. Notably, this study also indicated that SWCNTs can prolong the duration of DNA vaccine *in vivo*. Similar consequences were reported in numerous studies: higher parental DNA uptake and transgene expression in Chinese hamster ovary

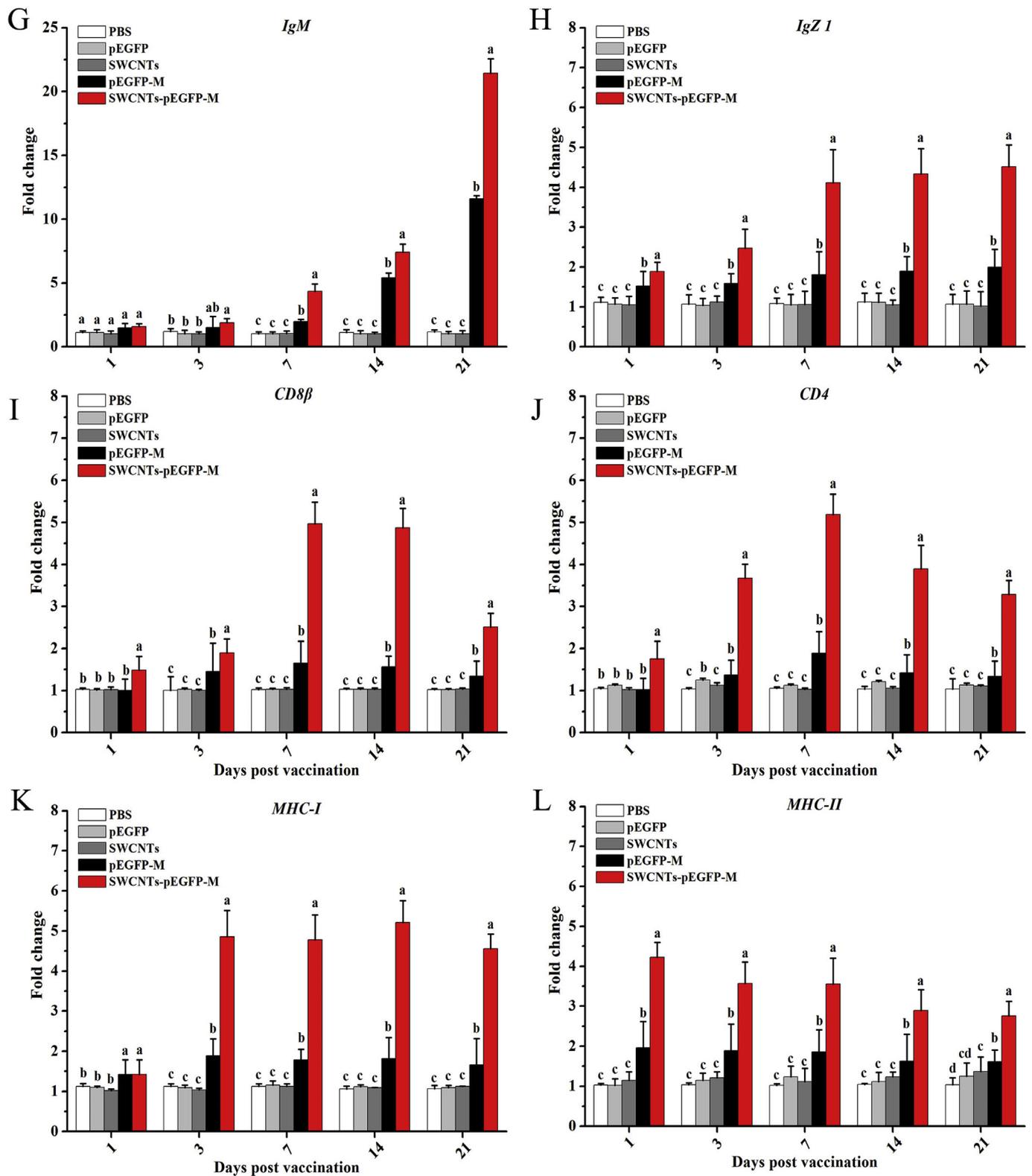


Fig. 5. (continued)

cells were observed by using SWCNTs as plasmid DNA vehicle (Pantarotto et al., 2003); There has also been a report of SWCNTs being used as antigen carriers, whereby Wilms' tumour antigen was solubilized onto single-walled CNT scaffolds which were rapidly internalized into antigen-presenting cells for recognition by T cells (Villa et al., 2011); There was also reported that immunized with peptide-functionalized carbon nanotubes enhances virus specific neutralizing antibody

response (Pantarotto et al., 2003). All these results above were corresponding with our study. As an effective immersion vaccine vehicle, SWCNTs can load more DNA vaccine into fish body. The biological function of vaccine could be carried out. These might be one of the reasons why SWCNTs can enhance the immunoprotective of our DNA vaccine via bath administration.

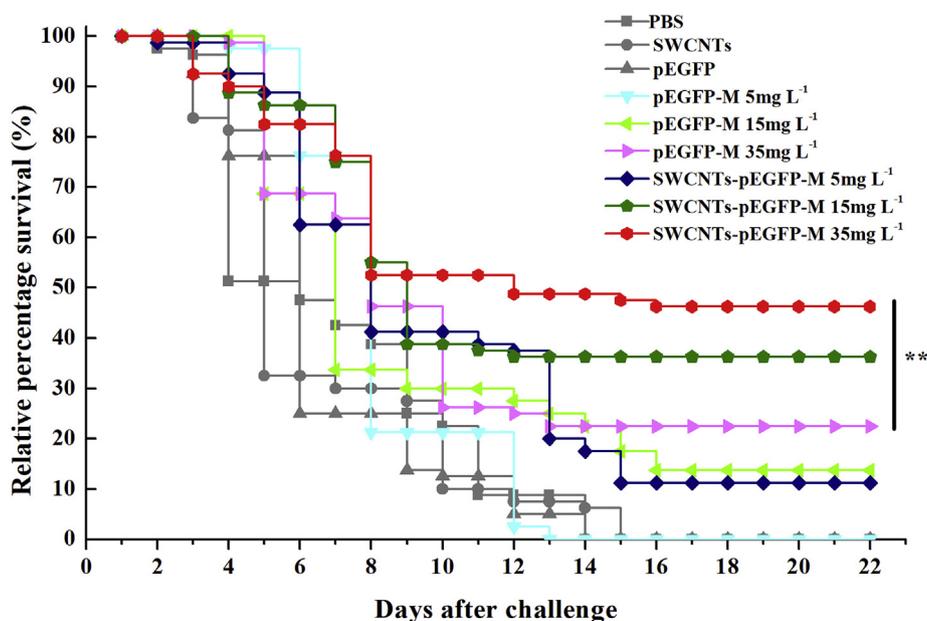


Fig. 6. Relative percentage survival after artificial challenging with SVCV in vaccinated common carp. The percentage survival was recorded daily and calculated at the end of the monitored period. *P* values were calculated by Log-rank (Mantel-Cox) Test (**P* < 0.05, ***P* < 0.01).

Table 2

Relative percentage survival (RPS) of fish challenged with SVCV.

Fish injected	RPS (22 d)
PBS	0%
SWCNTs	0%
pEGFP	0%
pEGFP-M 5 mg L ⁻¹	0%
pEGFP-M 15 mg L ⁻¹	13.8%
pEGFP-M 35 mg L ⁻¹	22.5%
SWCNTs-pEGFP-M 5 mg L ⁻¹	11.3%
SWCNTs-pEGFP-M 15 mg L ⁻¹	36.3%
SWCNTs-pEGFP-M 35 mg L ⁻¹	46.3%

Values are expressed as mean ± S.D; three replicates were set for the tests.

Cytokines play a key role in host innate immunity and are indispensable for recruitment and activation of macrophage, neutrophil, and lymphocyte to the infection sites for pathogen elimination (Chau-Berlinck et al., 2004; Paul and Seder, 1994). To further investigate the defense mechanisms induced by our SWCNTs-based immersion DNA vaccine against SVCV, we analyzed the immune genes expression in vaccinated fish. All these genes were significantly up-regulated (1.8–22.6 times) in vaccinated carps when compared with control groups. Moreover, fish vaccinated with SWCNTs-DNA vaccine induced higher levels of immune genes expression (1.2–3.6 times) than fish immunized with naked DNA vaccine alone. The induction of *TNF-α* was apparently up-regulated in different forms of vaccinated fish (Cerami, 2012). *TNF-α* is one of the main pro-inflammatory cytokines produced in response to a broad type of bacterial, viral and fungal infections, and has a crucial role in activating and orchestrating the immune response in order to protect the host organism from pathogens (Grasso et al., 2015). Besides, *TNF-α*, *IL-10* and type *I-IFN* have been reported play a significant role in the initiation and regulation of the inflammatory process and serve as an important component of innate immunity (Shibata et al., 1998). The type *I-IFN* is a complex group of cytokines and in charge of cell growth and differentiation in the hematopoietic and immune system, which serves as the first line of defense against viral infection (Grant et al., 2016; Ishikawa et al., 2009; Wang et al., 2010). *IFNγ2b* belongs to Type II IFN which is largely secreted by T cell and natural killer cells, and plays an essential part in natural immunity

of the host (Shibata et al., 1998). Thus, activation of innate immunity may condition the initiation of specific adaptive immune responses (Hashimoto et al., 2005). The *IgM* expression was increased significantly in vaccinated fish kidney. It is known that *IgM* is a major component of the humoral immune system of teleost fish, regarded as the first antibody (Medzhitov and J.C., 2016). Some investigators have reported that the *IgM* expression would be intensively increase in many tissues and organs from the second week after immunization and maintained almost one month, which is corresponding with our study (Tian et al., 2009). *MHC-I* and *MHC-II* are the markers reflecting the antigen presentation, the higher expression levels of *MHC-I* and *MHC-II* lead to increased advantages in terms of antigen presentation (Ding et al., 2017). The reason why SWCNTs could significantly enhance the cellular response and antigen presentation of our vaccine is possibly due to the increased numbers of cells (migration of head and leukocytes) participated in the process, in other words, SWCNTs make our DNA vaccine easier for attachment to specific target tissues and cells. These may partly explain the improved immune response in fish vaccinated with SWCNTs-pEGFP-M. Whereas, the regulatory mechanism requires further investigation. Therefore, the induction of cytokines, interferons, adaptive immune-related and antigen presenting response might lead to the improvement of our immersion DNA vaccine. However, the underlying mechanism of protection requires further investigation.

We recently reported a SVCV DNA vaccine encoding matrix protein which, when injected in the muscle at a dose of 2.5 μg/fish, confers up to 33.8% protection (Zhang et al., 2018). In this study, the highest protection rate of 46.3% was observed in carps immersed with 35 mg L⁻¹ SWCNTs-DNA vaccine. However, due to different factors, including the nature of the host, vaccine dose, environment and so on, it is hard to judge the immune effect between our previous intramuscular DNA vaccine and other SVCV DNA vaccines with the present immersion DNA vaccine. This is a primary study that focus on the immune response induced by DNA vaccine encoding matrix protein via immersion administration. Although the highest protection is barely 46.3%, the good immunogenicity of SVCV matrix protein and the excellent carrier properties of SWCNTs were confirmed in this study. In our further study, different parameters affecting vaccination such as vaccine delivery time, dose, duration of protection, booster regimes, immersion time will be optimized.

5. Conclusion

In summary, our results showed that functionalized SWCNTs loaded with DNA vaccine encoding the full-length matrix protein gene conferred long duration and significant protection to juvenile common carp against SVCV after bath administration. This study presents key findings that demonstrate the efficacy and commercial potential for this immersion DNA vaccine.

Conflicts of interest

The authors declare that they have no competing interests.

Consent for publication

All authors agree to be published.

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