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Chitosan and anisodamine improve the immune efficacy of inactivated infectious spleen and kidney necrosis virus vaccine in *Siniperca chuatsi*

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ABSTRACT

Siniperca chuatsi is an economically important fish in China, but infectious spleen and kidney necrosis virus (ISKNV) causes high mortality and significant economic losses. Currently, vaccination is the most promising strategy to prevent infectious diseases, while adjuvant can effectively enhance immune responses. In this study, inactivated ISKNV vaccine was prepared, then poly (I:C), chitosan, anisodamine and ims1312 were used as adjuvants to evaluate the effect on the immune responses and ISKNV replication. Chitosan could strongly boost the protection of liver and spleen tissues by pathological sections. In serum, poly (I:C) and chitosan group had protective effect on catalase, acid phosphatase, blood urea nitrogen. mRNA expressions showed these adjuvants induced the cytokines of early immune responses (*TNF-α*, *Viperin*) in both spleen and mesonephron by real time quantitative RT-PCR assays. Meanwhile, poly (I:C), chitosan and anisodamine were significantly improved the antiviral function and inhibited ISKNV replication. Chitosan and anisodamine played a significantly protective role in the immune protective rate test. The results indicated that all the four adjuvants are valid in the inactivated ISKNV vaccine, and chitosan is recommended preferentially. The present study provides reference for other animal vaccine adjuvants.

1. Introduction

Chinese perch (*Siniperca chuatsi*) is one of the most popular cultured fish in China because of delicious taste, abundant nutrition and high market value. However, with the rapid development of intensive agriculture, infectious diseases caused by a variety of viruses show a parallel growth trend [1,2]. In recent years, infectious spleen and kidney necrosis virus (ISKNV) has caused high mortality and significant economic losses in Chinese perch industry [1]. ISKNV is the typic species of the genus Megalocytivirus in Iridoviridae family, which can infect many marine and freshwater fish species. The diseased fish are often lethargic with abnormal swimming behavior. Symptom and pathology-dissection of the ISKNV infected fish include pale gill, enlarged spleen, pale liver, and red spot in liver [3–6].

Vaccination is one of the effective strategies for preventing pathogen invasion and is considered to be essential for reducing the use of antibiotic. Inactivated and live attenuated vaccines are widespread, while DNA vaccines and subunit vaccines are developing. Vaccines can induce innate and adaptive immune responses. However, a large number of clinical trials show that the lack of immunogenicity of these vaccines is the main obstacle to achieving an acceptable level of protection [7]. Thus, it is important to select the appropriate adjuvant to stimulate a strong and sustained immune response. Furthermore, a more effective adjuvant, is able to use a lower antigen dose and reduces side effects, and it will facilitate the use of vaccines [8].

Chitosan is a natural biodegradable polysaccharide obtained from crustacean shells. As a cationic polysaccharide, chitosan has attracted more and more attention in the field of medicine because of its non-toxic, biodegradable, sticky and other favorable biological properties. Increasing attention has been paid to chitosan as an adjuvant due to its

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attractive antibacterial, antiviral and antifungal biological properties. What's more, it also has other characteristics, such as bioadhesive, biocompatibility, biodegradability and penetration-enhancement properties [9,10].

Poly (I:C), the synthetic mimic of viral double-stranded RNA, can be used as adjuvant to enhance the innate and acquire immunity in animals [11–14]. The conjugation vaccines with poly (I:C) protect animals against homologous and heterologous virus infection over the long term and exhibit a high survival rate post virus challenge [15,16]. These findings imply that poly (I:C) may be a promising adjuvant of vaccines to prevent microbial infections in fishes.

Anisodamine, the first alkaloid extracted from the solanaceae plant, is an M-receptor antagonist in China [17]. Anisodamine can relax smooth muscle, stabilize cell membrane and dilate microvessel. Anisodamine can also restore and enhance the physiological function of mucous membrane and improve immunity, promote the growth and enhance the immune effect of vaccines in fishes [18–21].

Ims1312, an oil-in-water adjuvant, can facilitate antigen suspension, absorption of antigen and the diffusion of vaccine in fish gill. A study showed that ims1312 as an adjuvant improves the immune response of rainbow trout Yersinia vaccine by stimulating both adaptive and innate immune responses [22,23].

In this study, we researched the impact of chitosan, anisodamine, poly (I:C) and ims1312 as adjuvants on the immune responses in *S. chuatsi* against ISKNV. We divided the fish into six groups, and injected with physiological saline, vaccine, ims1312/vaccine mixture, anisodamine/vaccine mixture, chitosan/vaccine mixture and poly (I:C)/vaccine mixture respectively, challenged with ISKNV on day 15 post injection (dpi). The typical pathological changes in virus infection group were observed. The protective effects of four adjuvants mixed vaccine on different pathological tissues were examined. These adjuvants had a sustained effect on the acid phosphatase (ACP), catalase (CAT), blood urea nitrogen (BUN) in fish by the kinetic tests of serum enzyme activities. We also analyzed the expressions of *IgM*, *TNF-α* and *Viperin* in major immune tissues (spleen and mesonephron), and detected the viral *ISKNV007* gene to reflect the viral replication. Finally, the overall effect of adjuvants on vaccines was determined by measuring individual mortality. This study will provide a better understanding of chitosan, poly (I:C), ims1312, and anisodamine as promising candidate adjuvants for fish vaccines.

2. Materials and methods

2.1. Fish

S. chuatsi (100 g mean weight) were obtained from a fish farm in Huangshi (Hubei province, China) in April 2018 and were acclimated at 27 ± 1 °C. The fish were fed twice daily with commercial pellets for two weeks before experiments.

2.2. Preparation of vaccine, vaccination, challenge and sampling

ISKNV is kindly donated by professor Zeng, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. *S. chuatsi* was infected by injecting ISKNV into 10 fish with 150 μL and 250 μL virus suspensions respectively[38]. When there are obvious signs of pathological changes in fish, the tissues were cut with scissors and homogenized with 1.5 times of normal saline, then collected supernatant by filtrating with two layers of cotton and centrifuging at 8000 rpm for 30 min.

Six treatment groups about 600 fish were intraperitoneal (i.p) injection with 200 μL of inactivated ISKNV vaccine, vaccine formulated with 200 μg poly (I:C) [35,40], 100 μg chitosan [10,30], equal proportion of ims1312 [41] and 200 μg anisodamine [36] per fish on day 0 (D0) respectively. According to preliminary experiments, 100 fish were used in each group, 50 of them were fed separately to measure mortality, and the remaining 50 were used for sampling and all fish except control group were infected with ISKNV on 15 dpi (day post injection). Five fish of each treatment group (except ISKNV group) were sacrificed for harvesting spleen, mesonephron and serum on day 0.5 (D0.5), D1, D4, D7, D10, D14, D15, D20, D23 post injection, and the intestine tissues were randomly collected from each group on 23 dpi.

2.3. Protective efficacy study

Six groups of *S. chuatsi* (n = 50) were injected with 200 μL normal saline, inactivated ISKNV vaccine, vaccine mixed with 200 μg poly (I:C), vaccine mixed with 100 μg chitosan, vaccine mixed with equal proportion of ims1312, or vaccine mixed with 200 μg anisodamine, fed and changed water normally. On 15 dpi, all of them were challenged with 200 μL ISKNV virus and then, death events were monitored for the next 14 days.

2.4. Hematoxylin and eosin (HE) staining

The intestine tissues were dissected and fixed immediately in 10% neutral buffered formalin for 24 h, dehydrated, paraffin-embedded and sectioned. 4 μm sectioned samples were mounted on aminopropyltriethoxysilane-coated slides. Following the deparaffinization in xylene, sections were rehydrated, stained with hematoxylin and eosin (HE), and mounted with neutral gum, then the images were captured.

2.5. Serum biochemistry indexes

S. chuatsi were anesthetized with 3-Aminobenzoic acid ethyl ester methanesulfonate (MS222). Blood sample were collected from the caudal vein and were placed for 1 h at room temperature. After centrifugation at 4500 rpm (4 °C) for 15 min, the serum were gathered and stored at −80 °C. Acid phosphatase (ACP), catalase (CAT), blood urea nitrogen (BUN) were assayed by the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6. qRT-PCR of immune gene expressions

The total RNAs of mesonephron and spleen tissues were isolated with TRIzol reagents (Aidlab, China) according to the instruction, the quantity of RNA was determined by measuring absorbance at 260 and 280 nm, and its integrity was tested by electrophoresis in 2% agarose gel. mRNA were reverse-transcribed into cDNA respectively with M-MLV reverse transcriptase, RNase inhibitor (Thermo Fisher Scientific, USA), hexamer random primer. The primers for qRT-PCR analyses were listed in Table 1.

2.7. Statistical analysis

The statistical analysis was performed by using GraphPad Prism 7.0 software. Statistical significance was assessed using Student's two-tailed *t*-test in each group relative to vaccine group. Significance (*P*-value) was indicated as: *($P < 0.05$), **($P < 0.01$). All data were presented as means ± SE.

3. Results

3.1. Anatomical comparison of S. chuatsi infected with ISKNV

In order to study the anatomical changes of each organ after ISKNV infection *in vivo*, *S. chuatsi* in control group and infected group were distinguished and the changes between the two groups were observed. The results showed that there was a significant difference between the two groups (Fig. 1). Firstly, compared with the control group, we observed typical pathological symptoms on the surface of the fish, including obvious bleeding symptoms at the bottom of the gill and the

Table 1
Primers for qRT-PCR in the present study.

Gene name	Primer name	Primer direction	Sequence (5′-3′)	Accession No.
ISKNV007	VF545	Forward	GACATCTCCGCGTTTGATGC	NC_003494
	VR546	Reverse	TTAATGAGGTAGTCGCCGCC	
TNF-α	TnfF520	Forward	CAGGAGGACAGCTACAGAGC	DQ486758
	TnfR521	Reverse	AGAAAGTCTTGCCCTCGTCG	
IgM	IgF490	Forward	GGAAACGAAATGGCTGGTGC	AF327363
	IgR491	Reverse	ATCCCCTTGCTCCATTCGTC	
Viperin	VIF488	Forward	CAGGTGTGATTCGGTCGGTT	AY395718
	VIR489	Reverse	AACAGGTGGAACGGGTGAAG	
β-actin	ACF500	Forward	CAATGAGAGGTTCCGTTGCC	AY885683
	ACR501	Reverse	TGTTGTAGGTGGTCTCGTGG	

base of the fin (Fig. 1A). Secondly, we found that the gills became white and swollen when we dissected the fish (Fig. 1B). Afterwards, the liver of the infected *S. chuatsi* showed obviously white swelling (Fig. 1C). Then, the mesonephron of the infected group indicated the organ was hyperemia and swelling (Fig. 1D). Moreover, in the abdominal cavity, the infected group appeared a large amount of yellow ascites, in contrast, there was no ascites in the control group (Fig. 1E). In summary, the virus group manifested typical anatomical symptoms of ISKNV, which were the basis of the experiment.

3.2. Histological observation of liver and spleen after infectious spleen and kidney necrosis virus attack

The liver and spleen are the main diseased organs, so we made a histopathological observation. The liver of the control group exhibited normal cell morphology and arrangement in the compact network of fibrous tissue (Fig. 2A). Melano-macrophage centers (MMC) and tissue inflammation (TI) appeared in infected group (Fig. 2B). In vaccine group, MMC and TI could be alleviated (Fig. 2C). What's more, when vaccine mixed with four kinds of adjuvants like poly (I:C), ims1312, chitosan and anisodamine (Fig. 2D, E, F, G), these mixtures could obviously protect the body from damage. Among these mixtures, the liver tissue sections demonstrated that poly (I:C) and chitosan had the strongest protective effect on the body.

The spleen in the control group showed normal cell distribution and compacted arrangement (Fig. 3A). However, MMC and TI appeared in infected group of spleen (Fig. 3B). Tissue inflammation and macrophage centers was significantly reduced in the vaccine group (Fig. 3C). The effect of mixed vaccine and adjuvant obviously improved the health level of fish. All four kinds of adjuvants could boost the protective effect of the vaccine (Fig. 3D, E, F, G). According to the results of the slices, chitosan had the greatest protective effect on MMC and TI. The statistical results of histological alterations in the liver and spleen were shown in Table 2.

On the basis of observations and statistics, these results explained that chitosan could enhanced the protective effect of liver and spleen tissues.

3.3. Serum catalase, acid phosphatase, blood urea nitrogen could be positively improved by poly(I:C) and chitosan

To analyze the effects of poly (I:C), chitosan, anisodamine or ims1312 on serum biochemistry, ACP, CAT and BUN were determined with specific commercial kits (Fig. 4). Our study showed that CAT levels were higher than vaccine group after vaccination with four different adjuvants, especially chitosan and poly (I:C). When the virus invaded the body, a large amount of CAT was used and degraded to protect the fish (Fig. 4A). On the other hand, the level of ACP in the poly (I:C), chitosan and ims1312 groups were dramatically higher than the vaccine group during the trial period (Fig. 4B). Serum urea content was measured by blood urea nitrogen (bun) level, and the level was increased during kidney inflammation or virus invasion. Our data indicated that these four adjuvants had a strongly protective effect on fish health, in especial the poly (I:C) and chitosan group (Fig. 4C).

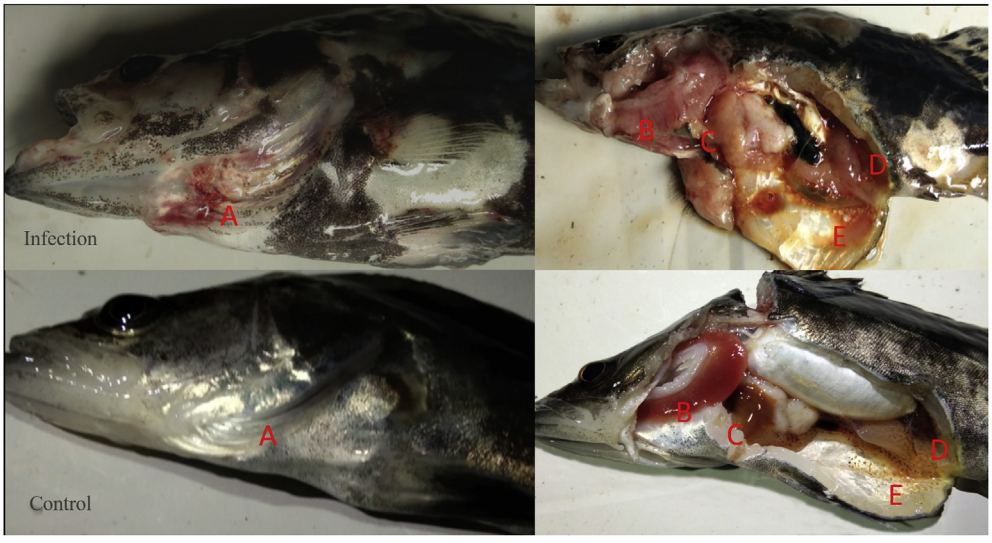

Fig. 1. Anatomical comparison of *S. chuatsi* post ISKNV infection. Fish were injected with normal saline and ISKNV respectively. Pathological changes were observed in gill (A), gill silks (B), liver (C), mesonepgron (D), abdominal cavity (E) respectively.

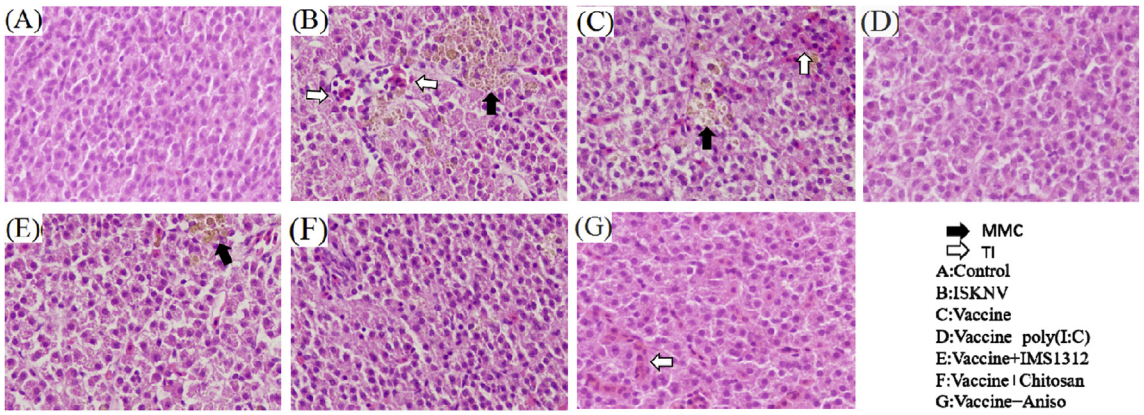

Fig. 2. Liver tissue sections post ISKNV infection. *S. chuatsi* were peritoneally injected with normal saline (B), vaccine only (C), poly (I:C)/vaccine mixture (D), ims1312/vaccine mixture (E), chitosan/vaccine mixture (F) and anisodamine/vaccine mixture (G) on 0 dpi, and challenged with ISKNV on 15 dpi, liver tissues were collected on day 23, then treated by HE staining. MMC and TI were the two main signs. Healthy liver tissue was used as blank control (A).

3.4. The cytokines of early immunization are induced by poly(I:C), chitosan and anisodamine

To detect the protective effect of adjuvants in the early stage of immunization, we specifically selected *TNF-α*, *Viperin* to test changes in every group. Compared with the group of vaccine alone, the mRNA expressions of *TNF-α* in spleen and mesonephron were all significantly increased on 4 dpi in the conjugation vaccine with four adjuvants groups, but the mRNA expressions were distinctly decreased on 10 dpi, when the virus challenged, it was rapidly upregulated on 15 dpi (Fig. 5A and B). Although the expression of spleen began to increase at 12 h, it began to increase in the mesonephron at 24 h. All adjuvants significantly improved the effectiveness of the vaccine. And from the datas, we can found the better groups of adjuvants were poly (I:C) and chitosan. *Viperin* could inhibit virus proliferation by interacting with viral proteins and some intracellular proteins [39], so it had broad-spectrum antiviral activity of early immunization. In our study, to explore the enhancement effect of adjuvants to the vaccine, we tested the amount of *Viperin* changes in the spleen. In the early stage of immunization, all the four adjuvants could improve the mRNA expression within 24 h, especially poly (I:C), chitosan and anisodamine. The mRNA expression quickly decreased on 4 dpi, when infected by ISKNV, it would rapidly recovered to defend fish (Fig. 5C). These results clearly demonstrated that the cytokines of early immunization responses were induced by administration of vaccine with poly (I:C), chitosan and anisodamine.

Table 2
Statistical results of histological alterations in liver and spleen of *S. chuatsi* on day 7 post ISKNV infection.

	Liver		Spleen	
lesion	MMC	TI	MMC	TI
Control	-	-	-	-
ISKNV	+++	+++	+++	+++
Vaccine	++	++	++	++
Vaccine + poly (I:C)	-	-	+	++
Vaccine + ims1312	+	+	-	++
Vaccine + chitosan	-	+	-	-
Vaccine + anisodamine	-	+	-	++

−, none; +, mild; ++, moderate; +++, severe. n = 3.

3.5. IgM expressions are induced by poly(I:C), chitosan, ims1312 and anisodamine

To determine whether these four adjuvants could strengthen the mRNA expressions of *IgM* in major immune tissues (spleen and mesonephron), the expressions of *IgM* were examined by qRT-PCR. Compared with vaccine alone, after vaccination with poly (I:C), chitosan, ims1312 or anisodamine, mRNA expressions of *IgM* were significantly increased in mesonephron on 7 dpi and kept rising on 21 dpi. According to these adjuvants effects, poly (I:C) and chitosan were more effective (Fig. 6A). In addition, chitosan was significantly increased in the spleen. There was no evident difference in the enhancement effect of other adjuvants except chitosan group. (Fig. 6B).

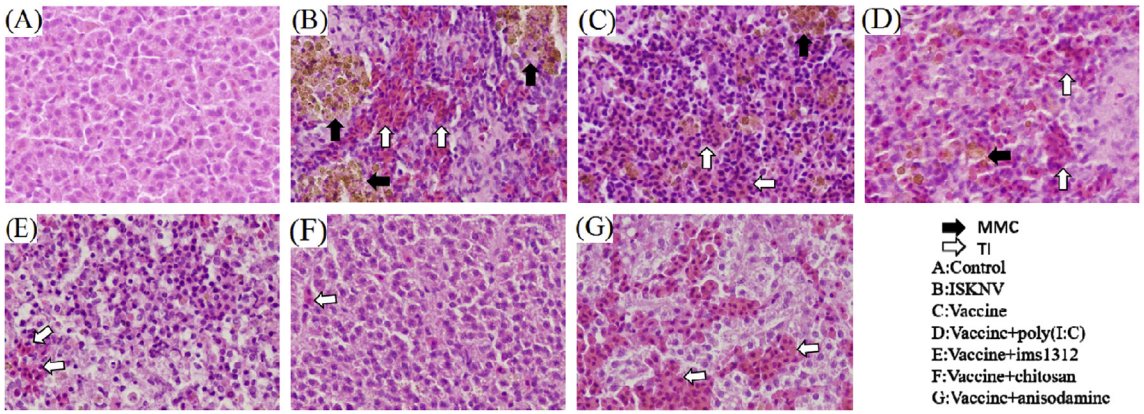

Fig. 3. Spleen tissue sections post ISKNV infection. *S. chuatsi* were peritoneally injected with normal saline (B), vaccine only (C), poly (I:C)/vaccine mixture (D), ims1312/vaccine mixture (E), chitosan/vaccine mixture (F) and anisodamine/vaccine mixture (G) on 0 dpi, and challenged with ISKNV on 15 dpi, spleen tissues were collected on 23 dpi, then treated by HE staining. MMC and TI were the two main signs. Healthy spleen tissue was used as blank control (A).

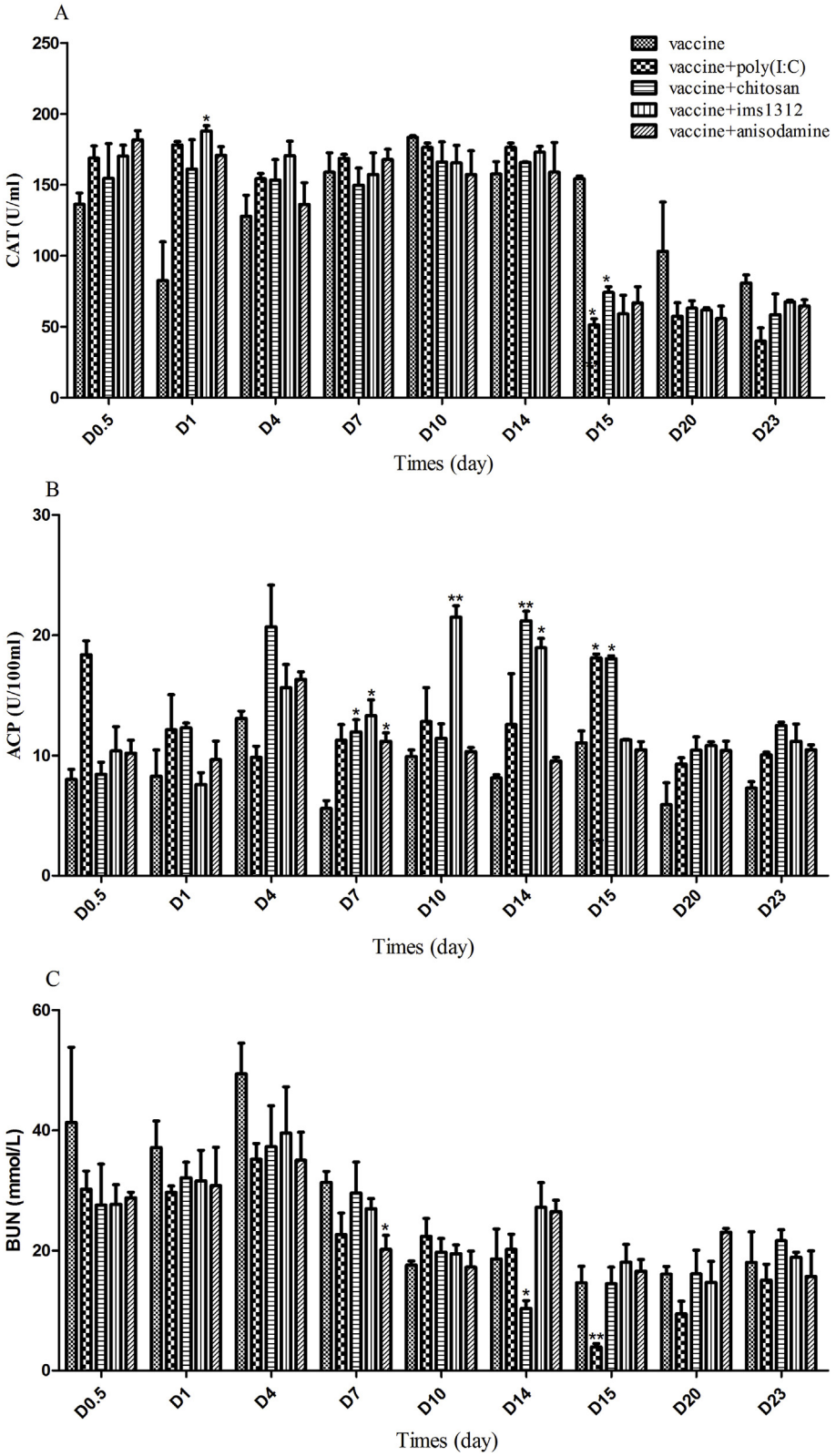

Fig. 4. Serum biochemical indexes of ACP, CAT, BUN. Acid phosphatase (A), catalase (B) and blood urea nitrogen (C). Serum biochemical indexes of ACP, CAT, BUN were determined by commercial kits. Data were presented as means ± SE (n = 4).

3.6. Virus replication was inhibited by chitosan and anisodamine supplement

To evaluate the role of these adjuvants in inhibiting viral replication, we examined the expression of mRNA in *ISKNV007*, one of the major capsid protein of ISKNV. Compared with the control group, the vaccine group reduced the replication of the virus, and these adjuvants more strongly inhibited the replication of the virus. As can be seen from the diagram, the replication of virus in each group increased over time, however, virus could be reduced by the admixture of adjuvants and

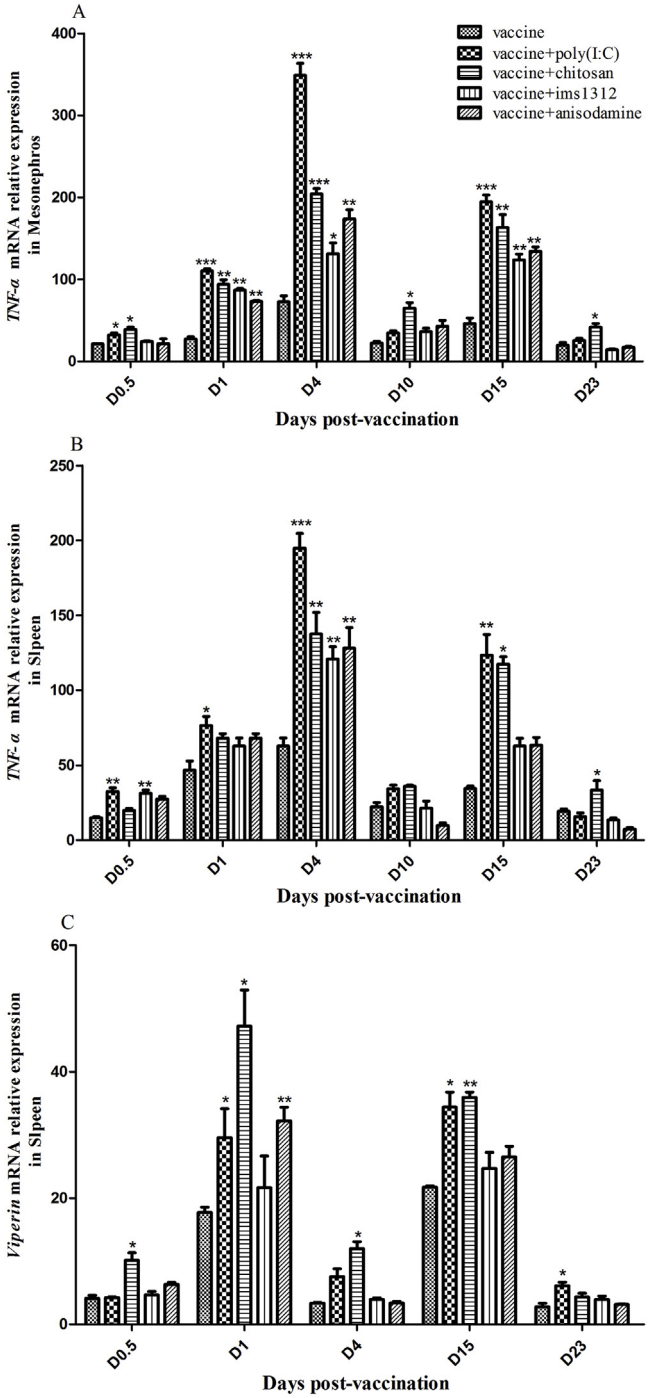

Fig. 5. mRNA expressions of *TNF-α* and *Viperin* in spleen and mesonepgron. They were examined by qRT-PCR. mRNA expressions of *TNF-α* in mesonepgron (A) and spleen (B) and *Viperin* in spleen (C). *β-actin* gene was used as a reference gene. Data were presented as means ± SE (n = 4). Statistical analysis was performed by unpaired student's *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

vaccines in mesonephron and spleen. In these tissues, chitosan and anisodamine were the better adjuvants to enhance the vaccine, but the other two groups had no significant effect in the mesonephric tissue (Fig. 7A and B).

3.7. The protective effect of chitosan and anisodamine

To prove poly (I:C), chitosan, ims1312 and anisodamine could aid the vaccine to provide better protection to *S. chuatsi*, every group were challenged by ISKNV on 15 dpi, and then calculated the immune protection rate (Fig. 8). We could see the protective effect of these adjuvants from the survival rate. When every group challenged with ISKNV, the rate of survival of the ISKNV group was 13.3%, but the survival rate of chitosan and anisodamine groups were raised to 58% and 52% respectively, the other adjuvants improved less significantly. The results indicated that chitosan and anisodamine play important role in the immune protection rate.

4. Discussion

Fish have a relatively sound immune system, which is the basis of fish vaccines to prevent fish diseases. Drug residues such as antibiotics have been around for a long time, and it is necessary and conscious to change the current state of disease prevention and treatment. As an important option, fish vaccine attract the attention by relevant government departments [23]. Adjuvant can enhance the immunization efficacy via innate and adaptive immunity [24]. In earlier studies, many researchers demonstrated poly (I:C), chitosan, ims1312 and anisodamine could be used as vaccine adjuvants to elevate efficacy of vaccine in protecting fish from pathogen invasion [10,11,17,22]. In the present study, we immunized *S. chuatsi* by ISKNV inactivated vaccine with poly (I:C), chitosan, ims1312 and anisodamine respectively, evaluate the efficacy of these adjuvants in *S. chuatsi* against ISKNV.

We infected *S. chuatsi* with ISKNV by injection, and observed typical pathological symptoms on the surface of the fish, such as obvious bled symptoms at the bottom of the gill and the base of the fin. It was found that the gill and liver of the fish were white and swollen, the symptom of the mesonephron was congestive and swollen, in the meantime, a large amount of yellow ascites appeared in the abdominal cavity [3,4]. Pathological section intuitively reflected the pathological injury of pathogenic infection. Spleen and liver were the major target tissues of ISKNV in *S. chuatsi*. In our study, we found that Melano-macrophage centers and tissue inflammation appeared in the infected group. Moreover, poly (I:C), chitosan, ims1312 and anisodamine groups greatly protected the tissues. These histopathologic slide results proved that the four adjuvants could improve the protective effect of vaccine [25].

The measurement of serum biochemical indexes had been widely used in the clinical diagnosis of fish physiology to determine the general health status of fish [26]. Acid phosphatase (ACP), catalase (CAT) and blood urea nitrogen (BUN) are extraordinarily important indicators of enzyme activity in serum, they usually change in different physiological and pathological condition [27–29]. Our results showed high levels of ACP, CAT after vaccination with poly (I:C), chitosan, ims1312 and anisodamine were consistent with the results of survival rates. When the virus invaded, fish could produce an immediate stress response, the number and vitality of ACP increased. ACP destroys and degrades the invasion of heterogenous substances to protect fish [27]. The difference between ACP and CAT is that CAT is consumed heavily after the attack [28]. BUN is the content of urea in the serum. When the kidney inflames or the virus invades, the urea content is increased in the serum [29]. BUN contents of the four adjuvant groups were obviously down-regulated, which revealed adjuvants could enhance the protective effect of the vaccine.

Innate immunity is the first line of defense against pathogen invasion. *TNF-α* is one of the pivotal early pro-inflammatory cytokines, which plays an important role in regulating immune response and inducing a series of inflammatory reactions to infections. *Viperin* inhibits virus proliferation by interacting with viral proteins to protect some intracellular proteins, and has broad-spectrum antiviral activity in early immunization [30,31]. In present study, we found that mRNA expressions of *TNF-α* in spleen and mesonephron were significantly increased on 4 dpi in conjugation vaccine with four adjuvants compared with the group of vaccine alone, but mRNA expressions were significantly

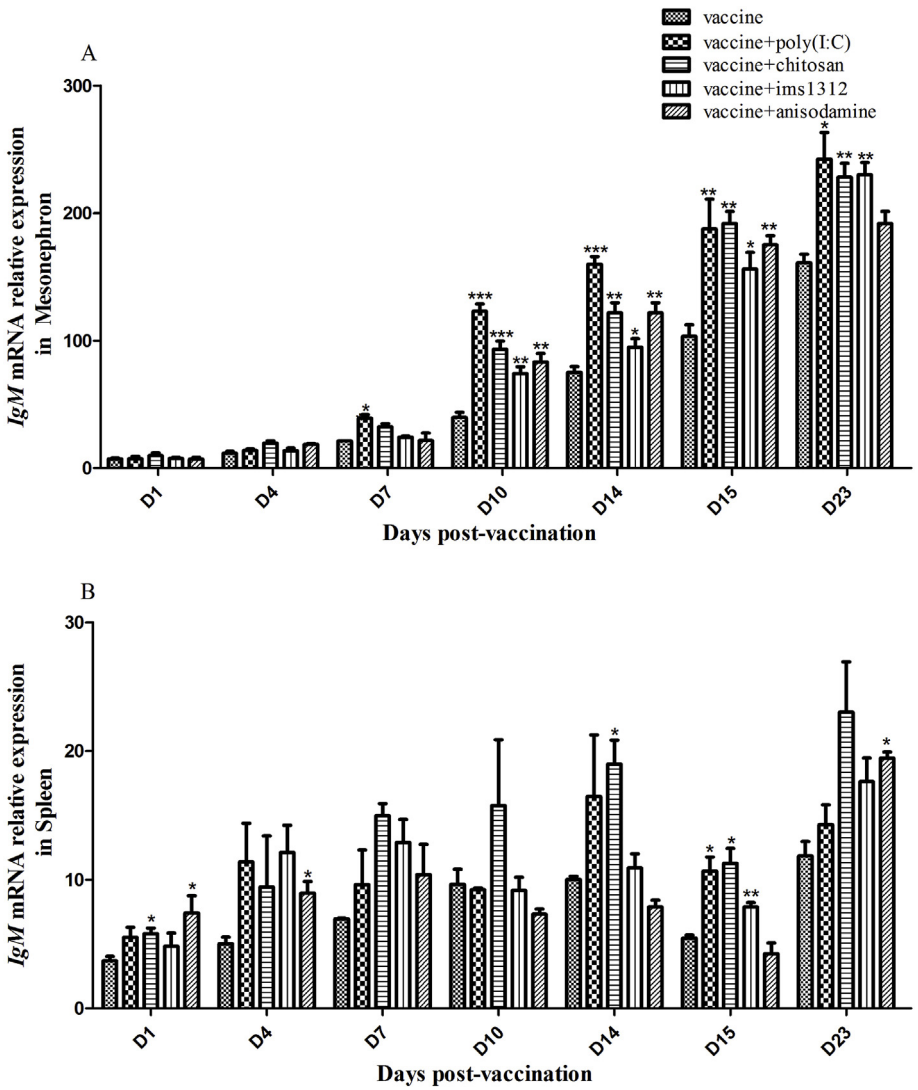

Fig. 6. mRNA expression profiles of *IgM* in spleen and mesonepgron. They were checked by qRT-PCR. mRNA expressions of *IgM* in mesonepgron (A) and spleen (B). *β-actin* gene was used as a reference control. Data were presented as means ± SE (n = 4). Statistical analysis was performed by unpaired student's *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

decreased on 10 dpi, when virus challenged, it was rapidly upregulated on 15 dpi. Meanwhile, in the early stage of immunization, four adjuvants improved mRNA expressions of *Viperin* on 24 h, when infected by ISKNV, it would rapidly recovered to defend fish. These results clearly demonstrated that the cytokines were induced by administration of vaccine with poly (I:C) and chitosan in early immune responses.

Then we wanted to detect the functions of these four adjuvants on immune protection and antiviral activity. In previous study, poly (I:C) as an adjuvant enhanced both humoral and cellular immune responses in *rhesus macaques* [32]. Chitosan as a vaccine adjuvant in mice also significantly enhanced levels of serum *IgM* [10,33,34]. Anisodamine could promote the growth of fish body and enhance the immune effect of vaccine in fishery production [21], ims1312 adjuvant increased the efficacy of Yersinia vaccine in rainbow trout by not only stimulating specific immunities, but also stimulating innate immune responses [23]. Our study showed mRNA expression of *IgM* in spleen and mesonephro increased over time, when ISKNV infected, it would quickly improve to protect fish. And testing mRNA expression of *ISKNV007*, we found that the expression in chitosan and anisodamine groups obviously decreased when the virus attacked.

Immune protection rate is the most important data to prove the functions of these adjuvants. poly (I:C) can improve the mice survival ratio when it serves as a vaccine adjuvant [35]. Anisodamine increases efficacy of *Aeromonas hydrophila* inactivated vaccine in *Carassius auratus gibelio* by immersion immunization [36]. ims1312 could be a contributing adjuvant to protect the gibel carp survival under *Aeromonas hydrophila* challenge [37]. The conjugation of PCV2 subunit vaccine with chitosan also had great influence in immune responses [34]. Poly (I:C), chitosan, ims1312 and anisodamine were verified to aid the vaccine to integrally provide better protection to *S. chuatsi* in present study, and chitosan and anisodamine were better than the other adjuvants.

In summary, inactivated ISKNV vaccine, mixed with poly (I:C), chitosan, ims1312 or anisodamine respectively as adjuvant, can protect liver and spleen tissues of *S. chuatsi* post ISKNV infection, and chitosan is the best choice. The enzyme activity in serum shows excellent effect on virus resistance by poly (I:C) and chitosan. Early immune cytokines can be significantly improved by poly (I:C), chitosan and anisodamine supplement. Chitosan and anisodamie play excellent protective role. These adjuvants are valid in the inactivated ISKNV vaccine, and chitosan is recommended preferentially.

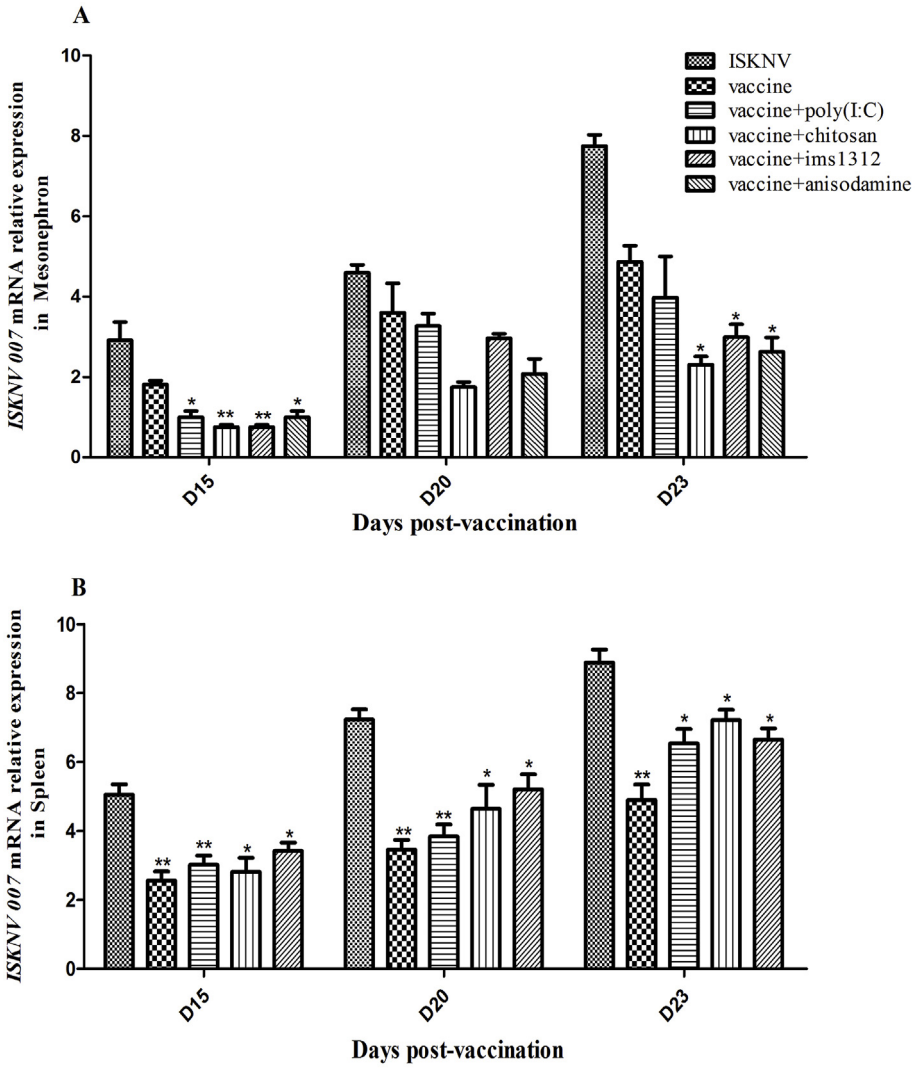

Fig. 7. mRNA expression profiles of *ISKNV007* in spleen and mesonepgron. They were determined by qRT-PCR. mRNA expressions of *ISKNV007* in mesonepgron (A) and spleen (B). *β-actin* gene was used as a reference gene. Data were presented as means ± SE (n = 4). Statistical analysis was performed by unpaired student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

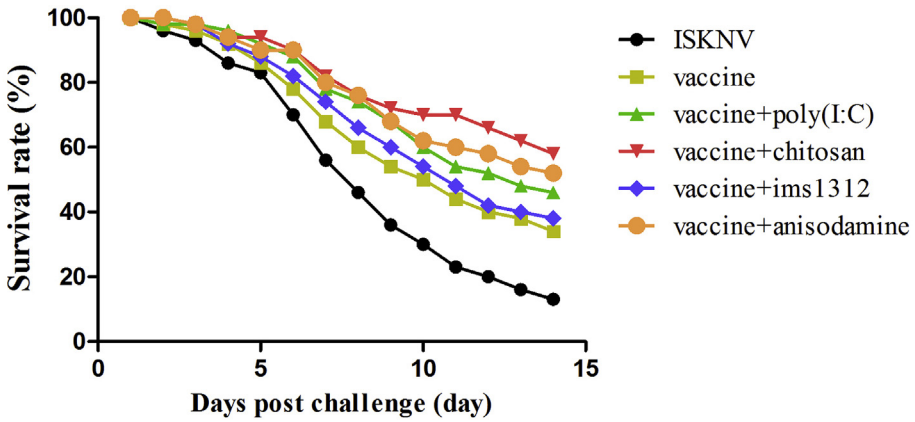

Fig. 8. Survival rates of *S. chuatsi* against ISKNV infection. Fish were peritoneally injected normal saline, vaccine only, poly (I:C)/vaccine mixture, ims1312/vaccine mixture, chitosan/vaccine mixture and anisodamine/vaccine mixture on 0 dpi, and challenged with ISKNV on 15 dpi. On day 15, fish in each group (n = 50) were challenged with ISKNV, and death events in each group were monitored on the next 14 days.

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