Effects of *Chlorella vulgaris* on blood and immunological parameters of Caspian Sea salmon (*Salmo trutta caspius*) fry exposed to Viral Nervous Necrosis (VNN) virus

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Abstract:

In the present study, the effects of *Chlorella vulgaris* on blood and immunological parameters of Caspian salmon (*Salmo trutta caspius*) before and after exposure to Viral Nervous Necrosis (VNN) virus were examined. In this regard, four treatments in triplicate were chosen. Groups included one control and 3treatments (T_1, T_2 and T_3). Fish in control group, T_1, T_2 and T_3 were fed diets supplemented with 0, 1×10^8, 2×10^7 and 3×10^6 chlorella/450 g of food respectively, for sixty days. In addition, a virus supernatant was prepared from infected wild golden grey mullet (*Liza auratus*) and used for virus challenge of *S. trutta caspius*. Virus was injected intraperitoneally and blood samples were collected before and 14 days after the challenge. Immunological (IgM, C_3, C_4, total protein, respiratory burst, albumin and lysozyme) and changes in blood parameters (RBC, WBC, Htc, Hb, MCH, MCHC and MCV) were also measured. Results showed that *C. vulgaris* could act as a natural immunestimulant. Also, the alteration trend in hematological and immunological parameters showed that experimental fish could be considered to be resistant to VNN virus after exposure and fish treated with *C. vulgaris* were more resistant in comparison to those in the control group. The dose used in T_1 (1×10^8 chlorella/450 g food) was the most effective approach with significant differences.

Keywords: *Chlorella vulgaris*, Blood parameters, Immunological parameters, *Salmo trutta caspius*, Viral Nervous Necrosis virus

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Introduction

Viral Nervous Necrosis (VNN) (Yoshikoshi and Inoue, 1990), also known as viral encephalopathy and retinopathy (VER; OIE, 2003), is caused by piscine nodaviruses. It was first described in 1990 in hatchery-reared Japanese parrotfish (*Oplegnathus fasciatus*) in Japan (Yoshikoshi and Inoue, 1990) and after that in more than 50 species all around the world (Johnsen *et al*., 2002; Munday *et al*., 2002; Nakai *et al*., 2009; Crane and Hyatt, 2011) including wild golden grey mullet (*Liza auratus*) in the Caspian Sea in Iran (Zorriehzahra *et al*., 2005 & 2016). Affected juveniles and older fishes show a variety of erratic swimming behaviors such as spiral, whirling, belly-up at rest with inflation of swim bladder (Zorriehzahra, 2004).

Histopathologically, the disease is characterized by severely extended necrosis and vacuolation of the central nervous system (brain, spinal cord) and retina, but sometimes fish in early larval stages lack tissue vacuolation (Munday *et al*., 2002).VNN is highly resistant to various environmental conditions and can survive for a long time in sea water (Arimoto *et al*., 1996). The disease is easily reproduced in healthy fish by co-habitation with infected fish, immersion in a virus suspension, or injection (Neguyen *et al*., 1996; Grotmol *et al*., 1999; Peducasse *et al*., 1999). Therefore occurrence of VNN in the Caspian Sea raised concerns about the infection of other fish in this region including Caspian Sea salmon (*Salmo trutta caspius*).

Caspian salmon is considered as an endangered species and has been listed as a protected species in the Iranian environmental regulations since August 1999 and is also included in the Red Data Books of Kazakhstan and Turkmenistan (Vera *et al*., 2010). Its natural populations have declined drastically in recent decades as a result of over-fishing, poaching, river pollution, destruction of natural spawning areas, and drought (Abdoli, 2000; Niksirat and Abdoli, 2009). It is anadromous and migrates up rivers to spawn. Captive breeding and conservation programs have been initiated to produce, restore, and protect populations (Sarvi *et al*., 2006; Jalali and Amiri, 2009). More than 900,000 fingerlings are produced annually through mixed milt fertilization of wild breeders and also from long-time hatchery breeders due to the decline of wild populations (Annaual report of IFO, 2010). It takes 2 years before the fingerlings reach a weight of 15–20 g, corresponding to a length of 10–15 cm, which is considered suitable for releasing (Vera *et al*., 2010). It is important to know if Caspian Sea Salmon is at risk or if there is any approach to improve fish immune system against VNN. Furthermore, natural immunestimulants such as microalgae are always an appropriate option to test. Nowadays, these microscopic organisms are consumed as food supplements such as *Chlorella vulgaris* (Fradique *et al*., 2010). *C. vulgaris* is a spherical microscopic cell with a 2–10 μm diameter (Yamamoto *et al*., 2004). This microalga has a rapid

growth rate and is ideal for production because it is remarkably resistant against harsh conditions and invaders (Safi *et al*., 2014). It's dry weight contains (42–58% of protein), (5–40% of lipid), and (12-55% of carbohydrate), pigments such as chlorophyll and carotenoid, vitamins and minerals which are used for different purposes like biofuels, human nutrition, wastewater treatment and animal feed (Safi *et al*., 2014). Consequently, the effect of *C. vulgaris*, as a natural immunestimulator was examined in Caspian Sea salmon exposed to VNN virus.

Materials and methods

Fish

A total of 800 Caspian Sea salmon fingerlings (mean individual initial weight of 8-10 g) were transferred from center of propagation and culture of Salmonids (Kelardasht, Iran) to the Caspian Sea Ecology Research Center (Sari, Iran). Fish were adapted to new conditions for a week and they were fed on a basal diet during acclimation.

Fish health examination

Prior to the experimental challenge, 40 fish specimen that were selected randomly from the mentioned population were screened for nodavirus by RT-PCR. All samples were negative and no VNN infection was observed (Gomez *et al*., 2004). The fish were acclimatised for one week and normal feeding behaviour was observed before the start of the challenge.

Microalgae

A pure culture of *C. vulgaris* was provided by the Caspian Sea Ecology Research Center. *C. vulgaris* cells was counted using a neubauer slide and light microscopy (Liu *et al.*, 2007) and was added to fish food according to determined concentrations for treatments.

Treatments design

720 fish were divided into 12 groups, in the form of four treatments (one control group and three experimental groups) with three replicates, each containing 60 fish. Treatments were named control, T_1, T_2 and T_3 and were fed on a diet supplemented with different concentrations of *C. vulgaris*.

Rearing condition

The mean water pH, dissolved oxygen (DO) and temperature were 7.5, 8 mg/L and 15°C respectively during the course of the experiment.

Food and feeding

Food was prepared once every ten days. To prepare the food, at first *C. vulgaris* cells were counted using a neubauer slide and were then added to ground food at a certain concentration for different treatments. The mixture of food and microalgae were dried in an oven. The control group was fed on the basal diet without *C. vulgaris*, while fish in T_1, T_2 and T_3 were fed with the diets supplemented with 1×10^8, 2×10^7 and 3×10^6 chlorella/450 g food respectively. The feeding trial lasted for 60 days. Fish were feed two times a day (8:00 a.m, and 14:00 p.m) at a daily

feeding rate of 8% of body weight in the first 2 weeks and then 6% and 4% of body weight in the second and final three weeks, respectively.

Supernatant preparation

A supernatant of the virus was prepared using brains and eyes of infected wild golden grey mullet. According to protocl provided by Kokawa *et al* (2008), brain tissues were mixed with HBSS (Hanks balanced salt solution), then centrifuged at 1610 g for 20 min at 15ºC and filterd through a 0.45 nm filter. The prepared supernatant was stored at -80°C for use to challenge with *S. trutta caspius*.

Virus challenge

Sixty days after the start of the feeding experiments, 10 fish were selected randomly from each treatment. In this study, the mentioned treatment groups kept at 18°C were challenged with an intraperitoneal injection (IP) and 1 group was kept as a control. The RGNNV used for the challenge was propagated in a cell line (SSN-1) derived from the striped snakehead *Channa striatus*. The Caspian salmon were challenged with 100 µL RGNNV infected SSN-1 cell culture supernatant (1.0×10^7 $TCID_{50}$ mL^{-1}). The control groups were mock challenged with an injection of supernatant from uninfected SSN-1 cells (Húsgaro, *et al.,* 2001). Each experimental group consisted of 60 fish. Virulence of mentioned supernatant was examined in a trial challenge in guppy fish (*Poecilia reticulata*), (Nazari, 2014).

Blood sampling and measurements

Blood samples were collected before and 14 days after the challenge. Fish were anaesthetized with clove extract (Farahi *et al.,* 2011). Blood was collected from the caudal vein using 1mL sterile disposable plastic syringes. Blood was transferred into 1.5 mL heparinated tubes (Trittau, Germany) for hematological study and also in non-heparinated tubes for plasma biochemistry analysis. Heparinated blood samples were placed in a refrigerator at 4°C. Non-heparinated samples were immediately centrifuged at 4°C at 1500 ×g for 5 minutes. Plasma was collected with a micropipette and stored at −80°C until analysis. Blood parameters were measured which included Red Blood Cells (RBC), White Blood Cells (WBC) (Torfi Moazenzadeh *et al.*, 2015), Haematocrit (Hct; %) (Barros *et al*., 2002), Haemoglobin (Hb; gL^{-1}) (Drabkin, 1945), MCH (pg/cell), Mean Corpuscular Volume (MCV) (fL) and Mean corpuscular hemoglobin concentration (MCHC) (g/dL) (Dacie and Lewis, 2001). Immune parameters were analysed using an autoanalyser (Mindray BS-200, China), with commercial clinical investigation kits (Pars Azmoon Kit, Tehran, Iran). Measured immunological parameters included total protein (TP), albumin (ALB), IgM, C_3, C_4, respiratory burst and lysozyme (Torfi Moazenzadeh *et al*., 2015).

Statistical analysis

The mean and standard error of three replicates and control were reported for

all parameters at the level of α=0.05. Paired sample t-test was used to determine the effect of each concentration of *C. vulgaris* on mean values of hematological and serum parameters in comparison to the control groups before and after virus exposure. The Pearson ratio was used to analyze the correlation of whole parameters together. Pearson's correlation co-efficient was used to determine an association between the hematological and immunological parameters, pairwise. All statistical analyses were performed using the SPSS ver. 18.0.

Results

The results of measured hematological and immunological parameters and paired comparisons of their alteration trends before and after exposure to VNN virus are presented in Tables 1 and 2 and also in Figs. 1-4. No significant differences were observed in amount of Hct, Hb, RBC, WBC, MCHC, respiratory burst, C_3, albumin (ALB), total protein (TP) and lysozyme in control groups and all experimental groups before the virus challenge (p>0.05) (Table 1). The highest amount of MCH was observed in T_1 but it showed no significant differences with control groups. Amount of IgM in T_1 was significantly more than other treatments. In addition, the amount of C_4 in T_1, T_2 and T_3 were significantly more than in the control group (p<0.05), yet no significant differences were observed between those treatments (p>0.05).

Results of measured parameters after VNN virus exposure showed that MCV in T_1 and T_2 were significantly more than T_3 and control group (p<0.05). The amount of MCHC and IgM in T_1, T_2 and T_3 were significantly less than control group (p<0.05), yet no significant differences were observed between those treatments (p>0.05). Also, C_3 in control group was significantly more than experimental groups (p<0.05). In addition, significant differences were observed in C_3 of all experimental groups, so that C_3 increased follow by increasing the amount of *C. vulgaris* used in treatments.

Table 1: The effect of different concentrations of *Chlorella vulgaris* on some hematological parameters of Caspian salmon before and after VNN virus exposure (*n*=36) and paired test .

		Control	T_3	T_2	T_1
Hct (%)	Before	36.33±0.88	38.5±1.72	35.82±2.44	35.83±1.99
	After	33.33±1.174	30.17±0.872	31.5±0.992	33.5±1.47
	Sig.	0.045	0.001	0.203	0.462
	R	0.429	0.761	0.43	-0.414
Hb (gL^{-1})	Before	6.93±0.25	6.83±0.30	6.56±0.32	6.68±0.30
	After	6.83±0.152	6.03±0.264	5.83±1.66	6.41±0.452
	Sig.	0.679	0.010	0.149	0.704
	R	0.479	0.761	-0.460	-0.527
RBC ($\times 10^6$ µL)	Before	0.858±0.04	0.96±0.05	0.90±0.075	0.83±0.63
	After	1.06±0.041	0.99±0.067	0.91±0.024	0.99±0.068
	Sig.	0.022	0.624	0.921	0.195
	R	0.706	0.528	-0.044	-0.278
WBC (µL)	Before	7066±877	8433±1819	7633±743	7483±727
	After	8200±302	8116±675	9366±1213	10666±1630
	Sig.	0.333	0.877	0.321	0.163
	R	-0.486	-0.013	-0.249	-0.2.6
MCV* (fL)	Before	427±16.56	400±7.39	399±11.99	435±16.66
	After	312±5.59 ^{b}	306 ±11.47 ^{b}	345±3.40 ^{a}	341±11.14 ^{a}
	Sig.	0.000	0.001	0.005	0.005
	R	0.604	-0.100	0.334	0.334
MCH* (pg $cell^{-1}$)	Before	81.41±2.58 ^{a}	71.18±1.12 ^{b}	73.88±3.59 ^{b}	81.03±2.63 ^{a}
	After	64.13±1.18	60.85±1.82	63.96±0.58	64.81±1.11
	Sig.	0.548	0.879	0.046	0.001
	R	0.311	-0.081	-0.096	0.460
MCHC* (gL^{-1})	Before	19.05±0.29	17.78±0.29	18.46±0.44	18.65±0.32
	After	20.55±0.36 ^{b}	19.91±0.40 ^{a}	18.55±0.16 ^{a}	19.05±0.59 ^{a}
	Sig.	0.009	0.019	0.888	0.464
	R	0.425	-0.611	-0.670	0.523

* In each row, shows significant difference at least between 2 groups, before or after VNN virus exposure or both of them ($p< .05$). In each row, different superscripts show significant difference (α=0.05). Sig.s less than 0.05 (p<0.05), in paired compare between a parameter before and after VNN virus exposure shows significant difference."*r*" shows Pearson correlation of a parameter before and after VNN virus exposure.

Table 2: The effect of different concentrations of *Chlorella vulgaris* on some immunological parameters of Caspian salmon before and after VNN virus exposure (*n*=36) and paired test.

		Control	T_3	T_2	T_1
Respiratory Burst	Before	1482±168	1279±91	1491±119	1314±65
	After	3064±102	2744±324	2129±211	2830±206
	Sig.	0.000	0.009	0.002	0.000
	r	0.282	-0.172	-0.095	0.430
IgM* (mg L^{-1})	Before	107.26±10.2 ^{cd}	129.36±9.45^{bcd}	142.23±11.45 ^{bc}	170.46±5.90^{a}
	After	131.16±9.58 ^{b}	83.20±6.34 ^{a}	82.18±5.23 ^{a}	83.96±4.69 ^{a}
	Sig.	0.224	0.024	0.009	0.000
	r	-0.668	-0.670	-0.434	0.714

Continued Table 2:

	Control	T_3	T_2	T_1	Control
C3* (mg L^{-1})	Before	29.95±3.32	29.60±1.45	35.35±3.42	28.81±2.57
	After	50.40±3.95 ^{bd}	20.93±1.34 ^{bc}	27.55±1.38 ^{bc}	40.81±4.22 ^{a}
	Sig.	0.002	0.008	0.068	0.010
	R	0.538	-0.059	0.244	0.710
C4* (mg L^{-1})	Before	19.51±1.77 ^{bc}	24.50±1.06^{abc}	29.25±4.84 ^{abc}	34.88±5.29 ^{abc}
	After	17.91±1.80	17.23±1.85	16.46±1.19	16.28±1.65
	Sig.	0.558	0.015	0.028	0.024
	R	-0.013	0.129	0.639	-0.179
ALB (gL^{-1})	Before	2.13±0.21	1.98±0.07	1.90±0.06	2.23±0.15
	After	2.84±0.27	1.95±0.22	2.08±0.10	2.68±0.21
	Sig.	0.135	0.842	0.150	0.108
	R	0.704	0.945	0.241	0.285
TP (gL^{-1})	before	3.63±0.19	3.95±0.45	3.66±0.12	3.86±0.26
	After	3.88±0.37	3.03±0.10	3.41±0.21	4.33±0.60
	Sig.	0.432	0.101	0.269	0.389
	R	0.620	0.114	0.389	0.594
Lysozyme (IU/ml)	Before	1.23±0.15	1.61±0.21	1.31±0.37	1.24±0.17
	After	1.70±0.15	1.59±0.49	2.18±0.58	3.18±0.82
	Sig.	0.033	0.979	0.143	0.074
	R	0.490	-0.358	0.534	-0.096

*In each row, shows significant difference at least between 2 groups, before or after VNN virus exposure or both of them (p<0.05).In each row, different superscripts show significant difference (α =0.05). Sig.s less than 0.05 (p<0.05), in paired compare between a parameter before and after VNN virus exposure shows significant difference. "r" shows Pearson correlation of a parameter before and after VNN virus exposure.

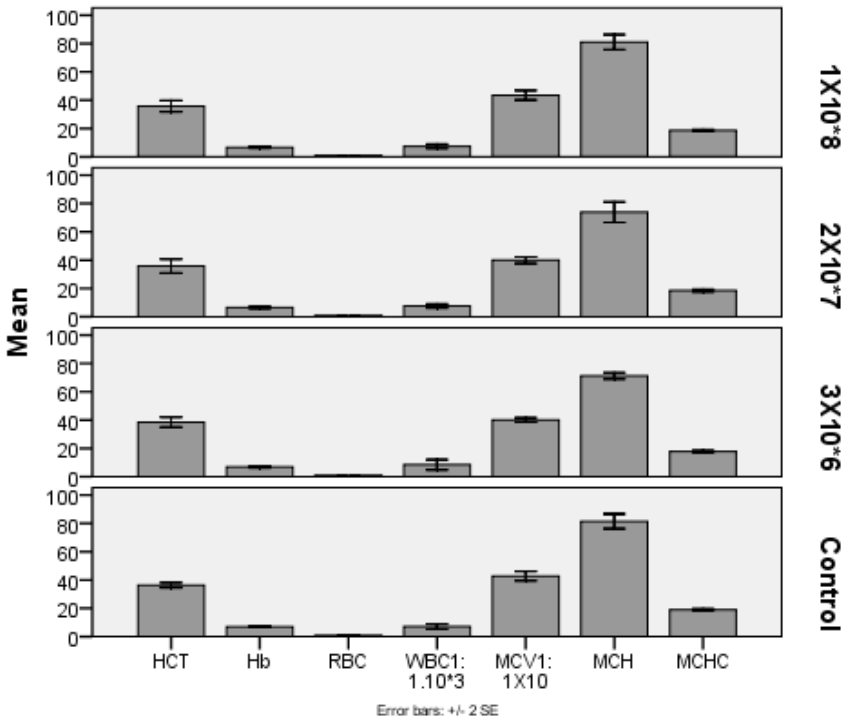
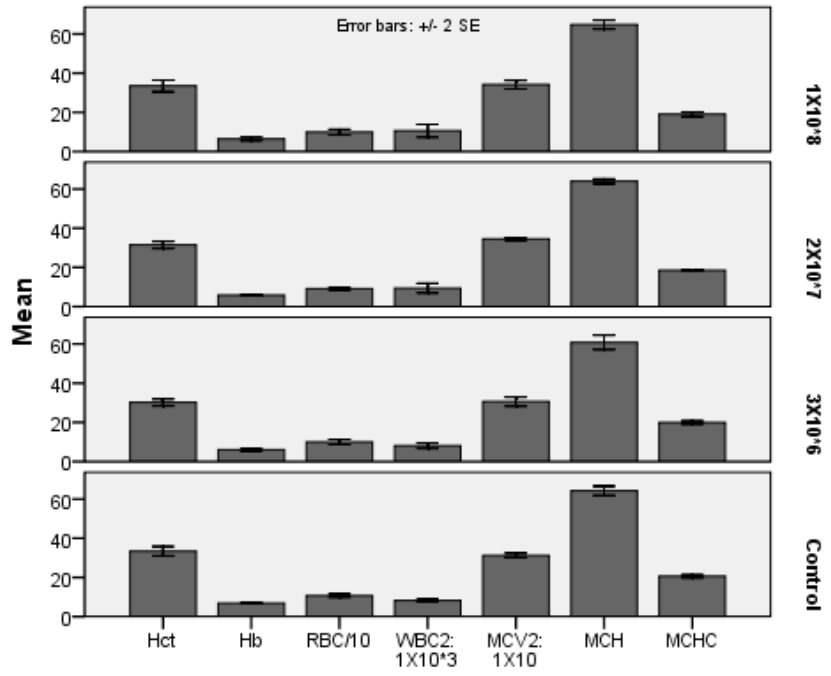


Figure 1: Effect of different concentrations of *Chlorella vulgaris* on blood parameters before exposure to VNN virus.



Error bars: +/- 2 SE

Figure 2: Effect of different concentrations of *Chlorella vulgaris* on blood parameters after exposure to VNN virus.

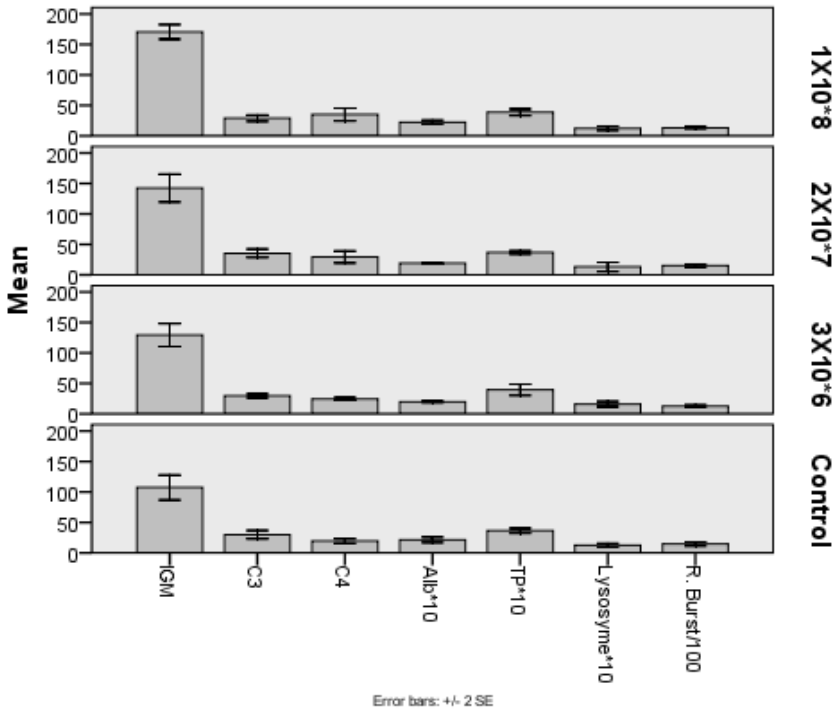


Figure 3: Effect of different concentrations of *Chlorella vulgaris* on immunological parameters before exposure to VNN virus.

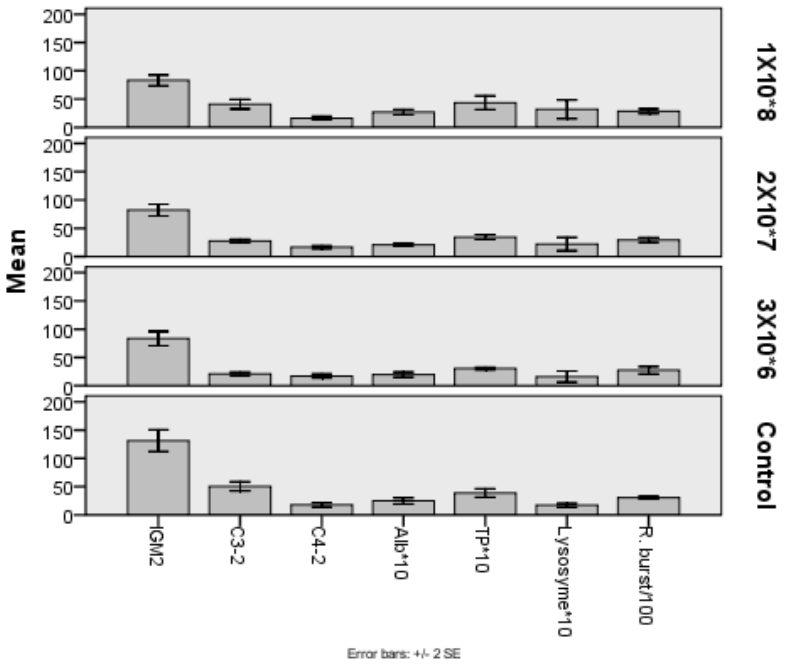


Figure 4: Effect of different concentrations of *Chlorella vulgaris* on immunological parameters after exposure to VNN virus.

Discussion

Research in fish immunostimulants is developing and many of these agents are currently in use in the aquaculture industry. Immunostimulants such as synthetic chemicals or natural ingredient like microalges, increase resistance to infectious diseases, not by enhancing specific immune responses, but by enhancing non-specific immune defence mechanisms. Use of these immunostimulants is an effective means of increasing the immunocompetency and disease resistance of fish (Sakai, 1999; Klesius *et al*., 2001; Subasinghe, 2009). Results of the present study showed that *C. vulgaris* could have acted as a natural immunestimulator in Caspian salmon. Different studies have been shown that microalgae such as *Spirulina platensis* (Ibrahim *et al*., 2013), *Nannochloropsis oculata* (Yanuhar *et al*., 2011), *C. minutissima* (Katharios *et al*., 2005) and also *C. vulgaris* (Xu *et al*., 2014) are effective agents in improving fish immune system. Crude polysaccharide extracts from the microalgae *Chlorella stigmatophora* (Chlorophycae) and *Phaeodactylum tricornutum* (Bacillariophycae) have shown strong anti-inflammatory and immunomodulatory activities both *in vivo* and *in vitro* (Guzman *et al.*, 2003).

No mortality or disease symptoms were observed in fish 14 days after they were challenged with VNN virus which indicates that Caspian salmon (*S. trutta caspius*) could be considered as a resistant species to VNN virus or fish need longer time to show probable disease symptoms. Results of cell culture, IFAT and RT-PCR of challenged fish were also negative (Data will be revealed in the next article

soon) that confirmed *S. trutta caspious* is resistant to VNN virus.

However, the most effective concentration of *C. vulgaris* to improve fish immune system was observed in T_1 (1×10^8), so that IgM and C_4 in *C. vulgaris* treated fish were significantly more than that in the control group and IgM in T_1 was significantly more than that in other groups.

C. vulgaris had a definite influence on the fish immune system in that it increased in all experimental groups even in T_3 with the lowest *C. vulgaris* concentration. Paired comparison of IgM levels before and after the challenge in treatments showed that the factor decreased after challenge in fish treated with *C. vulgaris* but increased in the control group. It seems that *C. vulgaris* treated fish were prepared against VNN virus in terms of IgM and the challenge caused IgM consumption, yet the humoral system was not active in the control group. IgM was produced just after the challenge and it would be consumed in a longer time (longer than the duration of the experiment).

In teleost fish, evaluating the complement system as a humoral component is an essential part of the innate immune system. A specific immunoglobulin or IgM is triggered by the binding of an antibody to the cell surface but can also be activated by acute phase proteins such as ligand-bound CRP or directly by viruses, bacteria and virus-infected cells (Balfry and Higgs, 2001; Aoki *et al*., 2008).

C_4 is a Non-specific humoral molecule and is a part of complement system that has an effective role in the fish immune system (Kum and Sekkin, 2011).

Results showed that *C. vulgaris* has no effect on blood parameters before VNN virus exposure; however it seems that higer the amounts of MCV in T_1 and T_2 after virus exposure is related to consuming more *C. vulgaris* before the challenge. In addition, MCHC increased in all treatments after expousure in that significant difference were observed between the control group and other experimental treatments but significant alteration of MCHC before and after the challenge were only observed in the control group and T_3 that were treated with no and a low concentration of *C.vulgaris*, respectively. It seems that consuming higher concentrations of *C. vulgaris* helps in reducing changes in MCV and MCHC after virus exposure.

C.vulgaris has no significant effect on Hct and Hb of fish before the challenge. Similarly, spirulina causes no significant differences in Hct and Hb levels in olive flounder (*Paralichthys olivaceus*) (Kim *et al*., 2013). Paired test results showed that higer concentrations of microalgae (concentration used in T_1 and T_2) cause lesser impact of virus on Hct and no significant differences were observed in T_1 and T_2 Hct levels not only before but also, after virus exposure. In addition, VNN virus affected Hct levels in fish in T_3 and in the control group which recieved less concentration or no *C.vulgaris*. Significant differences in Hb levels were only observed in T_3 suggesting no effect of *C. vulgaris* on Hb during the experiment.

In addition, fish start showing resistance to the virus after virus injection in that significant increase was observed in the respiratory burst trend before and after the challenge in all treatments that is in agreement with the results of studies by Kim *et al.*, (2013), Watanuki *et al*. (2006) and Abdel-Tawwab and Ahmad (2009). They found spirillina causes significantly higher respiratory burst activity in olive flounder (*P. olivaceus*), common carp (*Cyprinus carpio*), and Nile tilapia (*Oreochromis niloticus*), respectively.

Also, changes in other immune parameters such as C_4, C_3, and IgM showed that fish had begun to show resistance against VNN virus. The alteration trend in C_3 and following that in MCV and IgM showed that fish in T_1 were more ready than other treatments to deal with the virus. If fish blood and immunological parameters had been studied for a longer time, it would have been possible to see significant increase in WBC, TP and ALB that would suggest that *C. vulgaris* concentration in T1 had the best effects against VNN virus.

It seems that C_3 and lysozyme alteration trends were not caused by the virus but may have been accidental.

Lysozyme activity was not changed by *C. vulgaris* supplementation. Paired tests showed that lysozyme in T_1 and in the control group increased significantly and that it was accidental and not due to *C. vulgaris* or the virus effect. On the contrary, Kim *et al* (2013) suggested spirulina's influence on lysozyme activity of olive flounder (*P. olivaceus*) and Promya and Chitmanat (2011) showed a significant increase in serum lysozyme activity in African sharptooth catfish (*Clarias gariepinus*) fed 3% or 5% dietary spirulina. Differences observed in the present study and other mentioned studies might depend on fish species, nutrition, environmental condition, age (Adams *et al*., 2004; Adams and Thompson, 2008) and many other factors that were dissimilar in special experimental condition.

Immune systems affected by drugs such as immunostimulants, should act through the enhancement of the innate immune response (Austin and Brunt, 2009; Nayak, 2010) that was also observed in many measured factors in the present study.

The antiviral effect of microalgae is due to the existence of sulfated polysaccharides, which can inhibit viral infection and/or replicate (Fabregas *et al*., 1999). Some functions of immune parameters like C4, IgM, lysozym, etc include promoted binding of microbes to phagocytes, promoted inflammation at the complement activation, that cause osmotic lysis or apoptotic death. Also, change in the surface charge of microbes to facilitate phagocytosis, and haemolytic and antivirucidal effects (Kum and Sekkin, 2011) get improved due to the use of natural immunestimolants such as *C.vulgaris*.

In conclusion, by experimentation done in this study we can suggest that Caspian salmon should be considered as resistant to VNN virus. Also we can not deny the fact that since the exposure time was only 14 days in the present study, it might be possible that the virus

might be able to cause symptoms in this fish in longer periods of time. However, most of the studies about the possible outbreak of VNN in challenged fishes were done for longer than 14 days. For example after 20 days in Sevenband grouper (*Epinephelus septemfasciatus*) (Tanaka *et al*., 1998) and after 30 days in guppy, zebra, oscar, and gold fish (Zorriehzahra *et al*., 2013). However other than time, there were other differences in these experiments such as fish species, mode of challenge, virus titration and water temprature that should be considered. However Lopez-Jimena *et al* (2012) focused on time and sampled European seabass (*Dicentrarchus labrax*) on days 3, 10, 17, 24, 31 and also 2 months after the challenge. Althought VNN was previously reported in European seabass (Breton *et al.*, 1997), they reported antigenic proteins detected by the use of immunohistochemistry and also vacuolation in brains and retina tissues in the fish 3 days after the challenge.

Furthermore, it is important to emphasize that the trial infection was performed at a very low temperature (15°C), and according to recommendation of other researchers challenge trials at higher temperature should be recommendable before making final conclusions (A. Toffan, personal communication, May 18, 2015). On the other hand, the experimental species Caspian salmon (*S. trutta caspius*) is a coldwater species and the optimum temperature for them would be 10-16°C (trial temperature was 15ºC). When water temperature reaches above 18 °C, some physiological disorders may happen leading to undesirable mortality that would occur naturally. In fact, high temperatures more than 20-22°C would be hazardous for them.

Finally, according to the obtained results in this study, and the lack of findings from similar examinations in Caspian salmon, based on the effects of time and rearing temperature, which was set at 15ºC, it may be concluded that the Viral Nervous Necrosis Virus (VNNV) cannot cause morbidity and mortality in Caspian Salmon under these conditions. However, we cannot definitely claim that Caspian salmon is a resistant species to VNN at higher degrees of temperature or longer time of exposure. Therefore, it is suggested to consider the effect of longer time on morbidity and mortality of VNN in challenged *S. trutta caspius* in future studies.

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