EFFECTS OF DIETARY CHITOSAN-SELENIUM NANOPARTICLES (CTS-SENPS) ON ZEBRAFISH (*DANIO RERIO*) IMMUNE SYSTEM

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PhD

The Hong Kong Polytechnic University

2019

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

July 2018

CERTIFICATION OF ORIGINALITY

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6th May 2019 (Date)

ABSTRACT

Selenium (Se) is an essential micronutrient for human and all animals. There are 25 selenoproteins in human and they are primarily related to antioxidant functions. Effect of Se to the body is complex: it is dependent on the administration dose, the subject's baseline Se status, the administered form of Se and its subsequent metabolism.

Recently, there was increasing interest in the bioactivity of selenium nanoparticles (SeNPs). They are small particles (typically <100nm) of elemental Se and are typically stabilized with polysaccharides and/or proteins at the surface to enhance stability and dispersibility in water. In this study, chitosan stabilized selenium nanoparticles (CTS-SeNPs) were synthesized by using controllable reduction methods. immunostimulation activity of CTS-SeNPs was explored in zebrafish (*Danio rerio*) innate and adaptive immune systems. This thesis aims to explore the immune effects of CTS-SeNPs and its underlying mechanism.

In **Chapter 2**, zebrafish were fed different doses of CTS-SeNP for 3-60d. Immune biomarker assays and bacterial challenge were conducted to evaluate the immunostimulation effects of CTS-SeNP. CTS-SeNP showed immunostimulation effect from 5 to 20 μg/g but with the best outcome at 10 μg/g. Immunostimulation effect were rapidly induced after 3-9d and can sustain to 60d. The zebrafish fed with 10 μg/g CTS-SeNP also had survivalship 26.7 % higher than the control after intraperitoneal injection of common bacterium *Aeromonas hydrophila*. Our results suggested that CTS-SeNP is an effective immunostimulant to fish and has potential application in aquaculture.

In **Chapter 3**, mechanism of immunostimulation effects of CTS-SeNP was explored with proteomics approach on fish serum. Protein expression profiles of fish fed on CTS-SeNP and control diet under 1) non-infection (normal) condition or 2) under bacterial infection condition were compared. Molecular network analyses indicated in non-infection condition, CTS-SeNP activated free radical scavenging network by increasing the expression of two key regulator, SOD1 and NF-κB. It suggested that SeNP might be anti-inflammatory under normal CTS-SeNP supplementary condition. Under infection condition, complement pathways were more

activated in zebrafish supplied with CTS-SeNP. Improvement of complement activation indirectly enhance the downstream immune responses.

In **Chapter 4**, to test the hypothesis that immunomodulation of CTS-SeNP was driven by improvement in redox condition, effects of CTS-SeNP was compared with Trolox at equal antioxidant capacity. CTS-SeNP and Trolox offered similar protection effect on bacterial infection, but CTS-SeNP has better redox regulation and immune biomarker responses. These effects were likely related to functions of selenoproteins, indicating that CTS-SeNP was biologically metabolized to improve both the immune system.

In **Chapter 5**, transcriptome analysis of zebrafish kidney was carried out by RNAseq. Results again demonstrated that CTS-SeNP affect pathways involved in immune system, and also lipid metabolism and environmental adaptation. Findings echoed results in Chapter 2 that many genes involve in T-cell regulation and innate immunity was up-regulated by CTS-SeNP.

Finally, in **Chapter 6** an overall summary of CTS-SeNPs immunostimulation effects was presented and potential immune mechanism of SeNP was discussed. Limitations of the study, future research need and potential applications of this NP were also presented.

ACKNOWLEDGEMENTS

I want to thank my supervisor Dr. Kevin Kwok. He is a remarkable supervisor who interested in my research and help me on questions and experiments. He is the most patient person I`ve even met. He taught me how to think and how to schedule those experiments. His enthusiasm and encouragement have been major driving forces through my graduate career at The HK Polytechnic University. I never thought I could be that lucky. I will treasure this experience and encourage myself to be better in future.

I would also like to thank Dr. Ka-Hing Wong and his research team. They not just helped me on synthesizing selenium nanoparticles but also shared the laboratory with us. In addition, I have to thank Prof. Samuel Lo and Dr. Hang-Kin Kong and their research team. They gave me the tremendous help in proteomic studies and bioinformatic analysis. Also, I have to thank my lab-mates Kwong-Sen Wong, Manhui Wu, Dr. Weixuan Cheng and John Kwok for their generous help.

Finally, a special thanks to my family. I cannot use words to express how grateful I am to my father (Xia ZanShu), my mother (Deng JinHong) and my wife (Chen MeiLing) and my daughter (Xia Le). My father is my real doctor in my life and I love him.

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List of Abbreviations

ACN	Acetonitrile
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
BP	Biological Process
CAT	Catalase
C	Carbamidomethyl
C3	Complement component 3
C5	Complement component 5
C6	Complement component 6
C8	Complement component 8
C9	Complement component 9
CC	Cellular Component
$CD4^+$	Cluster of differentiation 4
CTS-SeNPs	Chitosan selenium nanoparticles
DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DEGs	Differential expression of genes
DTT	Dithiothreitol
ConA	Concanavalin A
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DMSO	Dimethyl sulfoxide
DTNB	5, 5'-Dithiobis (2-nitrobenzoic acid)
EDX	Energy dispersive X-ray
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FBS	Fetal bovine serum
FA	Formic acid
FPKM	Fragment per kb per million fragments
GO	Gene ontology
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCD	Higher-energy collisional dissociation
HCl	Hydrochloric acid
HOCL	hypochlorite
H_2O_2	hydrogen peroxide
IAA	Iodoacetamide
iBAQ	Intensity based absolute quantification
ICP-MS	Inductively coupled plasma mass spectrometry
idotp	isotope dot product
IFN-γ	Interferon γ
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL-1β	Interleukin 1 beta
IL-1	Interleukin 1
IL-10	Interleukin 10

IL-12	Interleukin 12
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
IL-4	Interleukin 4
TNF	Tumor necrosis factor
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
LC-MS/MS	Liquid chromatography–mass spectrometry
LOD	Limit of detection
LPS	Lipopolysaccharides
MAC	Membrane attack complex
MF	Molecular Function
MS	Mass spectrometry
MS-222	Ethyl 3-aminobenzoate methanesulfonate
MS/MS	Tandem mass spectrometry
MTT	Multi-walled carbon nanotube
NK	Natural killer
NaCl	Sodium phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NCBInr	National Center for Biotechnology Information non-redundant
OD	Optical density
NFκB	Nuclear factor-kappa B
NGS	Next generation sequencing
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxide species
RNS	Reactive nitrogen species
RT-PCR	Reverse-transcription polymerase chain reaction
O_2	oxygen
$O_2^{-\cdot}$	Superoxide
SD	Standard deviation
SGD	Sleepy Grouper Disease
SeNPs	Selenium nanoparticles
Se	Selenium
SOD	Superoxide dismutase
TRs	Thioredoxin reductases
TEM	Transmission electron microscopy
Th1	T helper 1 cell
TFA	Trifluoroacetic acid
TMB	3,3', 5,5' tetramethylbenzidine
TNB	2-nitro-5-thiobenzoate anion
UHPLC	Ultra-High Performance Liquid Chromatography

CHAPTER 1

General Introduction

1.1. Aquaculture and Fish diseases

Aquaculture is the fastest developing sector in food production with an average annual production growth rate of 8.6% from 1990 to 2012 [1]. In 2014, global aquaculture production was 73.8 million tonnes with an economic value of US$160.2 billion [2]. In 2015, aquaculture products supplied for 17% animal protein for 3.2 billion people in the world and provide jobs for 769.4 million people [3].

China has the largest aquaculture production in the world. In 2017, aquaculture production reached to 51.4 million tonnes, with year-on-year growth of 4.14 percent [4]. In terms of production volume, China contributes to 66.7% of freshwater aquaculture products and 33.2% of saltwater products to the world's farmed food fish production [3]. According to annual report from Ministry of Agriculture (MOA) and rural Affairs of China, China produced 19.6 million tonnes freshwater aquaculture products and 31.7 million tonnes saltwater products. Over eight hundred million hectares aquaculture area mainly operated in coastal areas of China. One of the biggest aquaculture produced provinces is GuangDong province. In 2014, aquaculture production in GuangDong province reached 6.67 million tonnes and increased to 6.89 million tonnes in 2015 [5]. Aquaculture production in GuangDong benefits customers around this area including Hong Kongers with quality and nutritious seafood products.

Fish diseases are one of the major obstacles in aquaculture development and caused major production and economic loss in aquaculture industry. For example, an outbreak of 'Sleepy Grouper Disease' (SGD) in brown-spotted groupers caused in more than 90% mortality in Singapore in 1998 [6]. Infectious salmon anemia (ISA) disease outbreaks in Chile caused 85% salmon production loss from 2007 to 2010 [7]. To date, there are more than 500 kinds of aquatic diseases recorded, causing a loss of over US$ 1 billion and 15-20% of production annually [8]. Common aquaculture diseases are caused by infection with either bacteria or viruses. Office International des Epizooties (OIE) recorded 35 common aquaculture diseases in 2003 and Quarterly Aquatic Animal Disease Reporting System (QAAD) recorded 32 common diseases in 2005 [9]. Many

of these diseases are specific to cold water species and are not commonly farmed in China. Fish diseases commonly found in China and other Asian countries are mainly caused by four bacterial pathogens, namely *Vibrio* spp., *Streptococcus* spp., *Edwardsiella tarda* and *Aeromonas hydrophila* [10], and seven viruses, including *Infectious pancreatic necrosis virus* (IPNV), *Betanodaviruses*, *Iridoviruses*, *Hirame rhabdovirus* (HRV), *Viral hemorrhagic septicemia virus* (VHSV), *Spring viraemia of carp virus* (SVCV) and *Grass carp hemorrhagic virus* (GCHV)/*Grass carp reovirus* (GCRV) [9, 11, 12] as shown in Table 1.1.

Table 1.1. Common aquaculture diseases of aquaculture fish species in China.

Name of diseases	Pathogens	Host	Pathogen features	Ref.
Aquaculture diseases induced by bacteria				
Vibriosis	*Vibrio harveyi* *Vibrio anguillarum* *Vibrio paraheamolyticus*	Atlantic salmon, rainbow trout, Asian sea bass, turbot, sea bream, Japanese eel, ayu and groupers	Gram-negative bacteria, curved-rod shape	[13]
Streptococciosis	*Streptococcus iniae* *Streptococcus agalactiae* *Lactococcus garvieae*	Rainbow trout, grey mullet, Japanese eel, tilapia, Japanese flounder and striped threadfin bass	Gram-positive, coccus (spherical)	[14]
Edwardsiellosis (EDW)	*Edwardsiella tarda*	Japanese eel, red sea bream, Japanese flounder, yellowtail, channel catfish, carp, turbot and tilapia	Gram-negative, straight rods with flagella	[15]
Motile aeromonads	*Aeromonas hydrophila*	Ayu, carp, channel catfish, Japanese eel, goldfish, golden shiner, snakehead fish, tilapia, trout and chinook salmon	Gram-negative, straight rods with rounded ends	[16]
Aquaculture diseases induced by virus				
Infectious pancreatic necrosis (IPN)	*Infectious pancreatic necrosis virus* (IPNV)	Atlantic salmon, rainbow trout, yellowtail, Atlantic cod, loach, pike	Non enveloped and icosahedral, 60 nm size	[17]
Nervous necrosis virus (NNV)	*Betanodaviruses*	Japanese parrotfish, barramundi, turbot, sea bass and red spotted grouper	Non enveloped, icosahedral 20-35nm	[18]
Iridoviral disease	*Iridoviruses*	Red sea bream, sea bass and grouper	Some enveloped, icosahedral	[19]
Hirame rhabdovirus (HRV)	*Hirame rhabdovirus* (HRV)	Flounder, ayu, black sea bream, Japanese red sea perch, stone flounder	Single-stranded, -ve sense RNA, 120–350 nm	[20]
Viral hemorrhagic septicemia (VHS)	*Viral hemorrhagic septicemia virus* (VHSV)	Rainbow trout, brown trout, white fish and turbot	Enveloped, bullet shaped, ~70 nm X 180 nm.	[21]
Spring viraemia of carp (SVC)	*Spring viraemia of carp virus* (SVCV)	Common carp, grass carp, silver carp, bighead carp, crucian carp, goldfish, tench and sheatfish	Enveloped, bullet shaped	[22]
Grass carp hemorrhagic virus (GCHV)/ Grass carp reovirus (GCRV)	*Grass carp hemorrhagic virus* (GCHV)*/Grass carp reovirus* (GCRV)	Grass carp, black carp, silver carp, bighead carp and common carp	Icosahedral virus, 65-72nm	[23]

Fish farmers are treating these diseases by antibiotics in aquaculture. Abuse of antibiotics in aquaculture leads to food safety issues and environmental contamination. Studies have shown that more than one type of antibiotics was detected in single fish in market [24]. For example, malachite green was detected in fish repeatedly in Hong Kong in the past 10 years even though it is not allowed to be used in food production [25]. A recent risk analysis showed that chronic exposure to low environmental concentrations and legal aquaculture doses of antibiotics cause systemic adverse effects in Nile tilapia and provoke differential human health risk [26].

Another strategy against fish diseases is vaccination. Vaccines have provided an effective protection for human and some farm animal against some common diseases. Fish vaccines against *Aeromonas salmonoides*, cold water vibriosis and Furunculosis has been widely heralded as a success story [27]. However, fish vaccines are generally still highly limited and there are less than 150 licensed fish vaccines globally [28]. Moreover, the predominant vaccine administration method is still manual intraperitoneal injection [29]. This is both time consuming and costly to perform. Further development of fish vaccines may not be a timely and practical solution for fish diseases. First of all, fish diseases are more numerous than other animal diseases. Secondly, aquaculture involved far more species of fish than all land farm animals combined, and these fish species also have widely different immune system biology [30]. Therefore, developing vaccines for fish diseases would be require much time and resources.

1.2. Immunostimulant

Ideally, we want to identify compounds that can help fish to fight against a broad spectrum of diseases, is easily administered in aquaculture setting and safe for human consumption. Immunostimulant is a good candidate for these “ideal” compounds. Immunostimulant are substances (artificial chemicals, animals or plants extracts, bacterial polysaccharides, nutritional factors, minerals and cytokines) that can increase efficiency of the immune system to fight against pathogens by activating or enhancing activity of any part of immune system [31]. It is used widely in animal farming and aquaculture to control diseases as shown in Table 1.2. Because they focus on modulating host immune response instead of directly targeting specific pathogens, they

can be simultaneously effective against pathogens of viral, bacterial, parasite, fungal origin [31]. Different immunostimulants have various potential mechanisms in immune system. For example, the immunostimulant, lipopolysaccharide (LPS) extracted from Gram-negative bacteria, could be recognized by toll-like receptor 4(TLR4) and CD14 on immune cell surface to deliver multiple signaling components to activated proinflammatory responses in host [32]. Another common used immunostimulant is β-glucan. It is comprised by major structural components of yeast and fungal cell walls. The potential mechanisms of β-glucan in immunostimulation has been explored by previous studies. People believed that various receptors such as complement component 3 and TLR1/6 were the targeted receptors. However, the mechanism of β-glucan immunostimulant in fish is still unknown [33]. In addition, antioxidant activity of immunostimulant such as herb extracts, vitamins and minerals contribute to their immunostimulation functions [34]. Previous study reported that antioxidant activity enhanced immunity through removing of excess reactive oxygen species (ROS) during infection thus more active phagocytes or other immune cells could involve in immune responses [35].

Most commonly used immunostimulants now are polysaccharides. For example, β-glucan can effectively enhance antibody production and up-regulate immune-related gene expression in wide range of fish species [36]. In fish culture, chitosan can generally improve innate immunity and resistance to thermal stress [37, 38]. Other immunostimulants proposed includes vitamins[39], garlic extract[40], bacterial lipopolysaccharide[41], herbal extracts[42] and minerals (e.g. selenium)[43]. However, the existing immunostimulants reported previously showed their limits gradually. For example, vitamin C supplementation in fish diet could increase the phagocytic capacity of leukocytes but the effective time is highly limited due to oxidation of vitamin C in fish culture condition [39]. Polysaccharide such as chitosan (CTS) require for a high dose (0.2-1%) to be effect and can be problematic in fish feed formulation [44-46]. These existing immunostimulants here still have their limitation in preventing from aquaculture diseases. Therefore, an immunostimulant with board-spectrum effects in immunomodulation is urgently required in aquaculture development.

Table 1.2. Reported immunostimulants for fish.

Immunostimulants	Dose	Time administration	Fish species	Immunological effects	Challenge study	Ref.
Garlic	0.1 %	14 days	Rainbow trout (*Onchorynchus Mykiss*)	Enhanced hematological parameters (RBC, WBC, monocytes, thrombocytes, lymphocytes), respiratory burst of blood leucocytes, and lysozyme activity	Increased relative percent survival= 68.0 % (*A. hydrophila ip* injection)	[47]
Astaxanthin	100 mg/kg	30 days	Common carp (*Cyprinus carpio*)	Enhanced hematological parameters (RBC, WBC, Hct, hemoglobin, total protein, albumin) Phagocytic ratio, phagocytic index, superoxide production by blood leucocytes, lysozyme activity serum bactericidal activity, serum anti-protease activity	Increased relative percent survival= 76.5 % (*A. hydrophila ip* injection)	[48]
Ginger (*Zingiber officinale Roscoe*)	0.5-1 %	14 days	Rainbow trout (*Onchorynchus Mykiss*)	Enhanced hematological parameters (RBC,WBC, Hct, monocytes and neutrophils proportion), leucocytes superoxide production, lysozyme activity, phagocytic ratio, serum bactericidal activity, anti-protease activity, globulin.	Increased relative percent survival= 94.0 % (*A. hydrophila ip* injection)	[49]
Green tea (*Camellia sinensis L*)	0.25-2.0 g/kg	12 weeks	Nile tilapia (*Oreochromis niloticus*)	Enhanced hematological parameters (RBC, WBC, lymphocytes count, monocytes, granulocytes, glucose, total lipids, total proteins, albumin, globulin), superoxide production (NBT)	Increased relative percent survival rate (*A. hydrophila ip* injection)	[50]
β-glucan	1-10 g/kg	2 weeks	Gilthead bream (*Sparus aurata*)	Spleen-macrophage respiratory burst activity, phagocytic activity	Increased relative percent survival rate (*Photobacterium damselae* subsp. *Piscicida*, 5 min bath)	[51]
Chitin	25-100 mg/kg	6 weeks	Gilthead bream (*Sparus aurata*)	Natural haemolytic complement activity, HK-leucocyte respiratory burst activity, cytotoxic activity	N/A	[52]
Yeast cell walls (*Saccharomyces cerevisiae*)	500 and 20,000 mg/kg	72 days	Japanese seabass (*Lateolabrax japonicus*)	Enhanced hematological parameters (albumin, total protein, total cholesterol), superoxide production (NBT), complement 3, and IgM.	Increased relative percent survival rate (*Aeromonas veronii im* injection)	[53]
LPS (*Aeromonas salmonicida* lipopolysaccharide)	0.01-0.03 %	62 days	Atlantic salmon (*Salmo salar L.*) fry	Total Ig amount	Increased relative percent survival rate (*Vibrio anguillarum* and *Aeromonas salmonicida*, 1 h bath, respectively)	[54]
Vitamin C	1000 mg/kg	60 days	Rohu (*Labeo rohita*)	Enhanced hematological parameters (Hct, total protein, albumin), respiratory burst activity, bactericidal activity	Increased relative percent survival rate (*Aeromonas salmonicida*, 7d bath)	[55]

RBC: red blood cells; **WBC**: white blood cells; **Hct**: hematocrit; **HK**: head-kidney; ***ip***: intraperitoneal; ***im***: intramuscular; **IgM**: immunoglobulin M; **NBT**: nitroblue tetrazolium; **N/A**: not available.

1.3. Selenium

Selenium (Se) is an essential trace element for animals [56]. At least 25 selenoproteins have been identified in human and animals, including important enzymes such as glutathione S-transferase [57]. Despite the exact mechanism is not fully understood, Se was shown to be key for both the innate and adaptive immune systems. Studies have shown that Se status often are lower in allergic asthma populations [58]. *Human immunodeficiency virus* (HIV-1) patients also have lower serum Se concentration compared with healthy individuals. Many researchers have suggested that Se benefit the immune system indirectly through antioxidant activities of selenoproteins [58]. Recently, Se metabolite methylselenol was shown to play important role in immune system by triggering immune activation through lymphocyte receptor NKG2D [59]. This suggested that Se metabolism also has the potential to modulate the immune system directly.

Table 1.3 Summary of immunostimulation effects by Se

Immune systems	Immune responses	Se status		Regulation		Ref.
Innate immune response	Killing capacity of neutrophils	Deficiency	↓	Sufficiency	↑	[60]
	Neutrophil numbers	Deficiency	→			[60]
	Natural killer (NK) cell activity	Deficiency	↓	Sufficiency	↑	[61-67]
	Phagocytosis/Respiratory burst activity			Sufficiency	↑	[68]
	Lysozyme activity			Sufficiency	→	[69]
Adaptive immune system	T lymphocytes proliferation	Deficiency	↓	Sufficiency	↑	[64-67, 70-73]
	Plaque forming cells/B-cell function	Deficiency	↓			[74, 75]
	Antibody concentration	Deficiency	↓			[74, 75]
	Immunoglobulin M (IgM)	Deficiency	↓	Sufficiency	↑ or →	[76-80]
	Immunoglobulin G (IgG)	Deficiency	↓	Sufficiency	↑ or →	[76-78, 80]
Cytokine	Interleukin2 (IL-2)	Deficiency	↓ or →	Sufficiency	↑ or →	[64, 71, 72, 81, 82]
	High-affinity IL-2 receptors			Sufficiency	↑	[72, 82]
	Interleukin-1 (IL-1)	Deficiency	↓ or →		↑ or →	[70, 71]
	Interferon γ (IFN-γ)			Sufficiency	↑	[64, 73]
	Interleukin 4 (IL-4)			Sufficiency	→	[64]
	Interleukin10 (IL-10)			Sufficiency	↑	[64]

↑ : increase the immune response； →: no change the immune response；↓ : increase the immune response

Se could affect reproductive performance and its effect on the immune system was discovered during those experiments [83, 84]. Immunomodulation of Se has been widely reported in previous studies, we summarized the effects of Se in immunity as Table 1.3. Se immunomodulation function was reported in aquaculture species. For example, Channel catfish (*Ictalurus punctatus*) supplemented by sodium selenite or selenomethionine or selenoyeast for 9 weeks showed improved macrophage chemotactic activity and antibody production [85]. Dietary Se supplementation at 1.15 mg/kg for 10 days resulted in a significantly higher blood cell count (RBC), white blood cell count (WBC) and thrombocyte counts in pacu (*Piaractus mesopotamicus*) [86]. The immunomodulatory effects of Se have also been demonstrated in the rainbow trout in terms of elevated WBC count after exposure to 4.0 and 6.0 ppm sodium selenite [87]. A recent transcriptomic using rainbow trout indicated that signaling pathways involved in hematopoiesis and immunity were up-regulated in head kidney, and expression of key mediators of the antiviral defenses and cellular immune responses were also increased [88]. In addition, Se deficiency in catfish decreased diseases resistance and organic Se is more potent than inorganic Se such as selenite in restoring the immune defenses [69].

However, the effective dosage range of Se is narrow and overexposure could result in toxicity. For example, Se in the environment will biomagnify and when water Se concentration increased beyond 3µg/L, mortality and deformities of larvae/fry of Bluegills were observed [89]. With dietary exposure to organic or inorganic Se in Japanese Medaka at 10 mg/g also led to similar toxicity phenotypes [90]. Therefore, the toxicity of inorganic and organic Se should be carefully considered when applying Se as immunostimulant.

1.4. Selenium nanoparticles (SeNPs)

Selenium nanoparticles (SeNPs) is a novel chemical form of Se. It can be synthesized by reducing different valences of Se into elemental form and stabilized by polysaccharides and/or proteins at surface to maintain in nano-size scale (<100 nm) [91]. SeNP have versatile biomedical applications, including drug delivery[92], bone growth promoter[93], antibacterial and antifungal[94], wound healing, nutrition supplement, antimicrobial, anticancer and even as animal feed supplement [91]. One

of the main reasons behind the rising popularity of SeNPs reside its low toxicity and high absorption relative to other common Se species [95]. A number of studies have compared lethal toxicity of SeNP with other common Se species. The LD50 of SeNP was 26 times larger Se dioxide in mice [96]. Comparison of 14-day oral toxicity with mice on selenite, selenate, SeNP and a mix of organic selenium (consists of mostly selenomethionine and selenocysteine) also revealed that toxicity was in the order of selenate>selenite>>SeNP>organic Se [97]. In fish, toxicity of organic Se was much larger than selenite and in turn much larger than SeNP [65].

Research of SeNP on the immune system are still rudimentary. In mammals, there were two studies showing oral consumption of SeNPs on sheep have positive effect on the immune system, namely more effective respiratory burst activities and antioxidative activity in neutrophils, as well as higher number of white blood cells and neutrophils in serum [98, 99]. A study on mice also reported the immunomodulatory effects of SeNP such as upregulation of cellular components (granzyme B, IL-12, IFN-γ, and IL-2) [100]. In fish, similar effects have been observed. For example, Zhou *et al.* indicated that crucian carp (*Carassiusauratus Gibelio*) showed higher antioxidant status and glutathione peroxidase activity after fed the supplement of SeNPs [101]. Under supplemented with SeNP in diets, Juvenile mahseer (*Tor putitora*) increased red blood cell amount, total protein content, lysozyme activity and glutathione peroxidase activity [102]. Despite the immunomodulation of SeNP was reported in different species, the exact mechanism is not fully understood. There are two potential mechanisms have been proposed to explain SeNP immunomodulation. Firstly, SeNP could benefit the immune system indirectly through antioxidant activities of selenoproteins [58]. In addition, SeNP intake could induce the complement systems to activate several downstream immune responses [103]. More studies will be needed to understand these potential mechanisms.

1.5 Fish immune system

The immune system of fish is physiologically similar to vertebrates, including a fully developed innate and adaptive immune systems [104-106]. The innate immune system of fish contains the humoral immune response (included lysozyme activity,

complement activity and cytokines regulation) and the cellular immune response (as phagocytosis). Previous study reported that the mechanism of cell phagocytosis and antimicrobial molecules secretion in fish are similar to mammals. For example, antibacterial peptides and homologous receptors (Toll-like receptors, TLR homologues in mammals) use similar pathways of regulation and transcription with mammals [107]. In addition, the nonspecific cytotoxic cells in catfish were reported functionally similar with granular lymphocytes of mammals with different morphological features [108].

Adaptive immune responses involve a complex network of highly specialized, systemic cells and processes to eliminate invaded pathogens. In fish, components of the adaptive immune system such as immunoglobulin (Ig), T cell receptors (TCR) and products of major histocompatibility complex (MHC) for clonal selection of B and T cells [107]. However, there are also some differences between fish and mammals. However, in mammals, five isotypes of Ig have been described and are named IgM, IgD, IgG, IgA, and IgE. Among them, IgG is the major Ig in class in blood circulation of mammals. For example, immunoglobin in fish is limited to mainly immunoglobulin M (IgM) tetramer with eight antigenic combining sites. Recently, IgT and IgZ were found in the intestine of rainbow trout and zebrafish respectively [109, 110]. The observation in these studies indicated that IgZ could be a novel B cell receptor to further process adaptive immune responses.

1.6 Experiment models

1.6.1 Zebrafish immune model

Zebrafish is a common model organism in molecular biology, toxicology and medical research [111]. It is easily maintained in laboratory and has excellent bioinformatics support on its well-annotated genome. Specifically, zebrafish has been advocated to be used as an alternative to rodents for studying immunological and oxidative stress to nanomaterials [112]. Many researchers have advocated to use zebrafish to understand immunity [105, 113]. Immune system of zebrafish shares high similarity to human’s and both have components of a fully developed innate and adaptive immune system. For example, development of T cells and B cells in zebrafish are remarkably similar to those of mammals [104]. Common immunological parameters will be considered as evaluation index of a immunostimulant, and a series

of comprehensive examination of fish immune system will be applied including the innate immune system (phagocytosis and lysozyme activity) and the adaptive immune system (lymphocyte proliferation and immunoglobulin concentration) [114, 115]. It shows as Figure 1.1.

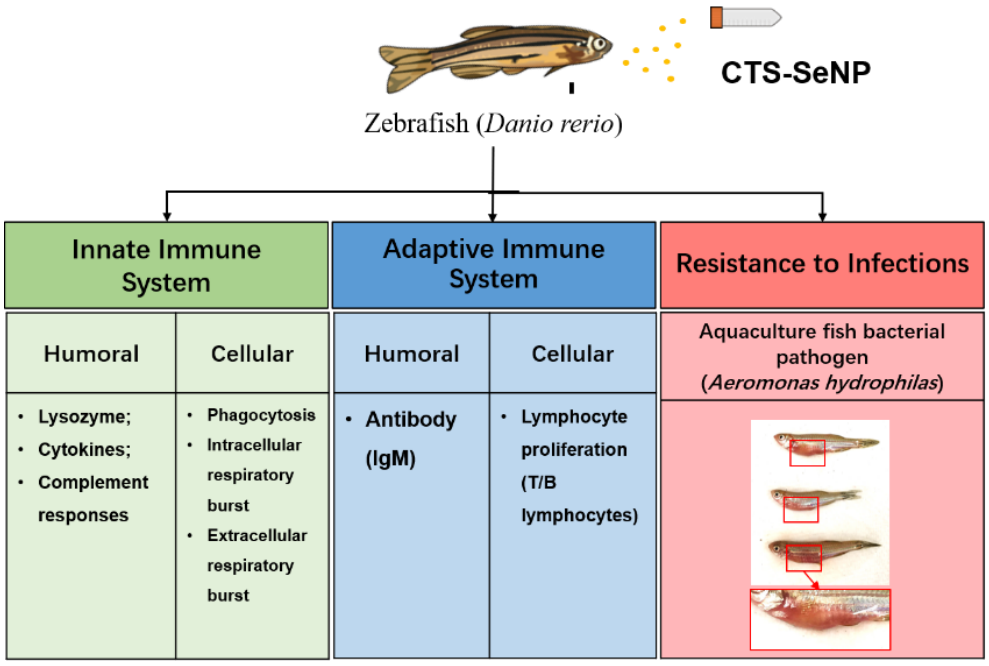

Figure 1.1 The comprehensive immune study platform in zebrafish.

Zebrafish has similar Se metabolism as mammals. At least 18 selenoproteins have been identified in the zebrafish, including glutathione peroxidases, thyroid hormone deiodinases, selenoproteins H, P, R and N [116]. Similar trends of higher uptake of organic Se than inorganic Se was also generally observed in fish as in mammals [90, 117]. In addition, proteomics and transcriptome analysis are powerful omics analysis tools widely applied in various biological studies. In this study, both proteomics and transcriptomes analysis will be performed to explore the mechanism of SeNP in immunomodulation.

1.6.2 Selenium nanoparticle (SeNP)

Combining a functional polysaccharide with SeNP produce excellent biological activity and immunomodulatory application potentials [118]. For instance, SeNP were synthesized by adding using mushroom polysaccharides–protein complexes (PSP) [118]. This PSP-coated SeNPs significantly inhibited the growth of MCF-7 human breast carcinoma cells through induction of apoptosis with the involvement of

Poly(ADP-ribose) polymerase (PARP) cleavage and caspase activation. In addition, using polysaccharide-protein complex (PSP) extracted from *Pleurotus tuber-regium* (PTR) as stabilizer could also prepared SeNP [93]. This PTR-SeNP was identified as an efficient bone-formation therapeutic. As polysaccharide, the amine and hydroxyl groups of chitosan can be used to generate size controllable and highly stable SeNPs in a simple redox system of sodium selenite and ascorbic acid [118, 119]. Therefore, in this study we combined chitosan (CTS), a commonly used immunostimulant, with SeNP to produce CTS-SeNP for further immunomodulatory studies.

1.7 Research questions

Three hypotheses regarding immunostimulation of CTS-SeNP were tested in this thesis.

1. Does CTS-SeNP have immunostimulation effects on zebrafish immunity to against pathogens?

2. Is effect of CTS-SeNP the same for normal healthy fish and fish under bacterial infection?

3. Is the immunostimulation effects related with antioxidant activity by CTS-SeNP?

1.8 Study aims and thesis structure

The thesis comprises four experimental chapters (i.e. **Chapters 2-5**) and focus on understanding impact of CTS-SeNP to fish immune system and the underlying mechanisms.

In **Chapter 2,** impacts of CTS-SeNP on fish immune system were investigated by using a number of biomarkers covering both innate and adaptive immune responses. Effects of dose and time were explored, and the optimum treatment time and dosage were determined to support its further application in immunomodulation. Finally, a within-host infection model was used to showed that CTS-SeNP offered true protection over bacterial infection. The results will have profound implications on immune responses of Se and its mechanism of immunomodulation.

In **Chapter 3**, proteomics combined with within-host infection model was used

to explore the mechanism of SeNP immunostimulation. After SeNP supplementary period, zebrafish were challenged by a common freshwater bacterium, *Aeromonas hydrophila*. After changed study, both non-infected and infected zebrafish were collected serum. Using LC-MS/MS based proteomics, we compared serum profiles and quantitate several interested proteins. A systematic analysis by using proteomics of serum could explore the mechanism of SeNP affects immunity.

In **Chapter 4**, the hypothesis which CTS-SeNP immunomodulation effect was through improving host`s redox status was explored. To test our hypothesis, another strong exogenous antioxidant, Vitamin E (Trolox) will be supplied to zebrafish and immune responses in fish will be studied. Redox regulation will be reflected by analyzed key antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR) to understand the glutathione regulation cycles in zebrafish.

Serum proteomics in **Chapter 2** cannot address cellular changes and responses in zebrafish after exposure to SeNP. In **Chapter 5,** I explored transcriptomics change in zebrafish kidney after CTS-SeNP exposure. The head kidney is an important hemapoietic organ and immune organ in fish. The results enable better understanding of changes occurring in immune cells in response to CTS-SeNP.

Finally, **Chapter 6** sums up the results from all the other chapters and discusses the immunomodulation function of CTS-SeNPs in the light of the results obtained in this study and potential application of CTS-SeNPs in aquaculture or as a health food. Recommendation for further studies are proposed while key research question requiring further investigations are outlined.

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CHAPTER 2

Dietary Chitosan-selenium Nanoparticle (CTS-SeNP) Enhance Immunity and Disease Resistance in Zebrafish*[1].

2.1 Introduction

Selenium (Se) is an essential micronutrient for human and animal health, and its biological functions are mainy exhibited through selenoproteins [1]. To date, 30 selenoproteins have been identified in humans, and many of them play an important role in redox balance and immunomodulation [2]. Interestingly, supranutritional Se levels are often associated with other health benefits such as reduced cancer risk and inflammatory diseases [1]. Immunomodulation of Se was widely studied with the eye on potential health application [3]. For example, researchers found that Se supplementation in polio patients increased T-lymphocyte proliferation and produced more T-helper cells [4]. In studies of thyroid disease, Se supplementation directly increased the number of T-lymphocytes [5] and reduced pro-inflammatory gene expression [6].

Different chemical forms of Se have different bioavailability and toxicity [7]. Se nanoparticles (SeNP) are drawing increasing attention of scientists due to their excellent bioavailability, lower toxicity and strong bioactivity in comparison with inorganic and organic Se [8, 9]. Combining a functional polysaccharide with SeNP often result in enhanced biological activities [10, 11]. For example, SeNP surface stabilized with polysaccharide from *Polyporus rhinoceros* effectively induced apoptosis and cell cycle arrest in A549 human lung adenocarcinoma cells [12]. SeNP combined with polysaccharide from *Ulva lactuca* exhibited anti-inflammatory activity and attenuated colitis in mice [13]. Therefore, SeNP with strong immunomodulation function may be possible by combing with immune-active polysaccharides such as chitosan (CTS). CTS is a linear polysaccharide and made by treating the chitin shells

[1] Published as:

I.F. Xia, J.S. Cheung, M. Wu, K.S. Wong, H.K. Kong, X.T. Zheng, K.H. Wong, K.W. Kwok, Dietary chitosan-selenium nanoparticle (CTS-SeNP) enhance immunity and disease resistance in zebrafish, *Fish Shellfish Immunol* 87 (2019) 449-459.

of shrimp and other crustaceans with an alkaline substance, like sodium hydroxide. It has been commonly recognized as a immunostimulant applied in agricultural and biomedical uses [14, 15]. Recently, chitosan based SeNP (CTS-SeNP) were successfully synthesized and its anticancer function and antioxidant capacities was studied [16, 17]. However, the immunomodulation function of CTS-SeNP has not been studied by these researches.

In this study, we explored the potential of CTS-SeNP as an immunostimulant for fish. CTS-SeNP were synthesized and mixed into a commercial fish feed at various concentrations. Zebrafish were fed with these feeds and their innate and adaptive immune responses were studied at different timepoints. Finally, zebrafish were challenged with a common fish pathogen *Aeromonas hydrophila* to understand the overall immune performance.

2.2 Materials and Methods

2.2.1 Animal ethics statement and fish maintenance

This study was carried out in strict accordance with the Animal (Control of Experiments) Ordinance Cap. 340 by Hong Kong Special Administrative Region (HKSAR) (license number of 15-157). The protocol was approved by Animal Subjects Ethics Sub-Committee (ASESC) (animal ethics 13/21).

Fish maintenance in the laboratory were carried out using the method described in described in previous study [7, 18]. Adult zebrafish (*Danio rerio*) (9-12 months) were maintained in 15-liter tanks flow-through system at temperature around 28 ºC and pH 7 under a 14:10 light-dark cycle. The fish were fed with commercial fish feed (Otohime B1: 6.5% moisture, 51.0% crude protein, 11.0% crude fat, 15.0% crude ash, 2.3% crude calcium, 1.5% phosphorus, USA) three times per day and brine shrimp nauplii once a day.

2.2.2 Selenium nanoparticle synthesis and characterization

Chitosan stabilized selenium nanoparticles (CTS-SeNP) were synthesized using controllable reduction methods as described in Shi *et al* [7]. Briefly, aqueous chitosan solution (0.25%) was mixed with freshly prepared ascorbic acid solution (100 mM)

with magnetic stirring. Aqueous sodium selenite solution (25 mM) was dropwise added in the mixture in the dark. The mixture was reconstituted to 25 mL by MilliQ water and allowed to react at room temperature for around 12 hours before extensive dialysis (Mw cut off: 8000). The characteristics of CTS-SeNP were analyzed by transmission electron microscopy (TEM; JEOL 2010 + Horiba EX-250, USA) and NanoSight NS300 (Malvern Instruments Limited, USA) for particle size distribution. In TEM, elemental composition of the SeNP was characterized by Energy Dispersive X-ray Spectroscopy (EDX). Nanoparticle size distribution were measured by taking average of 3 measurements in NanoSight. ICP-MS (Agilent 7500) was used to determined total Se concentration of this CTS-SeNP stock.

2.2.3 CTS-SeNPs diet preparation

Different amounts of CTS-SeNP were added to the base dry diet (Otohime B1, USA) to prepared low (5 µg/g), medium (10 µg/g) and high (20 µg/g) CTS-SeNPs diets using the method described in Shi *et al* [7]. Briefly, the appropriate amount of CTS-SeNPs was diluted with MilliQ water (Millipore, USA) to make a 10 mL solution. The suspension was thoroughly mixed with 10 g of dry food in a Petri dish to ensure the liquid was evenly distributed and well incorporated. The mixture was then freeze-dried overnight and then broken apart gently by passing through a 100 µm sieve to ensure the particle size was suitable for zebrafish. In addition, one control diet (with the addition of just MilliQ water) and two ingredient control diets were made using the same protocol. CTS ingredient diet (containing 0.025 µg/g chitosan only) was prepared using the same chitosan solution (0.25%) as nanoparticle synthesis. Sodium selenite ingredient diet (containing 10 µg/g Se only) were prepared using sodium selenite solution (25 mM) as nanoparticle synthesis. All the diets were stored in 50 mL centrifuge tubes at 4 °C until experiment. CTS-SeNP diets were also analyzed by TEM and EDX. Total Se concentration of the base diet and all experimental diets were determined by ICP-MS.

2.2.4 Exposure regime

2.2.4.1 Effective dose of CTS-SeNP

Zebrafish were fed with different dosage of CTS-SeNP diet (5, 10 and 20 µg/g Se) at a ration of 2% body weight with a feeding frequency of three time per day for 9

d. For each concentration there were 3 replicates of 9 fish. To comparison effects from different CTS-SeNP ingredients (CTS and selenite), CTS ingredient diet (containing 0.025 μg/g chitosan only) and sodium selenite ingredient diet (containing 10 μg/g Se only) were fed to zebrafish by using the same concentration of ingredient as 10 μg/g CTS-SeNP diet. All immune related organs of zebrafish as mentioned above will be collected for examine the immunomodulation effects of CTS-SeNP.

2.2.4.2 Effects of CTS-SeNP over time

To understand the effects of CTS-SeNP over time, a most effective dose of CTS-SeNP diet was fed to zebrafish at the same ration and frequency as previous describe. most effective dose of CTS-SeNP diet was identical to that of 2.4.1. Immune responses from both innate and adaptive immune parameters were studied at d3, d6, d9, d12 and d60. For each time point, there were 27 fish used. Serum, kidney and spleen in 9 individual zebrafish were collected and pooled as an experimental sample and triplicate measurements were carried out for all of the endpoints mentioned below.

2.2.5 Immune responses analysis

2.2.5.1 Zebrafish organs collection and immune cell isolation

Zebrafish immune organs collection and their immune cells recovery followed protocols published in previous studies [19, 20]. Briefly, zebrafish were euthanized by MS-222 (ethyl 3-aminobenzoate methane sulfonate, Sigma, USA) 1.0 mg/mL for 2 min. Zebrafish blood was collected by using a gentle centrifugation approach [21]. Firstly, the caudal peduncle of zebrafish was severed with a sharp scalpel (Swann Morton, UK). Fish with wound pointing down were individually put into a 0.5 mL micro-centrifuge tube with a small hole at the end. The 0.5 micro-centrifuge tube was then placed inside a 1.5 mL micro-centrifuge tube (Eppendorf, Germany) and the assembly was centrifuged at 700g for 5 min at 11 °C. Blood from 9 individuals was pooled into a 1.5 mL centrifuge tube and allowed to clot on ice for 30 min. Serum was separated with the cells by centrifugation (1200g, 10 min, 4°C) and stored at -80 °C.

Kidneys and spleens were removed and pooled from 9 individuals. Kidneys and spleens were placed separately in 1 mL of phosphate buffered saline (PBS). Single cell suspension were recovered from pooled tissues by using Micro-pestle homogenizer

(Sigma-Aldrich, USA). To remove red blood cells and tissue debris, these homogenates were then passed through a sterile syringe loosely packed with glass wool. The cell suspensions were centrifuged at 700g for 5 min at 4 °C. The kidney cells were resuspended in PBS for phagocytic respiratory burst assays. The cell pellet from spleen was resuspended in L-15 media (Sigma, USA) supplemented with 10 % fetal bovine serum (Gibco, USA), 1% penicillin/ streptomycin (Sigma, USA), 0.5% L-glutamine (Sigma, USA), and 15 mM HEPES buffer (Sigma, USA) for lymphocyte proliferation assay. Cell numbers/viability was determined by hemocytometer and trypan blue.

2.2.5.2 Lysozyme activity assay

Lysozyme is an antimicrobial enzyme abundant in host`s serum and its activity is an important part of the humoral immunity and the innate immune system [22]. Serum lysozyme activity was determined using the EnzChek Lysozyme Assay Kit (Molecular Probes, USA). DQ lysozyme substrate (fluorescein labeled *Micrococcus lysodeikticus*) stock suspension (1.0 mg/mL) and 1000 U/mL lysozyme stock solution were prepared according to the manufacturer's instruction. 10 µL serum was diluted with 40 µL reaction buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5) and incubated with 50 µL DQ lysozyme substrate for 30 min at 28 °C in the dark. The fluorescence was measured in a fluorescence microplate reader using absorption wavelengths of 494 nm and fluorescence emission wavelengths of 518 nm. Background fluorescence was corrected by subtracting the value from no-enzyme control. Serum lysozyme activity was calculated from a standard curve prepared with lysozyme from chicken egg white.

2.2.5.3 Phagocytic respiratory burst assay

Intracellular and extracellular respiratory burst activity are important for phagocytosis and digestion of foreign pathogens. Superoxide production in kidney cells was measured with and without addition of a stimulating agent, phorbol 12-myristate 13-acetate (PMA, Sigma, USA). Intracellular and extracellular superoxide production were measured as a change in absorbance resulting from reduction of Cytochrome C and oxidation of nitroblue tetrazolium (NBT) as described from previous studies [23]. For the extracellular superoxide production, 5×10^5 kidney cells in 50 µL supplemented L-15 medium and 100 µL cytochrome C (4 mg/mL were added to wells of a 96 wells

plate. In half of the wells, 0.5 µg/mL PMA was added. Superoxide production burst was measured with and without addition of 37.5 µg/mL superoxide dismutase (SOD). The plate was incubated at 28 °C for 60 min then was measure spectrophotometrically at 550 nm.

For intracellular superoxide production, 5×10^5 kidney cells were allowed to attach in wells of 96-well plate for 90 min. After attachment of cells, medium was removed, and the removed medium should contain less than 0.25 $\times10^6$ cell/mL. 1 mg/mL NBT (Sigma, USA) with or without SOD and PMA were added into four different wells. The plate was then incubated in 28 °C for 60 min. The formazan produced by NBT reduction in attached kidney cells was dissolved by DMSO and 2M KOH (Sigma, USA) and measured at a wavelength of 620 nm. To calculated both PMA-stimulated and non-PMA-stimulated superoxide production in both assay, the absorbance of wells with SOD were subtract the one without SOD. Total nmol of superoxide anion were calculated by multiplying final OD values by 15.87 [24]. The final extracellular and intracellular superoxide production were expressed as nmoles of $^{\cdot}O_2^-$ produced/5×10^5 cells/60 min.

2.2.5.4 Lymphocyte proliferation assay

For adaptive immune responses, lymphocyte proliferation assay was studied by using zebrafish splenocytes. Proliferation of lymphocytes in zebrafish spleen was determined by a microtiter assay with modification by using a Vybrant MTT Cell Proliferation Assay Kit (invitrogen, USA) [25]. Briefly, splenocytes (5×10^6 cells/well) in 100 µL L-15 medium were added wells of a 96 wells plate. Proliferation of B-lymphocytes and T-lymphocytes was determined in response to 100 µg/well of lipopolysaccharide (LPS, Sigma, USA) and 100 µg/well of concanavalin A (ConA, Sigma, USA) respectively. After 96-hour incubation at 28 °C, 10 µL 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) was added to each well for another 4-hour incubation at 28 °C. The reaction was ceased by adding 100 µL sodium dodecyl sulphate (SDS, Sigma, USA) solution (1 mg SDS in 10 mL of 0.01 M HCl) to each well. After incubation at room temperate for 10 min, the mixture containing soluble formazan crystals were read absorbance at 570 nm by using CLARIOstar Microplate Reader (BMG Labtech, Germany). Proliferation was

calculated as the change in absorbance at 570 nm.

2.2.6 Bacterial culture and challenge experiment

Overall immunity of zebrafish by CTS-SeNP was studied under bacterial challenge. After feeding on 10 μg/g CTS-SeNP diet, 0.025 μg/g CTS ingredient diet and 10 μg/g sodium selenite ingredient diet for 9 days, zebrafish were challenged by a common freshwater fish bacterium, *Aeromonas hydrophila* (ATCC 7699, USA) for 72 hours. After the optimal treatment time and the most effective dosage of CTS-SeNP diet were confirmed, a total 90 zebrafish were used in a bacterial challenge study. After 9 days of feeding, zebrafish on control and CTS-SeNP treatment diets were intraperitoneal (*ip*) injected with 2.5×10^6 cfu of *A. hydrophila* in 10 μL PBS. This bacterial concentration for zebrafish infection was well established in our laboratory and can induce mortality of ~80 % in 72 hours. A negative control was conducted with another 30 zebrafish individuals by *ip* injection of 10 μL PBS only. All zebrafish were maintained at 28 ± 1°C after injection and was observed for 72h. Survival rate of fish in each group was monitored every 8 hours.

2.2.7 Statistical analysis

Data were presented as mean ± SD and considered to be significantly different at $P \leq 0.05$ level. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism ver 6.00 (GraphPad Software, USA). Survivorship data of the bacteria challenge experiment were compared using Kaplan-Meier analysis coupled with log-rank test (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests in SPSS (ver 15.0, IBM SPSS Statistics, USA).

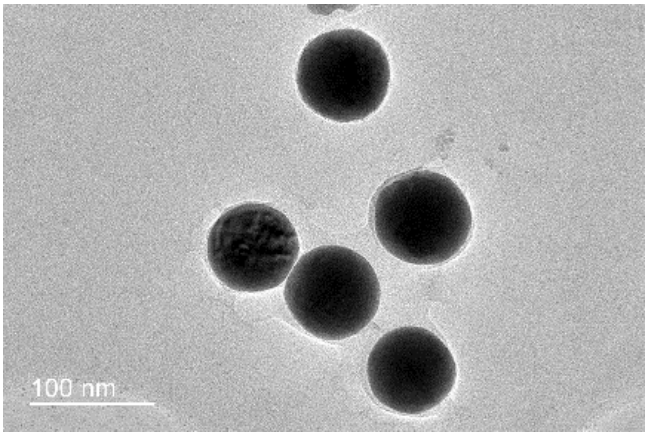
2.3 Results

2.3.1 Characterization of CTS-SeNPs and CTS-SeNPs diets

As shown in Figure 2.1.a, CTS-SeNP were largely spherical with a homogeneous structure. The average diameter was 72.1nm (SD = 2.27) under TEM. NanoSight showed average particle diameter of CTS-SeNP to be 117 nm (SD = 32 nm). TEM image showed that the distance between the two layers of Se atoms in the NP was 0.31 nm (SD = 0.06 nm) with a regular crystalline structure. Using EDX, CTS-SeNP was shown to contain 82.7% Se and some carbon (17.3%), which was likely from the

CTS surface stabilizer.

a)



b)

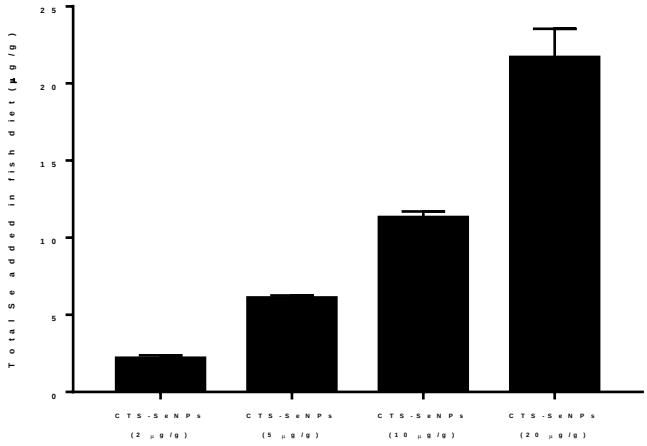


Figure 2.1 Chitosan stabilized selenium nanoparticles (CTS-SeNP): a) representative TEM image; b) measured concentration versus nominal concentration of CTS-SeNP fish diets as determined by ICP-MS.

Added Se concentrations in CTS-SeNP fish feeds were determined by using ICP-MS (Figure 2.1.b) and the concentration matched the nominal concentration well. The original Se concentration in base fish feed is 3.6 μg/g (sodium selenite), which is similar to the value reported by earlier studies using the same base fish feed [7, 26]. CTS-SeNP clearly attached to fish feed particulates while the control diet did not contain those similar nano-sized particles (black arrows, Figure 2.2.a and b). The CTS-SeNP in diet covered by fish feed release and showed slightly bigger than the CTS-SeNP stock. Elemental composition analysis by EDX indicated that Se concentration in CTS-SeNP diet was higher than control diet (Figure 2.2.c and d).

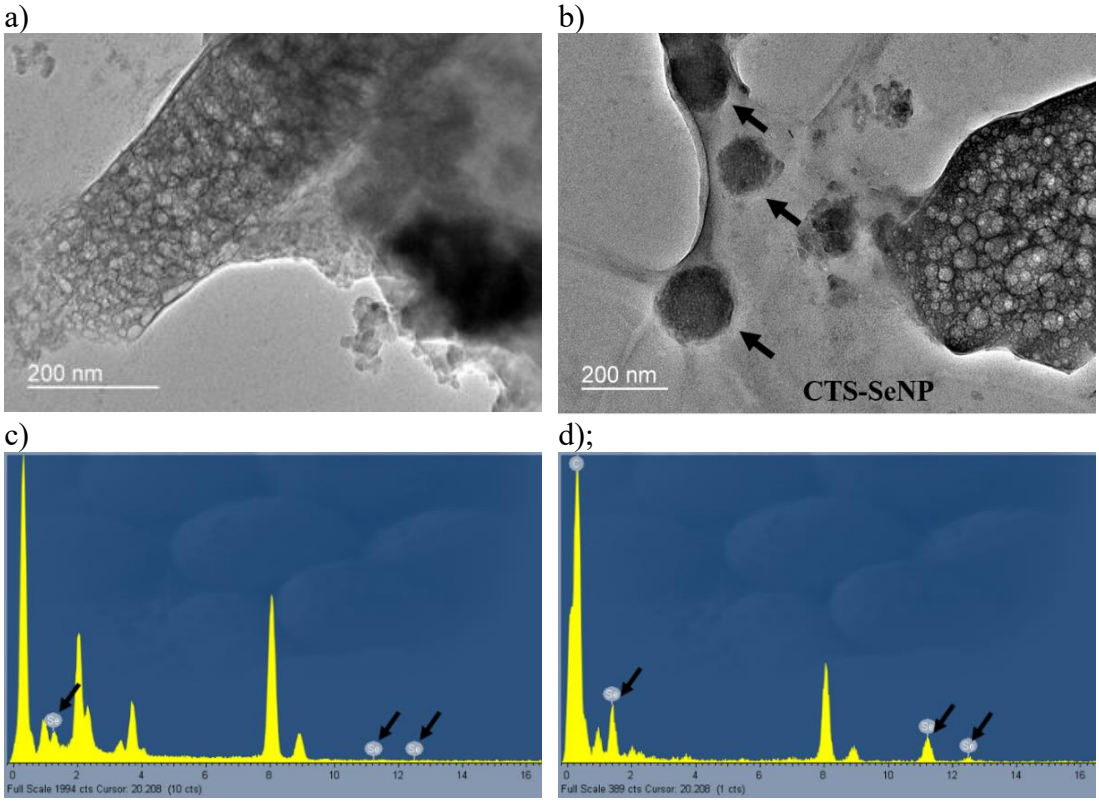
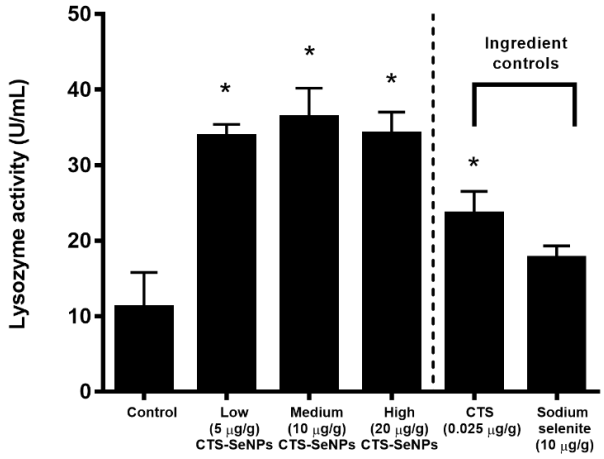


Figure 2.2 Representative TEM images of zebrafish pellet diet: a) control; b) after addition of CTS-SeNP (black arrows); c) corresponding EDX spectrum of control diet; corresponding EDX spectrum of CTS-SeNP diet. Arrows indicate spectra for Se element

2.3.2 Effective concentration of CTS-SeNP

Lysozyme activity increased approximately 3-fold compared to control for all concentrations of CTS-SeNP (Figure 2.3.a). In ingredient controls, CTS diet could double enhance this activity while sodium selenite diet could increase less. Intracellular and extracellular respiratory burst also improved under multiple concentrations of CTS-SeNP. Without PMA, both intracellular and extracellular superoxide production were slightly increased at the 20 µg/g but the change was not statistically significant (Figure 2.3.b and c). Intracellular respiratory burst after PMA stimulation showed more pronounced increase in all three concentrations while only 10 and 20 µg/g have significant effect on extracellular respiratory burst. Ingredient controls showed no changes in both extracellular and intracellular superoxide production.

a)



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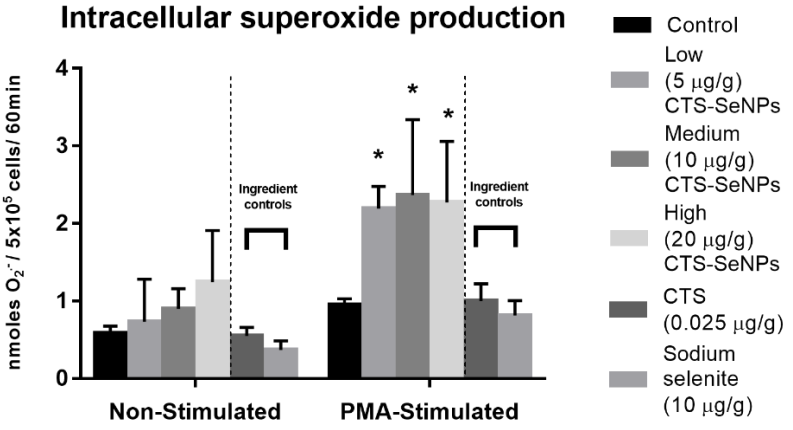


c)

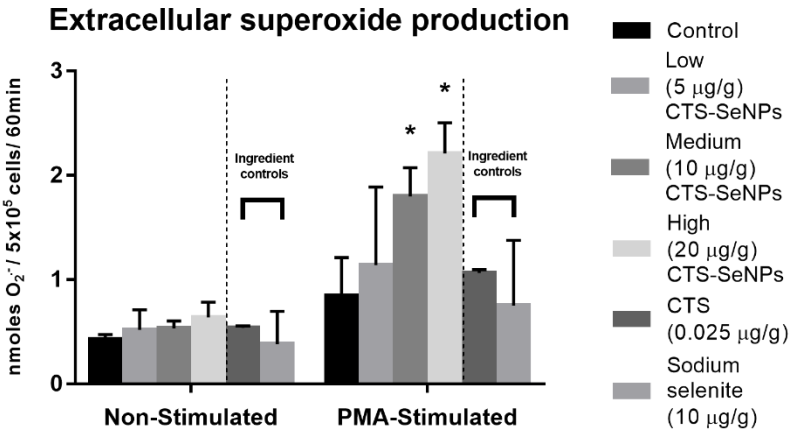
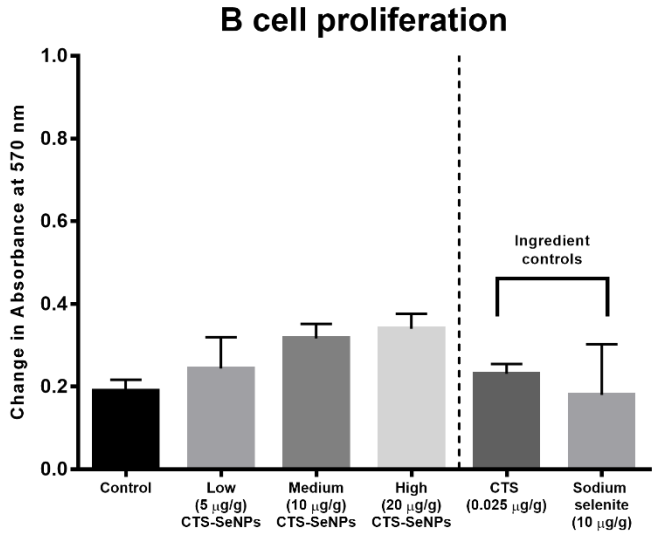


Figure 2.3 Innate immune responses in zebrafish after exposure to different concentration of CTS-SeNP and ingredient controls: a) lysozyme activity in zebrafish serum; b) intracellular respiratory burst activity; c) extracellular respiratory burst activity. Statistical significant difference from control was represented by asterisks.

For adaptive immunity, B cell proliferation towards LPS and ConA again

responded differently under the three CTS-SeNP concentrations (Figure 2.4.a and b). B cell proliferation towards LPS were not impacted significantly by CTS-SeNP while T cell proliferation towards ConA were significantly increased only at 10 μg/g. In addition, all dose of CTS-SeNP diets had higher response than those with ingredient diets.

a)



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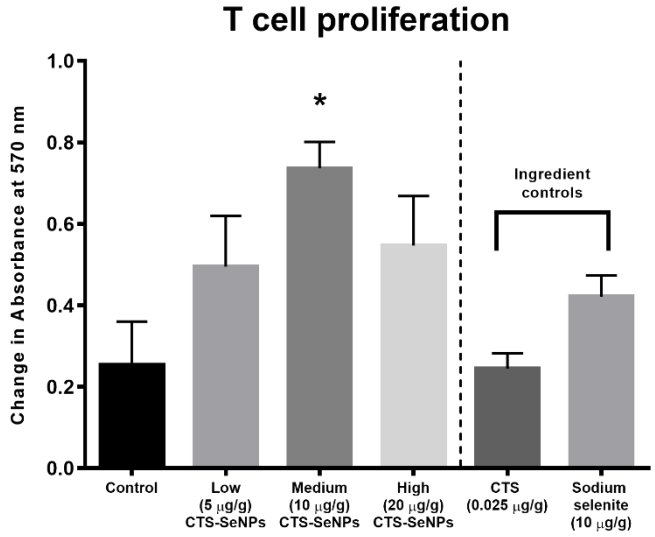


Figure 2.4 Lymphocyte proliferation assay in zebrafish splenocytes after at different concentrations of CTS-SeNP and ingredient controls: a) B cell proliferation towards LPS and b) T cell proliferation towards ConA. Statistical significant difference from control was represented by asterisks.

2.3.3 Effects of CTS-SeNP over time

Lysozyme activity was found to significantly increase after 6 days feeding on CTS-SeNP and reached a peak of 32 U/mL at d9 (Figure 2.5). Lysozyme activity remained high even at d60 at 29 U/mL.

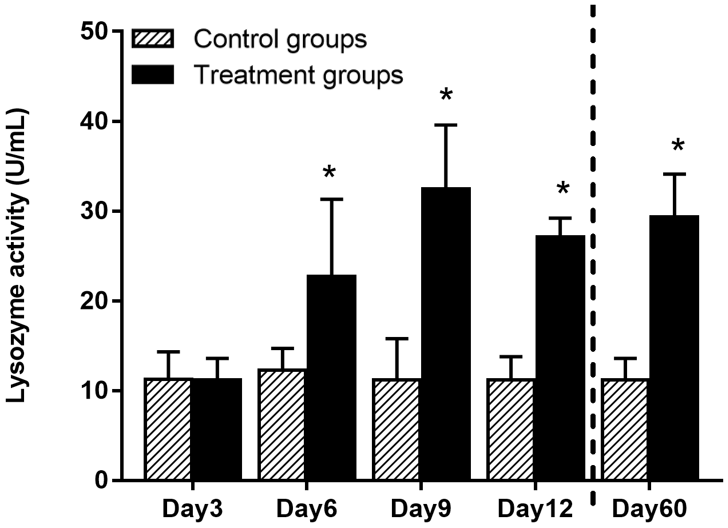
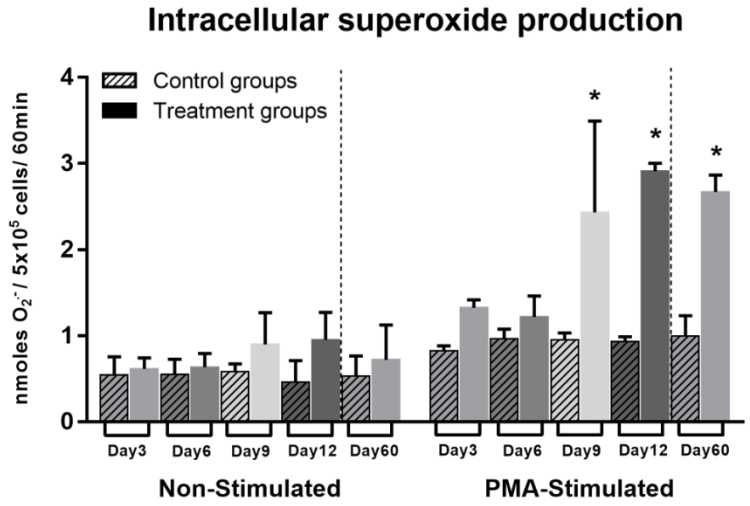


Figure 2.5 Lysozyme activity in zebrafish serum at different time points after dietary exposure to 10 μg/g CTS-SeNP. Statistical significant difference from control was represented by asterisks.

Without PMA stimulation, there was no difference between CTS-SeNP and control for both intracellular and extracellular responses. It indicated that CTS-SeNP might have a relative antioxidant capacity to keep the cellular redox status in balance. When PMA was present, both control and treatment groups were able to respond by producing superoxide anion intracellularly (Figure 2.6.a) and extracellularly (Figure 2.6.b). A more pronounced response was observed in extracellular superoxide production just after 3d of CTS-SeNP treatment and the increase was sustained even after 60d. For intracellular oxidative burst, a significant increase was observed after 9d and again the increase was sustained even after 60d.

a)



b)

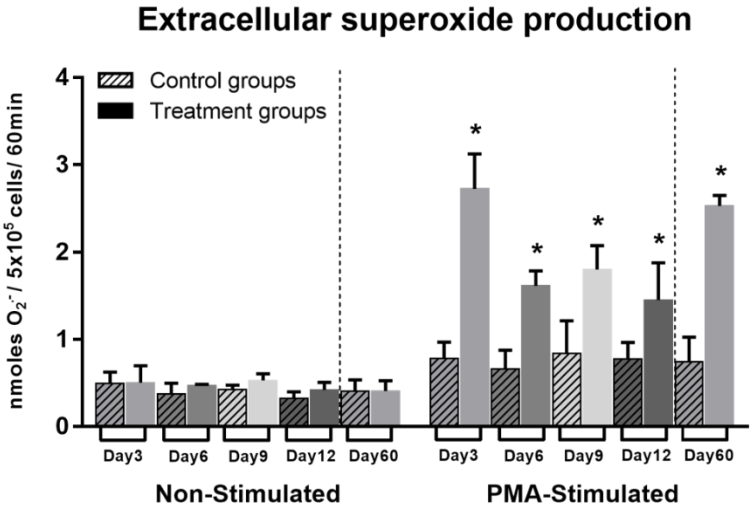
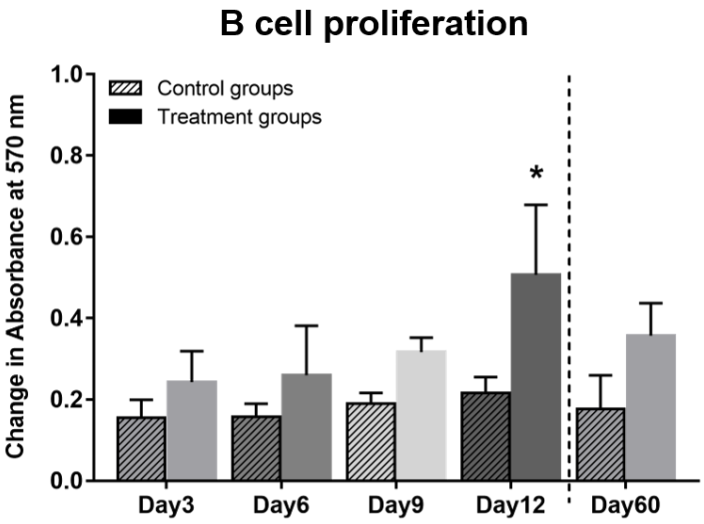


Figure 2.6 Phagocytic respiratory burst assay in zebrafish kidney cell at different time points after dietary exposure to 10 µg/g CTS-SeNP: a) intracellular respiratory burst activity and b) extracellular respiratory burst activity. Statistical significant difference from control was represented by asterisks.

LPS was used to stimulate B-lymphocyte proliferation. There was a general trend of increased proliferation in the CTS-SeNP treatment group and reached the highest at 12d with a 2.6-fold increase but there was no significant effect after 60d (Figure 2.7.a). On the contrary, T-lymphocyte proliferation was significantly increased just after 6d and the increase was sustained through 60d (Figure 2.7.b). T-lymphocyte proliferation reacted highest at 9d with a 2.9-fold increase comparing to control group.

10 μg/g CTS-SeNP have led to significant increase in lysozyme activity, phagocytosis and T-lymphocyte proliferation after a minimum treatment time of 3-9d. In next section, the effective dosage range of CTS-SeNP experiment would refer to these results and use 9d as the treatment period.

a)



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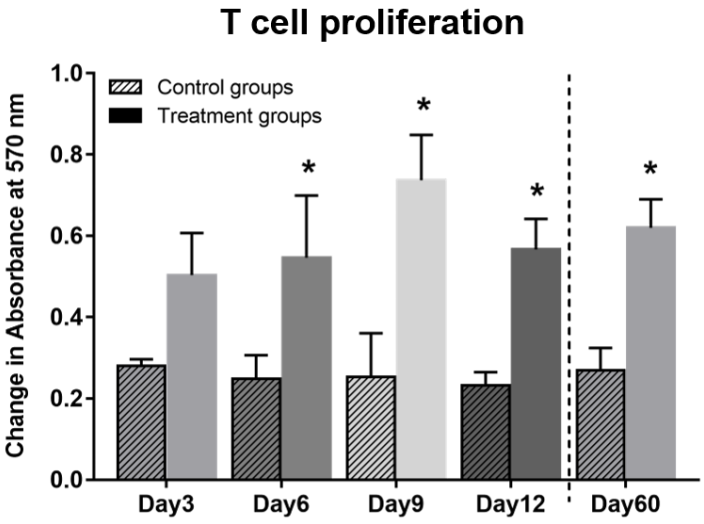

Figure 2.7 Lymphocyte proliferation assay in zebrafish splenocytes after at different time points after dietary exposure to 10 μg/g CTS-SeNP: a) B cell proliferation towards LPS and b) T cell proliferation towards ConA. Statistical significant difference from control was represented by asterisks.

2.3.4 Survival rate of zebrafish after bacterial challenge

There was no mortality in the PBS injected fish throughout the experiment. Zebrafish injected with bacteria developed phenotype of hemorrhagic septicemia and abdominal swelling (Figure 2.8.a), which are typical symptoms of *A. hydrophila* infection [27, 28]. In our study, infected zebrafish from control diet group were the first to display symptoms and mortality was recorded after just 4h. After 24h, survival rate of the group dropped to 26.7%, significantly lower than infected fish of the CTS-SeNP diet group which has a survival rate of 66.7% (Figure 2.8.b). After 72h, survival rate in control diet group (20%) were still significantly lower than the CTS-SeNP diet group (46.7%). Meanwhile, CTS diet group was 3.3% higher than control diet group but 23.4% lower than CTS-SeNP diet group. Survival rate in sodium selenite diet (16.7%) was even lower than the control.

a)

b)

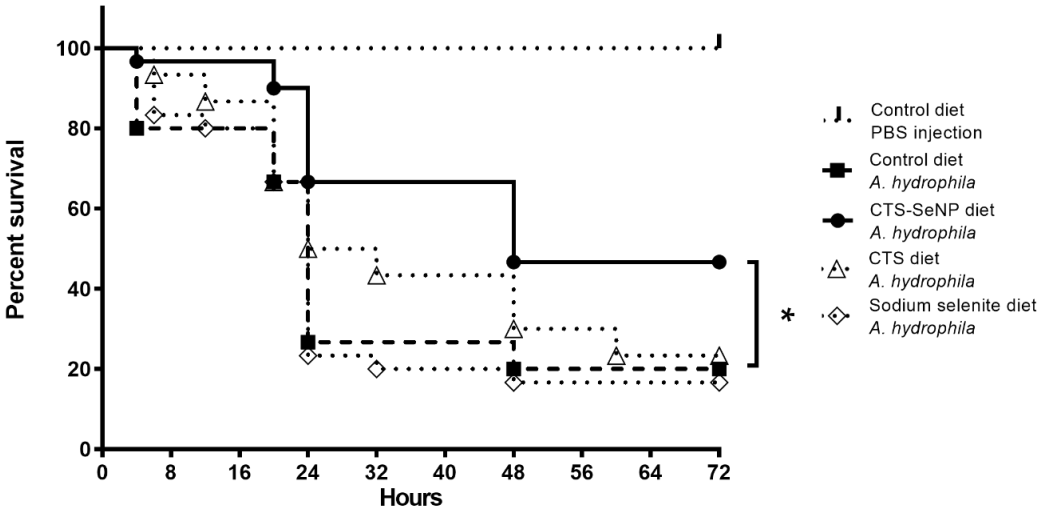

Figure 2.8 Bacterial challenge in zebrafish: a) zebrafish after *ip* injection of *A. hydrophila* showing typical symptoms of infection including hemorrhagic septicemia and abdominal swelling; b) 72h survival rate of zebrafish injection of *A. hydrophila*: dotted line represents the negative control which zebrafish injected with PBS; dashed line represents control zebrafish injected with *A. hydrophila*; solid line represents the CTS-SeNP treated zebrafish injected with *A. hydrophila*. Statistical significance was represented by asterisks.

2.4 Discussion

In this study, we explored immunomodulation effect of a nanoparticle which was produced from two known immunostimulants, namely Se and CTS. Through our experiments, we observed that CTS-SeNP have stimulatory effect on the innate and the cellular immune responses. These benefit to the immune system led to significant protection effect of CTS-SeNP against bacterial infection from *A. hydrophila.* The immunomodulation effects observed were more comprehensive than other immunostimulants or its primary ingredients inorganic selenite and CTS (Table 2.1). In general, polysaccharide immunostimulant only provide boost to innate immune responses. Inorganic Se and organic Se mainly enhances the immune responses in cellular immune responses such as phagocytosis. In our study, its primary ingredient CTS could significantly increase lysozyme activity and relatively increase extracellular superoxide production; Sodium selenite ingredient diet could not impact those innate immune responses, but it induced T cell proliferation towards ConA after 9d treatment. Therefore, both components in the CTS-SeNP have functional activity in fish, and CTS-SeNP is more comprehensive than other single component immunostimulants.

It is difficult to compare efficacy of CTS-SeNP with other traditional immunostimulants as this nanoparticle is a combined material from two immunostimulants. Comparing with organic and inorganic Se by using 0.2-4 mg/kg Se to develop immunomodulation effects (Table 2.1), CTS-SeNP in our study required a higher effective dose by using 10 μg/g Se. However, the stock CTS-SeNP contained at most 0.25% of the chitosan when produced (see section 2.2). Therefore, at its effective dose, there was only 0.025 μg/g of CTS. This concentration of CTS was 3-6 order of magnitude less than previous reports for polysaccharide immunostimulants to be effective (Table 1).

In terms of quick immunomoudaltion and chronic effect, CTS-SeNP compares favorably with other immunostimulants. Our observation found that CTS-SeNP could stimulate immune response in zebrafish after 9 days supplementary. Other studies reported that Kelp grouper supplemented with CTS constantly increased lysozyme activity and hematological indices as quickly as 6d. [29]. Fingerlings of channel catfish *Ictalurus punctatus* were supplemented by sodium selenite, selenomethionine or

selenoyeast for 9 weeks and showed improved antibody production and macrophage chemotactic activity. [30]. Previous study have also reported SeNP immunomodulation effects in fish. Juvenile mahseer (*Tor putitora*) fish supplemented with SeNP could increase red blood cell amount, total protein content, lysozyme activity and glutathione peroxidase activity in a 70d feeding trial [31]. We think the efficient uptake and stimulation effects of CTS-SeNP could due to mucoadhesion mechanism of CTS in zebrafish intestine. Previous study introduced that CTS could be effectively uptake through an ionic interaction between the positively charged amino groups of CTS and the negatively charged sialic acid residues in mucus [32]. Therefore, the timely protection by CTS-SeNP benefited from the efficient uptake of CTS in zebrafish intestine.

Table 2.1 Summary of immunomodulation effects of common immunostimulants.

Immunostimulants	Host species	Dosage and period	Innate Immunity			Adaptive Immunity		Pathogen Resistance	Ref.
			Lysozyme activity	Extracellular respiratory burst	Intracellular respiratory burst	Lymphocyte proliferation	Antibody production		
β-Glucans	*Oncorhynchus mykiss; Cyprinus carpio; Pseudosciaena crocea*	1.8% glucan for 21d; 0.5% glucan for 56d; 0.09% glucan for 56d;	↑	↑	↑	-	NA	*Flavobacterium columnare; Aeromonas veronii; Vibrio harveyi; Edwardsiella tarda*	[33-35].
Lipopolysaccharide	*Oncorhynchus mykiss; Labeo bata*	0.15mg/g for 14d; 100mg/kg for 30d	↑	-	↑	-	NA	*Aeromonas hydrophila; Edwardsiella tarda*	[36, 37].
Chitosan (CTS)	*Oreochrmis niloticus; Cyprinus carpio;*	1% CTS for 21d; 1% CTS for 90d; 0.6% CTS for 56d; 0.2% CTS for 56d	↑	NA	↑	NA	NA	*Aeromonas hydrophila; Vibrio harveyi;*	[34, 38-40].
Inorganic Se	*Ictalurus punctatus; Oncorhynchus mykiss*	0.4 mg/kg (sodium selenite) for 9 or 10 weeks	-	-	↑	NA	↑	*Edwardsiella ictaluri*	[30, 41, 42].
Organic Se	*Ictalurus punctatus; Oncorhynchus mykiss*	0.2 mg/kg (selenomethionine; selenoyeast) for 9 weeks; 4 mg/kg (Sel-Plex) for 10 weeks	-	-	↑	NA	↑	*Edwardsiella ictaluri*	[30, 41].
SeNP and vitamin C	*Tor putitora*	0.68 mg/kg (SeNP) and 100 mg/kg vitamin C for 60d	↑	NA	NA	NA	NA	NA	[43].
CTS-SeNP	*Danio rerio*	10 μg/g Se and 0.004% CTS for 9-60d	↑	↑	↑	↑	NA	*Aeromonas hydrophila*	this study

↑: increase the immune response

- : no change the immune response

NA: no available

It is also worthy to notice that both extracellular and intracellular respiratory burst were increased after supplementation of CTS-SeNP. Respiratory burst is a rapid immune response in activated phagocytes to release of reactive oxygen species (ROS) to eliminate foreign particles and bacteria to combat infections [44]. The mechanisms of extracellular and intracellular production of ROS in PMA-stimulated phagocytes are different [45]. Extracellular superoxide production mainly comes from NADPH oxidase from activated phagocytes to reduce molecular oxygen to superoxide anion radical [19]. Intracellular superoxide anion is mainly produced by mitochondria during redox regulation processes. Previous study reported that PMA-stimulated J774.4 macrophages released more superoxide in Se supplemented treatment, and Se deficient cells were partial perturbed the integrity of cellular membranes where the NADPH oxidase complex is assembled [46]. It was generally postulated that Se involved in cellular activity by synthesizing as antioxidant related enzymes such as glutathione peroxidase (GPx). GPx plays a key role in removing hydrogen peroxide (H_2O_2). We believe that one of the mechanism of the preventive effects of additional dietary Se supplementation could be potentiated GPx scavenging activity during removal of hydroperoxides diffused from phagolysosomes [47]. The increased GPx activity could reduce the production of H_2O_2 and promote the circulation of NADPH oxidases during phagocytosis [48].

Zebrafish splenocytes showed significantly better proliferation into T cell proliferation towards ConA and B cell proliferation towards LPS. Previous study reported the mechanism of T-lymphocyte proliferation was highly associated with GPx1-dependent control of intracellular ROS accumulation. In our study, Se supplementation in zebrafish by CTS-SeNP diet might regulate GPx activity to affect intracellular ROS accumulation [49]. Moreover, another mechanism of T-lymphocyte proliferation is through increasing IL-2 production. Previous study reported that IL-2 and IL-2 receptor were up-regulated at Se supplementation [49, 50]. Therefore CTS-SeNP could improve T-lymphocyte proliferation through these known pathways. On the other hand, there was little evidence showing that pathways regulating B-lymphocyte proliferation are influenced by Se. Significant increase in B-lymphocyte proliferation at 12d was more likely related to general improvement of redox status of the fish.

Increased lysozyme activity was normally associated with supplementation of antioxidants[51] or polysaccharides[33-40]. Rainbow trout supplemented with inorganic Se (sodium selenite) or organic Se (selenium yeast) did not have effect on lysozyme activity [41, 42]. The immunostimulation effects of CTS as was widely reported with lysozyme activation (Table 1). But the CTS used was 3-6 order of magnitude less than previously reported. Therefore, we believe that it is likely that CTS-SeNP as a combined material enhanced the efficacy of CTS, such as increased cellular entry. Further studies will be needed to elucidate the underlying mechanisms of immunostimulatory effect of CTS-SeNP. In addition, for the *A. hydrophila* challenge, mortalities were significantly reduced in all groups compared to controls, with the lowest mortality in immunostimulated fish fed with CTS-SeNP diet. It was considered that the dose of bacteria we used for challenge was relatively high, resulting in 80% mortality of control fish. Previous studies using *A. hydrophila* (1×10^6 cfu per fish) *ip* injected in tilapia also observed that fish started to die within 12 h with one quarter of the injected fish dying between 12 and 24 h post-infection [52]. In aquaculture, disease caused by *A. hydrophila* is most widespread in freshwater fish such as carp, channel catfish and tilapia. Vaccines are being developed against *A. hydrophila* but these are not yet commercially available [52]. Therefore, the use of CTS-SeNP as an immunostimulant in aquaculture could be an alternative on enhancing immunity and generally help the fish against pathogens infection.

2.5 Conclusion

These finding collectively suggest that CTS-SeNP has strong immunomodulation effects in host from both innate and adaptive immune system. Experiments supported that significant response was rapid (3-12d) and could be sustained to 60d. Overall immunity and survival rate also increased under assault of *A. hydrophila*. In addition, further mechanism of SeNP uptake could be worthy to explore in future study. CTS-SeNP showed great potential as immunostimulant to fish to help boost host immunity before pathogen infection.

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CHAPTER 3

Comparative proteomics study of selenium nanoparticle (SeNP) immunomodulation on male zebrafish serum and selenoproteins involved in immune responses[*2]

3.1. Introduction

In Chapter 2, we have reported that CTS-SeNP has positive effect on innate and the cellular immune biomarkers and can significantly improve bacterial resistance in zebrafish. The immunomodulation effects observed were more comprehensive than other immunostimulants or its primary ingredients inorganic selenite and CTS (Table 2.1). While we have observed rapid effects of CTS-SeNP in stimulating many components of the immune system in zebrafish, the underlying mechanism was not explored.

Blood is a readily accessible and obtainable body fluid in contact with most human cells and tissues. Biochemical changes in serum are often used as biomarkers reflecting health condition and immune status of an individual including key proteins, cytokines, salts, and metabolites [1]. The evaluation of serum proteins is a well-established laboratory approach in the diagnosis of many diseases in human and mammalian models [2, 3]. For example, serum immunoglobulin levels are frequently increased during pathogenic infection [4]. Complement components are highly existed in serum [5]. In addition, several seleoproteins exist in serum and widely used as markers of Se status in host. Two specific selenoproteins have so far been identified in human serum namely, GPx and selenoprotein P [6]. Similarly, selenoproteins are crucial in the antioxidant regulation and immune system [7]. Therefore, the variation of immune and antioxidant related proteins in serum reflect the heathy status of the host and Se intake and its physiological regulation.

With the advancement of mass spectrometry, LC-MS/MS based proteomic

[2] The presentation based on this work at ‘Mini-conference on Proteomics Research and Regenerative Medicine Workshop’ was awarded ‘The Best Poster Presentation’ .

could be used for establishing a global protein profile of host and quantitating interesting proteins with a limited biological sample. Previous zebrafish studies interested in serum proteomic profiles between different genders [8] or comparison with human being [9] or studied on immune proteomic changes by using larger organs such as gills [10] and skin [11]. Recently, proteomic techniques have been applied to discover bacteria-binding serum proteins, which are likely to be functionally related to antibacterial activities. By using proteomics, bacterial adaptation adaptive mechanisms were clarified about *Streptococcus pyogenes* in human plasma study [12]. It enlightens us to explore immunomodulation of SeNP and its interaction in bacterial infection in host.

In this chapter, we will combine proteomics with within-host infection model to explore the mechanism of SeNP immunomodulation. Firstly, SeNP were synthesized by using chitosan as stabilizer and fed to zebrafish. After SeNP supplementary period, zebrafish were challenged by a common freshwater bacterium, *Aeromonas hydrophila*. After changed study, both non-infected and infected zebrafish were collected serum. Using LC-MS/MS based proteomics, we compared serum profiles and quantitate several interested proteins. A systematic analysis by using proteomics of serum could explore the mechanism of SeNP affects immunity and antioxidant capacity and their interrelationship in zebrafish.

3.2. Materials and methods

3.2.1. Selenium nanoparticle (SeNP) and diet preparation

10 μg/g SeNP fish diet was prepared was the same batch of diet as in Chapter 2. It was made by adding SeNP stock to the base dry diet (Otohime B1, USA). Characteristics of SeNP diet and control diet were shown in Figure 2.2.

3.2.2. Fish maintenance

Animal ethics and husbandry were described in Chapter 2. Briefly, adult zebrafish (*Danio rerio*) were maintained in 15-liter tanks flow-through system at a temperature of 28 ± 0.5 ºC and pH 7.0 ± 0.2 under a 14:10 light/dark cycle. The fish were fed with live brine shrimp nauplii once a day and commercial fish feed (Otohime B1, USA) three times per day. The commercial fish feed contains 51.0% crude protein

with the size of 250 – 360 μm.

3.2.3. SeNP diet supplementary and bacterial challenges

To study dietary effect of SeNP on zebrafish serum, a total 120 male zebrafish were fed with the SeNP diet at a ration of 2% body weight while another 120 fish were fed with base diet(control) for 9 days. After 9 days of feeding, fish in each treatment were randomly separate into two groups (Figure 3.1). Half of the zebrafish were *ip* injected with 2.5×10^6 cfu of *Aeromonas hydrophila* (ATCC 7699, USA) in 10 μL and will be referred to the infection treatment. The other half of the fish were subjected to intraperitoneal injection of 10 μL phosphate-buffered saline (PBS) as a procedural control and will be referred to as the non-infection treatment. Survival rates of each treatment were recorded for 48h and zebrafish survived after 48h were sacrificed to collect serum.

Only male zebrafish were used in the experiment as our preliminary data showed that serum from female fish contained high concentrations of vitellogenin, masking detection of low abundant proteins in the serum. Survivorship of the treatments were compared using Kaplan-Meier analysis coupled with log-rank test (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests in SPSS (ver 15.0, IBM SPSS Statistics, USA).

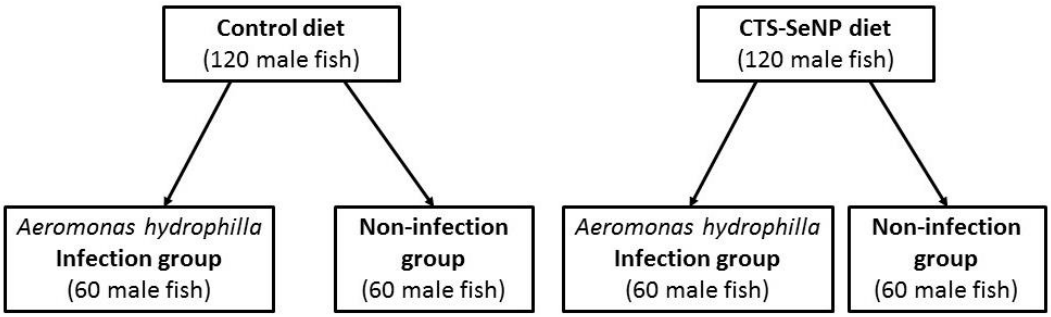


Figure 3.1 Experimental regime. Total 120 male zebrafish were fed with CTS-SeNP diet while another 120 fish were fed with control diet for 9 days. After dietary treatment, half of fish in each group were *ip* injected with *A. hydrophila. S*urvival fish were collected blood samples for serum proteomic analysis.

3.2.4. Serum collection

Serum samples were collected in zebrafish following a published protocol [8]. Briefly, zebrafish was euthanized quickly by immersion into 1.0 mg/mL MS-222 (ethyl 3-aminobenzoate methane sulfonate, Sigma, USA) for 2 min. The caudal peduncle of zebrafish was severed with a sharp scalpel (Swann Morton, UK). Fish with wound pointing down was individually put into a 0.5 mL micro-centrifuge tube with a small hole at the end. The 0.5 ml micro-centrifuge tube containing the fish was then placed inside a 1.5 mL micro-centrifuge tube (Eppendorf, Germany) and the assembly was centrifuged at 700g for 5 min at 11 °C. Blood from 5 individuals was pooled into a 1.5 mL centrifuge tube as a replicate and allowed to clot on ice for 30 min. Serum was separated with the cells by centrifugation (1200g, 10 min, 4°C) and stored at -80 °C. Triplicates serum samples were collected for each treatment.

3.2.5. Sample preparation for LC-MS/MS

Protein concentration in serum samples were determined by BCA protein assay (Thermo Scientific, USA). Ten µg serum protein was aliquot from each sample and diluted by 5 µL 6M urea buffer (6M urea, 50mM dithiothreitol (DTT), 10mM Tris-HCL, pH 8.0, Sigma-Aldrich, USA). The sample was then reduced by 5 µL 5 mM DTT in 25 mM ammonium bicarbonate for 45 min at 56 ºC, followed by alkylation with 10 µL 14 mM iodoacetamide (IAA, Sigma-Aldrich, USA) in 25 mM ammonium bicarbonate for 30 min at room temperature in dark. Protein in the sample was precipitated out with ice-cold acetone for 2 hours at -20 ºC and harvested by centrifugation at 14 000 rpm for 10 min at 4ºC. After removing the supernatant, the protein pellet was air dried for 30 min. Subsequently, the pellet was dissolved in 2 µL 6 M urea buffer. The protein sample was then digested with 1 µg trypsin (Promega, USA) in 20 µL 25 mM ammonium bicarbonate at 37 ºC overnight. The digestion was stopped by acidification (pH < 3) with 5% trifluoroacetic acid (TFA, Sigma-Aldrich, USA). Peptides were extracted by using C_{18} ZipTip (Millipore, USA) according to the manufacturer's protocol. Briefly, the C_{18} ZipTips was equilibrated with 0.1% TFA. After sample loading, the peptides were eluted with 80 % acetonitrile (ACN, mass spectrometry grade, Sigma-Aldrich) in 0.1 % TFA. Then, the peptides were dried by CentriVap Centrifugal Vacuum Concentrators (Labconco, USA). The peptides were resuspended in 10 µL of 0.1%

formic acid (FA, Sigma-Aldrich, USA). Prior to LC-MS/MS analysis, 1 µL of 1 fmol/µL tryptically digested alcohol dehydrogenase protein (*ADH*) from *Saccharomyces cerevisiae* (Sigma-Aldrich, USA) was spiked to each sample to serve as an internal standard. Each unique peptide concentration was normalized to the concentration of internal standard peptides [13, 14]. The internal standard added at the beginning of LC-MS/MS analysis procedure removed the variation from Orbitrap detection between runs.

3.2.6. LC-MS/MS analysis

Samples were analyzed by Ultra-High Performance Liquid Chromatography (UHPLC, UltiMate 3000 Rapid Separation Quaternary System, Thermo Scientific, USA) coupled with Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific, USA). Briefly, 2 µL sample (containing 2 µg digested serum sample with the internal standard) was loaded in a nanoflow C_{18} column (15 cm of length, 75 µm diameter, Thermo Scientific, USA) which was equilibrated in 0.1 % FA. Peptides were eluted with a linear gradient of 5-30% solvent B (80% ACN in 0.01% TFA) at a constant flow of 300 nL/min over 120 min. The eluted peptides were analyzed by Orbitrap tandem mass spectrometer in positive mode. The spray voltage was 2 800 V and the temperature of the capillary was set at 300 ºC. Data was acquired under both DDA and DIA modes. For DDA mode, full-scan MS spectra were acquired from 350 to 1 500 m/z with a resolution of 60 000 and automatic gain control (AGC) target was 400 000. The maximal injection time was 20 ms. For MS/MS scan, the activation type run on higher-energy collisional dissociation (HCD) type with collision energy of 30%. The Orbitrap MS/MS scan range mode was set at auto (m/z Normal) and a resolution of 15 000. The AGC target was 50 000. Ions were injected for all available parallelizable time and maximum injection time was 30 ms. For DIA mode, the scan range for MS scan was 400 to 1 000 m/z. The width of the isolation window was 20 m/z. The Orbitrap resolution was 60 000. AGC target was 400 000. The maximum injection time was 20 ms. For MS/MS scan, the resolution was 30 000. Scan range was set from 200 to 2 000 m/z. The AGC target was 500 000. Ions injection for all available parallelizable time was off and maximum injection time was 50 ms.

3.2.7. Proteomic profiles of zebrafish serum

Data collected from the DDA mode were searched against the National Center for Biotechnology Information non-redundant (NCBInr) database contained all zebrafish protein entries (143 725 sequences) (8 Nov, 2011) and the NCBInr database with taxonomy filter of *Saccharomyces Cerevisiae* by using Mascot v. 2.3.2 (Matrix Science, USA). The enzyme selected for searching was 'trypsin'. The variable modifications selected were 'Carbamidomethyl (C)' and 'Oxidation (M)'. The peptide mass tolerance was set at 20 ppm, and peptide charges were 2+, 3+, 4+. The mass tolerance for MS/MS was set at 0.1 Da. The search results with ion score less than 20 were discarded [15]. The false discovery rate (FDR) was set at 1%. Results were exported in Mascot DAT files and EXCEL files for further analysis.

Zebrafish serum proteins were analyzed by Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 in gene ontology (GO) functional annotation. All serum proteins from each group were combined and were analyzed by using GO functional annotations for cellular component, molecular function, and biological process to understand proteins classification in serum. Enrichment score was expressed as -log10 (Pvalue) to presented probability that the number of annotations in the protein list could have arisen by chance, assuming an underlying hypergeometric distribution.

3.2.8. Immune and antioxidant proteins quantitation

Skyline Targeted Proteomics Environment (MacCoss, USA) was used to quantitate target serum proteins using data collected from DIA [16]. In brief, the m/z range for DIA isolation scheme was 400 to 1,000 m/z with intervals of 20 m/z. The window of retention time for chromatograph extraction was 5 minutes. The Mascot DAT file of identified proteins in the zebrafish serum was imported into Skyline to build up a spectra library. A list of immune and antioxidant was prepared in a FASTA file and uploaded into Skyline. The enzyme for digestion was set as 'trypsin'. The modifications set were 'Carbamidomethyl (C)' and 'Oxidation (M)'. For quantitation of the proteins interested, the peak areas of the precursor ions (1+, 2+, and 3+) and their corresponding b, y type fragment ions were calculated. The precursor ions and the corresponding fragment ions with isotope dot product (*idotp*) over 0.9 were used for quantitation. The quantitation of the interested proteins was constructed on the

basis of the normalized peak areas of the five highest intensity fragment ions from the unique peptides. The results were exported from Skyline by using the Custom Reports in EXCEL format. Student *t*-test was used to compare SeNP and control groups in infection and non-infection treatment.

3.2.9. Differentially expressed proteins (DEPs) analysis

Non-gel based differentially expressed proteins (DEPs) analysis of serum proteins with LC-MS/MS was performed by MaxQuant-DDA[17] and Skyline-DIA[16] methods. MaxQuant-DDA quantitation method is one of the most frequently used platforms for MS-based proteomics analysis [17]. Parameters used in MaxQuant and its integrated search engine, Andromeda were described in Nature Protocol [17]. Briefly, DEPs were analyzed by MaxQuant v. 1.2.2.5 by using DDA raw files in each sample. The database acquired and quantitative parameters were the same as mention in section 3.2.7. The normalized intensity values (LFQ intensity) were calculated and exported for quantitation using Perseus v. 1.6.0.2. Student *t*-test was used to compare the LFQ values from the SeNP and control groups in infection and non-infection treatment in order to find out the differentially expressed serum proteins by SeNP. Skyline-DIA quantitated proteins as mentioned in section 3.2.8 by the same spectral library as MaxQuant-DDA [16]. Skyline-DIA method was used to quantitate those low abundant proteins such as immunoglobulins which cannot be analyzed by MaxQuant-DDA method. Therefore, more serum proteins could be quantitated by combining the results from both methods. Protein with a ≤ 0.80 or ≥ 1.20 fold difference were regarded as DEPs. DEPs were obtained from two pairs of comparison, namely between SeNP and control non-infection treatments, and between SeNP and control infections treatment.

3.2.10. Molecular pathway and network analyses

Molecular pathway and network analysis were generated using Ingenuity Pathway Analysis (IPA) core analysis (Ingenuity Systems, USA) using DEPs from non-infection groups and infection groups. The matched proteins encoding genes from Ingenuity Knowledge Base generated molecular networks according to biological and molecular functions. Analysis methods including canonical pathways and disease-based functional networks were performed. Right-tailed Fisher's exact test was utilized to determine the probability that biological function and/or disease was involved with

proteins. Pathways were also enriched in candidate categories, namely immune, metabolism and antioxidant related pathways.

3.3. Results

3.3.1 SeNP diet protected zebrafish from bacterial infection

Similar to our findings in Chapter 2, 10 µg/g SeNP provide protective effect to zebrafish against *A. hydrophila* infection. After supplemented with control or SeNP diets, zebrafish were *ip* infected with PBS or a common freshwater bacterium, *Aeromonas hydrophilla.* There was no mortality observed in the non-infection treatments throughout the experiment (Figure 3.2). Abdominal swelling, hemorrhagic sepcticemia and mortality were observed in infected fish. For the control-infection treatment, fish have significantly decreased survival rate 23.3% at 32h and only 13.3% fish survived after 48h. For SeNP-infection group, survivorship was 56.7% at 32h and 46.7%survived after 48h. Kaplan-Meier analysis showed that the infection treatments have statistically lower survivorship compared with the non-infection treatments, and control-infection treatment has statistically lower survivorship than the SeNP-infection treatment.

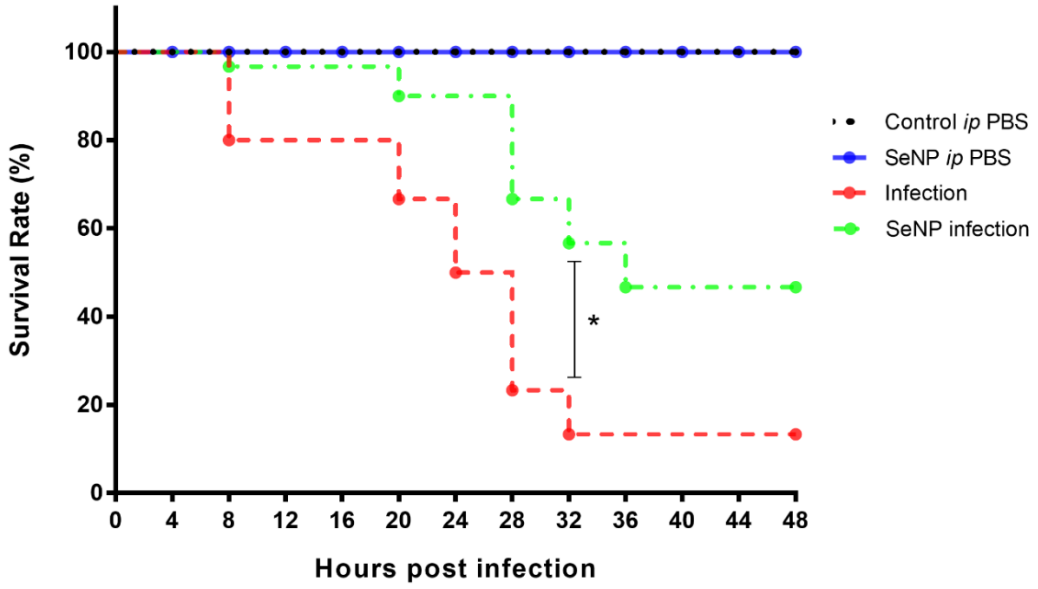


Figure 3.2 Survival rate of zebrafish challenged with *A. hydrophila* after different diets: black dotted line represents control diet fed zebrafish injected with PBS; blue solid line represents SeNP diet fed zebrafish injected with PBS; red dashed line represents control diet fed zebrafish infected with *A. hydrophila*; green dash dotted line represents SeNP diet fed zebrafish infection with *A. hydrophila*. Statistical significance was represented by asterisk.

3.3.2 Serum proteomic analysis

In non-infection group, a total of 352 protein families were identified in control diet group and 279 protein families in the SeNP diet. In infection group, a total of 468 protein families were identified in control diet group and 223 protein families in the SeNP diet (Figure 3.3). 157 protein families were common to all treatment groups and Control-infection showed most exclusive protein families (199) than others.

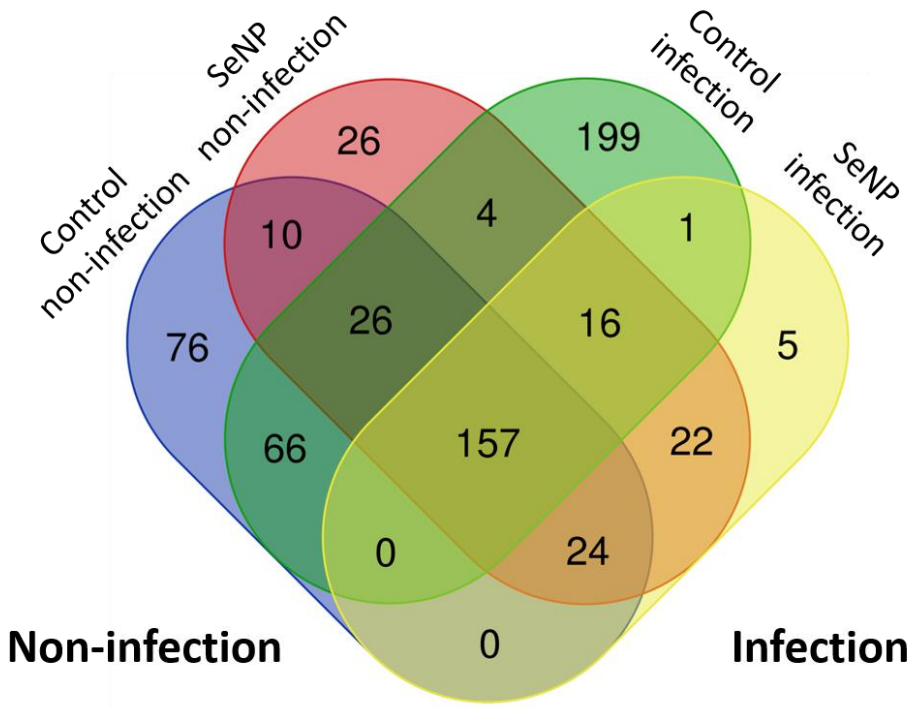


Figure 3.3 Venn diagram depicting total number of identified proteins families of zebrafish serum in control non-infection, SeNP non-infection, control-infection and SeNP-infection treatments based on average value of proteins obtained from three biological replicates of each treatment.

Proteomic profile of zebrafish serum was analyzed by Protein classification in IPA and Gene ontology (GO) annotation in DAVID. IPA classified most of serum proteins as enzyme (45.5%), peptidase (19.4%) and transporter (9.0%) (Figure 3.4.a). In DAVID, GO-term annotation classified all the serum proteins according to biological process, molecular function and cellular component. For biological process, cellular component organization or biogenesis had largest enrichment score (6.68), followed by primary metabolic process (Enrichment score 5.41), glycolysis (Enrichment score 4.70) and catabolic process (Enrichment score 4.03) (Figure 3.4.b). For molecular function, all top categories were enzyme activities such as peptide activity (Enrichment score 10.11), endopeptidase activity (Enrichment score 10.06), threonine-type endopeptidase activity (Enrichment score 8.30) and hydrolase activity (Enrichment score 7.01) (Figure 3.4.c). For cellular component, proteasome complex (Enrichment score 15.47), cytoplasm (Enrichment score 11.84) and extracellular region (Enrichment score 7.84) had highest enrichment scores (Figure 3.4.d).

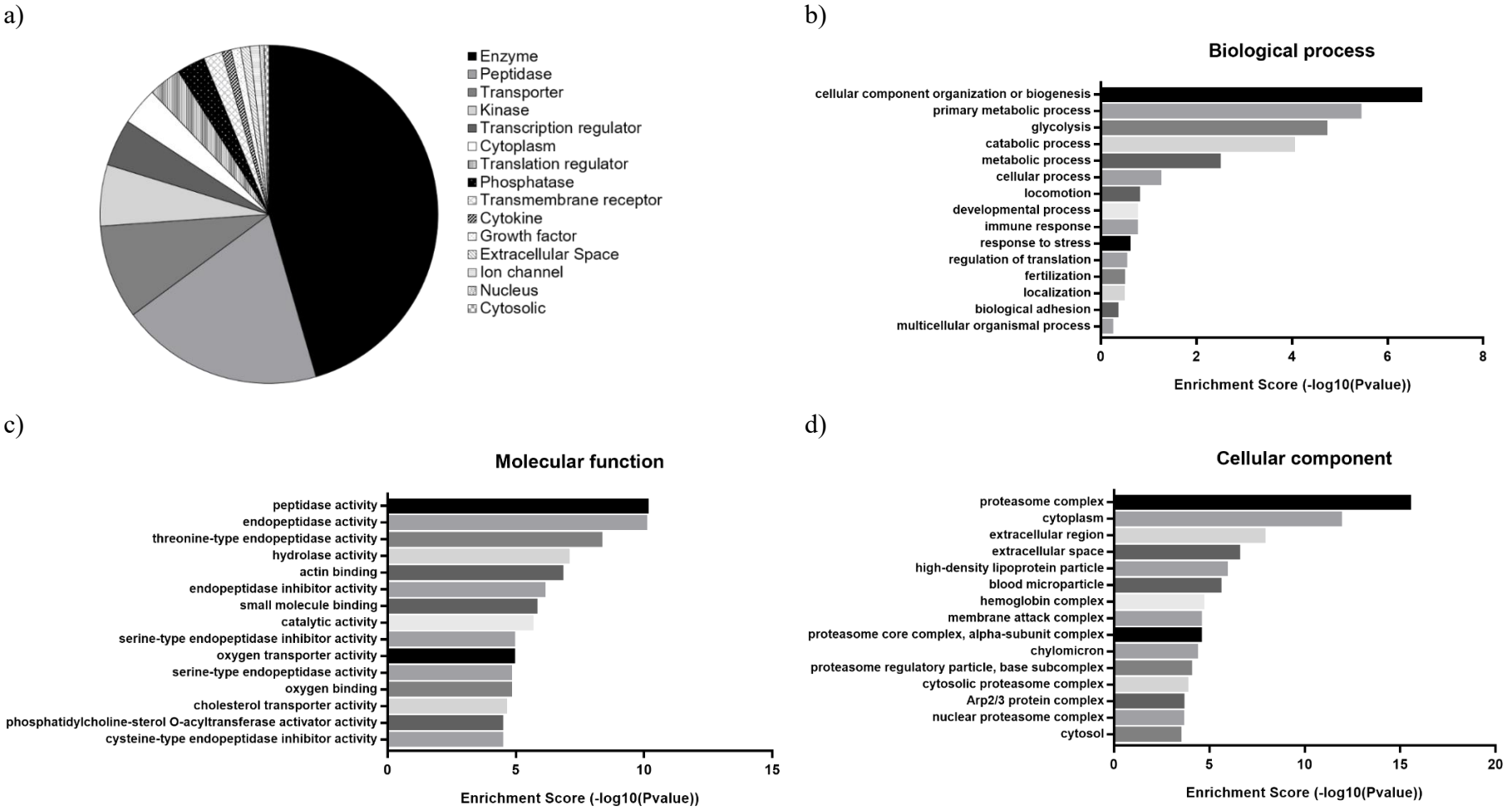


Figure 3.4 Classification of all identified proteins in zebrafish serum: a) Types of proteins as categoried by IPA; GO term annotation of 618 unique GI assession in zebrafish serum based on b) Biologicals process, c) Cellular component and d) Molecular function.

3.3.3. Quantitation of immune related proteins, antioxidant and selenoproteins

Selenium enrichment generally lead to better redox status and immunity of the animal [18] as well as increased expression of selenoproteins [19]. Therefore, we compared expression levels of these proteins of interest between the control non-infection and SeNP non-infection, and control-infection and SeNP-infection. Top proteins of interest were quantitated by using Skyline-DIA targeted quantitation method (30 immune related proteins and 19 antioxidant proteins) (Table 3.1). Between the non-infection treatments, the most differentially expressed immune-related proteins were adenylate kinase isoenzyme (5.603 fold change SeNP non-infection/control non-infection) followed by lysozyme g-like 1 (4.182 fold change SeNP non-infection/control non-infection) and complement factor H (0.274 fold change SeNP non-infection/control non-infection). Between the infection treatments, the most differentially expressed immune-related proteins were complement C4-B (13.569 fold change SeNP infection/control infection), followed by complement C1q-like protein (12.676 fold change SeNP infection/control infection) and immunoglobulin light chain constant region (5.977 fold change SeNP infection/control infection).

Table 3.1 Top immune-related or redox-related proteins (including 13 selenoproteins shown in **bold**) quantitated in zebrafish serum. Proteins quantitation was processed by using Skyline-DIA with the unique peptides. The data was processed in triplicate. (**Up:** An expression ratio of 1.2 (treatment/control) was used as a cut-off point to define differential up-expression; **Down:** An expression ratio of 0.8 (treatment/control) was used as a cut-off point to define differential down-expression.)

Accession IDs	Protein name	Peptide sequence	Peptide score (*idopt*)	Peptide mass to charge (m/z)	Parent ion retention time	Product ion	Non-infection (SeNP *vs* Control)		Infection (SeNP infection *vs* infection)	
							Differential expression	Fold change	Differential expression	Fold change
Internal standard										
gi|6324486	Alcohol dehydrogenase *ADH* (*Saccharomyces cerevisiae*)	*EALDFFAR*	0.90	484.74	47.43 ± 0.54	y6+, y5+, y4+, y3+, y4++	SeNP: 1.87E+08 Control: 1.79E+08		SeNP infection: 1.96E+08 Infection: 1.87E+08	
Immune-related proteins										
gi|51571925	adenylate kinase isoenzyme	*IVFVVGGPGSGK*	0.99	558.8242	34.32+/-2.05	y10+, y9+, y8+, y6+, b3+	Up	5.603	Down	0.233
gi|50540478	lysozyme g-like 1	*QMDPAVIAAIISR*	0.97	692.8845	71.92+/-3.38	y11+, y10+, y8+, y7+, y6+	Up	4.182	Up	1.797
gi|613410172	serotransferrin	*SAVVSFLSDIQSK*	1.00	809.0471	66.62+/-11.48	y10+, y9+, y8+, y7+, y6+	Up	3.077	Down	0.525
gi|47085873	beta-2 microglobulin precursor	*TTQTDLAFEQGWFFHLTK*	0.96	724.0250	79.71+/-2.38	y11+, y9+, y8+, y6+, y5+	Up	2.949	Up	1.654
gi|125852343	complement receptor type 1	*GELVPLDTVLDMIK*	0.96	671.3528	89.79+/-1.56	y9+, y8+, y7+, y5+, b3+	Up	2.656	Down	0.267
gi|227430295	intelectin 3 precursor	*NEFESGFITFR*	1.00	673.8224	57.02+/-1.96	y9+, y8+, y7+, y6+, b2+	Up	2.639	NS	1.171
gi|51011083	complement component C8 alpha chain	*NGYTGAACEETER*	0.99	729.3017	19.6+/-2.31	y10+, y9+, y8+, y7+, y6+	Up	2.626	Up	1.347
gi|14579217	immunoglobulin M heavy chain secretory form	*DLSDFVQYPAFGK*	0.99	743.8643	66.89+/-1.59	y8+, y7+, y6+, y5+, y3+	Up	2.487	Up	4.133
gi|363807314	complement component c3a	*IFAMANDLITIEEQVLCSALK*	0.97	793.7481	95.9+/-1.92	y10+, y9+, y7+, y6+, y5+	Up	2.113	NS	0.979
gi|312836777	T-complex protein	*VIDPATATSVDLR*	0.92	679.3697	35.66+/-1.72	y11+, y10+, y8+, y6+, y2+	Up	2.031	NS	1.048
gi|66773068	complement component C9	*AGYGINILGSGPR*	0.99	637.8462	43.28+/-1.19	y10+, y8+, y7+, y6+, y5+	Up	1.932	Down	0.510
gi|528480887	complement C5	*IITVNGEVLGDILSILNNPEGIK*	1.00	807.7915	94.71+/-2	y10+, y8+, y7+, y6+, y5+	Up	1.841	Up	1.637
gi|165972489	complement factor properdin precursor	*TCTNPSPSTAPPGR*	0.97	721.8383	18.02+/-1.48	y12+, y11+, y10+, y8+, y4+	Up	1.830	Up	1.576
gi|39795310	Ceruloplasmin	*EIQWDYAPSGK*	0.98	647.3091	35.95+/-2.63	y9+, y8+, y7+, y6+, y5+	Up	1.810	Up	1.287
gi|226823315	heat shock protein HSP 90-beta	*EDQTEYIEEK*	0.90	642.2855	18.85+/-1.66	y7+, y6+, y5+, y4+, y3+	Up	1.701	Down	0.402
gi|34740143	CDC48, partial	*VINQILTEMDGMSSK*	0.90	833.4129	58.4+/-1.96	y11+, y10+, y9+, y8+, y5+	Up	1.373	Up	1.332
gi|84626174	immunoglobulin light chain constant region	*SGQPALTAGDTVR*	0.97	636.8308	19.77+/-1.5	y10+, y9+, y8+, y7+, y6+	Up	1.365	Up	5.977
gi|656214630	complement C1q-like protein	*NELQEQNSLIQDLR*	0.99	850.4341	49.43+/-1.54	y10+, y9+, y4+, y2+, b2+	NS	1.197	Up	12.676
gi|41055345	complement component C6	*IDLANEPLYR*	0.89	602.3220	38.92+/-2.46	y9+, y8+, y7+, y6+, y4+	NS	1.155	Up	3.670
gi|75992716	complement component C3	*VGSSENVFVEAQDYSK*	0.98	879.9127	40.5+/-2.12	y11+, y10+, y8+, y9+, y7+	NS	0.88	Down	0.675
gi|439915901	C-reactive protein 3	*AVYYNQDNR*	1.00	571.7649	18.99+/-1.7	y7+, y6+, y5+, y4+, y2+	NS	0.801	Down	0.558

gi|37682087	heat shock 70kDa protein	*TTPSYVAFTDTER*	0.99	744.3543	33.38+/-0.96	y11+, y10+, y9+, y8+, y7+	Down	0.745	Up	4.990
gi|189528839	alpha-2-macroglobulin-like isoform 1	*NFAPLDQQYSSVVLEDSQDNR*	0.95	809.0471	53.99+/-1.51	y9+, y8+, y7+, y6+, b2+	Down	0.742	Up	2.491
gi|41054093	cell division control protein 42 homolog	*NVFDEAILAALEPPETKPK*	0.91	694.7088	80.82+/-2.94	y12+, y11+, y10+, y9+, y7+	Down	0.608	Up	1.432
gi|153792045	complement component c3b	*FYSPPEDK*	0.99	491.7295	18.3+/-1.27	y6+, y5+, y4+, b2+, b4+	Down	0.476	Up	1.850
gi|528523537	complement factor I	*IPPETNPEAAVEQTPDVNLEK*	0.99	764.3849	41.77+/-1.11	y8+, y7+, y6+, b9+, b9++	Down	0.393	Up	2.499
gi|18858247	complement factor B	*VLENGEVAPYQER*	0.99	752.3755	24.50 +/-2.44	y10+, y9+, y7+, y6+, y5+	Down	0.368	Up	2.197
gi|33416403	alpha-1-microglobulin/bikunin precursor	*AFVDLWAFDSSSGK*	0.99	765.3672	71.22+/-2.55	y11+, y9+, y8+, y7+, y5+	Down	0.341	Up	4.616
gi|125839308	complement C4-B	*NTGDLSVSPENTR*	0.94	695.3339	19.75+/-0.45	y11+, y8+, y7+, y6+, y5+	Down	0.302	Up	13.569
gi|313661372	complement factor H	*LNGENFAFGTTIR*	0.96	720.3675	47.04+/-1.87	y9+, y8+, y7+, y6+, y5+	Down	0.274	Up	2.269
					Redox-related proteins					
gi|56790262	superoxide dismutase (SOD)	*IEIEDAMLTLSGQHSIIGR*	0.93	695.03	72.76+/-1.52	y11+, y10+, y9+, y8+, y12+	Up	4.659	Down	0.344
gi|162287365	hemopexin	*GHHFLSITGDK*	0.99	404.54	22.76+/-3.66	y6+, y4+, y3+, b3+, b4+	Up	3.343	Down	0.203
gi|41054389	glycerol-3-phosphate dehydrogenase	*LPPNVLAVPDLLESVK*	0.98	568.66	81.77+/-1.36	y8+, y8++, b5+, b7+, b9+	Up	2.077	NS	1.126
gi|41393103	aldehyde dehydrogenase family 9 member A1-A	*EEIFGPVMSVLPFDTEEEVLQR*	0.98	855.42	90.38+/-1.65	y11+, y10+, y9+, y8+, y6+	Up	1.272	Up	1.468
gi|41152453	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	*YLAEVAVGEEK*	0.95	604.31	28.24+/-2.89	y9+, y7+, y6+, y4+, b2+	NS	1.042	Down	0.852
gi|41393155	isocitrate dehydrogenase	*AGSVVEMQGDEMTR*	0.93	755.33	27.68+/-1.42	y10+, y9+, y8+, y7+, y6+	Down	0.300	Up	3.475
gi|169403947	glyceraldehyde-3-phosphate dehydrogenase	*GASQNIIPASTGAAK*	0.98	693.37	24.67+/-4.81	y11+, y10+, y9+, y8+, b6+	Down	0.190	Up	2.427
gi|47086689	glutathione S-transferase mu	*MFEPACLDDFK*	0.93	686.7992	45.44+/-2.06	Y8+, y6+, y4+, y2+, b3+	Up	2.365	NS	0.920
gi|113682261	glutathione S-transferase rho	*LIPDNPAEMGLVYQR*	0.92	577.3029	58.05 ± 1.95	y8+, y7+, y5+, y4+, y3+	Up	2.297	Up	1.433
gi|18858197	glutathione S-transferase pi	*ENLVTFEEWMK*	0.92	713.3396	66.61 ± 2.66	y8+, y7+, y6+, b2+, b3+	Up	1.939	Up	1.334
gi|66911287	glrx protein	*AGHLELVDISAR*	0.99	640.8515	24.33+/-1.48	y9+, y8+, y4+, y1+, b3+	Up	3.133	Up	2.445
gi|41387146	**peroxiredoxin-6**	***AVDLVLNNAQLIPVLGNLR***	**0.97**	**678.0686**	**77.56+/-2.38**	**y7+, y5+, y4+, b3+, b4+**	**Up**	**3.701**	**Down**	**0.397**
gi|182636716	**selenoprotein Pa**	***QLFGGEGNGR***	**0.98**	**517.7543**	**32.9+/-10.93**	**y8+, y7+, y6+, y5+, y4+**	**Up**	**3.342**	**Up**	**2.058**
gi|29648601	**phospholipid hydroperoxide glutathione peroxidase B**	***SYNAEFDMFSK***	**0.90**	**669.7872**	**40.44+/-1.74**	**y9+, y7+, y3+, y2+, b2+**	**Up**	**3.284**	**Up**	**2.001**
gi|301500661	**selenoprotein J**	***FFGPGSEYDTPVNDPYR***	**0.95**	**490.9733**	**60.96+/-17.5**	**y9+, y7+, y6+, y5+, y4+**	**Up**	**2.697**	**Up**	**1.843**
gi|47086663	**pro-cathepsin H precursor**	***LNPVSFAYEVTSDFMHYK***	**0.99**	**716.6751**	**88.85+/-0.92**	**y10+, y8+, y7+, y3+, b2+**	**Up**	**2.456**	**Up**	**1.807**
gi|324021702	**serine (or cysteine) proteinase inhibitor**	***DLDADTVMMLINYMYFR***	**1.00**	**704.32**	**49.16 ± 3.09**	**y10+, y8+, y7+, y6+, y5+**	**Up**	**1.831**	**NS**	**1.057**
gi|41387146	**peroxiredoxin-5**	***FHEFLGNSWGILFSHPR***	**0.91**	**511.7602**	**55.84+/-2.06**	**y6+, y5+, y4+, b2+, b3+**	**Up**	**1.758**	**NS**	**0.936**
gi|40363541	**adenosylhomocysteinase**	***VPAINVNDSVTK***	**0.93**	**628.8459**	**27.20 ± 3.17**	**y10+, y9+, y8+, y11++, b3+**	**Up**	**1.374**	**Up**	**1.350**

For redox-related proteins, glyceraldehyde-3-phosphate dehydrogenase (0.190 fold change SeNP non-infection/control non-infection), isocitrate dehydrogenase (0.300 fold change SeNP non-infection/control non-infection) and superoxide dismutase (4.659 fold change SeNP non-infection/control non-infection) were the top differentially expressed proteins between the non-infection treatments. Between the infection treatments, the most differentially expressed proteins were hemopexin (0.203 fold change SeNP infection/control infection), superoxide dismutase (0.344 fold change SeNP infection/control infection) and isocitrate dehydrogenase (3.475 fold change SeNP infection/control infection).

Selenoproteins including glutathione S-transferase, phospholipid hydroperoxide glutathione peroxidase B, selenoprotein Pa and selenoprotein J were found in zebrafish serum (Table 3.1). They were generally upregulated in the SeNP treatments, especially in the non-infection treatment. Between the non-infection treatments, upregulated selenoproteins including peroxiredoxin-6 (3.701 fold change SeNP non-infection/control non-infection), phospholipid hydroperoxide glutathione peroxidase B (3.284 fold change SeNP non-infection/control non-infection) and Glrx protein (3.133 fold change SeNP non-infection/control non-infection). However, when comparing the infection treatments, expression pattern of selenoproteins became more complex. For example, peroxiredoxin-6 was downregulated in the SeNP-infection treatment (0.397 fold change SeNP infection/control infection), and serine (or cysteine) proteinase inhibitor and peroxiredoxin-5 were no longer differentially expressed (Table 3.1).

3.3.4. Identification of significant molecular networks through IPA interaction analysis

Based on the DEPs of non-infection and infection treatment groups, IPA network analysis identified a total of 9 networks that were significantly influenced.

Comparing non-infection treatments, the top three disease-based functional networks were related to free radical scavenging, lipid metabolism and the immune system respectively (Table 3.2). Comparing infection treatments, the top three disease-based functional networks were related to carbohydrate metabolism, lipid metabolism

and immune system respectively (Table 3.2). With or without infection, the SeNP diet led to DEPs related to lipid metabolism and immune responses. Interestingly, DEPs under the lipid metabolism molecular network were highly similar between the non-infection and infection treatments (19 out of 21 DEPs were common) (Table 3.2). On the contrary, for immune responses, only 6 out of 30 DEPs were shared between the non-infection and infection treatment (Table 3.2).

Table 3.2 Top three disease-based functional networks based on differentially expressed proteins (DEPs) comparing the two diets under non-infection and infection treatments respectively. DEPs that were indicated in **bold** text.

Analysis	ID	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
Non-infection (SeNP vs Control)	1	**A2M**, adhesion molecule, **AKR1B1**, **APOA1**, Cg, Cpla2, **F10**, **GGCT**, **GLRX**, glutathione peroxidase, **GPX1**, **GPX3**, **GPX4**, Growth hormone, GST, **GSTM3**, **GSTP1**, Hif, Ldh (complex), **LDHA**, **LDHB**, LDL, **MTPN**, NFkB (complex), peroxidase (miscellaneous), **PGAM2**, **PRDX1**, **PRDX2**, **PRDX5**, **PRDX6**, **PROC**, **SHBG**, Sod, **SOD1**, SYK/ZAP	45	22	Free Radical Scavenging, Small Molecule Biochemistry, Drug Metabolism
	2	**AHSG**, Alpha 1 antitrypsin, **APOA4**, **APOB**, **BHMT**, Ces, **CES1**, **CETP**, **CFH**, **CP**, creatine kinase, ERK1/2, **ESD**, Ferritin, **FGA**, **GC**, HDL, HDL-cholesterol, hemoglobin, **HPX**, **ITIH4**, **ITLN1**, MHC Class II (complex), Nos, Nr1h, PRKAA, **RBP4**, **SERPINA1**, **SERPINA9**, **SERPINC1**, **SERPINF2**, **SH3BGRL**, **TF**, VLDL, VLDL-cholesterol	43	21	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
	3	Akt, **C3**, **C5**, **C6**, **C7**, C8, C1q, C3-Cfb, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, **C8A**, **C8B**, **C8G**, **CFI**, Collagen Alpha1, Collagen type IV, Collagen(s), Complement, Complement component 1, **CSTB**, elastase, Fibrin, Fibrinogen, **HABP2**, **HBE1**, **HBZ**, Kallikrein, Laminin (complex), **LRG1**, MAC, **NME3**, Pdi, **PLG**, **SERPING1**	30	16	Developmental Disorder, Hereditary Disorder, Immunological Disease
Infection (SeNP infection vs Infection)	1	20s proteasome, **ACLY**, **AK1**, **ALDOA**, AMPK, Dynamin, EGLN, ENO3, ERK, **GAPDH**, **GAPDHS**, GOT, **GOT1**, Hif1, Ikb, Immunoproteasome Pa28/20s, **LDHA**, **LDHB**, **LRG1**, NDPK, **NME1**, **NME2**, **NME3**, **PGK1**, **PGM1**, **PRDX2**, **PRDX5**, Proteasome PA700/20s, PSMA, **PSMA4**, **PSMA5**, **PSMA8**, **PSMB3**, Rsk, **SOD1**	44	22	Carbohydrate Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
	2	**AHSG**, Alpha 1 antitrypsin, **AMBP**, **APOA4**, **APOB**, **CES1**, **CETP**, **CFH**, **CP**, creatine kinase, ERK1/2, Ferritin, **FGA**, **GC**, **HBZ**, HDL, HDL-cholesterol, hemoglobin, **HPX**, Iti, **ITIH3**, **ITIH4**, **ITLN1**, MHC Class II (complex), Nos, Nr1h, PRKAA, **RBP4**, **SERPINA1**, **SERPINA9**, **SERPINC1**, **SERPINF2**, **TF**, VLDL, VLDL-cholesterol	41	21	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
	3	**A2M**, **C6**, **C7**, C3-Cfb, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, **C8A**, **C8B**, **CAPNS1**, **CFB**, **CFD**, **CFI**, **CFP**, chymotrypsin, coagulation factor, **CORO1A**, Ecm, elastase, **F9**, **F10**, glutathione peroxidase, Hif, Kallikrein, Ldh (complex), **MASP2**, NFkB (complex), **PAPSS2**, **PLG**, **PROC**, **PRSS2**, Serine Protease, **SERPING1**, **SOD3**, trypsin	38	20	Developmental Disorder, Hereditary Disorder, Immunological Disease

The top disease-based functional network of each group was visualized as molecular network (Figure 3.5 and 3.6). Base on this network, it was observed that under non-infection condition, SeNP can enhance a cascade of antioxidant related proteins which in turn inhibit NFκB (Figure 3.5), suggesting the role of SeNP might be anti-inflammatory. Surprisingly, the top molecular network under infection condition was not related to immune responses but carbohydrate metabolism. It should be noted that this comparison is between fish under infection but with or without pre-exposure to SeNP. It was well-known that tissues under infection or stress commonly enter a hypermetabolic stress state that is associated with enhanced glucose uptake and utilization [20]. From the network, most of the proteins were downregulated (Figure 3.6). This might indicate that the infected animals were less stressed after feeding with SeNP.

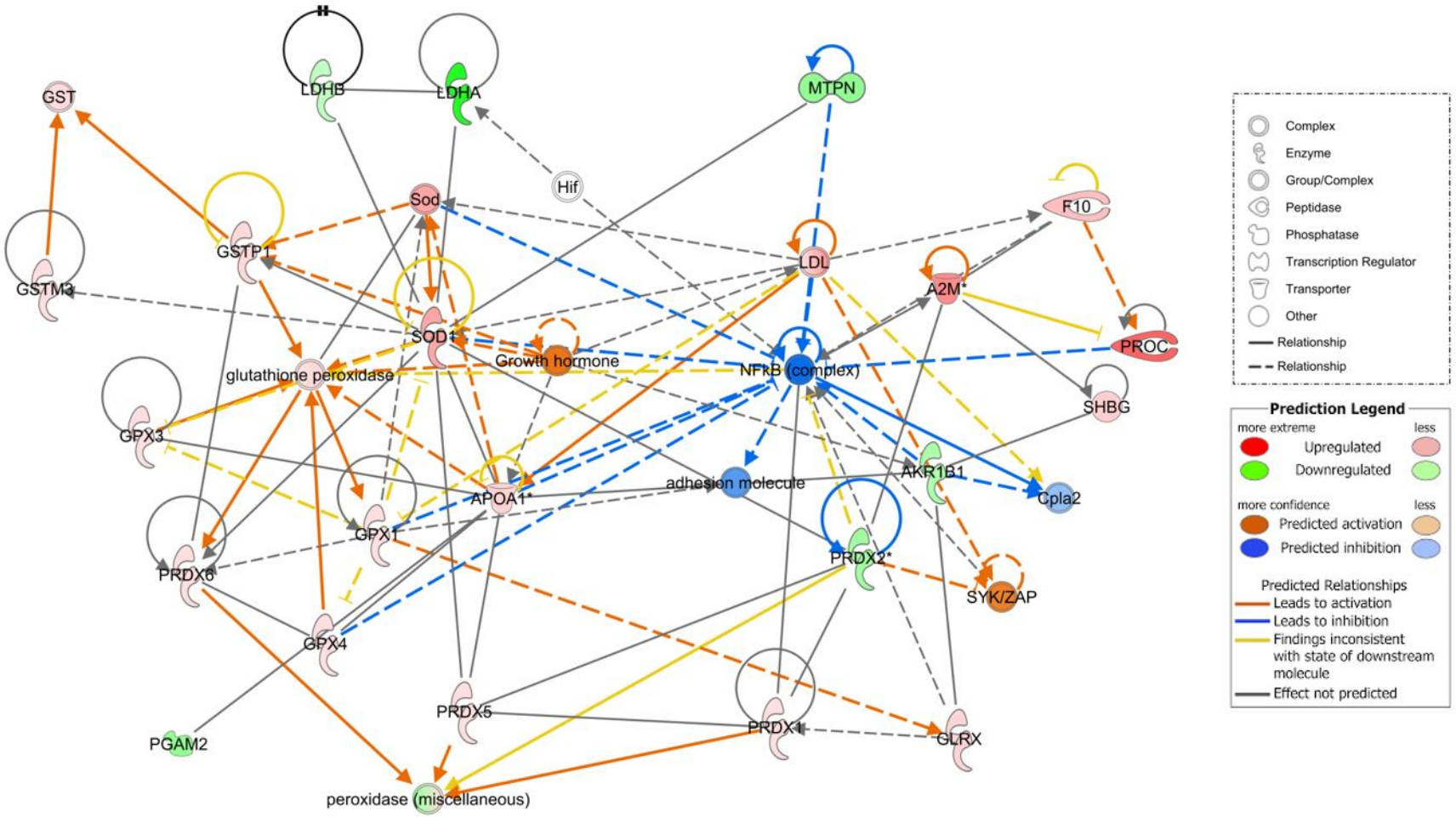


Figure 3.5 Top disease-based functional network in non-infection group involving free radical scavenging, small molecule biochemistry, drug metabolism. Overexpressed proteins were represented in red and under-expressed genes were represented in green.

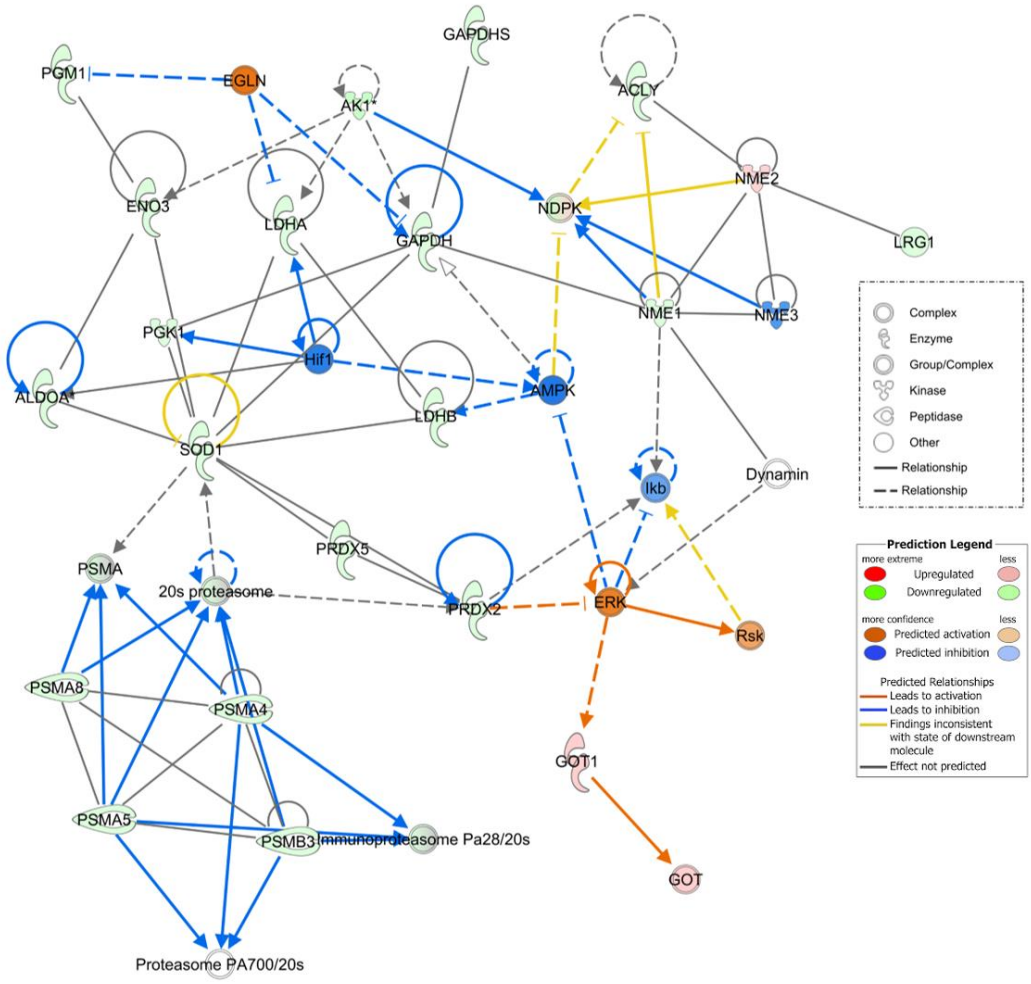
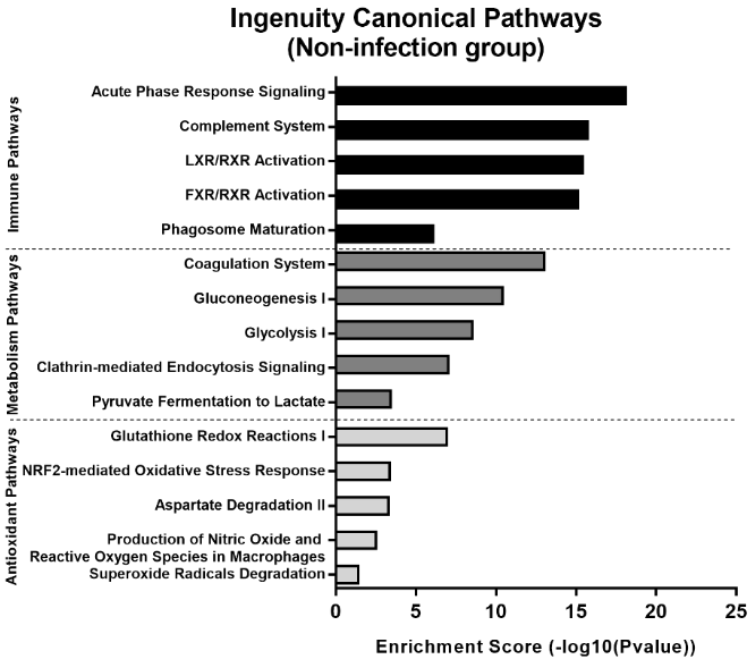


Figure 3.6 Top disease-based functional network in infection group involving carbohydrate metabolism, nucleic acid metabolism, small molecule biochemistry. Overexpressed proteins were represented in red and under-expressed genes were represented in green.

3.3.5. IPA pathway analysis reveals activation of key hubs in immunity, metabolism and antioxidation.

From IPA canonical pathways analysis of our experimental proteome datasets, 194 pathways were identified in the non-infection group while 235 pathways in the infection group. The top 5 significant enriched pathways related to immunity, metabolism and antioxidant activities were shown in Figure 3.7.

a)



b)

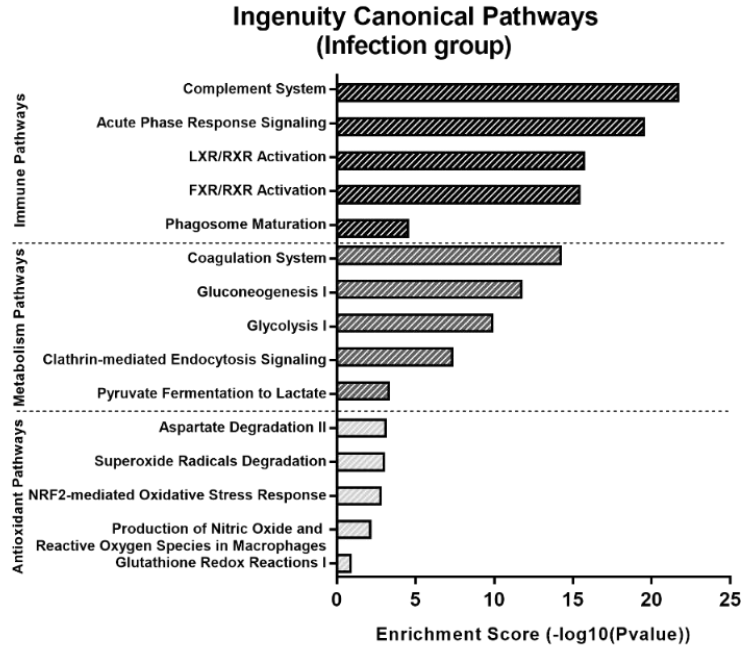


Figure 3.7 Top five enriched pathways in immune, metabolism and antioxidant activities identified in IPA from the comparison of SeNP effects on zebrafish serum in a) non-infection and b) infection groups. The pathways are indicated on the y-axis. On the x-axis, enrichment score (-log10(Pvalue)) for each pathway is indicated by the bars. Deep color bars indicated DEPs from non-infection group and light color bar indicated DEPs from the infection comparison.

For both infection and non-infection groups, immune canonical pathways were highly significant. The top two immune canonical pathways in both groups were acute phase response signaling (Figure 3.8) and complement system (Figure 3.9). For the infection group, these two pathways have a higher enrichment score than for the non-infection group (complement system: 21.70 vs 15.70; acute phase response signaling: 19.50 vs 18.10) while the other 3 pathways have similar enrichment scores between the two groups. This suggested that *A. hydrophilla* infection primarily led to changes in acute phase response signaling and complement system.

The top five pathways related to metabolism were the same for both infection and non-infection groups and were in the same order in terms of enrichment score: coagulation system, gluconeogenesis I, glycolysis I, clathrin-mediated endocytosis signaling, and pyruvate fermentation to lactate. The enrichment score for all five pathways were slightly higher in the infection group than in the non-infection group. This may suggest that fish have higher metabolic need under bacterial infection.

Antioxidant related pathways showed the largest differences between the infection and non-infection groups. For infection groups, the enrichment score of the antioxidant related pathways were generally lower than that for non-infection groups. While the top five pathways between the two groups were the same, the order was very different. For non-infection groups, the most significant pathway was glutathione redox reactions I (Enrichment score 6.94) but this pathway was less significant for the infection groups (Enrichment score 0.87).

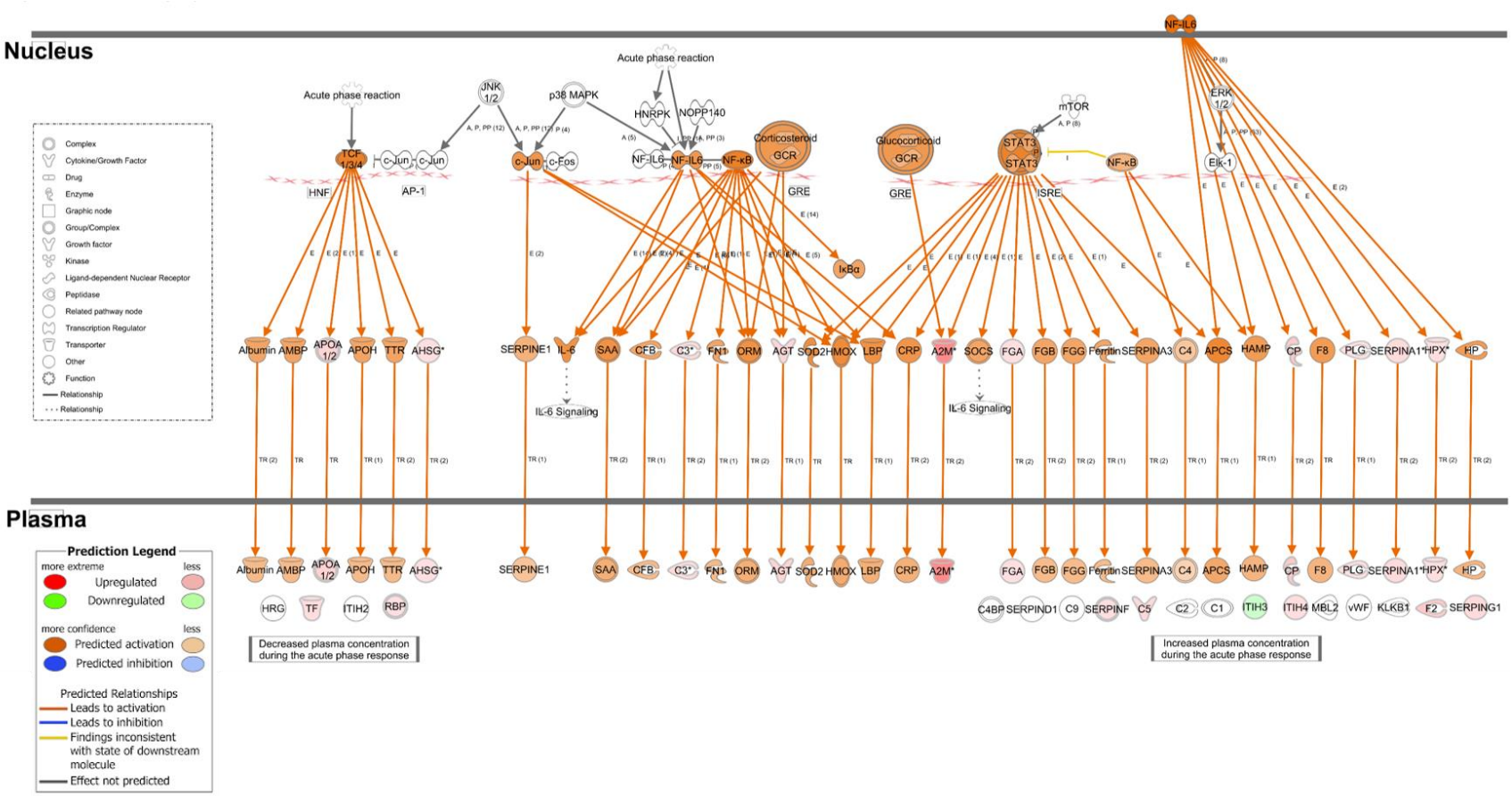


Figure 3.8 Prediction analysis by IPA on the expression of proteins related to acute phase response signaling pathway (Enrichment score 18.10) in non-infection group. In red are represented the overexpressed proteins (= hypomethylated promoters) and in green the under-expressed genes (= hypermethylated promoters). In Orange are represented the predicted activation and blue for the inhibition.

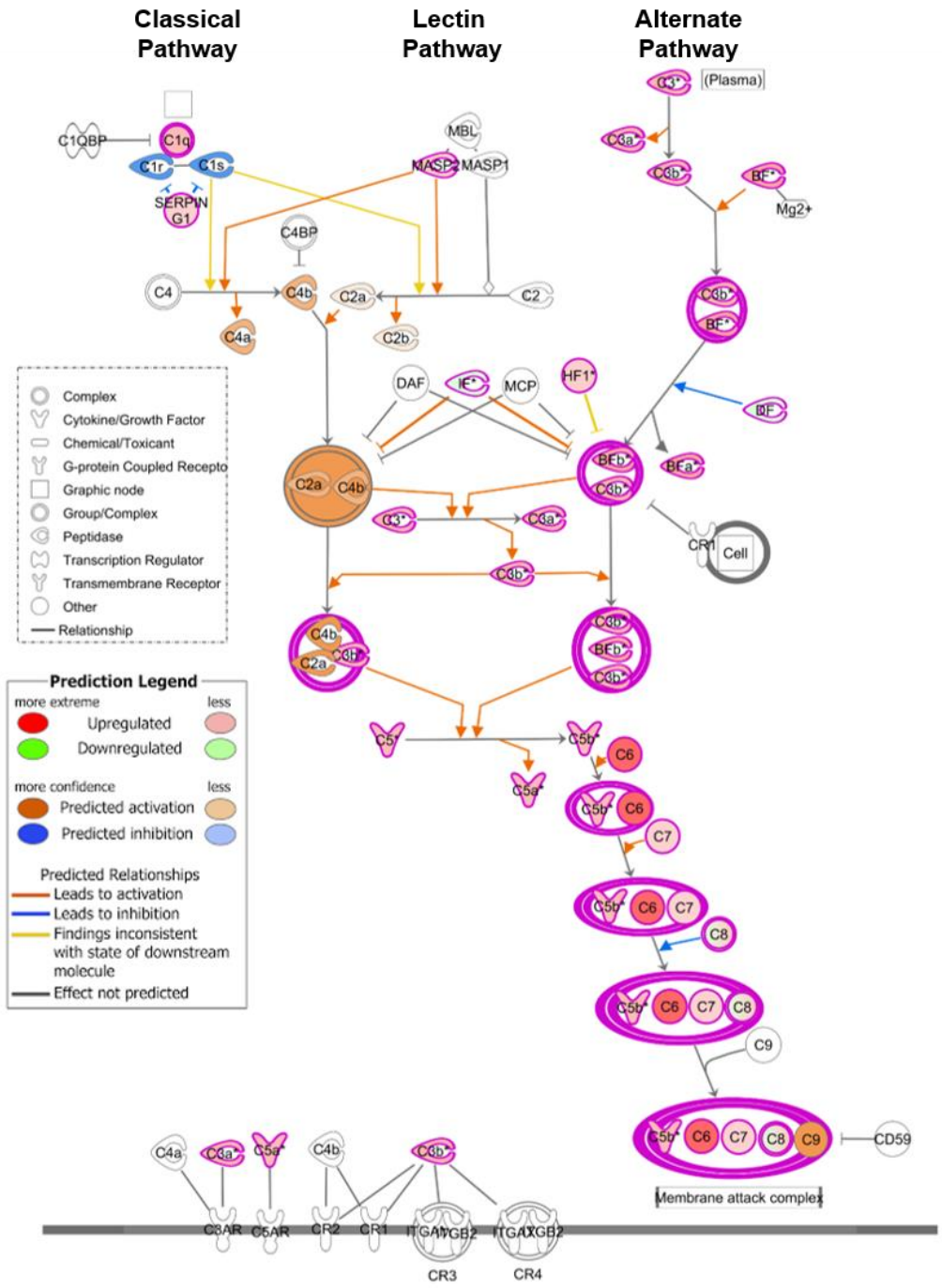


Figure 3.9 Prediction analysis by IPA on the expression of proteins related to complement system pathway (Enrichment score 21.70 in infection). In red are represented the overexpressed proteins (= hypomethylated promoters) and in green the under-expressed genes (= hypermethylated promoters). In Orange are represented the predicted activation and blue for the inhibition.

The top pathways were presented in Figure 3.8 and 3.9 to understand the interactions of the DEPs. Under normal condition, acute phase response pathway was the most enriched pathway. Almost every plasma protein in the pathway were upregulated (Figure 3.8). Since this analysis was based on serum, more upstream proteins cannot be detected and were based on prediction. Overall, this result suggested activated innate immune response in the SeNP treatment even without infection. During infection, the top molecular pathway was the complement system (Figure 3.9). Most of the proteins were upregulated. This suggested that the complement system was more activated in the SeNP treated fish under infection.

3.4. Discussion

Serum proteomics provided important insight into interactions of CTS-SeNPs with zebrafish under normal condition and bacterial infection. Large number protein families were enzymes, peptidases and transporters. Large number protein families were identified through this technique (Table 3.1) and when coupled with bioinformatics can provide insight into biological processes impacted by CTS-SeNP.

3.4.1 Effects of SeNP under normal non-infection condition

Using DEPs between control non-infection and SeNP non-infection it was found that proteins related to immune response, metabolism, redox regulation, as well as some selenoproteins were enriched for the CTS-SeNP treatment under normal conditions. This is confirmed by pathway analyses using all DEPs comparing control non-infection against SeNP non-infection treatment that top enriched functions and pathways were related to immunity, antioxidant regulation and metabolism (Tables 3.1 and 3.2). This finding is largely similar to those reported by studies supplementing other forms of Se [21]. It was also reported that SeNP diets cause increasing GPx [22] and GST [23] in mammals. In fish, antioxidant enzymes such as GPx, SOD and Glycerol-3-phosphate dehydrogenase were observed with overexpression after supplemented with SeNP [24]. This suggests that SeNP act similarly as other forms of Se under normal condition.

Examination of the first molecular network (Figure 3.5) showed that those

antioxidant proteins centered around SOD1 and NF-κB. SOD1 activation may induce ROS elimination [25], enforcing the antioxidant system which may in turn strengthen the defense ability of the fish. Meanwhile, the NF-κB inhibition were also predicted, suggesting that SeNP might be anti-inflammatory under normal non-infection condition. Examination of the second molecular network (Figure 3.6) on acute phase signaling showed that almost all serum protein related to the pathway was upregulated. These serum proteins could be roughly separated into two groups, those that increase in concentration during acute phase response, and those that decrease in concentration during acute phase response [26]. Interestingly, proteins from both groups showed overexpression (Figure 3.6). This indicated that the fish were not showing acute phase response, but the precise biological significance of this phenomenon will require further investigation.

3.4.2 Effects of SeNP under bacterial infection condition

Surprisingly, under infection condition, SeNP were still impacting similar proteins related to immune response, redox regulation and metabolism (Table 3.1). IPA also showed that the enriched pathways related to immune response and metabolism under infection and non-infection were similar (Tables 3.1 and 3.2). This might be due to that enriched immune pathways were mainly related to innate immunity (Table 3.2), which could also be expressed without infection. It is notable that two proteins, namely immunoglobulin M heavy chain secretory form and immunoglobulin light chain constant region were also upregulated (Table 3.1), indicating that SeNP also have the potential to improve the adaptive immune system.

Examination of the first molecular network showed that (Figure 3.6) SOD1, 20s proteasome, ERK and GAPDH were highly affected their expression as central interaction molecules. During infection, SOD1 showed an opposite regulation with non-infection condition. It resulted in incapability in free radical scavenging, meanwhile inhibited the action of GAPDH. Previous study indicated that GAPDH significantly suppressed during pathogen infection. It caused the suppression of glycolysis in host [27]. Therefore, the activity of infected fish showed low mobility and weak. The second molecular network was on complement system

(Fig. 3.9). The complement system represents an efficient first line of defense against microbial infection. During acute stress or bacterial infection, an immunosuppressive effect is caused by a reduction in complement activities (both the classical and alternative pathways) in fish serum [28]. Previous study reported that the key factor of complement system, C3, seems to be related to Se status and several serum biochemical measurements in host health [29]. In this study, overexpression of C3 at the alternative pathway and C1q of the classical pathway appeared to be initiators of activation of the complement system. Activation of complement system resulted in overexpression of downstream C5b, C6, C7 and C8 to triggered membrane attack complex synthesis. We have also recorded the overexpression of C5 and C8 (Table 3.1), confirming prediction of IPA.

3.5. Conclusion

In conclusion, SeNP protected zebrafish from bacterial infection and improve the survivability in *A. hydrophila* challenge. Using proteomics on fish serum, it was found that SeNP influenced antioxidant regulation, metabolism and immune functions in the host. The impact of SeNP on the host was largely consistent with or without bacterial infection. Our observation supported a common argument that the role of Se in redox regulation is linked to its immunomodulation capacity.

3.6. Reference

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CHAPTER 4

Improved Redox regulation as a potential mechanism of selenium nanoparticle induced immunomodulation

4.1 Introduction

4.1.1 Selenium and selenium nanoparticles

One of the most important function of selenium (Se) is to regulate redox balance in the host. A total 25 of selenoproteins were identified in human being and many of them were involved in redox regulation [1]. Different chemical forms of Se has their unique biological activity and functions. Se nanoparticle (SeNP) containing elemental form Se has excellent bioavailability, lower toxicity and better immunomodulation activity comparing with organic and inorganic forms of Se [2-6]. Although Se plays a certain role in immune functions, the molecular action of this element and the mechanism of those beneficial effects of health is still unclear.

4.1.2 Redox regulation and Immunity

Redox regulation plays an important role in the regulation and maintenance of several key immunological functions of the body. Reactive oxygen species (ROS) are common in both redox regulation and cellular immunity, particularly for neutrophils and macrophages in response to bacterial and fungal pathogens [7]. During phagocytosis, the redox regulation and oxygen dependent bactericidal mechanism of phagocytes were well studied [8] (Figure 4.1). When pathogens were detected in the host, phagocytes would engulf the pathogenic particles through phagocytosis. Activated NADPH oxidase will then produce ROS, including superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2). This process is also called phagocytic respiratory burst [9]. Then, myeloperoxidase produces hypochlorite (HOCL) which contributes to destruction of bacteria.

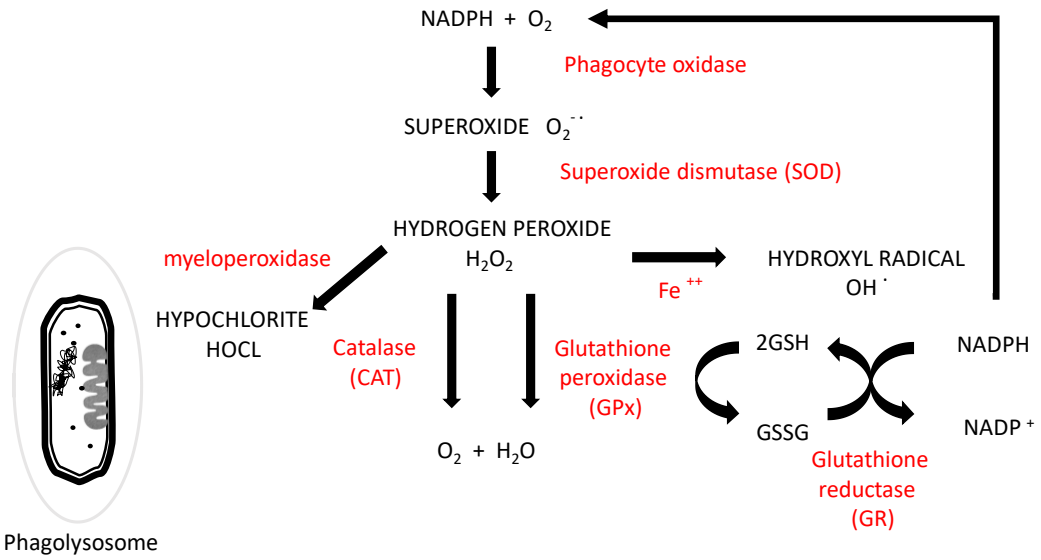


Figure 4.1 Oxygen dependent bactericidal mechanism of phagocytes and redox regulation by antioxidant enzymes.

There is a hypothesis proposed by many researchers that the immunomodulation effects of Se could be achieved by redox regulation [10]. Previous studies reported that Se-deficient J774.1 murine macrophages exhibited decreased phagocytic activity, superoxide production and cytokine secretion compared with Se-adequate group when the cells stimulated with mitogens (PMA and LPS). In Se-deficient rats, neutrophils reduced respiratory burst when long-term incubated with PMA or opsonized zymosan [11]. One possible explanation is that suppressed redox regulation leading to inefficient production of H_2O_2 and led to loss of activity of the membrane-bound NADPH-dependent superoxide-generating system. Adequate Se J774.1 cells exhibited higher activity of glutathione peroxidase (GPx) which would provide protection against H_2O_2 formed from SOD of the increased superoxide. Interestingly, levels of Se intake can influence the production of ROS and their downstream effects [12]. Therefore, antioxidant enzymes such as GPx, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) are commonly regulated by Se status in hosts [13]. Although the relationship of redox regulation and immunomodulation from inorganic and organic forms of Se has been studied, the unique bioactivity of SeNP in this interaction was unknown.

4.1.3 SeNP and Trolox roles in redox regulation and immunomodulation function

Therefore, to test our hypothesis that SeNP immunomodulation contributes from redox regulation, a parallel antioxidant experiment was conducted by using a typical exogenous antioxidant, Vitamin E. Previous studies demonstrated that Vitamin E could scavenge ROS by delivering an H atom, and thus protect cells or organs from oxidative damage. A typical exogenous antioxidant applied in clinic has been reported to be Vitamin E, and its water-soluble analogue Trolox is also widely used in research projects [14, 15]. Apart from redox regulation of Vitamin E, there have been a number of reports of its influence on immunomodulation effects in variety of species [16]. This paper will examine the roles that SeNP play in redox regulation and immunomodulation functions, and it will discuss the implications of antioxidant supplementation on fish health and immune responses. Zebrafish will be applied in our study. Se and Vitamin E supplement diets provide the same antioxidant capacity to the fish. Subsequently, zebrafish supplemented with CTS-SeNP and Vitamin E will exam their immune responses and redox status. Immune-challenge study will also be conducted by using a fish bacterial disease *Aeromonas hydrophila* to test the overall immunity of the host.

4.2 Materials and Methods

4.2.1 Fish maintenance and CTS-SeNP preparation

Zebrafish (*Danio rerio*) maintenance and CTS-SeNP synthesis and characterization were described in **Chapter 2**. Briefly, adult zebrafish (9-12 months) were maintained in 15-L glass tanks with flow-through system at water temperature of 28 ± 1 ºC and pH of 7.0 ± 0.2. The light-dark cycle was 14:10. The fish were fed by commercial fish diet (Otohime B1: 51.0% crude protein, 11.0% crude fat, 15.0% crude ash, USA) three times per day and brine shrimp nauplii once a day.

Selenium nanoparticles stabilized by chitosan (CTS-SeNP) were prepared by controllable reduction under room temperature. Briefly, chitosan solution (0.25%) was mixed with freshly prepared ascorbic acid (100 mM) with magnetic stirring. Sodium selenite solution was added in the mixture dropwise away from light. Next, MilliQ water was added in the mixture to 25 mL and allowed to react overnight (around 12 hours) at room temperature. After dialysis, CTS-SeNP characterizations were processed by transmission electron microscopy (TEM; JEOL 2010 + Horiba EX-250, USA) and NanoSight NS300 (Malvern Instruments Limited, USA) for particle size distribution.

4.2.2 Total antioxidant capacity assays and fish diet preparation

Total antioxidant capacity of CTS-SeNP were determined by using a Total Antioxidant Capacity Assay Kit (Sigma, USA). The ability of antioxidants in the samples to eliminate a certain amount of oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) was determined colorimetrically by an enzymatic reaction which involved conversion of Cu^{2+} ion to Cu^{+}. Trolox, a water-soluble vitamin E analog, serves as an antioxidant standard (Calbiochem, USA). A linear standard curve was obtained between 0 and 10 M Trolox ($y = 36.364x + 0.1054$, $R^2 = 0.9998$). The total antioxidant capacity of 1M CTS-SeNP suspension (containing 1M Se as determined by IPC-MS) was determined to be 348.9 mM Trolox equivalents. (Figure 4.2)

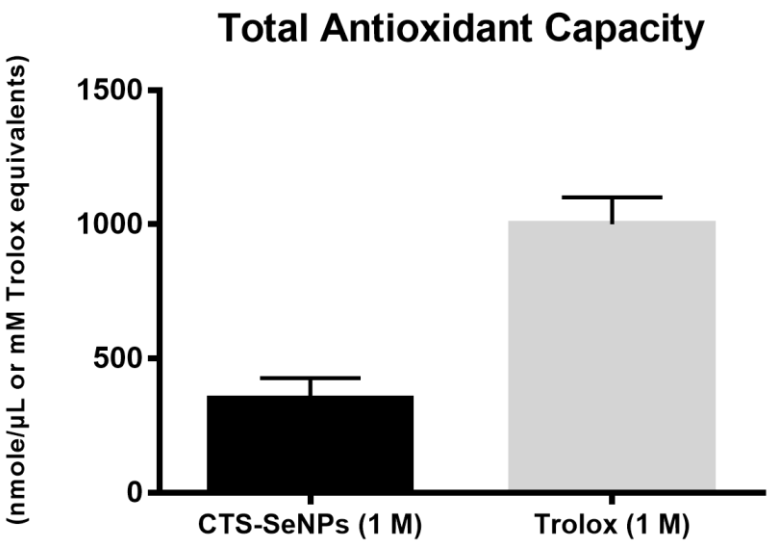


Figure 4.2 Total antioxidative capacity of CTS-SeNP and Trolox.

Our previous study demonstrated that CTS-SeNP has positive immunomodulatory effects in fish by using 10 µg/g Se in fish diet. To test our hypothesis that antioxidant capacity of CTS-SeNP is the potential driver of its immunomodulatory effect, an equal antioxidant capacity fish diet was prepared using Trolox. Fish diet preparation was followed the method described in **Chapter 2**. Briefly, 10 µg/g CTS-SeNP diet contains 126 nM Se and equal antioxidant capacity as 43.9 nM Trolox. The molecular weight (MW) of Trolox is 250.29 g/mol as the product

instruction. Therefore, 110 µg Trolox (43.9 nM) was dissolved in 10 mL MilliQ water was then added in 10 g commercial fish diet (Otohime B1, USA) to prepare a Trolox diet for treatment.

4.2.3 Experimental regime

Zebrafish were fed with three experimental fish diets (CTS-SeNP diet, Trolox diet and control) at ration of 2% body weight for 9 days. 90 adult fish were used for each diet. After dietary exposure, fish were sacrificed and immune biomarker responses, antioxidant enzymes activity and redox related gene expression were studied. Serum, kidney and spleen samples were collected for immune responses study while livers were used for antioxidant enzymes activity and gene expression study.

4.2.4 Immune biomarkers measurement

Tissues samples were collected as **Chapter 2** and blood sample was collected by using centrifugation method [17]. Serum lysozyme activity was analyzed by using the EnzChek Lysozyme Assay Kit (Molecular Probes, USA).

Intracellular and extracellular respiratory burst activity in zebrafish kidney cells [18]. Intracellular and extracellular superoxide production were measured as a change in absorbance resulting from reduction of Cytochrome C and oxidation of nitroblue tetrazolium (NBT) respectively as described from previous studies [19, 20].

Proliferation of B and T lymphocytes in zebrafish spleen was measured after stimulation by lipopolysaccharide (LPS, Sigma, USA) and 100 µg/well of concanavalin A (ConA, Sigma, USA) respectively and then determined by Vybrant MTT Cell Proliferation Assay Kit (invitrogen, USA) [21-23].

4.2.5 Bacterial culture and challenge experiment

Bacterial challenge in total 180 treated zebrafish was carried out as **Chapter 2** using *Aeromonas hydrophila* (ATCC 7699, USA). After 9d treatment by CTS-SeNP diet and Trolox diet, bacterial challenge was conducted. Briefly, the fish from control diet, CTS-SeNP diet and Trolox diet were intraperitoneal (*ip*) injected with 2.5×10^6 cfu of *Aeromonas hydrophila* in 10 µL phosphate buffered saline (PBS). A negative

control was carried out by *ip* injection of 10 µL only PBS in fish. The bacterial challenged zebrafish were maintained at 28 ± 1°C. Survival rate was recorded constantly until 72 hours. Survivorship of various treatments were compared by using Kaplan-Meier analysis (SPSS version 15.0, IBM SPSS Statistics, USA).

4.2.6 Reactive oxygen species (ROS) and reduced glutathione (GSH) concentration

Total cellular ROS in zebrafish liver samples were conducted by using commercial assay kit (Abcam, UK). Briefly, liver samples from 5 individuals were homogenized in 1 ml of PBS with pH 7.0 on ice with triplicates. The homogenized tissues samples were incubated with the redox sensitive dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) in a dark humidified chamber for 20 min at 28 °C. After incubation, ROS concentration was measure plate on a fluorescence plate reader at Ex/Em =485/535 nm in end point mode in the presence of compounds, media, or buffer. At last, the ROS concentration was normalized against protein concentration in samples.

Protein concentration in samples was measured by bicinchoninic acid (Pierce BCA, Thermo Scientific, USA) method. Briefly, albumin (BSA) standards were prepared by serial dilution with PBS. 25 µL of each standard or fish samples were mixed with 200 µL BCA working solution. The reaction was incubated at 30 °C for 30 min. The colorimetry was used for OD determination (562 nm) in this study by using CLARIOstar Microplate Reader (BMG Labtech, Germany).

GSH concentration in zebrafish liver samples were measured using commercial biomedical assay kit (Nanjing Jiancheng Bioengineering Institute, China). Briefly, the liver homogenates were centrifuged under 4000g for 10 min at 4 °C. The supernatants were aliquoted and incubated with 5,5-dithio-*bis*-(2-nitrobenzoic acid) to produce a yellow color 5-thio-2-nitrobenzoic acid (TNB). The mixed disulphide, GSTNB (between GSH and TNB) that is concomitantly prodced, is reduced by glutathione reductase (GR) to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH. Measurement of TNB at 405-414 nm were normalized by

protein concentration in samples.

4.2.7 Measurements of antioxidant enzyme activities

Activities of key antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) were measured by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China). Livers samples were homogenized and prepared for these assays as described in Section 4.2.6.

4.2.7.1 Measurements of glutathione peroxidase (GPx) activity

GPx activity was determined indirectly by measuring the rate of formation of oxidized glutathione (GSSG). Briefly, 5 μL homogenized liver samples were incubated with 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB at 100 μL) for 10 min to generate 2-nitro-5-thiobenzoate anion (TNB). Positive controls were performed by added 20 μmol/L GSH solution with 5μL. The reaction was measured by the increase in absorbance at 412 nm by colorimetry. The activity of GPx was expressed as U/mg protein.

4.2.7.2 Measurements of glutathione reductase (GR) activity

GR activity was determined by measuring the decrease in absorbance (340nm) due to oxidation of NADPH [24]. Briefly, the homogenized livers (10 μL) were added with 1mM NADPH solution (200 μL). NADPH oxidation was determined at 340 nm for 3 min. One unit of GR activity is defined as the amount of protein that oxidized 1 mM NADPH per min.

4.2.7.3 Measurements of superoxide dismutase (SOD) activity

SOD activity was measured the inhabitation of tetrazolium salt (WST-1) reduction. Basically, tetrazolium salt (WST-1) could be reduced by superoxide anion to produce a water-soluble formazan dye during the SOD involved xanthine oxidation reaction. SOD reduces the level of superoxide anion converting it to oxygen (O_2) and H_2O_2, thus inhibiting WST-1 reduction. Briefly, the liver homogenizers (20 μL) were incubated with the WST-1 solution (200 μL) for 20 min. Then, SOD activity was detected by colorimetry at 450 nm. One unit of SOD activity was defined as the amount

of enzyme required to inhibit the oxidation reaction by 50% and was expressed as U/mg protein.

4.2.7.4 Measurements of catalase (CAT) activity

CAT activity was determined by the amount of enzyme that catalysed the decomposition of 1 μmol of H_2O_2 per min. In brief, 10 μL homogenized liver samples were incubated with ammonium molybdate (220 μL) for 10 min. The residual H_2O_2 reacts with ammonium molybdate to generate a yellowish complex (OD with 240nm). One unit of catalase activity is 1 μmol H_2O_2 consumed/mg protein per sec.

4.2.8 RNA extraction and gene expression

Genes related to redox regulation and immune responses were studied in zebrafish liver after treated with CTS-SeNP diet and Trolox diet. Genes of interest studied related to redox included glutathione peroxidase (GPx), catalase (CAT) and ATP synthase F0 subunit 6 (ATPo6). Immune-related genes studied were complement component 3 (C3), interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α). Forward and reverse primer sequences were provided in Table 4.1. Liver from 5 zebrafish per dietary treatment with triplicates were placed in 1 mL of RNAiso Plus (TaKaRa BIO, Japan) in a 1.5 mL Eppendorf tube and were homogenized with tissue grinder. Lysed liver tissues were centrifuged at 12,000 g at 4 ℃ for 5 min, and the supernatant was isolated from the mixture and transferred to a new tube. 200 μL chloroform was added to the supernatant and vortexed until milky. The mixture were allowed to sit for 15 min under room temperature and centrifuged at 12,000 g at 4 °C for 15 min. The supernatant was transferred to a new tube again and was added an equal volume of isopropanol. This mixture was mixed and centrifuged at 12,000 g at 4 °C for 10 min. The RNA pellet was washed twice by using 1 mL 75% ethanol and treated with DNase (DNase I, TaKaRa BIO) to remove DNA impurities according to manufacturer's instruction.

RT-PCR was performed using SYBR PrimeScript RT-PCR kit II (TaKaRa BIO, Japan) with Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). Every reaction consisted of 20 μl final volume of 50 ng of isolated sample RNA, 0.8 μl of PrimeScript RT enzymes mix, 10 μl of 2×SYBR

RT-PCR buffer 4, and 0.5 μM of corresponsive forward and reverse primers. To evaluate formation of primer dimer and RNA contamination, a control reaction with no RNA template added (NTC) was conducted. The PCR reaction was processed as a single cycle of reverse transcription at 42 °C for 5 min and 95 °C for 10 s, 40 amplification cycles consist of 95 °C for 5 s for denaturation and 60 °C for 20 s for annealing and elongation, and a single cycle of melting process that consisted of an increase temperature from 65 °C to 95 °C over 10 min. A single peak in all melting curves verified the generation of a single amplicon for each primer pair. Expression of the genes of interest were normalized using expression of β-actin and fold change was calculated as $2^{-\Delta\Delta Ct}$.

Table 4.1 Primer sequences used in the study.

Assemble number	Genes	Sequences (F: forward primers; R: reverse primers)	Amplicon size (bp)
M24113	Beta-actin (β-actin)	F: TCAACACCCCTGCCATGTAT	108
		R: TCACACCATCACCAGAGTCC	
AW232474	Glutathione peroxidase (GPx)	F: AGATGTCATTCCTGCACACG	94
		R: AAGGAGAAGCTTCCTCAGCC	
AF170069	Catalase (CAT)	F: CACTCTCAGAAGCGCAATCC	499
		R: ATCCCAAACCATGTCCGGAT	
AC024175	ATP synthase F0 subunit 6 (ATPo6)	F: TTATCCTCGTTGCCATACTTC	118
		R: AGTTGGTTTGTGAATCGTCC	
AB004105	Complement component 3 (C3)	F: AGGGCTGATGTTTGAGTCGA	118
		R: TGTTGCTGTGATTGTGCGAA	
AM941672	Interleukin 1 beta (IL-1β)	F: CGTCATCCAAGAGCGTGAAG	105
		R: TGCGCACCAGAGACTTCTTA	
AY427649	Tumor necrosis factor alpha (TNF-α)	F: ACCAGGCCTTTTCTTCAGGT	108
		R: TGCCCAGTCTGTCTCCTTCT	

4.2.9. Statistical analyses

One-way analysis of variance (ANOVA) followed by a Tukey test were used (GraphPad Prism 6.00, USA). Data are presented as mean ± SD and considered to be significantly different at $P \leq 0.05$ level.

4.3 Results

4.3.1 Immune biomarkers responses

Lysozyme activity in fish serum significantly increased in both treatments but CTS-SeNP treatment (36.29 U/mL) had a higher activity than the Trolox (30.26 u/mL) (Figure 4.3).

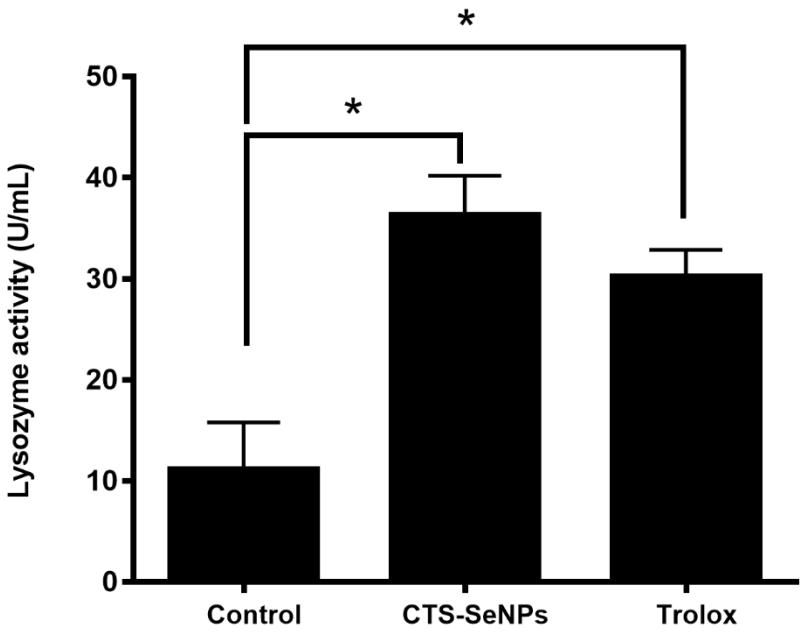
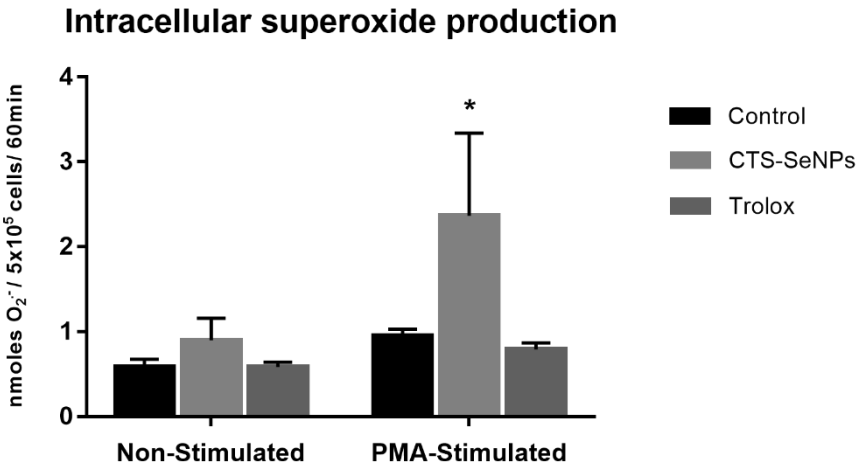


Figure 4.3 Lysozyme activity in zebrafish serum after supplementary of CTS-SeNP, and Trolox in fish diets. Statistically significant difference from control was represented by asterisks.

Without stimulation, CTS-SeNP has no significant effect on intracellular and extracellular superoxide production but Trolox significantly decreased extracellular superoxide production (Figure 4.4). After stimulation, Trolox fred fish showed no significant difference in intracellular and extracellular superoxide production when compared with control while CTS-SeNP fed fish have significantly higher superoxide production.

a)



b)

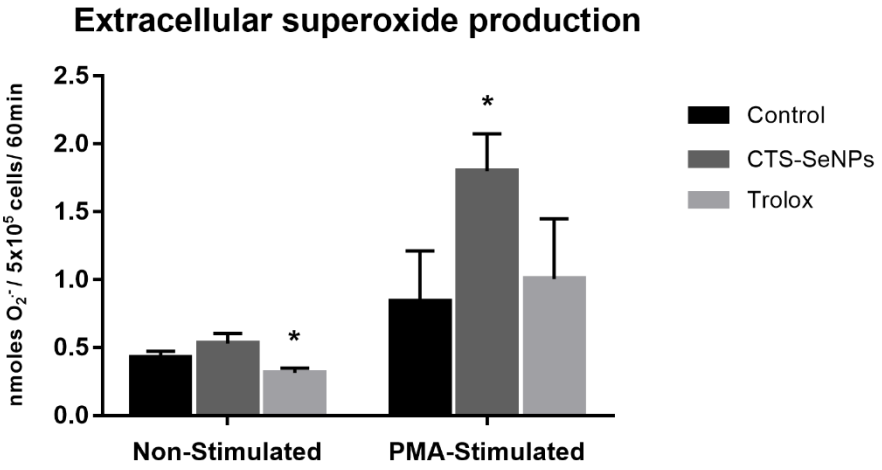
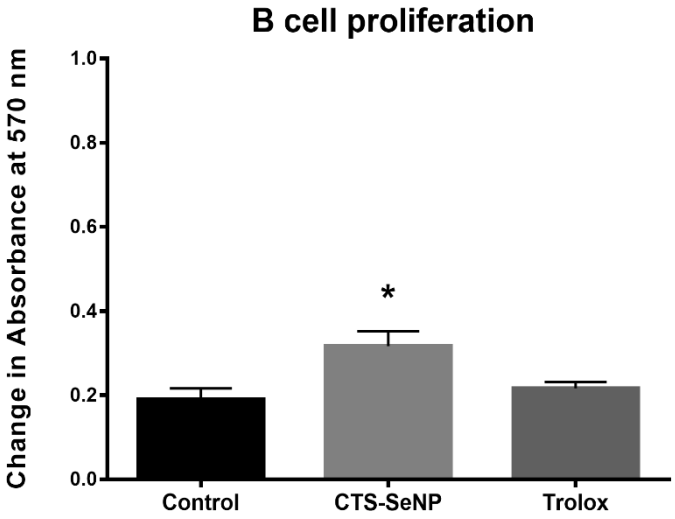


Figure 4.4 Phagocytic respiratory burst after supplementary CTS-SeNP and Trolox: a) intracellular respiratory burst activity; b) extracellular respiratory burst activity. Statistically significant difference from control was represented by asterisks.

Lymphocyte proliferation in zebrafish splenocytes were stimulated by using ConA and LPS. In splenocyte-responses toward LPS (B-lymphocytes proliferation) (Figure 4.5.a), both supplemented groups showed no change with the control. However, in splenocyte-responses toward ConA (T-lymphocytes proliferation) (Figure 4.5.b), CTS-SeNP diet significantly increased the responses with 2.91 fold against the control. Similarly, Trolox group significantly increased (1.96 fold) comparing with the control.

a)



b)

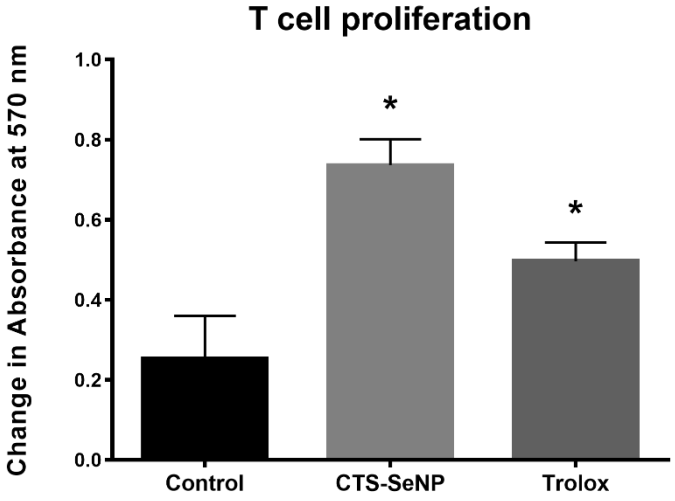


Figure 4.5 Lymphocyte proliferation assay in zebrafish splenocytes in CTS-SeNP and Trolox treatments: a) B cell proliferation towards LPS and b) T cell proliferation towards ConA. Statistically significant difference from control was represented by asterisks.

4.3.2. Bacterial resistance by CTS-SeNP and Trolox supplements

Overall immune responses in zebrafish were determined by survival test in bacterial infection as Figure 4.6. Mortality was first found in 4h after *ip* infection. Then the significant difference between CTS-SeNP and Trolox was at 48h. At the end of challenge test for 72h, over 80% fish died in control group, while CTS-SeNP group had 55% mortality and Trolox group had a similar percentage of 58%. Therefore, both CTS-SeNP and Trolox diet could provide protection for zebrafish during *A. hydrophila* infection but the earlier protective effects found in CTS-SeNP.

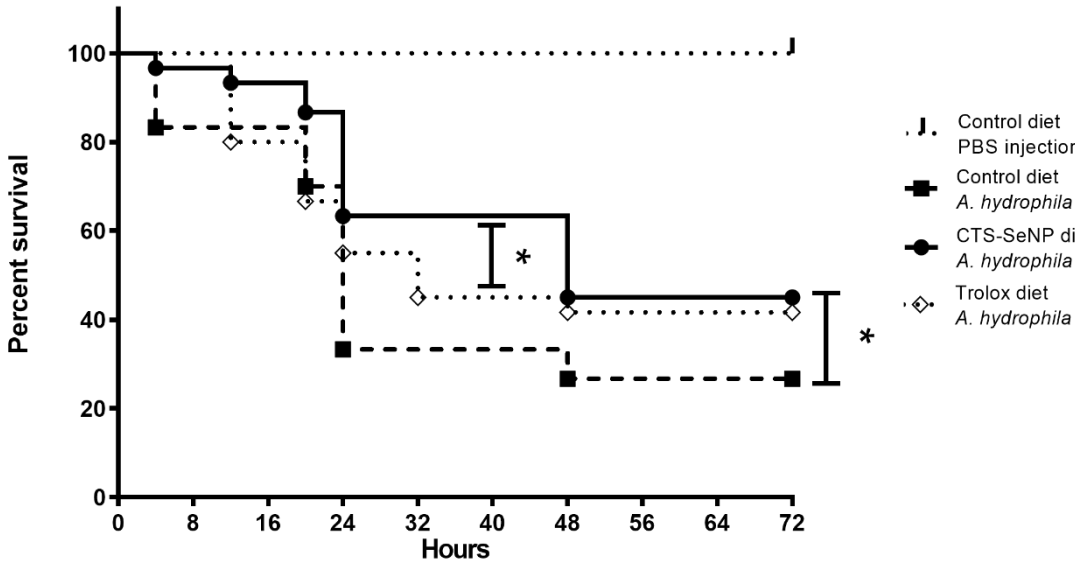
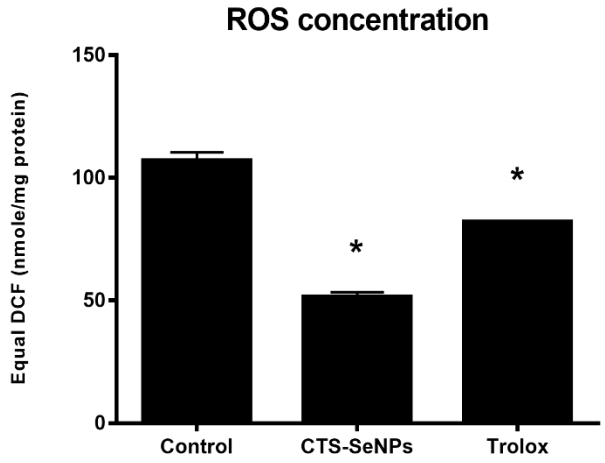


Figure 4.6 Bacterial challenge by *Aeromonas hydrophila* in zebrafish and the survival rate during 72h infection. Statistically significant difference from control was represented by asterisks.

4.3.3. ROS and GSH concentration

Total ROS concentration in fish livers for both CTS-SeNP (51.5 nmole/mg protein) and Trolox (82.1 nmole/mg protein) groups (Figure 4.7.a). On the other hand, CTS-SeNP significantly increased GSH by 0.1 nmole/mg protein while Trolox had no significant effect (Figure 4.7.b).

a)



b)

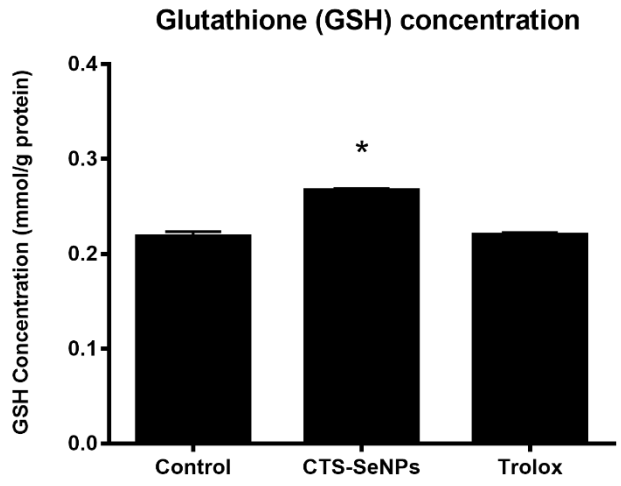


Figure 4.7 ROS and GSH concentration in zebrafish livers. a) ROS concentration and b) GSH concentration. Statistically significant difference from control was represented by asterisks.

4.3.4. Antioxidant enzymes activities

CTS-SeNP has significant effect on activities of all four enzymes. It has significantly increased GPx and GR activity and significantly decreased SOD and CAT activities (Figure 4.8). Trolox had similar effect on GPx and CAT as CTS-SeNP, but did not have significant effect on GR and SOD.

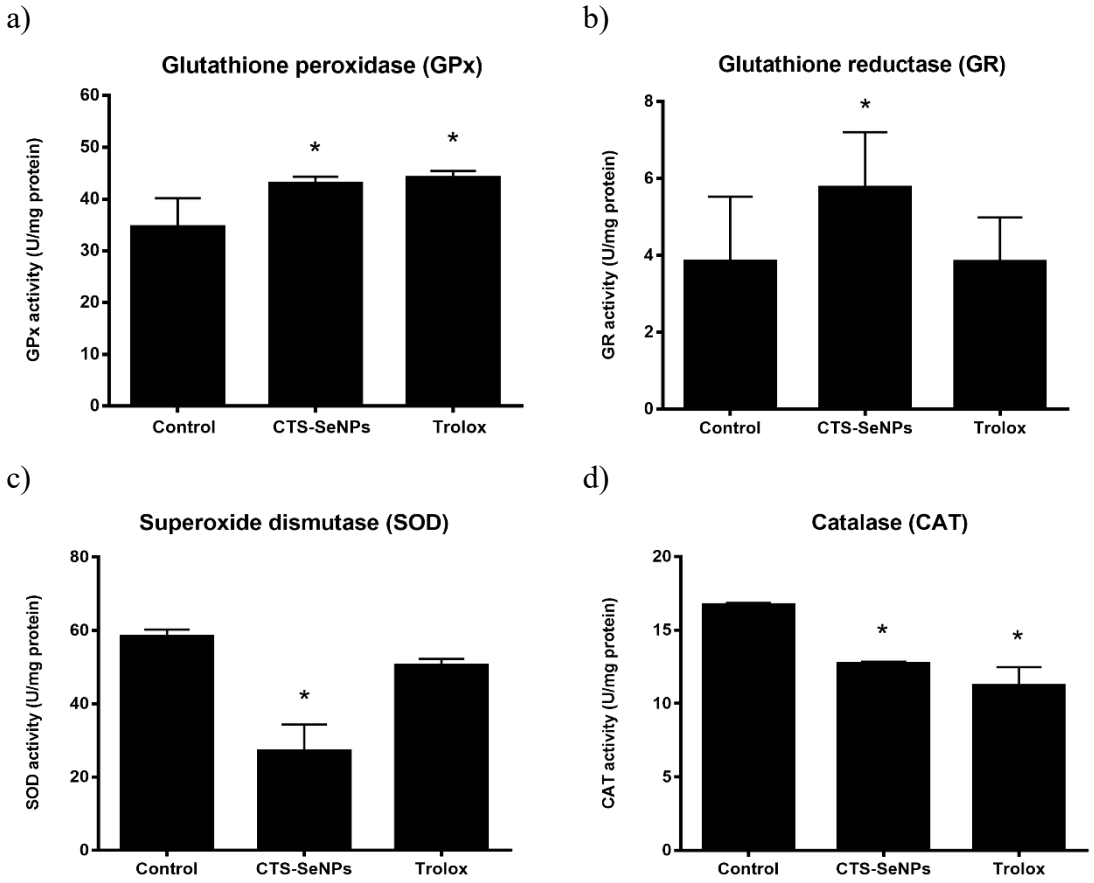


Figure 4.8 Antioxidant enzymes regulation in zebrafish livers: a) glutathione peroxidase (GPx) activity; b) glutathione reductase (GR) activity; c) superoxide dismutase (SOD) activity; d) catalase (CAT) activity. Statistically significant difference from control was represented by asterisks.

4.3.5. Gene expression patterns

As shown in Figure 4.9, the variation of reference gene (β-actin) ranged from 17.8 to 20.3 Ct value for the control, CTS-SeNP and Trolox treatments. It suggested that β-actin had a relatively consistent expression under different conditions. Gene expression patterns more complicated than other endpoints reported above. For GPx, although enzyme activity was significantly increased for both treatments, CTS-SeNP treatment caused a marginally significant increase in gene expression (Figure 4.10.a). For CAT, although enzyme activity was significantly increased for both treatments, gene expression was significantly increased by Trolox (Figure 4.10.b) while CTS-SeNP has not significant effect. For ATPo6, both treatments led to significant upregulation (Figure 4.10.c).

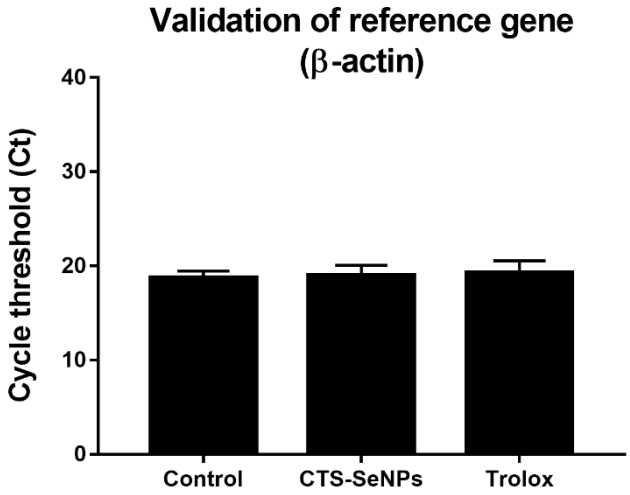


Figure 4.9 Validation of reference gene (β-actin) with the consistent Ct values range from 17.8 to 20.3 for the control, CTS-SeNP and Trolox treatments suggested that reference gene (β-actin) with a relatively constant expression under different conditions.

For immune related genes, only IL-1β were significantly upregulated by only CTS-SeNP treatment (Figure 4.10.d) while there was no significant change in expression of C3 and INF-α (Figure 4.10.e and f).

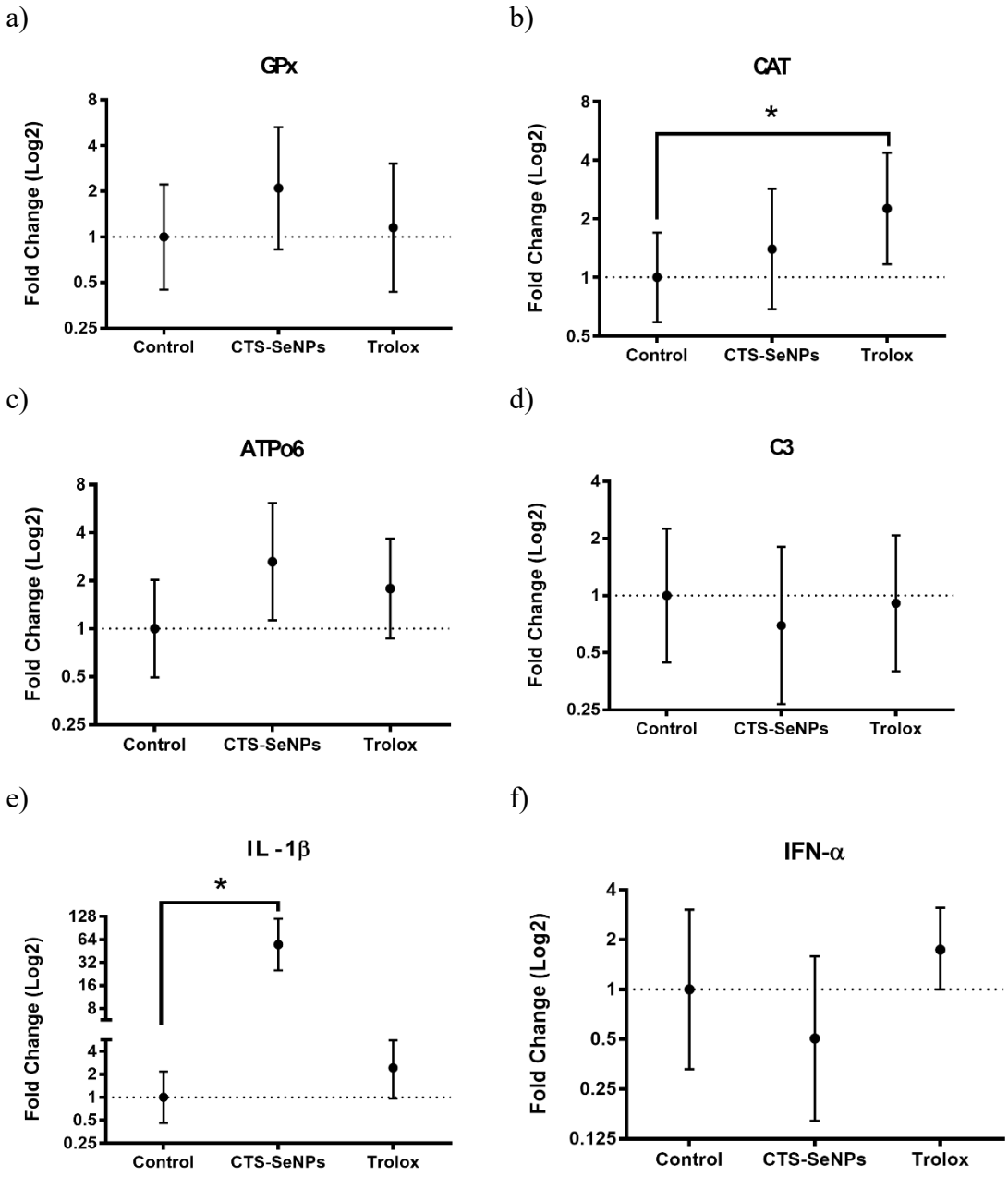


Figure 4.10 Expression of genes in redox and immune regulation normalized to β-actin expression (mean +1 SD; n=5 for each treatment) in zebrafish liver after 9d supplementary diet, a) glutathione peroxidase (GPx); b) catalase (CAT); c) ATP synthase F0 subunit 6 (ATPo6); d) complement component 3 (C3); e) interleukin 1 beta (IL-1β) and f) tumor necrosis factor alpha (TNF-α). Statistically significant difference from control was represented by asterisks.

4.4. Discussion

In this experiment we compared immune and antioxidant biomarker responses to dietary treatments of SeNP and Trolox at the same antioxidant capacity. Both treatments led to significant improvement in redox condition and immune biomarkers in fish. Improvement in redox condition were reported to correlate with improved immunity [25].

In terms of immune biomarkers, Trolox could only improve lysozyme activity and T cell proliferation. This fall short of the effect of SeNP that can also improve intracellular and extracellular respiratory burst and had better efficacy than Trolox in improving lysozyme activity and T cell proliferation. Based on these findings alone it seemed to suggest that antioxidant capacity alone cannot explain all the immunostimulatory effect of SeNP.

When comparing changes redox condition and regulation of the animals subjected to SeNP and Trolox, some differences could be observed (Figure 4.7 and 4.8). Total ROS in the animals were significantly decreased by both treatments but SeNP was better than Trolox (Figure 4.7a). Similarly, total GSH levels in the animals were only increased by SeNP but not Trolox (Figure 4.7b). Even though the SeNP and Trolox diets were having the same antioxidant capacity, SeNP was more effective than Trolox in removing oxidative stress. This could be because SeNP can also be metabolized to have additional biological effects beyond its initial role as a chemical antioxidant. This is evident in the activities of 4 key enzymes in regulating redox where SeNP can have effect on SOD and GR but not Trolox (Figure 4.8). Gene expression data also showed that SeNP but not Trolox can upregulate GPx expression (Figure 4.10.a). GPx and GR were selenoproteins and supplementation of Se will most likely lead to increased expression, producing addition protection against ROS in the SeNP fed fish [26]. These additional effects of SeNP on redox have impact on immunity and may explain some of the discrepancies observed in the different immune biomarker responses of the two treatments.

ROS are deleterious by-products of mitochondrial and endosomal metabolic activities. Neutrophils and macrophages also utilize respiratory burst activity to

produce large amount of ROS to destroy engulfed foreign organisms [27]. Both SeNP and Trolox caused decrease in CAT activity and increase in GPx activity. This might lead to build up of H_2O_2 in the cells, which is a prerequisite for respiratory burst activity [9]. Increased GPx activity could increase production of H_2O_2 and promote the circulation of NADPH oxidases during phagocytosis in both treatments [28-30]. In this manner, both treatments can lead to improved immunity in the host.

However, excess H_2O_2 can be toxic to cells causing cell death. Antioxidant protects the neutrophils and macrophages from being damaged by the ROS during respiratory burst [31] and can also help these cells to be more efficient in producing ROS [32]. One of these protective antioxidants in cells was GSH. GSH will sacrificially reduce ROS in cells to prevent oxidative damage by the action of GPx [33] and transformed into oxidized glutathione (GSSG). GR is important to recycle GSSG back to reduced glutathione (GSH). These two enzymes (GR and GPx) that were central to the GSH/GSSG cycle and both enzymes were selenoproteins. While both SeNP and Trolox can improve GPx activity, only SeNP increased GR activity and increased GSH. In addition, SeNP led to upregulation of GPx gene expression. Therefore, SeNP can be also improving efficiency of the GSH/GSSG cycle and offering additional protection to cells than Trolox.

Interestingly, when subjected to bacterial infection challenge, Trolox offered similar protective effect as SeNP (Figure 4.6). This might be due to Trolox activating other components of the immune system. For example, parrot fish (*Oplegnathus fasciatus*) supplemented with Vitamin E showed increase in intracellular phagocytosis and myeloperoxidase activities [34].

4.5 Conclusion

At an equal antioxidant capacity, Trolox and SeNP offered similar protection against bacterium *A. hydrophila* in zebrafish. Despite the same end result, we observed similarities and also some differences in their ability to interact with the immune and redox regulation systems. In terms of immune biomarkers, both treatments could significantly increase lysozyme activity and T cell proliferation. Both treatments could also decrease total ROS in the host and modulate GPx and CAT activities. However,

SeNP can also modulate activity of GR and SOD and increase GSH concentration in the host. These are likely related to the biological activity of Se which are unrelated to the antioxidant capacity of SeNP. Additional research will be needed understand how SeNP are metabolized and the subsequent biological activity.

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CHAPTER 5

Transcriptome profiling reveals the immune mechanism in zebrafish (*Danio rerio*) under chitosan selenium nanoparticles (CTS-SeNP) supplement

5.1 Introduction

Selenium (Se) immunomodulatory function has been reported widely, but the mechanism is still not fully understood [1, 2]. Although selenium nanoparticles (SeNP) different bioavailability and toxicity, its immunomodulatory activities was supported in researchers [3]. In our previous **Chapter 2-4**, chitosan stabilized SeNP (CTS-SeNP) was studied its immunomodulation and potential mechanism in serum proteomics. In this chapter, we would like to explore further its immune regulation function in most important hematopoietic organ as kidney in zebrafish by using transcriptome.

There are two potential mechanisms have been proposed to explain Se immunomodulation. The first is that Se exerts redox regulation roles take part in phagocytosis processes. Phagocytic respiratory burst is that activated leukocytes such as macrophages and neutrophils through different receptors to induce a series of rapid increase in reactive oxidant species (ROS) to eliminate invaded foreigners [4]. Adequate Se required for optimal activation and function of these phagocytes by rapidly produced superoxide radicals ($O_2^{-\cdot}$) from NADPH-dependent superoxide-generating system and strengthen the instantaneous respiratory burst by inhibiting the downstream regulation enzyme, superoxide dismutase (SOD) [5]. Further redox regulation by selenoproteins such as glutathione peroxidases (GPx) and thioredoxin reductases (TRs) can maintain glutathione and thioredoxin related redox balance [4, 6]. Another potential mechanism of Se immune regulation is cell-to-cell signaling regulation by ROS. ROS are important mediators of cell signaling and cell to cell communication for immune cells. Se exists in selenoproteins to alters the species and amount of ROS to regulate cellular signaling presents among immune cells [7]. Common cellular cytokines such as tumor necrosis factor a (TNF-a), interleukin 2 (IL-2), interferon γ (IFN- γ) are associated with Se status in hosts.

To understand the molecular mechanism of Se immunomodulation, analyzing transcriptomes of immune cells is an important method. The continuously changing cellular transcriptome is analyzed by using RNAseq which uses next generation sequencing (NGS) to reveal the presence and quantity variation of RNA in biological samples. Previous study revealed the transcriptome profiles of goose T cells under selenium stimuli. It demonstrated that selenoproteins were promoted by selenium stimulation and primarily involved in immune and environmental response, and infectious disease and genetic information processing related pathways were identified [8]. Zebrafish (*Danio rerio*) is a widely used immunological animal model, it has complete reference genomes to fully access transcriptome profiling [9]. In addition, a fully developed immune system including innate and adaptive immunity exists in zebrafish. Many immunological assays were well established in zebrafish models [10]. Previous study compared the transcriptome profiles of zebrafish brain and spleen infected with spring viremia of carp virus (SVCV). The results demonstrated that changes and tissue-specific influences caused by SVCV *in vivo* and Influenza A pathway and Herpes simplex infection pathway were regulated in brain and Tuberculosis and Toxoplasmosis pathways in spleen [11].

To explore the mechanism of Se immunomodulation, transcriptome analysis of zebrafish immune responses was performed in kidney cells. Kidney as the most important immune organs in fish, it is considered as bone marrow in vertebrates and the largest site of hematopoiesis [12]. Previous chapter analyzed the serum proteomics of zebrafish supplemented with CTS-SeNP. Therefore, this chapter will also reveal the hematopoiesis related organ and its transcriptome responses when the fish supplemented with CTS-SeNP.

5.2 Materials and Methods

5.2.1 Animal ethics statement and zebrafish maintenance

Fish maintenance and animal experiments were carried out in strict according to the Animal (Control of Experiments) Ordinance Cap. 340 by HKSAR and approved by Animal Subjects Ethics Sub-Committee (ASESC) (license number of 15-157 and ethic number is 13/21). Briefly, healthy adults were kept in 15-L tanks flow-through system at temperature 28 ± 1 ºC and pH 7.0 ± 0.2 under a 14:10 light-dark cycle. Fish

were fed with commercial fish feed (Otohime B1, USA) three times per day and supplemented by live brine shrimp nauplii once a day.

The commercial fish feed has ingredients as Krill Meal, Fish Meal, Squid Meal, Potato Starch, Wheat Flour, Fish Oil, Brewers Yeast, Calcium Phosphate, Guar Gum, Soy Lecithin, Betaine, Licorice Plant, Apple Extract, Wheat Germ. The crude protein content is 51.0% while the crude fat content is 11.0%.

5.2.2 CTS-SeNP diet preparation and fish exposure

Chitosan stabilized selenium nanoparticles (CTS-SeNP) were synthesized using controllable reduction methods as described in Shi *et al* [3]. and in **Chapter 2**. Zebrafish were fed with 0 (control) or 10 μg/g CTS-SeNP at a ration of 2% body weight for nine days. For each diet 30 adult fish were used. At the end of the nine day exposure, zebrafish were euthanized in MS222 and necropsy was performed to collect the kidney as described in **Chapter 2**. Briefly, zebrafish were euthanized in MS222 (1.0 mg/mL) then dried by tissue papers. The whole zebrafish kidney was collected using dissection forceps under Nikon SMZ18 stereo microscope including head kidney, trunk and tail parts. Tissues of nine individuals per treatment were pooled in a 1.5 mL Eppendorf tube as one experimental sample. All subsequent analyses were conducted in duplicate.

5.2.3 RNA isolation and manipulation

Total RNA was extracted from the kidney samples using a RNeasy Mini kit (Qiagen, Netherlands) according to the manufacturer's instructions. Purity of RNA in each sample was determined using a NanoDrop 200 spectrophotometer (Thermo Scientific, USA). Quality of RNA samples were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Concentration of RNA samples were determined by Agilent RNA 6000 Nano Kit (Agilent Technologies, USA). All RNA samples used for RNAseq has OD260/280 = 1.8~2.2, OD260/230 = 1.8~2.2, without degradation and contamination, as well as RNA integrity number (RIN) value ≥ 7.0.

5.2.4 Sequencing library construction and illumine sequencing for transcriptomic analysis

Sequencing library construction and Illumina sequencing were performed.

Briefly, mRNA was enriched via purification with oligo (dT) magnetic beads and fragmented into short fragments using fragmentation buffer [13]. The First-stand cDNA was synthesized using random hexamer-primed reverse transcription and the fragmented mRNA as templates. Then the complementary second-strand cDNA was synthesized by using DNA polymerase I. The synthesized double strand cDNA was purified by magnetic beads, sequencing and adaptors were ligated to the end of purified DNA fragments. Next, these products were subjected to TAE-agarose, and further enriched by PCR amplification. Enriched fragments were purified by magnetic beads and dissolved in the appropriate amount of Epstein-Barr solution. Then the purified products were sequenced on an Illumina HiSeq 4000 platform.

5.2.5 Reads processing and identification of differentially expressed genes (DEGs)

Raw reads were generated with Sequence platform as Table 5.1. There are some adaptor sequences and/or low quality reads present in the raw reads, data filtering is carried out to obtain high quality reads as the clean reads (clean data). The quality of sequencing was presented by clean reads which showed a high ratio over 98% for all samples. To obtain clean data, low quality reads and adaptor sequences were removed. The clean reads were mapped to a reference genome *Danio rerio*.GRCz downloaded from Ensembl (ftp://ftp.ensembl.org) using hierarchical indexing for spliced alignment of transcripts (HISAT2).

Table 5.1 Statistical transcriptome data of zebrafish kidney with/without CTS-SeNP treatments.

Samples	Control 1	Control 2	CTS-SeNP 1	CTS-SeNP 2
Total Raw Reads	47299840	45898596	47911804	48837824
Total Clean Reads	46371986	45327760	47003492	48056628
Total Clean Reads Ratio (%)	98.04	98.76	98.10	98.40
Total Mapped Reads (%)	88.19	87.70	88.32	89.13
Unique Mapped Reads (%)	75.14	74.73	74.91	74.87
Reads Mapped in Paired (%)	82.20	81.40	82.00	83.06

HTSeq which is a Python package that provides infrastructure to process data from high-throughput sequencing assays to map the reads to genes efficiently and

accurately is used to locate reads to genes. Fragment per kb per million fragments (FPKM) method was used in calculating expression level. DESeq2 was used to detect the differential expressed genes (DEGs). Genes with an expression log fold change value larger than 1 and P-value less than 0.01 were considered as significant DEGs [14, 15].

Further, transcriptome profiles were integrated and the extent of overlap was presented in Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). We performed cluster analysis of DEGs with R package heatmap. For function analysis, GOseq (version: v1.16.2) was used to compare the DEGs to the all reference genes for GO enrichment analysis. Web Gene Ontology Annotation Plot (WEGO) was used for visualizing, comparing and plotting GO annotation results (corrected P-value < 0.01) [16]. KEGG was used to perform pathway enrichment analysis of DEGs. In addition, scatter plot can display the Rich factor, P-value and DEGs number of the enriched pathway. RichFactor is the ratio of differentially expressed gene numbers annotated in this pathway to all gene numbers annotated in this pathway. Greater RichFactor means greater intensiveness. Qvalue is corrected P-value ranging from 0~1, and its less value means greater intensiveness.

5.3 Results

5.3.1 Illumina sequencing quality and gene expression analysis

Zebrafish kidney samples from control and CTS-SeNP treatment groups were processed strictly on RNA extraction, library construction and sequencing to obtain high quality data. In this study, total raw reads obtained of all samples were from 45.89-48.83 million and over 98% of them were clean reads. Mapped reads with zebrafish reference genome were 88.19%, 87.70%, 88.32% and 89.12% for Control 1, Control 2, CTS-SeNP 1 and CTS-SeNP 2, respectively. Transcriptomic profiles of zebrafish samples indicated over 18, 983 genes (76.5%) expressed in both control group and CTS-SeNP treatment group. (Figure 5.1)

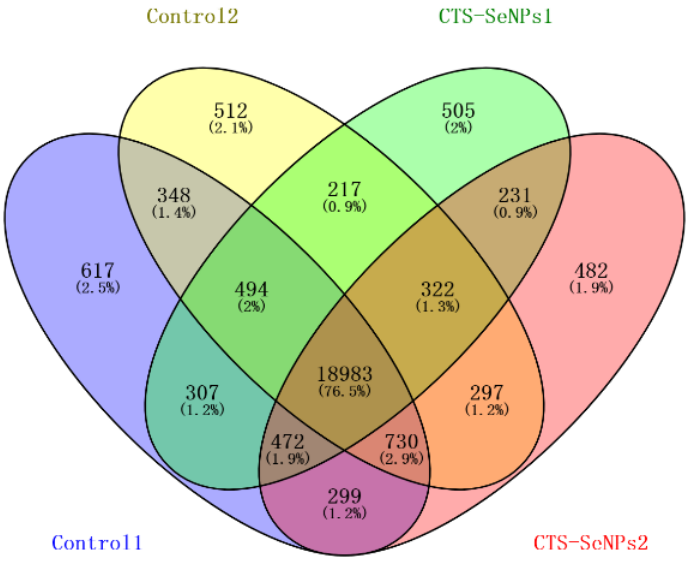
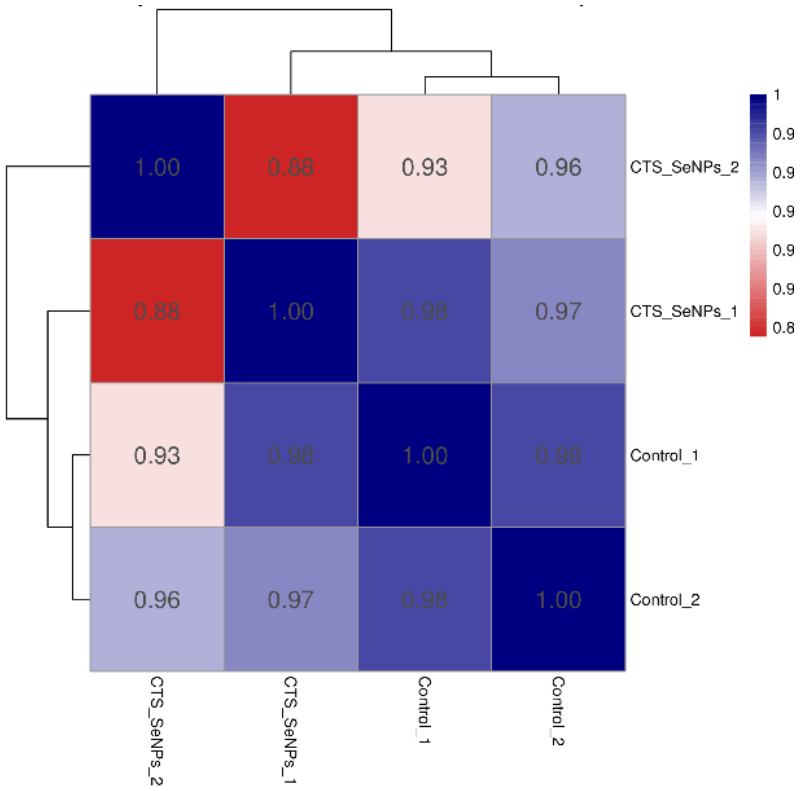


Figure 5.1 Overview of whole transcriptome data. Venn diagram showing the number of genes identified from different treatments and the extent of overlap.

Hierarchical clustering based on Pearson correlation coefficients showed high correlation (0.98-1.00) among samples in control group. Only 0.88 correlation was found among samples in CTS-SeNP group. (Figure 5.2.a) In addition, gene expression density was performed to observe the overall difference between these four samples. There was no significant difference found in among samples as Figure 5.2.b.

a)



b)

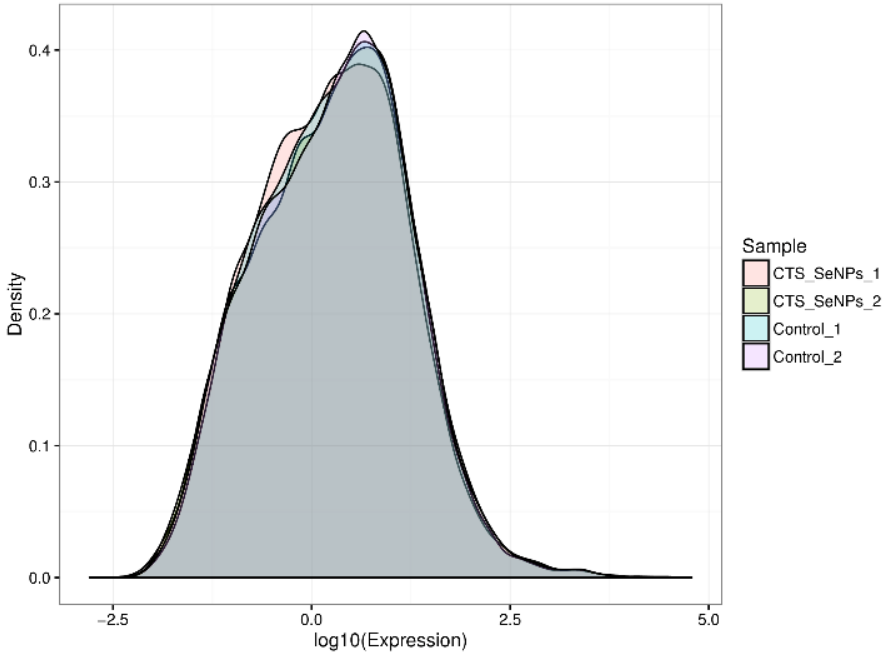
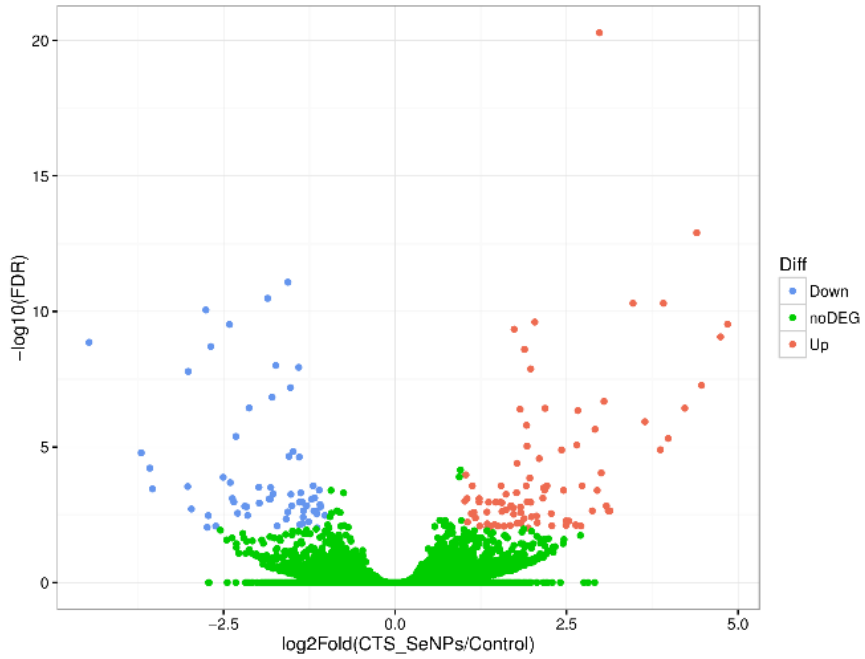


Figure 5.2 Transcriptomic profiles of zebrafish kidney in the control and CTS-SeNP treatment groups: a) Hierarchical clustering; b) gene expression density.

5.3.2 Analysis of differential expression of genes (DEGs)

All genes between CTS-SeNP treatment and the control were summarized by volcano plot as Figure 5.3.a. Within them, differentially expressed genes (DEGs) were identified by comparing to control group using the following thresholding criteria: log2 fold Change > 1.0, Qvalue < 0.5. Therefore, we identified total 156 genes were detected as DEGs (92 up-regulated and 64 down-regulated DEGs). (Figure 5.3.b). Among them, MHC (ENSDARG00000023203) was identified in DEGs with significant upregulated with 4.75 fold higher in CTS-SeNP treatment.

a)



b)

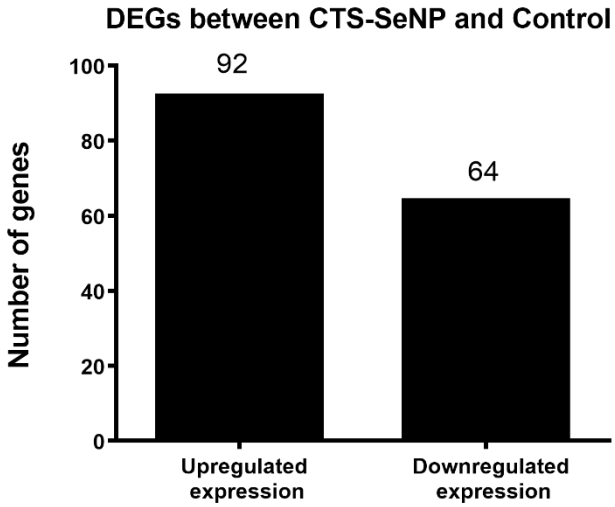


Figure 5.3 Expression difference analysis: a) volcano plot of all genes between two groups, X axis represent fold change (CTS-SeNP/Control, log2), Y axis represent Qvalue (-log10). Each dot represents a gene, blue and red dots represent DEGs, and red dots represent gene up regulation, blue dots represent gene down regulation. green dots represent genes with no change in two samples; b) DEGs between CTS-SeNP treatment and the control.

To explore the gene expression difference by CTS-SeNP in zebrafish kidney cluster analysis of DEGs were performed as heatmap (Figure 5.4). Overall expression was similar in each group, but there are three major clusters significantly altered in at least one transcriptome produced in responses to CTS-SeNP or control diets. These initial analyses revealed that the CTS-SeNP has specific and different effects in zebrafish kidney. Further functional analysis could reveal the responses in immune and antioxidant related gene expression and pathways.

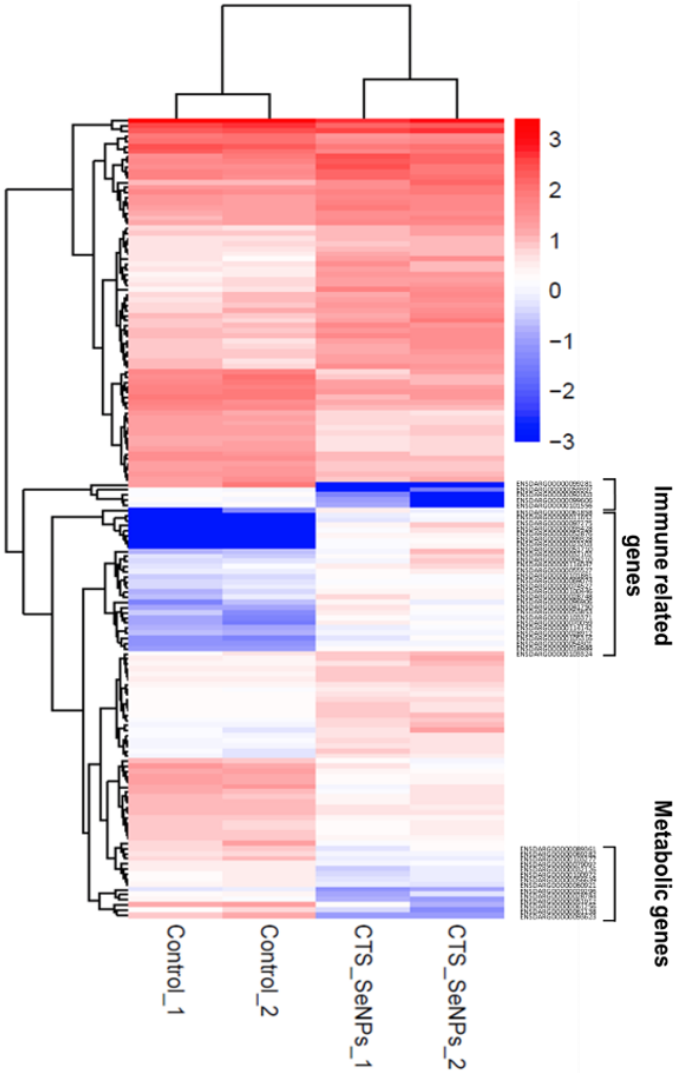


Figure 5.4 Cluster analysis of DEGs in heatmap following CTS-SeNP and control treatment. Each column represents transcriptome samples; each row represents a gene. Expression differences are shown in different colors. Red means upregulation and blue means downregulation).

5.3.3 Gene Ontology (GO) analysis for selected gene

GO enrichment analysis of the 156 DEGs revealed that there were 65 significantly enriched GO terms under filtering conditions (corrected P-value < 0.01). We compared the DEGs to the all reference genes of the GO terms in the three aspects (Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) shown as WEGO analysis in Figure 5.5. Generally, most of the DEGs and all genes expression were the same enrichment in GO term annotation. In BP, only growth, metabolic process and responses to stimulus were more enriched in DEGs than all genes. In CC, extracellular matrix, extracellular region, extracellular region part, virion and virion part were higher enriched percentage in DEGs than all genes. It might indicate that CTS-SeNP regulated genes expression in specific biological processes by secreting extracellular regulation genes.

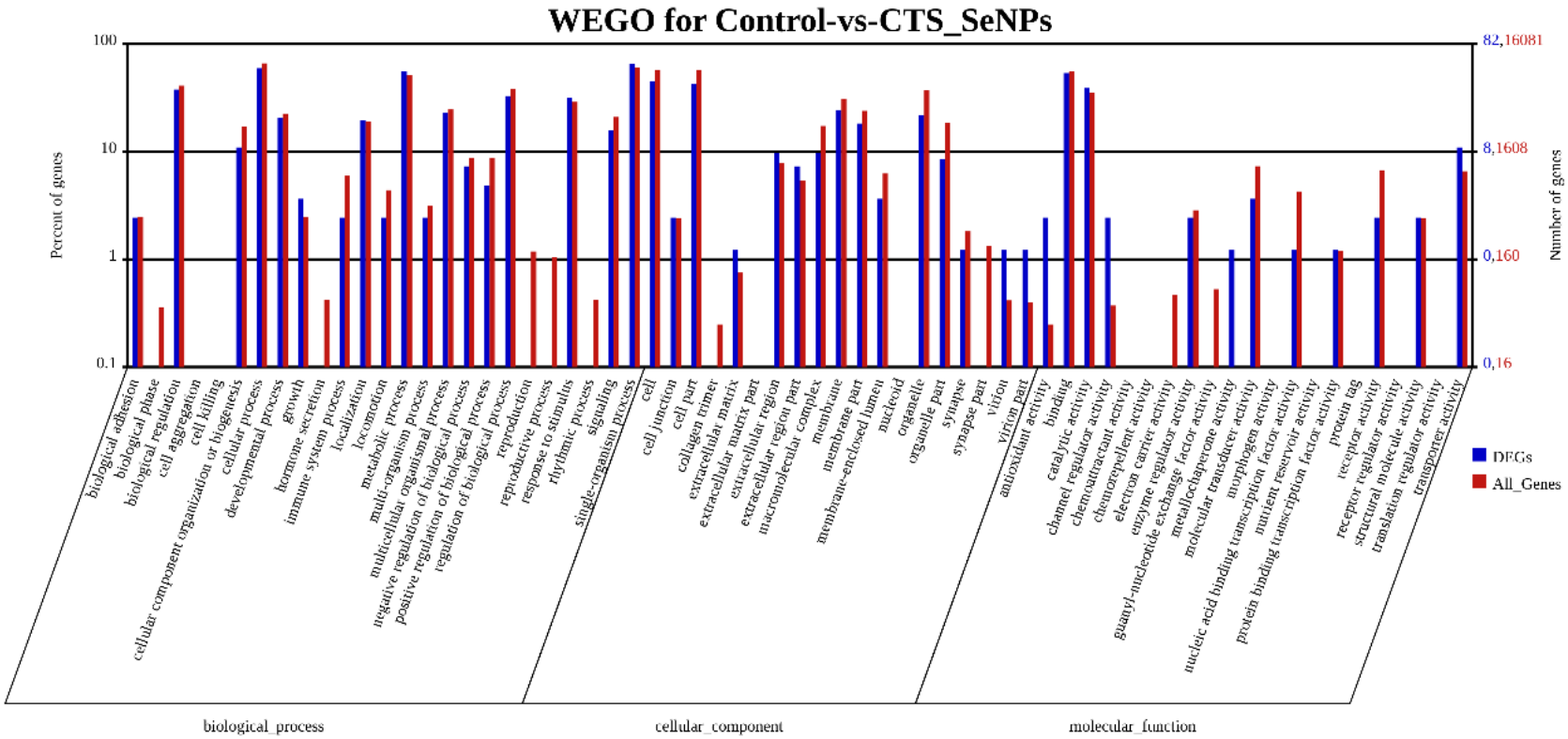


Figure 5.5 WEGO analysis of DEGs between CTS-SeNP treatment and the control. X axis represent GO classification (cellular component, molecular function and biological process); Y axis: left: percentage of genes (number of genes in this GO term/number of genes in all GO terms); right: number of genes in this GO term.

5.3.4 Pathway analysis by CTS-SeNP treatment.

Top 20 statistically enriched pathways in DEGs between CTS-SeNP treatment and the control were indicated in Figure 5.6 presenting as scatter plot. In the scatter plot figure, higher RichFactor and less Qvalue better intensiveness in the pathway.

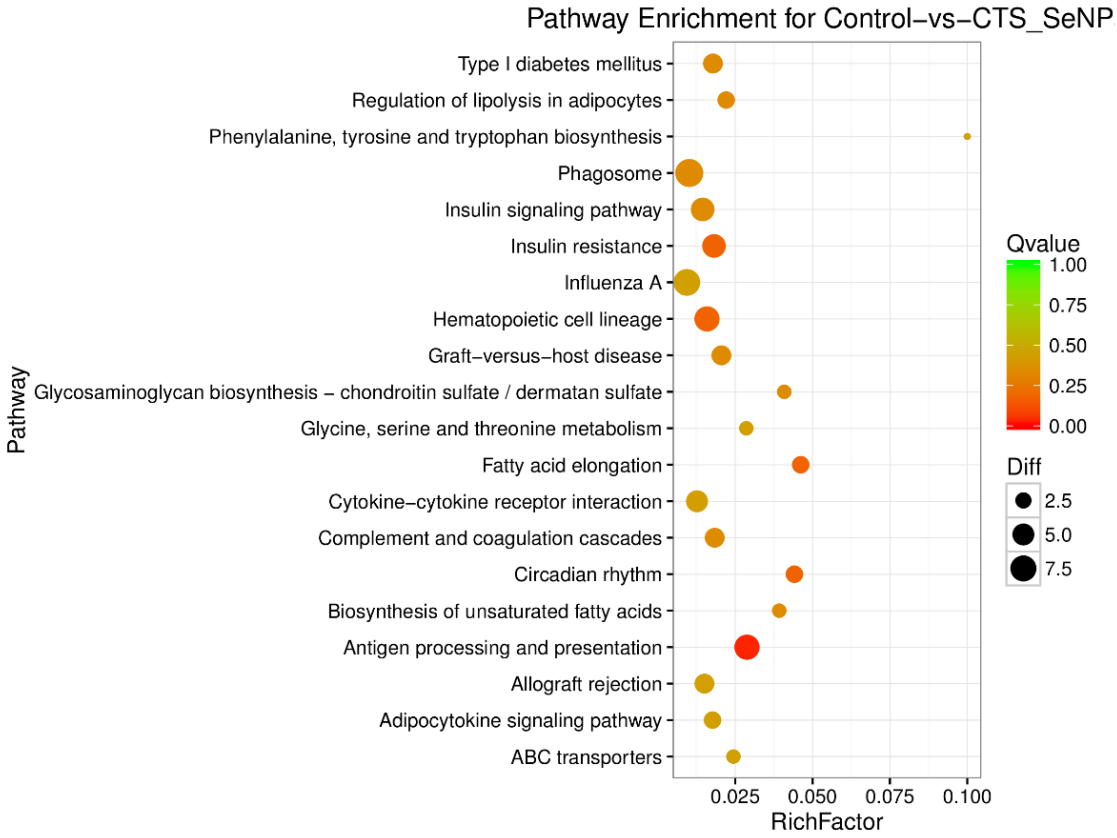


Figure 5.6 Scatter plot of DEGs between CTS-SeNP treatment and the control enriched pathway result (Y axis: Pathway term; X axis: RichFactor; the point represents DEGs number, the larger point means more DEGs number; the different colours of points means different Qvalue).

The most enriched pathway (Qvalue < 0.5) was ko04612 (Antigen processing and presentation) with RichFactor of 0.0288 and Qvalue of 0.0275 in Figure 5.7, followed by ko00062 (Fatty acid elongation) (Figure 5.8) and ko04710 (Circadian rhythm) (Figure 5.9). These pathways involved in immune system, lipid metabolism and environmental adaptation. Specifically, antigen processing and presentation

pathway involved in innate immune responses to identify any invading pathogen by major histocompatibility complex (MHC) I and MHC II pathways. In this pathway, MHC I (ENSDARG00000023203) upregulated with log2 fold change of 4.7. In ko00062 pathway (Fatty acid elongation), acetyl-CoA acyltransferase 2 (2.3.3.16) upregulated with log2 fold change of 1.4. The gene regulation in ko04710 pathway (Circadian rhythm) was represented by cryptochrome (ENSDARG00000069074) with downregulation of 2.1 fold change, class B basic helix-loop-helix protein 2 (ENSDARG00000041691) with downregulation of -2.3 fold change and nuclear receptor subfamily 1 group D member 1 (ENSDARG00000033160) with downregulation of -1.6 fold change.

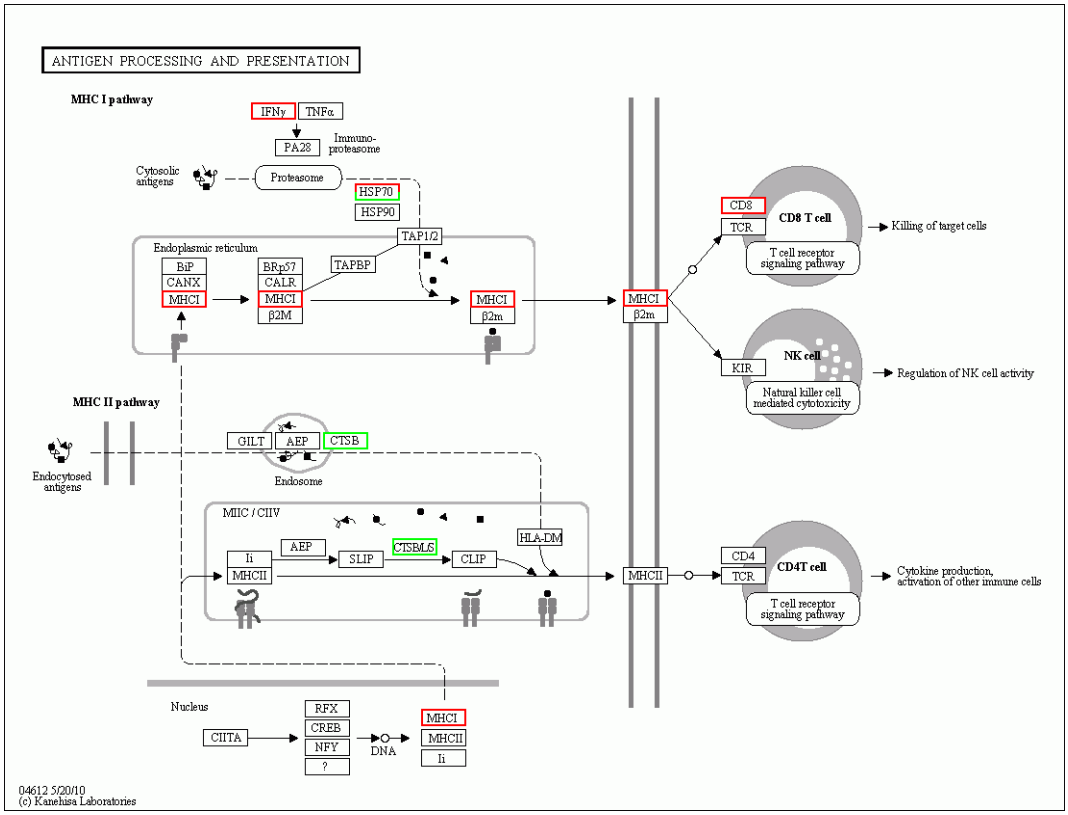


Figure 5.7 Top enriched KEGG pathway from DEGs between CTS-SeNP treatment and the control: ko04612 (Antigen processing and presentation pathway, with RichFactor of 0.0288 and Qvalue of 0.0275) with red of upregulation and green of downregulation.

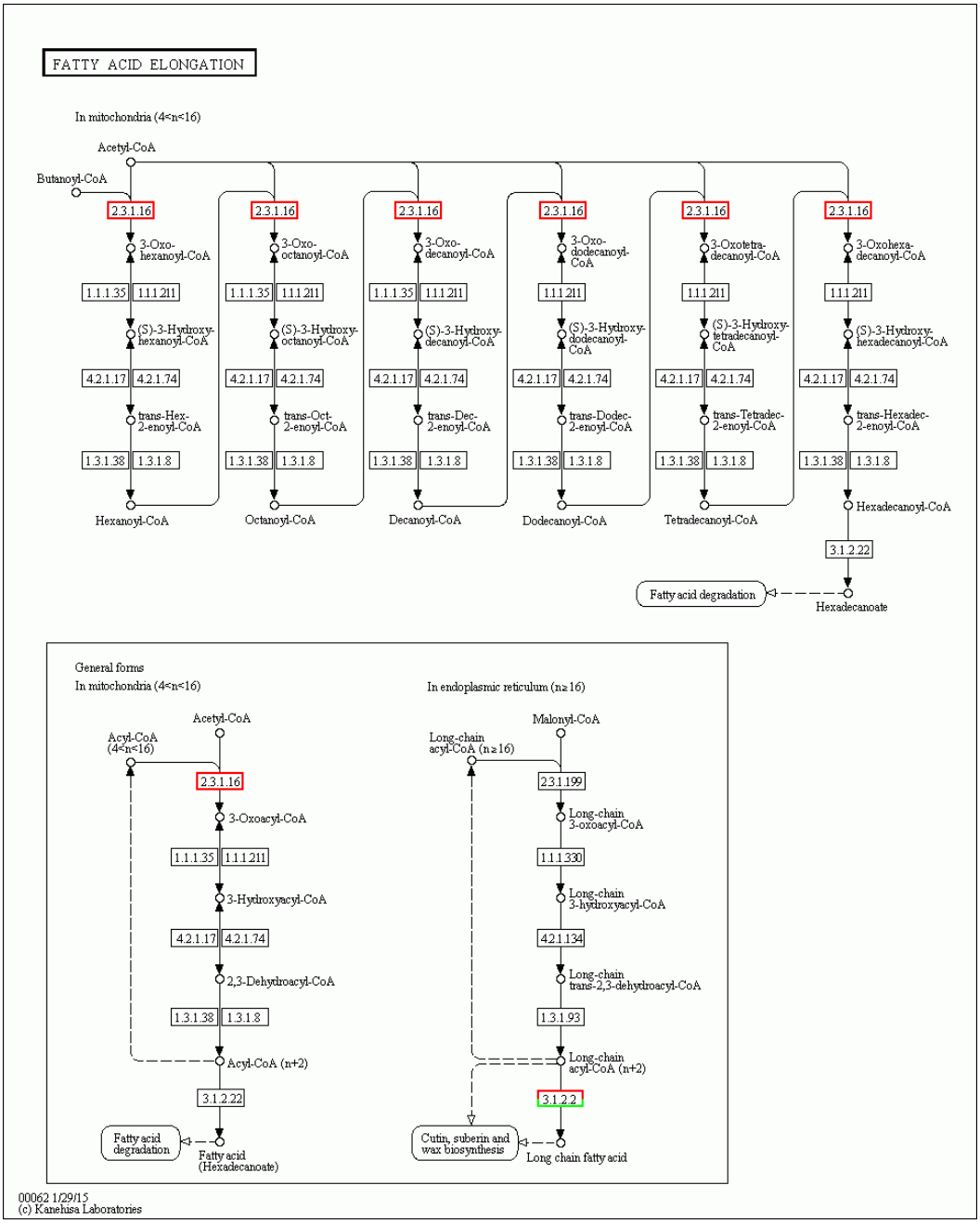


Figure 5.8 Second top enriched KEGG pathway from DEGs between CTS-SeNP treatment and the control: ko00062 (Fatty acid elongation pathway with RichFactor of 0.0461 and Qvalue of 0.0037) with red of upregulation and green of downregulation.

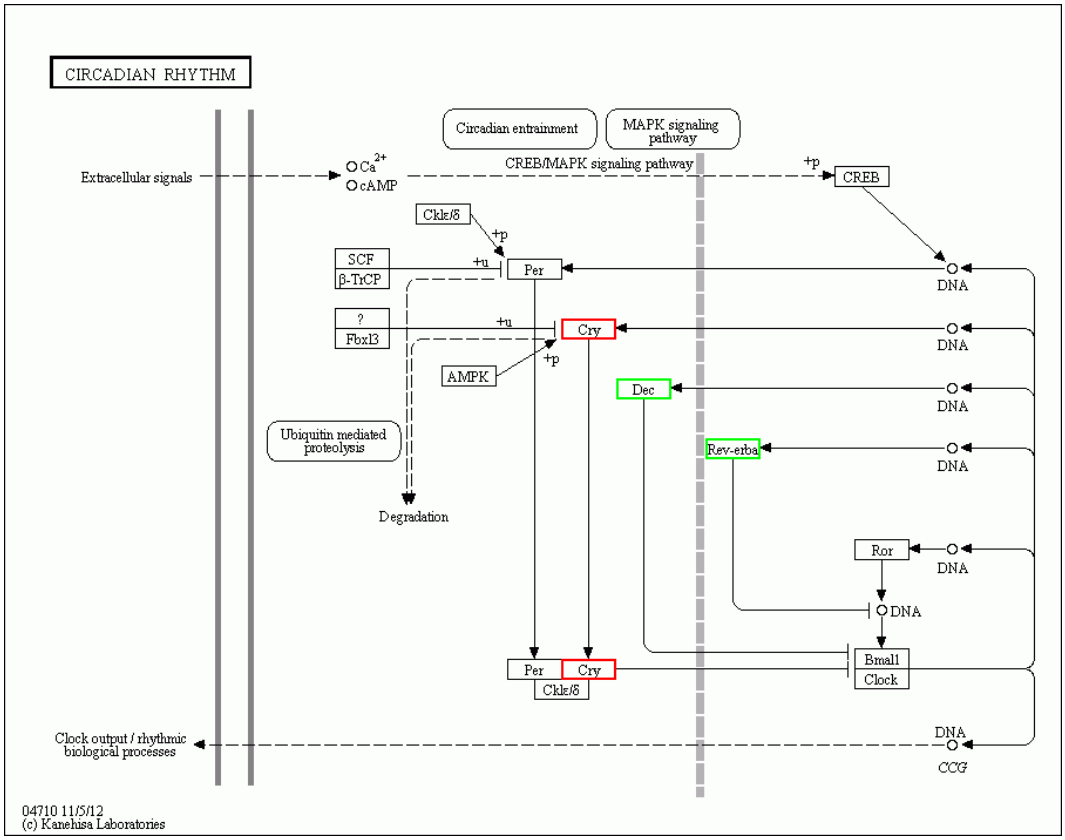


Figure 5.9 Third top enriched KEGG pathway from DEGs between CTS-SeNP treatment and the control: ko04710 (Circadian rhythm pathway, with RichFactor of 0.0441 and Qvalue of 0.171) with red of upregulation and green of downregulation.

In addition, ko04640 pathway (Hematopoietic cell lineage) was identified as the fifth enriched pathways in DEGs between CTS-SeNP and the control with RichFactor of 0.0158 and Qvalue of 0.1716 in Figure 5.10. This pathway involved in immune system by upregulation CD34 (ENSDARG00000100446) with 1.7 fold higher in CTS-SeNP treatment.

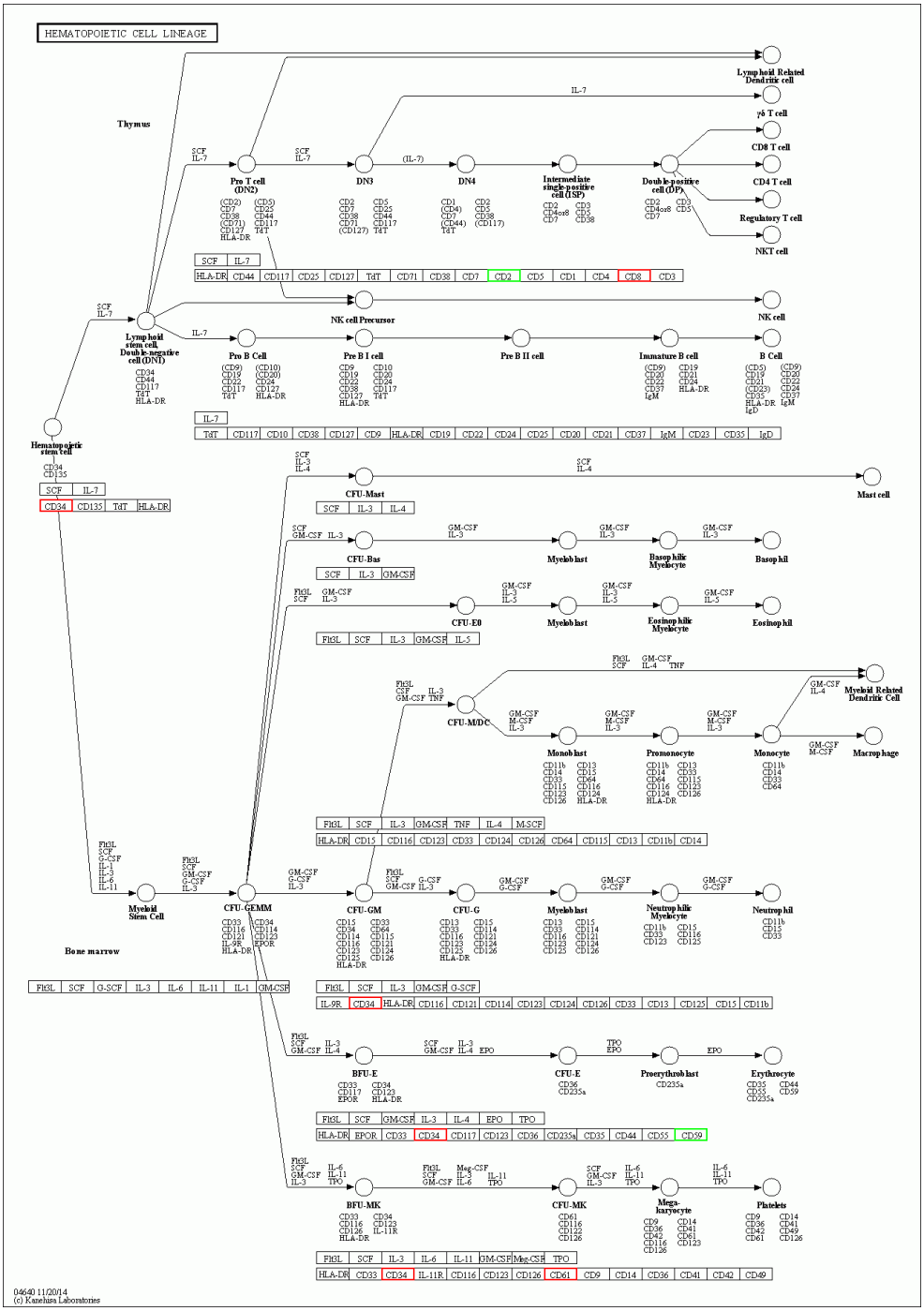


Figure 5.10 The fifth enriched KEGG pathway as ko04640 pathway (Hematopoietic cell lineage) with RichFactor of 0.0158 and Qvalue of 0.1716. Red represents upregulation and green represents downregulation.

5.4. Discussion

5.4.1 Overall transcriptome analysis of CTS-SeNP treated zebrafish

The RNAseq is a powerful analytic tool, and it provides all transcribed mRNA expression profiles from interested samples within a specific period. In this study, we studied the transcriptome of zebrafish kidneys to understand gene expression profiles by CTS-SeNP supplemented diets and its immunomodulation mechanism of CTS-SeNP in zebrafish. Due to limited sets of analyzed transcriptome data, the DEGs identified in zebrafish kidney after statistical analysis remained 156 transcript mRNA. Many Se related mRNAs were found in transcriptome profiles but showed with no significant expression difference. For example, glutathione peroxidases, thioredoxin reductases and iodothyronine deiodinases play important roles in redox regulation and antioxidant defenses [17]. Even so, many immune related and redox regulation related DEGs were identified.

5.4.2 DEGs Pathway Analysis

The first activated pathway in zebrafish kidneys after supplemented with CTS-SeNP is antigen processing and presentation (ko04612) pathway. In this pathway, MHC (ENSDARG00000023203) was identified in DEGs with significant upregulated. Major histocompatibility complex (MHC) is a set of proteins presented on the cell surface for recognition by the appropriate lymphocytes and avoided the immune responses to target its own cells [18]. Similar transcriptional regulation was found in B16F10 melanoma cells by organic Se (methylseleninic acid) [19]. It indicated that cellular glutathione is associate with the MHC class Ia dimer formation on the cell surface, meanwhile, Se level in the host is associate with the regulation of glutathione [20]. Previous study also supported that cellular redox regulation could modulate of the MHC class Ia expression level by the enzyme ER oxidoreductase (ERO-1a) through controlling the oxidative folding [21]. Moreover, the status of Se in the host upregulated some major components of the antigen processing machinery (APM) and the interferon (IFN) signalling pathway and accompanied by a reduced migration of B16F10 melanoma cells in the presence of Se supplements [19]. It supported our founding of upregulation of IFN-γ in antigen processing and presentation pathways. Therefore, a conclusion we can draw from the transcriptome profiles is that the mechanism of CTS-SeNP

immunomodulation function is upregulating the expression of MHC and IFN molecules and trigger the antigen processing and presentation pathway.

Another enriched KEGG pathways was fatty acid elongation in zebrafish kidney transcriptome analysis after supplemented with CTS-SeNP. First of all, fatty acid elongation pathway regulates the creation of fatty acids from cetyl-CoA and NADPH through the action of enzymes called fatty acid synthases [22]. Previous study reported that Se intake could regulate the expression of complement component 3 (C3) as an early marker of metabolic syndrome manifestations in human [23]. In that study, supplementary Se in human reduced C3 production in serum and regulated oxidative stress in host. As known, oxidative stress was a potential inducer of inflammation and susceptibility to develop obesity and related diseases [24]. Therefore, this supports our observation in fatty acid elongation pathway activation by CTS-SeNP in zebrafish. In addition, complement and coagulation cascades pathway (ko04610) was also identified in the enriched KEGG pathway. We suggested that CTS-SeNP exerted its biological function in the regulation of fatty acid elongation through regulating the complement component.

Hematopoietic cell lineage pathway was enriched in CTS-SeNP treated zebrafish. The development of hematopoietic cells progressed from a hematopoietic stem cell, which could work either self-renewal or differentiation into a multilineage committed progenitor cell, namely common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). CLP enhanced the lymphoid lineage of white blood cells, leukocytes, natural killer (NK) cells and T/B lymphocytes. In our **Chapter 2**, lymphocyte proliferation assay indicated that after supplemented 10 μg/g CTS-SeNP, T lymphocytes significantly increased its proliferation comparing with the control. The enriched hematopoietic cell lineage pathway in zebrafish kidney was associated with our previous observation in T lymphocyte increases in kidney cells proliferation assay. Within this pathway, CD34, CD61 and CD8 were upregulated but CD59 and CD2 were downregulated. CD 34 is cell surface glycoprotein and functions as a cell-cell adhesion factor. It is an important adhesion molecule and is required for T lymphocytes to enter lymph nodes [25]. In addition, elemental Se are well tolerated by normal CD34-positive hematopoietic stem and progenitor cells [26]. Therefore, it could be a potential

mechanism of CTS-SeNP immunomodulation and further immunoregulation study could process to explore the central regulator.

5.4.3 Further study on mechanism of SeNPs

Finally, some improvement of transcriptomic analysis is required in further study. First of all, the DEGs should be calculated by using more than two biological replicates and the difference between two gene expression level calculation methods should be reconsidered. Fragment per kb per million fragments (FPKM) method and counts of reads mapped to a specific gene method are two commonly used calculation methods to present DEGs [27]. DEGs might present different patterns by using these two methods. Another consideration of RNAseq analysis is Se related gene expression by CTS-SeNP. Even though the selenoproteins such as GPx and TRs did not show difference between control and CTS-SeNP treatment, one more specific analysis of selenoproteins profile by transcriptome analysis should be performed in the future study.

5.5 Conclusion

Given that CTS-SeNP have many favourable properties for immunomodulation and their potential applications expected to increase significantly over the coming decade, their immune mechanism cannot be overlooked. In this study, transcriptome analysis of CTS-SeNP treated zebrafish kidney samples were performed and potential immunomodulation mechanisms were discussed. In this study, CTS-SeNP affect pathways involved in immune system, lipid metabolism and environmental adaptation in zebrafish kidney. CTS-SeNP were reported with essential roles in redox regulation and involved in many selenoproteins expression. Antigen processing and presentation seems to be the major immunomodulation pathway CTS-SeNP modulated. The expression of MHC and IFN significantly motives the immunomodulation of CTS-SeNP. Further research will be needed to investigate other immune mechanism of Se and the potential application of CTS-SeNP as immunostimulants to fish or other animals.

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CHAPTER 6

General Discussion

This thesis explored the biological activity of chitosan selenium nanoparticles (CTS-SeNP) in fish, aiming to evaluate its potential to be used as an immunostimulant and understanding the mechanism behind its activity.

Se is an essential micronutrient in human and animal. Its biological functions were mostly related to the antioxidant function of selenoproteins. A literature review was conducted in **Chapter 1** to summarize studies and effects of organic and inorganic Se species on the hosts' immune and redox system. To date, several studies on effects of SeNP to the immune system has been published, demonstrating positive effects of SeNP on hosts' immune system [1-3]. However, there was no published study investigating the underlying biological mechanism.

6.1 Immunomodulatory effects of CTS-SeNP: dose and exposure time

The first question in this thesis is whether there are immunomodulatory effects from CTS-SeNP. In **Chapter 2**, effects of CTS-SeNP dose and exposure time on zebrafish immune system were explored. Four immunological biomarkers were used, covering innate and adaptive immune systems in zebrafish and different feeding dose (2, 5, 10 and 20 μg/g Se) and from short-term (3, 6, 9 and 12d) to long-term (60d) exposure period were studied. Results demonstrated that broad effect range of CTS-SeNP (5-20 μg/g), rapid onset of effects (starting from 3d) and the effects persisted even after 60d. No toxicity was observed in fish despite some toxicity was reported at similar dose using the same CTS-SeNP on a different fish species (Japanese medaka) [4]. This suggested that different fish species have different sensitivities to SeNP and administration dose need to be adapted for individual carefully when used as an immunostimulant in the future.

Another important observation was that CTS-SeNP was better than its raw components in stimulating the host's immune system. CTS-SeNP was designed to boost the immunity in host by combining two components (CTS and Se) that were known

immunostimulants. CTS-SeNP has more comprehensive immunomodulatory effects than other immunostimulants or its primary ingredients inorganic selenite and CTS. Of the 4 immune biomarker tested, selenite has no significant effect on any, CTS has significant effect on one (lysozyme activity) while CTS-SeNP has significant effect on three. Previous studies have shown that inorganic or organic Se have effect on cellular immune responses such as phagocytic respiratory burst (intracellular or extracellular responses) and lymphocytes proliferation [5]. However there were no reports of Se having effects on humoral immunity such as lysozyme activity. This suggested that CTS on the CTS-SeNP may be responsible for its stimulatory effect on lysozyme activity, as indicated by my results (Figure 2.3a).

6.2 Biological activities of CTS-SeNP

Two omics techniques were performed to explore immunomodulatory mechanism and biological function of CTS-SeNP in zebrafish (proteomics in **Chapter 3** and transcriptomics in **Chapter 5**). The two chapters were complementary. In **Chapter 3** I studied changes in fish serum, covering secretory proteins and humoral elements of the immune system. Serum is a body fluid carrying several biomarkers reflecting the health and immune status of the host by key proteins, cytokines and metabolites [6]. In **Chapter 5**, I looked at changes in cells of the kidney, an important immune organ and exert exhematopoiesis in fish [7], to cover changes inside the cellular environment.

Both techniques revealed that CTS-SeNP impact many biological processes in fish but primarily on redox regulation, general metabolism and innate immunity. These findings helped us to explain and understand the results of **Chapter 2**. For example, by exploring KEGG pathway in kidney transcriptome in **Chapter 5**, hematopoietic cell lineage pathway was enriched in CTS-SeNP treated zebrafish. Within this pathway, CD34, CD61 and CD8 were upregulated. All these genes were important to T-cell proliferation. This suggested CTS-SeNP could regulate cell surface glycoprotein to lead the expression of T lymphocytes entering lymph nodes for further proliferation, explaining increased T-cell proliferation observed in **Chapter 2**.

6.3 Redox regulation and immunomodulation of CTS-SeNP

IPA network and pathway analysis of serum proteomics in **Chapter 3** indicated that redox regulation could be a potential mechanism of CTS-SeNP immunomodulation. Se is widely considered as an endogenous antioxidant to regulate the expression of selenoproteins such as GPx, GR and SelP. Therefore, we conducted a comparing study between CTS-SeNP and Trolox in immunomodulation function and redox regulation to insight the relationship between redox regulation and immumodulation in **Chapter 4**.

At an equal antioxidant capacity, Trolox and CTS-SeNP diet offered similar protection against bacterium *A. hydrophila.* Although similar bacterial protection was found, there were subtle differences in redox and immune regulation by Trolox and CTS-SeNP. For example, CTS-SeNP can improve redox condition of the host indirectly via modulation of the GSH/GSSG cycle while Trolox improved redox condition of the host via its antioxidant properties directly. This study provided critical insight into how CTS-SeNP improve redox and immune system in the host.

6.4 Potential applications of CTS-SeNP

Aquaculture is a key food production sector and is increasingly important to provide protein for the growing world population. This study showed that CTS-SeNP has good potential to be applied as an immunostimulant for aquaculture. In this study, we have demonstrated that CTS-SeNP has ability to stimulate broad spectrum of immune biomarkers and has protective effect against bacterial infection. This bioactivity is related to the ability of CTS-SeNP to simultaneously modulate both the redox and innate immune systems.

Immunostimulation effects of CTS-SeNP studied in zebrafish is worthy to explore the potential application in some commercial aquaculture species. For example, zebrafish belongs to *Cyprinidae* family of freshwater fish, that includes the carps, the minnows and the Koi [7]. Most of *Cyprinidae* fish are stomach-less fish without tooth jaws. The digestion system is composited by intestine and digestive gland. In worldwide, Grass Carp (*Ctenopharyngodon idella*) is the fish species with the largest reported production in aquaculture [8]. It was also reported that *Aeromonas hydrophila* as a common pathogen in warm water aquaculture and is associated with many diseased

widespread in carps in Asia [9]. In our study, the resistance of *A. hydrophila* infection by CTS-SeNP showed over 200% survival rate increase. Therefore, it obviously tells the immune benefits in fish by this CTS-SeNP immunostimulant. The application of CTS-SeNP as an effective and efficient immunostimulant in aquaculture could help fish farmers or large global aquaculture companies to minimize the loss during the disease exposure.

Another potential application of CTS-SeNP immunostimulant is for aquarium species such as koi (*Cyprinus rubrofuscus*) which also belongs to the *Cyprinidae* family [10]. People are interested in the coloured and patterning variants of koi and take them as their pet. The unique coloured and patterning, scalation of koi worth with valuable price. Therefore, CTS-SeNP as immunostimulant pet food could provide protective function to these unique and valuable fish.

Since zebrafish innate immune system and selenium metabolism is highly similar to that of mammals, results from this study also suggest that CTS-SeNP may have similar effects on human. This will open up other possibility to other applications, such as anti-stress or anti-cancer supplements. Future research should explore these possibilities.

6.5 Limitations of study and recommendations for further research

Due to time limitation of this PhD study, further investigation of immunomodulation of SeNPs and their immune mechanism could not be carried out. As reported by a number of nanomaterial literature, nanoparticles different coating materials on the same core materials could have different biological activities [11]. Therefore, it is recommended to carry out more SeNPs and compare their immunomodulation effects.

Another limitation in the current study is the constraint of study models of Se immune mechanism. While using *in vivo* model has the benefit of exploring the full repertoire of immune response, the model is too complicated to tease out precise molecular mechanisms. An additional *in vitro* model such as the THP-1 cell line or peripheral blood immune cells RBL-2H3 will certain help in understanding the

underlying mechanism.

While omics technique has a lot of advantages to measure expression of a large number of targets simultaneously, every omics analysis only offer a snapshot of what is happening inside the animal model. If time and resources allow, it would be better to do measurements at multiple time points to detect trends and directions of change of these targets. This will provide us much stronger evidence to the biological processes taking place after the CTS-SeNP treatment and under bacterial challenges.

In conclusion, CTS-SeNP showed great potential as an immunostimulant in fish. CTS-SeNP with a board-spectrum immunomodulation function enhanced the immunity. Quick immunomodulation and persistent effects could protect cultured fish species against diseases in time windows of different lengths. The mechanism of CTS-SeNP immunomodulation is not fully understood but is related to simultaneously modulating both the redox and innate immune systems.

6.6 Reference

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