MOLECULARLY TOXICOLOGICAL AND IMMUNOLOGICAL RESPONSES OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*, LINNAEUS) TO SILVER NANOPARTICLE INDUCTION

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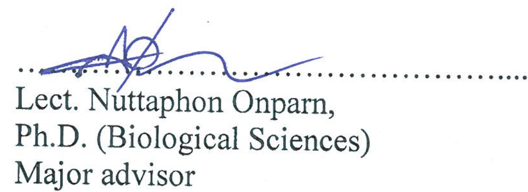

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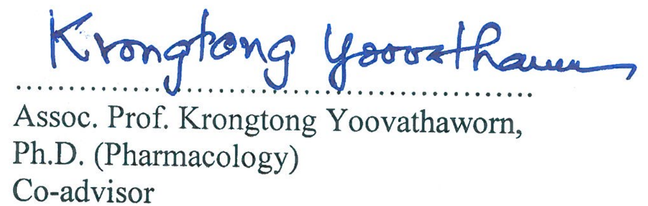

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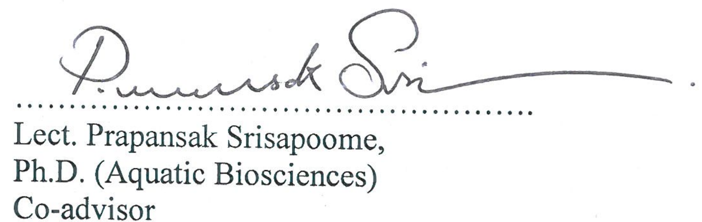

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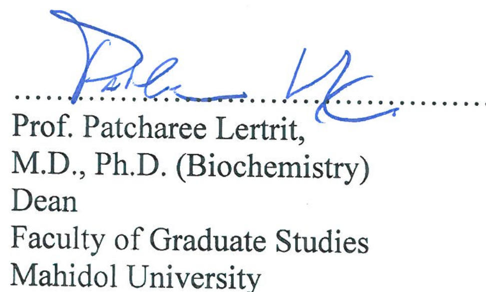

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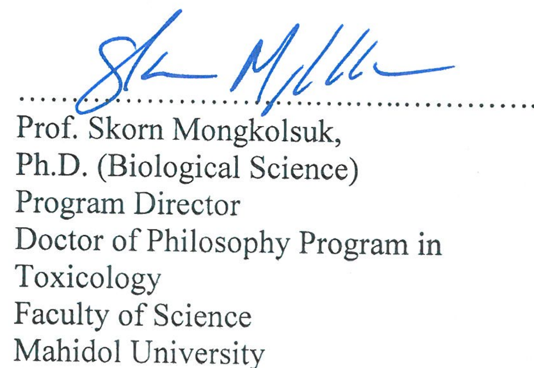

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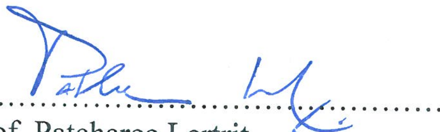

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ABSTRACT

Nile tilapia thioredoxin-interacting protein (*On*-TXNIP) and selenoprotein P (*On*-SEPP) cDNAs were cloned and characterized. The full-length cDNA of *On*-TXNIP cDNA was 2,260 bp and consisted of 116 bp of 5' untranslated region (UTR), 1,188 bp of open reading frame (ORF) (equal to 396 amino acid residues), 925 bp of 3'UTR and 28 bp of a poly A tail. The calculated theoretical isoelectric point (p*I*) and molecular weight (MW) of *On*-TXNIP were 7.81 and 43.95 kDa, respectively. The full-length cDNA of *On*-SEPP was 2,427 bp and consisted of 99 bp of 5'UTR, 1,239 bp of ORF (equal to 413 amino acid residues), 1,086 bp of 3'UTR, and 29 bp of a poly A tail. The calculated p*I* and MW of *On*-SEPP were 6.27 and 46.36 kDa, respectively. Evolutionary analyses of both the *On*-TXNIP and *On*-SEPP genes revealed that these genes were closely related to the TXNIP and SEPP genes in zebrafish (*Danio rerio*). A normal tissue distribution analysis indicated that the *On*-TXNIP and *On*-SEPP genes were ubiquitously expressed in all tissues examined, and the highest expression levels of these genes were observed in peripheral blood leukocytes (PBLs) and the trunk kidney, respectively. The expression analysis of cellular stress response genes using real-time reverse transcription polymerase chain reaction (real-time RT PCR) in fish acutely and chronically exposed to 1, 10 and 100 mg Ag NPs/kg fish by intraperitoneal (i.p.) injection revealed that significant up-regulation of *On*-TXNIP and *On*-SEPP transcripts was in the liver, spleen, and head kidney at the early phase of Ag NP exposure (hours 6 through 48). Down-regulation of *On*-SEPP transcripts was clearly observed in the liver at weeks 1 to 4. The statistically up-regulated *On*-HSP40B9, *On*-HSP90α and *On*-HSP90β transcripts were observed in all tested tissues of fish exposed to Ag NPs at hours 6 and 12 and down-regulated *On*-HSP70 and *On*-MT genes were found in the liver at hours 6 to 48. Histopathological analysis demonstrated that the fish livers exhibited a dramatic infiltration of Kupffer cells, elevated bi-nucleated cells, expanded sinusoidal blood congestion and severe necrosis in a dose-dependent manner. Thickening of the capsule layer of the spleen was a predominant indicator of chronic Ag NP exposure under i.p. induction. Innate and adaptive immunological responses and hematological parameters of fish exposed to various doses of Ag NPs revealed that phagocytic activity, amounts of red blood cells (RBCs) and the percentage of hematocrit (%Hct) significantly decreased at week 1 after exposure. However, amounts of white blood cells (WBCs) were not significantly changed at all-time exposure ($P>0.05$). Additionally, antibody titer of fish immunized with *Streptococcus agalactiae* vaccine and simultaneously exposed to Ag NPs at 10 and 100 mg/kg were found to effectively decrease at only early phase. Finally, in the challenge test, all vaccinated fish with Ag NPs-exposed groups were still protected against *S. agalactiae* infection. Based on these findings, the expression analysis of cellular stress response genes, histopathological observation, and compromising of innate immune response of fish exposed to Ag NPs could be reliable for the assessment of Ag NP contamination in teleost fish.

KEY WORDS: NILE TILAPIA / CELLULAR STRESS RESPONSES / GENE EXPRESSION / SILVER NANOPARTICLES / IMMUNOLOGICAL RESPONSES

120 pages

การตอบสนองทางพิษวิทยาและภูมิคุ้มกันวิทยาระดับโมเลกุลของปลานิลที่ถูกเหนี่ยวนำด้วยอนุภาคนาโนของธาตุเงิน
MOLECULARLY TOXICOLOGICAL AND IMMUNOLOGICAL RESPONSES OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*, LINNAEUS) TO SILVER NANOPARTICLE INDUCTION

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บทคัดย่อ

ลำดับนิวคลีโอไทด์ของ Complementary DNA (cDNA) ของยีน Thioredoxin-interacting protein (*On*-TXNIP) และ selenoprotein P (*On*-SEPP) ในปลานิลได้ถูกโคลนเพื่อศึกษาคุณลักษณะโครงสร้างโปรตีน ความสัมพันธ์เชิงวิวัฒนาการและการแสดงออกภายใต้สภาวะต่าง ๆ โดยพบว่าลำดับนิวคลีโอไทด์ทั้งหมดของ cDNA ของยีน *On*-TXNIP มีความยาวเท่ากับ 2,260 คู่เบส ซึ่งประกอบด้วยตำแหน่ง 5’ Untranslated region (UTR) ยาวเท่ากับ 116 คู่เบส ตำแหน่ง Open reading frame (ORF) ยาวเท่ากับ 1,188 คู่เบส (เท่ากับลำดับกรดอะมิโน 396 หน่วย) ตำแหน่ง 3’ UTR ยาวเท่ากับ 925 คู่เบส และตำแหน่ง poly A tail ยาวเท่ากับ 28 คู่เบส ค่าไอโซอิเล็กทริก (p*I*) และน้ำหนักโมเลกุล (MW) ของ *On*-TXNIP ที่คำนวณได้เท่ากับ 7.81 และ 43.95 กิโลดาลตัน ตามลำดับ ขณะที่ลำดับนิวคลีโอไทด์ทั้งหมดของ cDNA ของยีน *On*-SEPP มีความยาวเท่ากับ 2,427 คู่เบส ซึ่งประกอบด้วยตำแหน่ง 5’ UTR ยาวเท่ากับ 99 คู่เบส ตำแหน่ง ORF ยาวเท่ากับ 1,239 คู่เบส (เท่ากับลำดับกรดอะมิโน 413 หน่วย) ตำแหน่ง 3’ UTR ยาวเท่ากับ 1,086 คู่เบส และตำแหน่ง poly A tail ยาวเท่ากับ 29 คู่เบส ค่าไอโซอิเล็กทริกและน้ำหนักโมเลกุลของ *On*-SEPP ที่คำนวณได้เท่ากับ 6.27 และ 46.36 กิโลดาลตัน ตามลำดับ การวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการเผยให้เห็นว่า ยีนทั้ง 2 ชนิดนี้มีความสัมพันธ์ใกล้ชิดกับยีน TXNIP และยีน SEPP ในปลาม้าลาย (*Danio rerio*) มากที่สุด การวิเคราะห์การแสดงออกของทั้ง 2 ยีนนี้ในเนื้อเยื่อต่าง ๆ ของปลานิลปกติ แสดงให้เห็นว่า มีการแสดงออกของยีนทั้ง 2 นี้ในทุก ๆ อวัยวะที่นำมาตรวจสอบ ซึ่งพบการแสดงออกสูงสุดในเม็ดเลือดขาวที่ไหลเวียนอยู่ในกระแสโลหิตและไตส่วนหลังตามลำดับ การศึกษาการแสดงออกของยีนที่ตอบสนองต่อความเครียดระดับเซลล์โดยใช้เทคนิค Real-time reverse transcription polymerase chain reaction (real-time RT PCR) ในปลานิลที่ได้รับอนุภาคนาโนของธาตุเงิน (Ag NPs) ที่ 1 10 และ 100 มิลลิกรัมต่อกิโลกรัมโดยการฉีดเข้าทางช่องท้อง ในสภาพเฉียบพลันและเรื้อรัง เผยให้เห็นว่ามีการเพิ่มการแสดงออกอย่างมีนัยสำคัญของยีน *On*-TXNIP และยีน *On*-SEPP ในตับ ม้ามและไตส่วนหน้าของปลานิลที่ได้รับอนุภาคนาโนของธาตุเงินในระยะแรก (ชั่วโมงที่ 6-48) ขณะที่มีการลดการแสดงออกของยีน *On*-SEPP ในตับที่สัปดาห์ที่ 1 ถึง 4 และมีการเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติของยีน *On*-HSP40B9, *On*-HSP90α และ *On*-HSP90β ในทุกเนื้อเยื่อทดสอบของปลานิลที่ได้รับ Ag NPs ที่ชั่วโมงที่ 6 และ 12 และมีการลดการแสดงออกของยีน *On*-HSP70 และ *On*-MT ในตับที่ชั่วโมงที่ 6 ถึง 48 การวิเคราะห์พยาธิสภาพของเนื้อเยื่อตับของปลานิลแสดงให้เห็นว่ามีการแทรกผ่านของ Kupffer cells อย่างเด่นชัด มี Bi-nucleated cells เพิ่มสูงขึ้น มีการคั่งของเลือดบริเวณ Sinusoid และมีการตายของเนื้อเยื่อตับสัมพันธ์กับปริมาณของอนุภาคนาโนของธาตุเงินที่ได้รับ นอกจากนี้ยังพบว่าพยาธิสภาพของเนื้อเยื่อม้ามบริเวณชั้น Capsule layer หนาขึ้น ซึ่งเป็นดัชนีชี้วัดที่ชัดเจนของการได้รับสัมผัสกับอนุภาคนาโนของธาตุเงินอย่างเรื้อรังโดยการฉีดเข้าทางช่องท้อง การศึกษาการตอบสนองทางภูมิคุ้มกันแบบไม่จำเพาะเจาะจงและแบบจำเพาะเจาะจงรวมทั้งการวิเคราะห์ค่าพารามิเตอร์ของโลหิตวิทยาในปลานิลที่ได้รับอนุภาคนาโนของธาตุเงินที่ปริมาณต่าง ๆ แสดงให้เห็นว่า ค่ากิจกรรมการกลืนกินสิ่งแปลกปลอมโดยกระบวนการ Phagocytosis ปริมาณของเม็ดเลือดแดงและเปอร์เซ็นต์ฮีมาโตคริตมีค่าลดลงอย่างมีนัยสำคัญที่สัปดาห์ที่ 1 แต่อย่างไรก็ตาม ปริมาณของเม็ดเลือดขาวไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญที่ทุกช่วงเวลาของการทดลอง (P>0.05) นอกจากนี้ เมื่อปลานิลที่ได้รับวัคซีนเชื้อตายของ *Streptococcus agalactiae* และได้รับอนุภาคนาโนของธาตุเงินควบคู่กันที่ 10 และ100 มิลลิกรัมต่อกิโลกรัม พบว่ามีค่าไตเตอร์ของแอนติบอดีลดลงในช่วงแรก และเมื่อทำการฉีดเชื้อ *S. agalactiae* พบว่า ปลาที่ได้รับวัคซีนในกลุ่มที่ได้รับอนุภาคนาโนของธาตุเงินยังคงมีความคุ้มโรคในการต่อต้านการติดเชื้อ จากการค้นพบในครั้งนี้แสดงให้เห็นว่า การวิเคราะห์การแสดงออกของยีนที่ตอบสนองต่อความเครียดระดับเซลล์ พยาธิสภาพของเนื้อเยื่อและการตอบสนองทางภูมิคุ้มกันแบบไม่จำเพาะเจาะจงที่ลดลงในปลานิลที่ได้รับอนุภาคนาโนของธาตุเงิน มีความน่าเชื่อถือสำหรับใช้ในการเป็นดัชนีเพื่อการประเมินการปนเปื้อนของอนุภาคนาโนของธาตุเงินในปลากระดูกแข็ง

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
bp.	Base pair
pH	Potential of hydrogen ion
sp.	Species
nM	Nanometer
e.g.	Example given
et al.	At alli (and others)
etc.	Et Cetera (and so forth)
g	Gram
kg	Kilogram
mg/l	Milligram per liter
µg/l	Microgram per liter
$\mu g/cm^2$	Microgram per square centimeter
C3b	Complement component 3subunit b
LC_{50}	Median lethal concentration
EC_{50}	Median effective concentration
$AgNO_3$	Silver nitrate
Ag^+	Silver ion
Hg	Mercury
NADPH	Nicotinamide adenine dinucleotide phosphate
UV	Ultraviolet
On	*Oreochromis niloticus*
Cd	Cadmium
Co	Cobalt

LIST OF ABBREVIATIONS (cont.)

Cu	Copper
Ni	Nickel
Pb	Lead
Zn	Zinc
Mg	Magnesium
$^{\circ}C$	degree Celsius
CDS	Coding sequence
EDTA	Ethylenediaminetetraacetic acid
dNTP	Deoxynucleotidetriphosphate
C_T	Cycle threshold
CFU	Colony forming unit
rpm	Round per minute
Fig.	Figure
ORF	Open reading frame

Nucleotides

A	Adenine
T	Thymine
C	Cytosine
G	Guanine
U	Uracil

LIST OF ABBREVIATIONS (cont.)

Amino acid codes

1-letter	3-letter	Amino acid	Codons
A	Ala	Alanine	GCA, GCC, GCG, GCT
C	Cys	Cysteine	TGC, TGT
D	Asp	Aspartic acid	GAC, GAT
E	Glu	Glutamic acid	GAA, GAG
F	Phe	Phenylalanine	TTC, TTT
G	Gly	Glycine	GGA, GGC, GGG, GGT
H	His	Histidine	CAC, CAT
I	Ile	Isoleucine	ATA, ATC, ATT
K	Lys	Lysine	AAA, AAG
L	Leu	Leucine	CTA, CTC, CTG, CTT, TTA, TTG
M	Met	Methionine	ATG
N	Asn	Asparagine	AAC, AAT
P	Pro	Proline	CCA, CCC, CCG, CCT
Q	Gln	Glutamine	CAA, CAG
R	Arg	Arginine	AGA, AGG, CGA, CGC, CGG, CGT
S	Ser	Serine	AGC, AGT, TCA, TCC, TCG, TCT
T	Thr	Threonine	ACA, ACC, ACG, ACT
U	Sec	Selenocysteine	UGA
V	Val	Valine	GTA, GTC, GTG, GTT
W	Trp	Tryptophan	TGG
Y	Tyr	Tyrosine	TAC, TAT
X	X	Any amino acid	N
*	*	stop codon	TAA, TAG, TGA

CHAPTER I
INTRODUCTION

Nanotechnology is a novel branch of chemical synthesis of nanomaterials (particles typically less than 100 nanometers) to accomplish the innovatively desirability characteristics of nanometals, which have been drawn scientific interests in the world (Wijnhoven *et al.*, 2009). Silver nanoparticles (Ag NPs) are widely used in medicine, physics, material sciences, and chemistry. Because of a broad spectrum of bactericidal activity of Ag NPs, many medical products are coated with them for applications, for example, contraceptive devices, surgical instruments, bone prostheses, and dental alloys (Kruszewski *et al.*, 2011). As their bactericidal capacity and application, Ag NPs become strongly popular in consumer products leading to expanding their utilizations and ready for elevation of environmental contamination, especially in aquatic environments, which are important habitations for algae, planktons, invertebrate and vertebrate aquatic animals.

Ag NPs affected the early development of zebrafish (*Danio rerio*) embryos, which were observed as developmental retardation, increased heart rates, neurodevelopmental effects, sluggish circulation, pericardial oedema, and eye malformations (Yeo and Kang, 2008). In adult zebrafish, there was evidence of uptake of Ag NPs via the gills that caused specific changes in gene expression and increased mortality (Griffitt *et al.*, 2009). Oedema was also the noticeable malformation in fathead minnows (*Pimephales promelas*) after larval exposure (Laban *et al.*, 2010). Increased mortality was also observed in adult Japanese medaka (*Oryzias latipes*) accompanied by induction of DNA damage (Wu *et al.*, 2010). Chromosomal aberrations and DNA damage were clearly found and induction of proliferation arrest in cell lines of zebrafish (Wise *et al.* 2010).

Nowadays, the use of aquatic organisms as experimentally animal models is increasing continuously. It becomes challenge to scientists to investigate the responses of these animals on environmental pollutants and to further earn some benefits of these models in risk assessment. Not only zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) are usually used as fish models in scientific research (Norrgren, 2012), but Nile tilapia (*Oreochromis niloticus*), a tropical teleost fish, is also preferably selected as a model to study the consequences of chemical insults including heavy metals, pesticides, etc.

A great number of factors including water qualities, pathogen infections and toxic chemicals are capable to threaten fish health status resulting in a mass of death and loss of economic values. Nile tilapia is a freshwater fish significantly considerable as an importantly and economically aquatic animal in the global countries (Shoko *et al.*, 2011). Up to date, Nile tilapia production in Thailand was 139,263 tonnes in 2011 following the data from fisheries movement document, fisheries economic division (http://fishco.fisheries.go.th/fisheconomic/). Therefore, Nile tilapia becomes the first priority of aquatic animal that has potential impacts on economic trading of Thailand export value.

According to a less number of information on toxicities of Ag NPs in finfish, especially in Nile tilapia, this study was carried out to delve important evidence providing a basic knowledge involved in pathological effects, hematological changes, cellular stress and immunological responses of Nile tilapia exposed to Ag NPs and to consider the possibility of using cellular stress biomarker genes for evaluation of Ag NPs contamination in freshwater fish. This study may further be applied for setting some stress response genes as the candidates of molecular biomarkers to monitor the environmental contamination of Ag NPs of fish in aquatic ecosystem.

CHAPTER II
OBJECTIVES

2.1 Rationale

Many manufacturers in worldwide utilize silver nanoparticles (Ag NPs) as strongly bactericidal agent in many applicable products leading to the release of Ag NPs directly into environmental habitats including aquatic environments which are important sources for algae, planktons, invertebrate and vertebrate aquatic animals. Nile tilapia (*Oreochromis niloticus*), which is an importantly economic aquatic animal of Thailand, was selected as a fish model for investigating the effects of Ag NPs on the expression level of cellular stress response genes and its immune responses. This study was conducted to get more information and better understanding of the effects of Ag NPs on fish immunity, cellular stress response and molecular aquatic immunotoxicology. This information would be necessary for investigating and exploring the proper explanation of the impacts of Ag NPs on fish pathophysiological changes, immunological responses and hematological parameters. All of these studies would be a crucial clue to further setting up the parameters used in risk assessment.

2.2 Objectives

2.2.1 To clone and characterize the full-length cDNAs encoding Nile tilapia thioredoxin interacting protein (*On*-TXNIP) and selenoprotein P (*On*-SEPP) genes

2.2.2 To evaluate the evolutionary relationship of *On*-TXNIP and *On*-SEPP genes in Nile tilapia compared to other known organisms

2.2.3 To study the normal distribution of *On*-TXNIP and *On*-SEPP genes in various tissues using quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) in healthy Nile tilapia

2.2.4 To study the effects of Ag NPs on histopathology of internal organs in Nile tilapia

2.2.5 To study acute and chronic effects of Ag NPs on expression of cellular stress response-related genes in Nile tilapia using real-time RT-PCR analysis

2.2.6 To evaluate hematological parameters in Nile tilapia exposed to Ag NPs

2.2.7 To determine the effects of Ag NPs on innate and adaptive immune responses of Nile tilapia

CHAPTER III
LITERATURE REVIEW

3.1 Nile tilapia (*Oreochromis niloticus*)

3.1.1 History

Nile tilapia (*Oreochromis niloticus*) was one of the first fish species cultured. Illustrations from Egyptian tombs, which showed the fish held in ornamental ponds, suggest that they were cultured more than 3,000 years ago (Popma and Masser, 1999).

In Thailand, on March 25, 1965, Emperor Akihito, as His Royal Highness Crown Prince of Japan at that time, sent, as the royal tribute to His Majesty the King Phumipol, 50 Nile tilapias (or called “Pla Nil” in Thai). Nile tilapia is a kind of fish that is easy to care for, has a good taste, bear a lot of fry, and has a rapid growth. It was His Majesty’s intention to breed this species for the sake of his populace. Therefore, on March 17, 1966, His Majesty bestowed a royal name to the fish as "Nile tilapia", and, sent 10,000 of them with body lengths of 3 to 5 centimeters to the Department of Fisheries to feed and breed them at the experimental and breeding plan on central Bang Khen campus, and other 15 fishery stations throughout the kingdom. After the donated fish had grown enough population, they could be distributed to the people to feed and breed as they liked (http://www.fisheries.go.th/freshwater/web3/index.php?option=com_content&view=article&id=55&Itemid=61).

Currently, the aquaculture of Nile tilapia has expanded throughout the country. Up to date, Nile tilapia production in Thailand was 139,263 tonnes in 2011 following the data from fisheries movement document, fisheries economic division (http://fishco.fisheries.go.th/fisheconomic/). This creates various kinds of jobs and professional careers through the cycles of fish nurseries, fish feed manufacturers, fish farmers, production suppliers, fishermen, freezing storage, and market traders becoming the surpassing industry over other aquatic animals.

3.1.2 Scientific classification and general biology

Nile tilapia is the generic name of a group of cichlids firstly found in Africa. It is classified in family Cichidae, order Perciformes, class Actinopterygii and phylum Chordata. The scientific name of the Nile tilapia has been given as *Tilapia nilotica*, *Sarotherodon niloticus*, and currently as *Oreochromis niloticus*. They are laterally compressed and deep-bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spined. The spines are also found in the pelvic and anal fins (Popma and Masser, 1999).

Nile tilapia is tropical fish species preferring temperature ranges from 28 to 33 degree Celsius. It is an omnivorous fish and able to filter feed by entrapping suspended particles, including phytoplankton, on mucous in the buccal cavity. Sexual maturity in ponds is reached at an age of 5 to 6 months (http://www.fao.org/ fisheries/species/3217/en). The breeding process starts when the male establishes his territory, digs a crater-like spawning nest and guards his territory. The mature female spawns in the nest, and after fertilization by the male, then female collects the eggs into her mouth. The female incubates the eggs in her mouth and broods the fry after hatching until the yolk sac is absorbed. Incubating and brooding is accomplished in 1 to 2 weeks, depending on temperature. After fry are released, they may swim back into her mouth if danger threatens. Egg number is proportional to the body weight of the female. A 100 g female will produce about 100 eggs per spawn, while a female weighing 600-1000 g can produce 1000 to 1500 eggs. Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg (http://www.fao.org/fisheries/species/ 3217/en).

3.2 Diseases in Nile tilapia

A great number of factors, for example, super-intensive cultures, water qualities including pH, temperature, dissolved oxygen, ammonia, nitrite, nitrate, ectoparasites and internal parasites, are capable to threaten fish health status (Rottmann *et al.*, 1992). If animals cannot maintain their balance or homeostasis, they

will be weak and easier to be infected by many infectious diseases. Nile tilapia diseases can be sub-divided in two main causes. The first is infectious diseases which may arise from bacteria, parasites, fungi and viruses (Magnadottir, 2010). The second one is non-infectious diseases resulting from improper environments, unsuitable diets and poor handling and transporting (Rottmann *et al.*, 1992). The infectious diseases caused by pathogenic invasion have been mostly and generally found in aquatic environments or in carriers. These diseases can be contagious and needed to manage immediately to restrain spreadbility. Infectious diseases mostly found in Nile tilapia are:

3.2.1 Ectoparasites or external parasites

A great number of species of ectoparasites have ability to irritate and make the wound along the sites of adhesion as such gills, skin and fins. In case of gills, these parasites can affect the gas exchange system of Nile tilapia leading to insufficiently oxygen supply. A way that fish tries to get rid of these parasites is the production of more mucus to peel them. Signs and symptoms definitely seen are the wounds or spots along the body, more or less coloring, unsettled swimming and loss of appetite.

3.2.1.1 Protozoa

This group preferably harms hatchery and fry rather than adult fish. The species often found are *Trichodina* sp., *Chilodonella* sp., *Ichthyophthirius multifilis, Epistylis* sp., *Scyphidia* sp., *Apiosoma* sp. and *Ichthyobodo* sp. (Pantoja *et al.*, 2012). Most of these parasites come to adhere the gills and skin, especially *Trichodina* sp., which is easily and abundantly found in Nile tilapia cultured in earthen pond.

3.2.1.2 Monogenetic trematodes

The members are composed of *Gyrodactylus* sp. and *Dactylogyrus* sp. (Lerssutthichawal, 2008). Two of these favor to stick around gills and make gill epithelial membrane thicker or more swelling resulting in obstruction of respiratory system. Monogene that is usually found in Nile tilapia is *Cichlidogyrus* sp. (Sanchez-Ramirez, 2007).

3.2.1.3 Crustaceans

Ergasilus sp., *Lernaea* sp. and *Lamproglena* sp. are members of this group (Kuperman, 1992; Avenant-Oldewage, 2003). Mostly, they have spike-like unique structure embedded in fish flesh to support their adhesion and/or to take the host blood and nutrients. They are greatest found in pond and cage culturing system.

3.2.2 Endoparasites or internal parasites

Generally, these parasites are frequently found along the side of gut-intestinal tract and are harmless to host.

3.2.2.1 Protozoa

Trypanosoma sp. is a member of this group and typically found in blood stream of Nile tilapia. The heavy infection induces anemia because of hemolysins secreted by live trypanosomes which lyse the red blood cells and lead to mortality (Adam *et al.*, 2009).

3.2.2.2 Metazoa

This group consists of digenetic trematodes or digenea, cestodes or flatworms, nematodes or roundworms and acanthocephalan or spiny-head worms (Pantoja *et al.*, 2012). Fish infected with these parasites is usually thin and loss of appetite leading to be easily infected by opportunistic pathogens.

3.2.3 Bacteria

Definitely signs and symptoms of fish infected with pathogenic bacteria are able to be noticed as hemorrhagic skin, wounding body, erosive fin, uncontrolled swimming, ascites and loss of appetite. There are three virulent pathogenic bacteria which are the main cause of death in Nile tilapia.

3.2.3.1 Columnaris disease

Flavobacterium columnare is a player in this disease. It is a Gram's negative long rod bacterium and gliding motile, which is an obviously

characteristic. This disease is mostly found in immediately climatic changes, in water with high organic matters, during transportation and in condition of stress induction. Signs and symptoms of infected fish are slowly swimming, loss of appetite, erosive fin, gill infarction and white erodent lesion on saddleback. In the present time, columnaris disease brings to a bulk of economic loss in Nile tilapia culture (Sebastiao *et al.*, 2011).

3.2.3.2 Motile aeromonad septicaemia (MAS)

The performer in this disease is *Aeromonas hydrophila*, which is a Gram's negative short rod bacterium. Slowly swimming, loss of appetite, erosive fin, skin hemorrhage, deep wound, ascites, yellowish liver and intestinal bleeding are signs and symptoms of this disease (Yardimci and Aydin, 2011).

3.2.3.3 Streptococcosis disease

The disease outbreak usually occurs during high temperature season resulting in massive loss of death of Nile tilapia in farms and brings economic disadvantages to farmers. The criminal of this disease is *Streptococcus agalactiae*, which is a Gram's positive coccal bacterium. External signs and symptoms can be noticeable as more intenseness of body color, slow swimming, unagitated buoyancy along the edge of the pond, loss of appetite, hemorrhage along the body including operculum or gill opening, base of swimming fins, mouth and anal pore, swelling of caudal peduncle, ascites, bleeding with mucus releasing from anal pore, abscess around jaw, screw or twist motility, erodent and ripping fins, inflammation around and/or within eyes rendering bulging eyes or exophthalmia and eye opacity (Rodkhum *et al.*, 2011).

3.3 Fish immune system

Generally, immune system helps protect organisms from invading pathogens against infectious diseases by identifying and eliminating non-self molecules. The mechanism discriminating self from non-self is sophisticated components located on cell membrane of immune cells. The final outcome of immune

response reaction is the induction of inflammation. Fish immunity is simple and rather similar to those of mammals. Basically, immune system is divided into two major groups; innate immunity and adaptive immunity (Janeway and Medzhitov, 1998; Du Pasquier, 2001; Du Pasquier, 2004).

3.3.1 Innate immunity

Innate or non-specific immunity is the first line defense regarding as a primitive immune response against infection from various substances including parasites, microbes, viruses and toxic chemicals. It exists in vertebrates, invertebrates and plants. This system is fated and committed in the germ line making fish can produce receptors, which enable to recognize a main group of pathogenic structures called "pathogen associated molecular patterns or PAMPs", for example, lipopolysaccharide (LPS), peptidoglycan and beta-glucan. In addition, the responses of this innate immunity are rather fast and readily immediate function. Naturally, innate immunity is subdivided into three categories; physical, cellular and humoral defenses (Magnadottir, 2010).

3.3.1.1 Physical defenses

The physical barriers such as mucus and epithelial cells lining in the buccal cavity, skin and scale are fundamental barriers potentially protecting fish from harmfully external agents comprising particulate matters, corrosive substances and ectoparasites (Aoki *et al.*, 2008).

3.3.1.2 Cellular defenses

The cellular defenses of fish innate immunity compose of cells involving in non-specifically antigenic clearance abilities. They consist of phagocytic and non-specific cytotoxic cells. Off these, macrophages are not only able to phagocytose foreign antigens, but also act as antigen presenting cells (APCs). The mechanism utilizing to get rid of invading pathogenic determinants is the generation of respiratory or oxidative burst which is a process of creating reactive intermediate molecules notably reactive oxygen intermediates (ROIs) (Ellis, 2001). The mechanism of stimulation of cytokine production, which is a signal molecule of cell-cell

communications and is an essential linkage of cross talking between innate and adaptive immune responses, is not able excluded. All of these events enable to promote the elimination and discrimination of non-self from self-antigens.

3.3.1.3 Humoral defenses

The humoral parameters are referred to secreted soluble components including complements, bactericidal enzymes, proteases, anti-bacterial peptides, anti-viral proteins, acute phase proteins, etc. The complement system comprises alternative, lectin and classical pathways. All of three pathways can terminate the invasion of pathogenic bacteria by attacking cell membrane and urging cell lysis or enhance phagocytic activity by using C3b protein as opsonin in the process of opsonization of the pathogens (Nonaka and Smith, 2000). In the other hand, humoral responses can also produce a variety of proteins and glycoproteins to destroy or inhibit growth of infectious pathogens. For example; α2-macroglobulin and α1-antitrypsin can block lytic bacterial enzymes; transferrin can inhibit or delay bacterial growth; and interferon can act against viral infection (Bowden *et al.*, 1997; Stafford and Belosevic, 2003; Furnes *et al.*, 2009).

3.3.2 Adaptive immunity

Adaptive or acquired or specific immunity is naturally potential and sophisticated immune responses against pathogenic infection. Normally, it is mainly referred to a group of antigen-specific T and B lymphocytes and it also includes the development of populations of memory cells, which immediately reacts to secondary infection of the same epitopes. It is fundamentally classified into two majorities consisting of humoral immunity and cell-mediated immunity. B lymphocytes (B cells) are normally involved in the humoral responses while T cells are responsible for the cell-mediated responses (Du Pasquier, 2004).

3.3.2.1 Humoral defenses

The important parameter in humoral defenses is immunoglobulins (Igs) or antibodies, which are specifically produced by B cells and its derivatives. In fish, there are three types of Igs; IgM, IgD and IgZ/T (Hikima *et al.*,

2011). The immunoglobulins are expressed either as B-lymphocyte receptors in forms of IgM and IgD membrane-bound Igs or secreted form releasing into the blood stream. IgM in teleosts is rather different from mammals by forming a tetrameric structure, while a pentameric form is usually found in mammals. Following activation by a specific antigen, either in soluble form or by antigen presenting cells (APCs), the B cells proliferate and differentiate into memory cells and plasma cells, which secrete the specific antibodies against specifically to that antigen. Although this response is more effective and potent than innate immunity, but it needs to spend a long period of time to generate the specific antibodies.

3.3.2.2 Cellular defenses

T cells, using a specific receptor called "T-cell receptors (TCRs)", recognize pathogens only in association with the major histocompatibility complex (MHC) markers on the membrane of APCs. Recognition leads to the production of memory T cells, which will persist after infection and are ready to expand on re-encounter. The MHC cell surface structure is the key tool involved in the rejection of a pathogen. The MHC class I is expressed on nearly all cells and is primarily involved in presenting intracellular viral or any non-self proteins to specific T cells while MHC class II is expressed by specific APCs and is involved in presenting opsonized and degraded pathogen components to B and T cells. The expression of these molecules has been established in several fish species and unlike the situation in mammals they appear to be present on different chromosomes in fish. This suggests a separate evolutionary lineage of the traits for immune defense against viral infection (MHC class I) and bacterial infection (MHC class II) (Van Muiswinkel *et al.*, 1999).

3.4 Silver nanoparticles (Ag NPs)

Silver, a heavy metal historically found at low concentrations in environments, is rapidly being incorporated into new consumer and medical products as nanoparticles (NPs) (Wijnhoven *et al.*, 2009). Nanotechnology is dynamically developing filed involving in manipulation of substances at the nanoscale of around 1

to 100 nm (Piotrowska *et al.*, 2009). Nanoproducts and nanomaterials have novel and size-related physicochemical properties differing significantly from the larger particles. The novel properties of silver nanoparticles (Ag NPs) have been widely applied for use in food technology (e.g., food processing equipment, packaging materials, food storage), textiles/fabrics (e.g., antimicrobial clothing), medical applications (e.g., wound care products, implantable medical devices), cosmetics, paints, etc (Wijnhoven *et al.*, 2009). Ag NPs have broad spectrum bactericidal activity by inhibiting DNA synthesis and cell replication, by elevating oxidative stress, and by interfering with ion homeostasis and electron transport required for energy utilization. As their bactericidal capacity and application, Ag NPs are extremely popular in a diverse range of consumer materials leading to increasing their market value and ready to elevated human and environmental exposures.

3.4.1 Effect of Ag NPs on the environment

Silver can be present in four different oxidation states: Ag^0, Ag^{1+}, Ag^{2+} and Ag^{3+}. The former two are the most abundant ones; the latter two are unstable in the aquatic environments. In the environment, silver is found as a monovalent ion together with sulfide, bicarbonate or sulfate or more complex with chlorides and sulfates adsorbed onto particulate matter in the aqueous phase (Wijnhoven *et al.*, 2009). Ag NPs may be released into the environments by several routes, including during synthesis, during manufacturing and incorporation of the nanoparticles into goods, during the use phase of the goods containing the nanoparticles, and while recycling or disposal of goods and Ag NPs (Wijnhoven et al., 2009). It also leaks into the aquatic environments via municipal and industrial water treatment plants.

3.4.2 Bioaccumulation of Ag NPs in aquatic animals

Bioaccumulation is an important process to understand hazard and risk assessment of Ag NPs. It is also a direct way to evaluate the processes that influence bioavailability, where bioavailability is defined as the pollutant concentration that an organism takes up from environmental media including water and food (Luoma and Rainbow, 2005). Marine bivalve mollusks accumulate silver more strongly than algae and some mollusks can even accumulate silver from sediments. Oysters, gastrops and

arthropods, all can incorporate silver; the quantity in which this occurs depends on the biological availability of silver and age, size, sex, reproductive stage, general health and metabolism of the organisms (Wijnhoven *et al.*, 2009). Experiments with rainbow trout showed that the uptake of silver took place via a sodium-ion channel located on the branchial apical membrane (Bury and Wood 1999).

3.4.3 Toxicity of Ag NPs to aquatic organisms

3.4.3.1 Toxicity to fish

Griffitt and co-workers (2009) determined the response of the gills to Ag NPs in adult female zebrafish (*Danio rerio*). Fish was exposed to Ag NPs at 24 and 48 hours. At various times, gills were examined for changes in transcriptional activity, gill morphology, and total metal content. The result showed that exposure to Ag NPs also significantly increased whole body silver content and elevated gill silver levels. Surprisingly, no change in gill filament was observed. Furthermore, a number of differentially expressed genes detected by microarray also increased. At hour 48, nanosilver exposures produced expression profiles involved in osmoregulation in the gills very different from control profiles.

The toxicity of Ag NPs was reported to affect the early developmental stage of zebrafish embryos (Yeo and Kang, 2008). The embryos were observed deformities at different time points until 72 hours post fertilization (hpf). The groups exposed to Ag NPs, flies were very seriously injured with abnormality notochord development showing very short and curved in both 10 and 20 ppt groups. Furthermore, the eyes were not normally developed in some flies. Additionally, there were increased pericardial oedema and weak heartbeats in the 20 ppt Ag NPs exposed group. It was concluded that the commercial Ag NPs caused serious damage to the development of zebrafish embryos.

Laban and colleagues (2010) also investigated the effect of Ag NPs on fathead minnow (*Pimephales promelas*) embryos. Eggs were collected 24 hours after exposure to 5 mg/L of Ag NPs. The result showed that exposure of fathead minnow embryos to Ag NPs induced a variety of developmental abnormalities in hatching larvae. These included absence of noticeable air sac, development of

moderate and severe pericardial and yolk sac oedema, hemorrhages to the head and pericardial area, and lordosis or bending upwards of the vertebral column. Transmission electron microscopy (TEM) images revealed that Ag NPs were easily detectable in embryos. The photographs showed that Ag NPs firstly attached in large amounts to the surface of the egg chorion and later embedded throughout fathead minnow embryos.

Assessment of developmental toxicity of early-life stages in fish is known to be accepted by environmental protection agency (EPA). Thus, Wu and co-workers (2010) used this model to explore in detail of the potential reproductive and developmental toxicity of Ag NPs in Japanese medaka (*Oryzias latipes*). In this study, the investigator utilized the early-life stages test to estimate growth retardation and morphological abnormalities in embryonic and larval medakas exposed to Ag NPs. The result revealed that exposure to 400-1000 μg/L Ag NPs produced significantly adverse effects on the developmental process of embryonic medaka, presenting as delayed development, reduced pigmentation and decreased maximum width of the optic tectum. Microscopic observation suggested that the expression of pigmentation was inhibited by Ag NPs treatment on 5 days post fertilization (dpf). Low expression of melanophores, xanthophores and leucophores in exposed embryonic larvae was observed. The findings suggested that significant increase in heart rate and various morphological abnormalities were associated with Ag NPs induced sluggish circulation (arrest of blood flow), hemorrhage and hemostasis in fish body. The non-linear or U-shaped dose–response patterns of growth retardation and morphological abnormalities in the treated fish indicated the complex toxicological mechanisms of silver nanoparticles.

In 2010, Wise and partners conducted the experiment to investigate the cytotoxicity and genotoxicity of Ag NPs using medaka cell line. They used OLHNI2 cells, which is a medaka cell line established from adult fin tissue. Genotoxicity was assessed by measuring chromosomal aberrations in metaphase. The result revealed that LC_{50} of Ag NPs for cytotoxicity, which was measured by a colony forming assay, was 0.33 μg/cm^2 with appearance of clonogenic effect. The Ag NPs resulted in a concentration-dependent genotoxic effects with the spectrum of damages including chromatid lesions, isochromatid lesions, chromatid exchanges and

centromere spreading. Additionally, Ag NPs also induced a concentration-dependent increase in the percentage of aneuploid cells.

3.4.3.2 Toxicity to invertebrates and algae

The toxicity of Ag NPs was investigated not only in lower vertebrates, but also in invertebrates. The potential adverse effect of Ag NPs on oyster embryos, *Crassostrea virginica*, was carried out by Ringwood and co-workers in 2010. The aims of this experiment were to characterize the toxicity of Ag NPs to the embryonic development of oysters and to compare the relative sensitivity of embryos to adults. For the embryonic studies, 48 hours developmental assays were conducted with Ag NPs concentrations ranging from 1.6 to 0.0016 μg/L. The result indicated that a dramatic drop in normal embryonic development at the highest concentration (1.6 μg/L) was observed. In the adult oysters, statistically significant adverse effects on lysosomal destabilization rates that exceeded 35-40% were associated with a high level of impaired gamete viability and reproductive failure.

Roh and partners (2009) studied ecotoxicity of Ag NPs that was investigated in *Caenorhabditis elegans*, which is a terrestrial nematode, using survival, growth, and reproduction, as the ecotoxicological endpoints, as well as stress response gene expression. For microarray analysis, *C. elegans* were exposed to 0.1 mg/L of Ag NPs for 24 hours. Survival and growth of the worms were also assessed. Reproduction was investigated by 72 hours exposures. The microarray indicated that genes involved in nuclear signaling and transport pathways were found to be significantly up-regulated, whereas genes involved in behavior-related pathways such as response to stimulus, locomotive behavior, feeding behavior, and reproductive behavior pathways were significantly down-regulated. The survival, growth, and reproduction of Ag NPs exposure did not alter the worm mortality rate or growth, but it seriously decreased reproduction potential. Based on gene expression data and reproductive endpoint, the investigator concluded that Ag NPs induced dramatic reproductive failure in *C. elegans* likely associated with oxidative stress.

In 2008, Navarro and colleagues reported study which aimed at examining whether Ag NPs were toxic to freshwater algae, using *Chlamydomonas reinhardtii* as a model organism, and whether toxicity of Ag NPs was related to

release of Ag^+ from the Ag NPs, or to specific effects of Ag NPs. The result revealed that increasing concentrations of $AgNO_3$ and Ag NPs reduced the algal photosynthetic yield. $AgNO_3$ showed similar half maximal effective concentration (EC_{50}) values upon 1 or 2 hour(s) of exposure: at 188 ± 61 nM and 184 ± 81 nM, respectively. Differently, Ag NPs toxicity was time-dependent during the first 2 hours, showing an average EC_{50} of 3,300 ± 572 nM after 1 hour, and 1,049 ± 396 nM after 2 hours. It was concluded that based on total Ag concentration, $AgNO_3$ displayed higher toxicity than Ag NPs. However, based on Ag^+, Ag NPs appeared to be more toxic than $AgNO_3$.

3.5 Stress

3.5.1 Definition of the stress

Stress is defined as physical or chemical processes that cause bodily reactions that may contribute to disease and death (Rottmann *et al.*, 1992). Hans Selye, who is the father of stress study, defined the term of stress as "the nonspecific response of the body to any demand made upon it" and called this response the general adaption syndrome (GAS) (Watts, 1990).

3.5.2 Mechanisms of the stress

General adaptation syndrome, or GAS, is a term used to describe the body's short-term and long-term reactions to stress. The GAS consists of three stages: 1) the alarm reaction stage, 2) resistance stage, 3) exhaustion stage. The alarm reaction is the immediate reaction to a stressor. In the initial phase of stress, animals release hormones and neurotransmitters stimulating themselves for a "fight or flight" response. Cortisol and catecholamines are classified as the major components directly responsible for this stage. The resistance stage or the stage of adaptation occurs when the stress continues, the body adapts to the stressors. Changes at many levels take place in order to reduce the effect of the stressor. Finally, the stage of exhaustion, the stress has continued for some time. The body's resistance to the stress may gradually be reduced, or may collapse quickly. In the other word, the organism is unable to cope with the stressor (Watts, 1990; Roberts *et al.*, 2010).

3.6 Stress responses in fish

Fish is subject to a broader variety of stressors because their homeostatic mechanisms are highly dependent on prevailing conditions in their immediate surroundings. Examples of additional stressors for fish include fluctuations in water salinity, pH, hardness, alkalinity, dissolved solids, water level or current, and exposure to waterborne pathogens or toxicants (Harper and Wolf, 2009).

Under stressful conditions, fish possesses immediate primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of the stress hormones, cortisol and catecholamines (adrenaline and epinephrine) into the bloodstream by the endocrine system (Reid *et al.*, 1998). Secondary responses occur as a consequence of the released stress hormones, causing changes in the blood and tissue chemistry, e.g. an increase of plasma glucose (Begg and Pankhurst, 2004; Martinez-Porchas *et al.*, 2009). However, these parameters are not good reliable indicators because of so many factors rendering fluctuation of actually basal level. For example; metabolic changes of individual fish as an adaptation or acclimation, unconscious researchers during manipulation of experimental animals and inadequate control of variable factors, all of these make the indicators unreliable determinants.

3.6.1 Cellular stress response

Cells respond to stress in a variety of ways ranging from activation of pathways that promote survival to eliciting programmed cell death that eliminates damaged cells (Fulda *et al.*, 2010). At the cellular level the cellular stress response (CSR) is a defense reaction to a stress imposed by environmental force(s) on macromolecules (Kultz, 2005). That stress normally results in DNA damage, lipid peroxidation, protein denaturation and other macromolecule disturbances if cells cannot maintain degree of capacity to counteract with harmfulness. The CSR is characteristic of all cells. It targets a defined set of cellular functions, including cell cycle control, protein chaperoning and repair, DNA and chromatin stabilization and repair, removal of damaged proteins, and certain aspects of metabolism (Kultz, 2003 and 2005). In essence, if the stress stimulus does not go beyond a certain threshold, the cell can deal with it by responding an appropriate protective cellular response, which

ensures the cell survival. Conversely, the failure to activate or maintain a protective response, for example, if the stressful agent is too strong, results in activation of stress signaling cascades that eventually fuel into cell death pathways (Fulda *et al.*, 2010).

3.6.2 Oxidative stress response

Cell survival requires appropriate proportions of molecular oxygen and various antioxidants. Normally in cells there exists equilibrium between pro-oxidant species and antioxidant defense mechanisms such as ROS-metabolizing enzymes including catalase, glutathione peroxidase, and superoxide dismutases (SODs) and other antioxidant proteins such as glutathione (GSH) (Fulda *et al.*, 2010). Oxidative stress occurs when there is a disturbance in this pro-oxidant and antioxidant balance and it has been implicated in several biological and pathological processes (Trachootham *et al.*, 2008). Although most oxidative insults can be overcome by the cell natural defenses, sustained perturbation of this balance may result in either apoptotic or necrotic cell death (Orrenius *et al.*, 2007).

3.7 Cellular stress response-related genes

3.7.1 Thioredoxin interacting protein (TXNIP)

Thioredoxin interacting protein (TXNIP) also known as thioredoxin binding protein 2 (TBP-2) or vitamin D3 up-regulated protein 1 (VDUP-1) is an endogenous inhibitor of cellular thioredoxin (TRX), which is one of the key molecules in anti-oxidant systems, and inactivates TRX anti-oxidative function by binding to the redox-active cysteine residues in its consensus sequence (Trp-Cys-Gly-Pro-Cys), which can be reversibly reduced by the action of TRX reductase and NADPH. The TXNIP locates in the cytosol and is strongly induced by stress responses. TRX–TXNIP interaction, therefore, plays a crucial role in the redox regulation in stress responses (Li *et al.*, 2009). TXNIP expression is also readily inducible in response to various cellular stresses including oxidative stress, UV, heat shock and hydrogen peroxide. These characteristics of TXNIP render cells more vulnerable to oxidative stress. Its anti-TRX function and expression pattern imply that the TXNIP is a key

modulator for stress responses to modify the redox status in the cells (Junn *et al.*, 2000).

3.7.2 Selenoprotein P (SEPP)

Selenoprotein P (SEPP) is a secreted glycoprotein circulating in the blood stream (Burk and Hill, 2009). It is able to bind and carry selenium, which is absorbed by gastro-intestinal (GI) tract, to peripheral tissues in order to maintain selenium homeostasis in the body (Saito *et al.*, 2002). The mRNA of selenoprotein P is characterized by the presence of two unusual patterns: an inframe UGA, which is normally used as a stop codon, and the selenocysteine-insertion sequence (SECIS), which directs the translational machinery to decode UGA as selenocysteine (Steinbrenner *et al.*, 2006). It has been considered as an antioxidant molecule by protecting centrilobular endothelial cells against diquat-induced oxidative injury resulting in liver necrosis in rat (Burk *et al.*, 1995; Atkinson *et al.*, 2001). The study of SEPP expression in zebrafish revealed that SEPP mRNA transcripts were highly up-regulated in fertilized eggs and in the yolk sac of developing embryos suggesting that the protein may play a role in protecting embryonic tissue from damage by oxidants or heavy metals in the environment (Tujebajeva *et al.*, 2000). Yoneda and Suzuki in 1997 reported that rat SEPP was able to chelate heavy metal, especially mercury, to be formed (Hg-Se)-SEPP complex detected by high performance liquid chromatography /inductively coupled argon plasma-mass spectrometry (HPLC/ICP-MS) method. Steinbrenner and partners (2006) attributed the function of SEPP by involving in the protection of human astrocytes against tert-butyl hydroperoxide (t-BHP)-induced oxidative damage. Specific downregulation of SEPP expression by small interfering RNA decreased cell viability of human astrocytes and made them more susceptible to t-BHP-induced cytotoxicity.

3.7.3 Metallothionein (MT)

Metallothionein (MT) is a group of ubiquitous protein with low molecular mass containing cysteine-rich intracellular metal binding protein and its function to regulate essential metals such as copper and zinc, and in detoxification of non-essential metal ions such as cadmium and lead (Cheung *et al.*, 2004). Because of high

thiol-group, it can bind to a large number of metals including cadmium, mercury, platinum and silver (Haq *et al.*, 2003). As its capacity of metal binding, it can protect cells from metal toxicity and also play a protective role as antioxidant protein against hydroxyl free radicals (Thirumoorthy *et al.*, 2007). Cheng and co-workers (2005) successfully cloned and characterized MT gene of *Oreochromis mossambicus* and *Oreochromis aureus* and found that MT was up-regulated by dose-dependent manner when fish was administered with Cd^{2+}, Co^{2+}, Cu^{2+},Ni^{2+}, Pb^{2+} and Zn^{2+}. Northern blot analysis showed that liver gave the highest fold induction of MT gene expression following the administration of heavy metal ions. These indicated that hepatic MT mRNA level in tilapia is a potential sensitive biomarker of exposure to various metal ions.

3.7.4 Heat shock proteins (HSPs)

Heat shock proteins (HSPs) or stress proteins were firstly named by Tissiers and co-workers who observed that the exposure of fruit fly (*Drosophila melanogaster*) to heat shock resulted in the synthesis of a new set of these proteins in salivary glands (Tissiers *et al.*, 1974). The HSPs are a family of conserve proteins found in all living organisms (Basu *et al.*, 2002). These proteins assist the correctly conformation and folding of beginning or nascent polypeptide chains of amino acid residues, act also as molecular chaperones or nursing proteins, and compromise the amendment of misfolded proteins and degradation of denatured proteins (Gupta *et al.*, 2010). Although elevated temperature is the classic inducer of heat shock proteins, it is now clear that a variety of other stresses including pesticides, heavy metals and solvents can induce heat shock proteins expression (Gupta *et al.*, 2010). In eukaryotes, HSPs are categorized into several families according to their function, sequence homology and molecular mass (Roberts *et al.*, 2010). The families primarily include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several smaller Hsp groups (Table 1).

According to a direct correlation between the level of stress response and the amount of HSPs expression and also higher conservation across the taxa, a possible idea may be the use of stress genes or proteins expression as an indicator or a biomarker for determining stress response and, in particular, as a tool to evaluate the level of toxicity (Gupta *et al.*, 2010). The application of Hsp70 as a bio-indicator of

environmental pollution in environmental risk assessment has drawn the interests by many researchers (Rossner *et al.* 2003; Varo *et al.* 2002). The induction of cytosolic chaperones of Hsp70 and DnaJ families or Hsp90 (or both) was found in carbon tetrachloride (CCl_4)-treated rat liver to protect the hepatocytes from the damages (Lee *et al.*, 2004). Increased levels of Hsp70 were detected in the experiment of rainbow trout (*Oncorhynchus mykiss*) exposed to beta-naphthoflavone (BNF), an industrial chemical (Vijayan *et al.* 1997). Zebrafish (*Danio rerio*) embryo exposed to a mixture of chlorpyrifos and nickel indicated that the Hsp70 was sensitive marker as well as histological damages responding to toxicity (Scheil *et al.* 2010)

Table 3.1 Major HSP families of organism and their characteristics

HSP groups	**Monomer mass (kDa)**	**Localization**	**Function**
HSP 100	80–110	Cytoplasm, nucleolus, nucleus, chloroplast	Thermotolerance, ethanol tolerance, long-term spore viability
HSP 90	82–96	Cytoplasm, nucleus	Essential for viability; Increased concentration required for growth at high temperatures
HSP 70	67–76	Cytoplasm, nucleus, mitochondria, chloroplasts, endoplasmic reticulum	Chaperone required for protein assembly, secretion, protein import into the endoplasmic reticulum and organelles; growth at high temperature
HSP 60	58–65	Mitochondria, chloroplasts	Chaperonin, assembly of oligomeric proteins and folding of monomeric proteins; high concentration required for growth at elevated temperature
Small HSPs	18–40	Cytoplasm, nucleus	Protection from stress, apoptosis inhibition

Data source: Modified from Roberts *et al.* (2010)

CHAPTER IV
MATERIALS AND METHODS

4.1 Experimental animals

One thousand juvenile Nile tilapias (*Oreochromis niloticus*) were purchased from Nile tilapia farm in Chachoengsao province, Thailand. The weights of the juvenile fish were between 30 - 40 g. Fish was pooled together in an indoor blue tank at Department of Aquaculture, Faculty of Fisheries, Kasetsart University. Tilapias were daily fed with commercial feed twice a day. Before starting the experiment, fish was acclimatized at least 7 days at room temperature in experimental blue tank containing 150 liters tap water and continuously aerated by using air stone. During the experimental period, excretion and feces were removed everyday and water was changed every two days. Photoperiod would be maintained at a 12:12 hour light-dark cycle.

4.2 Cloning and characterization of full-length cDNAs encoding *On*-TXNIP and *On*-SEPP genes

4.2.1 Searching nucleotide sequences of *On*-TXNIP and *On*-SEPP genes from expressed sequence tags (ESTs) library

Partial cDNAs encoding *On*-TXNIP (Srisapoome *et al.*, 2008. Accession number FF280875, FF280295, FF280155, FF280089, FF279889, FF280874, FF280418, FF280136, FF279936 and FF279562) and *On*-SEPP (Srisapoome *et al.*, 2008. Accession number FF280665, FF280708 and FF280634) genes of Nile tilapia, which were deposited in NCBI database (http://www.ncbi.nlm.nih.gov/), were retrieved and checked their fidelity using BLAST program in NCBI database. Forward and reverse primers (Table 2) of these two genes were designed from these partial

cDNAs for further being used in the polymerase chain reaction (PCR) of 5’ and 3’ rapid amplification of cDNA ends (RACEs).

Table 4.1 Sequences of primer used

Target gene	Accession number	Primer name	Sequence (5’ to 3’)	Purpose	Amplicon size (bp)
Beta-actin	XM_003443127	*On*-Beta-actin F	ACAGGATGCAGAAGGA GATCACAG	Real-time PCR	155
		On-Beta-actin R	GTACTCCTGCTTGCTGA TCCACAT	Real-time PCR	
HSP40 subfamily B member 9	XM_003450707	*On*-HSP40B9 F	CGCCGAAGCCAAGTTCA GAGAAAT	Real-time PCR	160
		On-HSP40B9 R	TGCTGGTTAAAGTCGTA GTCTCCTCC	Real-time PCR	
HSP40 subfamily C member 3	XM_003447288	*On*-HSP40C3 F	GCGAGCAGAGTGTTTCA TTCAG	Real-time PCR	150
		On-HSP40C3 R	TTGAGGGACATCTCATG GTCTC	Real-time PCR	
HSP70	XM_005466767	*On*-HSP70 F	GGTATTGATCTTGGGAC CACCTAC	Real-time PCR	183
		On-HSP70 R	GAAGACTGTGTTTGTGG GGTTC	Real-time PCR	
HSP90 alpha	XM_003440644	*On*-HSP90α F	GGATACATGGCAGCAAA GAAGCAC	Real-time PCR	171
		On-HSP90α R	CTGTGGGTCATCCAGGG TGAATCC	Real-time PCR	
HSP90 beta	XM_013271984	*On*-HSP90β F	TCTCTGTCACCAAAGAG GGTCTGG	Real-time PCR	153
		On-HSP90β R	CCAGTCTGTTTGACACC GTCACCT	Real-time PCR	
MT	XM_003447045	*On*-MT F	GCCAAGACTGGAACCCG CAACTGC	Real-time PCR	136
		On-MT R	TTCCTTTGCACACGCAG CCAGAGG	Real-time PCR	
TXNIP	XM_003455234	*On*-TXNIP F	GGTAGTGATAAGCTGAT CCTGGAG	Real-time and RACE PCR	142
		On-TXNIP R	GTTCAGAAGGCATCCTG AGAGACA	Real-time and RACE PCR	
SEPP	XM_013271867	*On*-SEPP F	ACTGGCTGGAGAGAAA GACGACTT	Real-time and RACE PCR	181
		On-SEPP R	GCACTCCTCTGGGATCT CAGCACT	Real-time and RACE PCR	

4.2.2 Construction of 5' and 3' RACE templates

4.2.2.1 Total RNAs extraction

Total RNAs of head kidney and spleen of 0.5 kg healthy Nile tilapia were extracted by using TRIZOL reagent (Molecular Research Center, USA). Briefly, 100 mg of these two tissues was pooled together in 1 ml TRIZOL reagent followed by tissue homogenization using FastPrep® homogenizer (MP Biomedicals, USA) for 40 seconds (s). The homogenized tissues were centrifuged at 4°C, 13,000 xg for 5 minutes (min) by using high speed refrigerated microcentrifuge (TOMY DIGITOL BIOLOGY, Japan). Supernatant containing total RNAs was transferred into 1.5 ml microcentrifuge tube and incubated at room temperature for 5 min. After that, 200 µl of chloroform was added into the tube and vigorously mixed by inverting for 15 s followed by incubated at room temperature for 3 min. The tube was centrifuged at 4°C, 13,000 xg for 15 min. After centrifugation, the solution was separated into 3 phases. The total RNAs appearing in the upper phase were transferred into a new 1.5 ml Eppendorf tube. Five hundred microlitter of isopropyl alcohol was added to the tube and vigorously mixed by inverting for 15 s followed by incubated at room temperature for 10 min. Then, the tube was centrifuged at 4°C, 13,000 xg for 10 min. The total RNAs were settled down in the pellet. The supernatant was discarded and 1 ml of 75% ethanol was added into that tube for washing the pellet followed by centrifuged at 4°C, 5,800 xg for 5 min. After that, the supernatant was discarded and the pellet was dried on air at room temperature. Subsequently, the pellet was dissolved with 50 µl of DEPC (diethylpyrocarbonate, DNase- & RNase-free) solution and kept at -80°C until use.

4.2.2.2 Isolation of messenger RNA (mRNA)

The mRNA was extracted from the product of total RNAs from 2.2.1 by using QuickPrep™ mRNA Purification Kit (GE Healthcare, UK). Briefly, 1 ml of oligo (dT) cellulose was put in 1.5 ml Eppendorf tube and centrifuged at 16,000 xg for 1 min. After that, the supernatant was discarded. Four hundred microlitter of extraction buffer and 750 µl of elution buffer were added into the

Eppendorf tube containing 50 µl of total RNAs from 2.2.1 and mixed gently. The mixture between the total RNAs and two buffers was transferred into the Eppendorf tube containing 1 ml of oligo (dT) cellulose and mixed well for 3 min. Then, the mixture solution tube was centrifuged at 16,000 xg for 10 s and the supernatant was discarded again. The pellet was washed with 1 ml of high salt buffer for 5 times and spun down at 16,000 xg for 10 s. After that, the pellet was later washed with 1 ml of low salt buffer for 3 times and spun down at 400 g for 2 min. After discarding the supernatant, 300 µl of low salt buffer was added into the tube and mixed gently. Subsequently, the solution was transferred into microspin column, which was put on the top of new Eppendorf tube, and centrifuged at 16,000 xg for 10 s and repeat washing with 300 µl of low salt buffer for 2 times. The microspin column was transferred to a new Eppendorf tube. The 200 µl of pre-warmed (65°C) elution buffer was added into the microspin column and spun down at 16,000 xg for 10 s. The eluted solution containing mRNA in the Eppendorf tube was precipitated by adding 10 µl of glycogen solution, 40 µl of potassium acetate solution and 1 ml of 95% ethanol. After that, the mixture solution was centrifuged at 16,000 xg for 5 min. The supernatant was discarded and the pellet was dried on air at room temperature. Finally, 10 µl of DEPC solution was added into the tube that contains the extracted mRNA and kept at -80°C until use.

4.2.2.3 Synthesis of 5’ and 3’ RACE templates

5’ and 3’ ready-to-use first strand cDNAs were synthesized by using BD Smart^{TM} RACE cDNA Amplification Kit (BD biosciences Clontech, USA) following the recommendation of company. Briefly, two microcentrifuge tubes were prepared, the first tube for 5’ RACE-Ready cDNA and the other tube for 3’ RACE-Ready cDNA. In the 5’ RACE-Ready cDNA tube, the components consisting of 1 µg of mRNA from 2.2.2, 1 µl of BD Smart II A oligonucleotide (5'-AAGCAGTGGTATCAACGCAGA GTACGCGGG-3') and 1 µl of 5’-CDS primers (5’-$(T)_{25}$ V N-3’, N = A, C, G or T; V = A, G or C) were added to synthesize nucleotide sequences at 5’ direction. On the other hand, 3’ RACE-Ready cDNA tube was composed of 1 µg of mRNA from 2.2.2 and 1 µl of 3’-CDS primers (5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ V N-3', N = A, C, G, or T; V = A, G

or C) to synthesize nucleotide sequences at 3' direction. Distilled water was added in each tube adjusting to 5 µl of total volume. After that, each tube was spun down rapidly, then incubated at 70ºC for 2 min and chilled on ice for 2 min. Subsequently, 2 µl of 5X First-strand buffer, 1 µl of 20 mM Dithiothretol, 1 µl of 10 mM dNTP mixed buffer and 1 µl of BD Power Script Reverse Transcriptase were added into each tube. The tubes were mixed well, spun down and incubated at 42ºC for 90 min in hot-lid thermal cycler (Takara, Japan). Finally, the products in each tube were diluted with 250 µl of tricine-EDTA buffer, incubated at 72ºC for 7 min, and kept at -20ºC until use.

4.2.3 DNA cloning

4.2.3.1 5' and 3' RACE polymerase chain reaction (PCR)

5' and 3' RACEs were conducted using PCR reaction in thermal cycler machine to amplify and fulfill the lacking information of nucleotide sequences at 5' and 3' directions. Specific primer pairs designed from partial cDNAs of *On*-TXNIP and *On*-SEPP genes (Table 2) from 2.1 and universal primers (UPM) were used in the reaction of touchdown PCR method. The PCR reaction was described as follow:

5' or 3' RACE first strand cDNA	1.25	µl
10X universal primer	2.5	µl
10 µM gene-specific primer	1	µl
10X LA PCR^{TM} buffer II (Mg^{2+} free)	2.5	µl
10 mM dNTP mix	0.5	µl
25 mM $MgCl_2$	2.5	µl
5 U/µl *LA Taq*^{TM} DNA polymerase	0.5	µl
Sterilized distilled water	14.25	µl
Total volume	25	µl

Target DNA was amplified under the PCR reaction by putting the mixture solution tube into the thermal cycler machine. The PCR reaction was attributed with thermal cycler condition with 5 cycles of 94ºC for 30 s, 72ºC for 3 min,

followed by 5 cycles of 94ºC for 30 s, 70ºC for 30 s, 72ºC for 3 min and 25 cycles of 94ºC for 30 s, 68ºC for 30 s, 72ºC for 3 min, respectively. The PCR products were kept at -20ºC until use.

4.2.3.2 DNA recovery

RACE PCR products and 2 µl of 6X loading dye were loaded on 1% agarose gel and run electrophoresis under 1X TBE (Tris-Borate-EDTA) buffer using 100 voltages. Ethidium bromide (EtBr) solution (0.5 µg/ml) was used for gel staining and the suspected PCR bands were observed and taken photograph to check their approximate sizes under ultraviolet (UV) light by using a transilluminator (Hoefer, USA). The expected bands were cut and transferred to a new 1.5 ml Eppendorf tube for DNA purification using HiYield^{TM} Gel/PCR DNA fragments extraction kit (Real Biotech, Taiwan). Briefly, 500 µl of DF buffer was added into the tube containing DNA fragment, then mixed by vortex, and incubated at 55ºC for 15 min until a piece of gel containing DNA fragment was completely dissolved. After that, DF column was used and placed on collection tube for preparing the step of DNA binding. Next, 800 µl of dissolved DNA mixture from the previous step was loaded into the DF column and centrifuged at 15,300 xg for 30 s. The supernatant at the bottom of the collection tube was discarded and the DF column was placed back. Subsequently, 600 µl of washing buffer (ethanol added) was put into the DF column and centrifuged at 15,300 xg for 30 s. The solution in the collection tube was discarded and the DF column was centrifuged again at 15,300 xg for 2 min to make the column matrix dried. The dried DF column was moved into a new 1.5 ml microcentrifuge tube. Finally, 15 µl of elution buffer was added around the central area of the DF column tube, stand for 2 min, centrifuged at 15,300 xg for 2 min for eluting the purified DNA product and kept at -20ºC.

4.2.3.3 DNA ligation

Three microliter of purified PCR products from 2.3.2 was ligated into 1 µl of pGEM$^{®}$-T easy vector (Promega, USA) (Fig. 1) in 0.2 ml of microcentrifuge tube containing 1 µl of T4 DNA ligase and 5 µl of 2X Rapid ligation

buffer at the total volume of 10 µl. The reaction was incubated at 4ºC for 16-18 hours (h).

4.2.3.4 Transformation

Firstly, competent cells (*Escherichia coli* strain DH5 alpha) were prepared as described by Sambrook and Russel (2001). Briefly, the *Escherichia coli* strain DH5 alpha was cultured in 2 ml of enrichment media Luria-Bertani (LB) broth and incubated at 30ºC for 16-18 h. After that, 1 ml of bacterial solution was continuously sub-cultured in 9 ml of LB broth at 37ºC for 3 h. After the end of incubation, the bacterial suspension was chilled on ice for 10 min, separated into 6 tubes of 1.5 ml microcentrifuge tube and centrifuged at 800 xg, 4ºC for 3 min. The supernatant was discarded and 625 µl of 100 mM $CaCl_2$ was added, mixed well and chilled on ice for 30 min. Subsequently, the suspension was centrifuged at 800 xg, 4ºC for 3 min, then, the supernatant was discarded, and 375 µl of 100 mM $CaCl_2$ in 15% glycerol was added. After the solution was totally mixed, they were kept at -80ºC until use.

Five microliter of ligation product from 2.3.3 was used for transformation (heat shock transformation) by putting into the new 1.5 ml Eppendorf tube containing 100 µl of the DH5 alpha competent cells. The solution was mixed well, further chilled on ice for 25 min and immediately transferred to certain 42ºC water for 50 s. After that the tube was quickly transferred on ice for 3 min. Nine hundred micro liters of super optimal culture (SOC) medium was added. The tube was moved to incubate in incubator shaker at 37ºC for 1.30 h. Subsequently, the tube was centrifuged at 800 xg, 25ºC for 5 min. The supernatant was discarded and the remaining part about 100 microliters was mixed thoroughly by using micropipette. The mixed solution was spread on AX agar plate that contained 0.01% of ampicillin, 20 µl of 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 100 µl of 100 mM IPTG (isopropylthio-β-D-galactoside). The AX agar plate was incubated at 37ºC for 16-18 h.

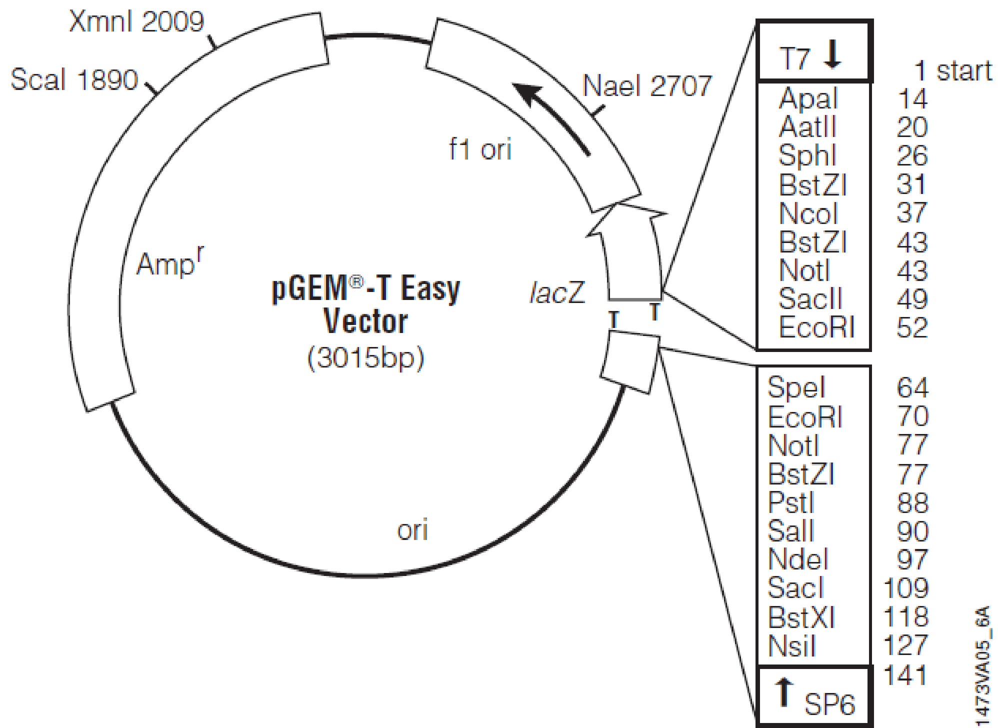

Figure 4.1 Physical map of DNA cloning vector and restriction enzymes in multiple cloning site of pGEM®-T easy vector

Source: http://www.promega.com (2012)

4.2.3.5 Colony screening

The colonies appeared on the AX agar plate from 2.3.4 were collected based on blue-white colony screening. Fundamentally, the white colonies contained the DNA fragment. All white colonies were sub-cultured on a freshly prepared AX agar plate and incubated at 37ºC for 16-18 h serving as a master plate for stocking and keeping at 4ºC. The selected white colonies were conducted for PCR colony screening technique using M13 forward and M13 reverse primers. The colony PCR reaction was described as follow:

10X Dream*Taq*™ Buffer	1.5	μl
2.5 mM dNTP	1.6	μl
5 mM M13 forward primer	1.5	μl
5 mM M13 reeverse primer	1.5	μl
25 mM $MgCl_2$	1.875	μl
5 U/μl Dream*Taq*™ DNA polymerase	0.2	μl
Distilled water	6.825	μl
Total volume	15	μl

The selected white colonies were picked up by pasteurized micropipette tip and put in the prepared reaction solution tube. The plasmid containing DNA fragment was amplified by the thermal cycler machine. The PCR reaction was attributed with thermal cycler condition with 95ºC for 5 min in the first period followed by 25 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 1.5 min. The last period was set as 72ºC for 5 min. The PCR products and 2 μl of 6X loading dye were loaded on 1% agarose gel and run electrophoresis under 1X TBE (Tris-Borate-EDTA) buffer using 100 voltages. Ethidium bromide (EtBr) solution (0.5 μg/ml) was used for gel staining and the suspected PCR bands were observed and taken photograph to check their approximate sizes under ultraviolet (UV) light by using a transilluminator.

4.2.3.6 Plasmid DNA extraction

White colonies that showed positive result of colony PCR were collected to extract the plasmids. Briefly, the white colonies from master plate were sub-cultured in 3 ml of LB broth containing 0.01% ampicillin and incubated at 37ºC for 16-18 h. After that, the bacterial suspension was mixed by vortex mixer and divided into 1.5 ml for each Eppendorf tube. The tubes were centrifuged at 13,000 xg at 25ºC for 3 min. The supernatant was discarded. The plasmids were extracted by using Plasmid DNA Extraction Manual Kit (Bio Excellence, USA). Two hundred microliters of cell suspension solution was added and mixed well by pipetting. After that, 200 μl of cell lysis solution was added and mixed well by inverting the tubes, followed by adding 200 μl of neutralization solution and mixed well by inverting the

tubes. Subsequently, the tubes were centrifuged at 9,100 xg at 25ºC for 5 min. Only supernatant was allowed to transfer into new 1.5 ml microcentrifuge tubes. Then, 600 µl of isopropanol was added, mixed well and centrifuged at 9,100 xg at 25ºC for 5 min. The pellet was allowed to keep on and washed with 1 ml of 70% ethanol. After well mix, the tubes were centrifuged at 9,100 xg at 25ºC for 5 min. The supernatant was discarded and the pellet was dried on air. Finally, 20 µl of Tris-EDTA (TE) buffer was added and kept at -20ºC.

4.2.3.7 Restriction enzyme digestion

In order to check the interested insert on plasmid, the extracted plasmid was cleaved with *Eco* RI (Fermentas, USA), restriction endonuclease enzyme. The reaction solution was described as follow:

Component	Volume	Unit
Plasmid DNA	2	µl
10X Buffer *Eco* RI	2	µl
Eco RI	0.5	µl
Distilled water	5.5	µl
Total volume	10	µl

All of these components were added into 0.6 ml microcentrifuge tube and incubated at 37ºC overnight. Interested DNA was demonstrated by 1% agarose gel electrophoresis with 2 µl of 6X loading dye in 1X TBE (Tris-Borate-EDTA) buffer using 100 voltages. Ethidium bromide (EtBr) solution (0.5 µg/ml) was used for gel staining and the suspected DNA fragment was observed and taken photograph to check their approximate sizes under ultraviolet (UV) light by using a transilluminator. The DNA fragment was compared with standard DNA ladder 100 bp plus (Thermoscientific, USA).

4.2.3.8 DNA sequencing

Nucleotide sequence analysis of plasmid containing DNA fragment was performed using M13 forward and M13 reverse primers by Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham pharmacia biotech) from Macrogen, Inc. (Korea).

4.2.4 Characterization of full-length *On*-TXNIP and *On*-SEPP cDNAs

Nucleotide sequences of *On*-TXNIP and *On*-SEPP cDNAs in both 5’ and 3’ RACE directions were observed to analyze the fidelity and the vector and adapter nucleotide sequences appeared in the RACE products were excised using VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/Vecsreen.html) and Genetyx® version 7.0. After that, the nucleotide sequences were subsequently conducted for homology with sequences available in GenBank database using both BlastX and BlastN programs (http://www.ncbi.nlm.nih.gov/). The nucleotide sequences in both 5’ and 3’ directions were multi-aligned with each other. The overlapping region of nucleotide sequences was represented the full-length cDNAs of *On*-TXNIP and *On*-SEPP genes. Hydrophobic leader peptide sequence was proved by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP).

4.2.5 Multiple alignments

Deduced amino acid sequences in the region of open reading frame (ORF) of *On*-TXNIP and other known organism TXNIP cDNAs, which were available in GenBank database, were multi-aligned using ClustalW (http://www.clustal.org) in order to compare and examine the important motifs. In parallel, the *On*-SEPP cDNA was aligned with other known organism SEPP cDNAs as the similar means described for *On*-TXNIP cDNA.

4.3 Evolutionary evaluation of Nile tilapia *On*-TXNIP and *On*-SEPP genes compared to other known *On*-TXNIP and *On*-SEPP genes of other organisms

4.3.1 Identity and similarity of nucleotide and amino acid sequence

Full-length cDNAs encoding Nile tilapia *On*-TXNIP and *On*-SEPP genes were trimmed the sequences of 5' and 3' untranslated regions (UTRs) out using Genetyx® version 7.0 in order to get only open reading frame (ORF). The identity and similarity of amino acid sequences of Nile tilapia *On*-TXNIP and *On*-SEPP cDNAs were separately constructed by comparing with TXNIP and SEPP sequences of other living organisms, which were available in GenBank database, by ClustalW and MatGat 2.02 (http://bitincka.com/ledion/matgat) programs.

4.3.2 Phylogenetic tree construction

In order to conduct the phylogenetic tree analysis, the open reading frame of Nile tilapia *On*-TXNIP and *On*-SEPP amino acid sequences was separately aligned by the ClustalW with other known TXNIP and SEPP genes of other living organisms available in GenBank database. The phylogenetic tree was ultimately constructed by MEGA 5.05 available program (http://www.megasoftware.net) using UPGMA method. The reliability of the obtained tree was assessed by bootstrapping with 1,000 bootstrap replications.

4.4 Messenger RNA (mRNA) expression level analyses of *On*-TXNIP and *On*-SEPP genes in different organs of a healthy Nile tilapia by quantitative real-time PCR

4.4.1 Sample collection and total RNAs isolation

In order to examine the expression pattern of *On*-TXNIP and *On*-SEPP genes in various organs of a healthy Nile tilapia (approximately 30 g), thirteen organs consisting of brain, gills, gonad, heart, anterior kidney (head kidney), intestine, liver, muscle, peripheral blood leukocytes (PBLs), skin, spleen, stomach and posterior kidney (trunk kidney) were collected and kept in Trizol reagent for total RNAs extraction as described in 2.2.1.

4.4.2 First strand complementary DNA (cDNA) synthesis

One microgram of total RNAs obtained from different organs was used and first strand cDNA synthesis was performed using RevertAid^{TM} first strand cDNA synthesis kit (Fermentas, USA) as the recommendation of manufacturer. Firstly, one microgram of total RNAs was treated with DNase I enzyme to get rid of genomic DNA contamination. The reaction solution was described as follow:

Total RNAs (1µg)	A	µl
DEPC water	8-A	µl
10X buffer with $MgCl_2$	1	µl
DNase I	1	µl
Total volume	10	µl

The mixture solution above was incubated at 37ºC for 30 min. After that, 1 µl of 25 mM EDTA was added into the tube and incubated at 65ºC for 10 min. Subsequently, first strand cDNA was further synthesized. The reaction solution was described below:

DNase I treated-total RNAs	11	µl
Oligo (dT) primer	1	µl
5X reaction buffer	4	µl
Ribolock™ Ribonuclease inhibitor	1	µl
10 mM dNTP mix	2	µl
RevertAid™ reverse transcriptase	1	µl
Total volume	20	µl

The mixture solution was incubated at 42ºC for 60 min followed by terminating the reaction at 70ºC for 10 min. The first strand cDNA was kept at -20ºC until use.

4.4.3 Real time RT-PCR analyses of *On*-TXNIP and *On*-SEPP genes

Expression level analyses of *On*-TXNIP and *On*-SEPP genes in different organs of healthy Nile tilapia were conducted by quantitative real time RT-PCR using 2X Brilliant®II SYBR® Green QPCR Master Mix (Stratagene, USA). First strand cDNA obtained from 4.2 was used as template. Specific primers for *On*-TXNIP, *On*-SEPP and beta-actin genes (Table 2) were used to evaluate relative expression ratio of *On*-TXNIP and *On*-SEPP genes normalized with beta-actin gene. One reaction consisted of following components:

First strand cDNA	0.5	µl
Brilliant®II SYBR® Green QPCR Master Mix	6.25	µl
10 µM Forward primer	0.5	µl
10 µM Reverse primer	0.5	µl
Distilled water	4.75	µl
Total volume	12.5	µl

Each reaction with three replicates was loaded into thermal 96-well polypropylene plate. The same reaction with housekeeping gene (beta-actin gene), which acted as internal control, was used laterally. The expression levels of *On*-TXNIP and *On*-SEPP mRNAs in Nile tilapia were analyzed by using MxPro qPCR software, Mx3005P real-time PCR system (Stratagene, USA). The real time PCR condition was attributed with the first period at 95ºC for 10 min, the second period with 40 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min and the final period with one cycle of 95ºC for 1 min, 55ºC for 30 s followed by 95ºC for 30 s. Threshold cycle or C_T values were recorded for further calculating and analyzing expression pattern of *On*-TXNIP and *On*-SEPP genes in each organ of Nile tilapia. The relative expression ratio could be calculated by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

$$\text{Relative expression ratio} = 2^{-\Delta\Delta C_T}$$

ΔC_T was a difference between C_T of gene of interest (GOI) and of housekeeping gene in each time interval. The formula used to calculate ΔC_T could be written as:

$$\Delta C_T = C_T \text{ of GOI} - C_T \text{ of housekeeping gene}$$

ΔC_T of initial time $[(\Delta C_T)_{\text{Time } 0}]$ was used as calibrator to adjust the ΔC_T of each time interval $[(\Delta C_T)_{\text{Time X}}]$ in order to normalize the values. The values after normalization were assigned as $\Delta\Delta C_T$, which could be written as:

$$\Delta\Delta C_T = (\Delta C_T)_{\text{Time X}} - (\Delta C_T)_{\text{Time } 0}$$

The $\Delta\Delta C_T$ was represented in the formula of relative expression ratio above.

4.5 Effects of Ag NPs on cellular stress responses in Nile tilapia

4.5.1 Silver nanoparticles (Ag NPs)

A bottle containing 5 g of 99.5% Ag NP powder (No. 576832), with poly vinyl pyrrolidone (PVP) as a dispersant agent, was purchased from Sigma-Aldrich, USA. The Ag NP size was declared to be less than 100 nm.

4.5.2 Experimental design

After seven days of acclimatization, one hundred and twenty juvenile Nile tilapias were separated into four groups, thirty fish per group. In group I, fish was intraperitoneally injected with phosphate buffer saline (PBS) pH 7.4, which was a control group. In group II, III and IV, fish was exposed *via* the same route to the Ag NPs at various concentrations of 1, 10 and 100 mg/kg, respectively. The target organs of liver, spleen and head kidney from 3 fish were dissected, collected at time intervals of 6, 12, 24, and 48 hours post injection (hpi) as well as the 1st, 2nd, and 4th weeks post-injection (wpi) and kept in TRIzol reagent for total RNA extraction. The liver and spleen at the 1st, 2nd, and 4th wpi were also partly preserved in 10% normal buffered formalin for histological processing.

4.5.3 Histopathological observation

Histopathology of the tissue organs was followed by the guidelines of Department of Pathobiology, Faculty of Science, Mahidol University, Thailand. Liver, spleen and head kidney were collected at time intervals and preserved in 10% normal buffer formalin for 24 h and washed with 70% ethanol. The tissues were dehydrated in graded series of ethanol, cleared in xylene and embedded in a block using melted paraffin. Five micron sections were cut by a rotary microtome (Histo STAT, Reichert, USA) and stained with hematoxylin and eosin for the standard protocol of histopathological examination (Humason, 1972).

4.5.4 Quantitative real time RT-PCR of cellular stress response genes

Cellular stress response genes were chosen from Expressed Sequence Tag (EST) library, which was a DNA library of Nile tilapia (Srisapoome *et al.*, 2008). A group of genes were composed of thioredoxin-interacting protein (TXNIP), selenoprotein P (SEPP), metallothionein (MT), heat shock protein 40 subfamily B member 9 (HSP40B9), heat shock protein 40 subfamily C member 3 (HSP40C3), heat shock protein 70 (HSP70), heat shock protein 90 alpha (HSP90α) and heat shock protein 90 beta (HSP90β). Liver, spleen and anterior kidney were collected from control and treatment groups at time intervals described above. Total RNAs were extracted following the method in 2.2.1 and converted to first strand cDNAs mentioned in 4.2. Real-time RT-PCR was conducted as previously described in 4.3 to evaluate the expression levels of mRNA transcripts of target and beta-actin genes.

4.6 Effects of Ag NPs on immunological responses and hematological parameters in Nile tilapia

4.6.1 Effect of Ag NPs on non-specific immune response

4.6.1.1 Experimental animal design

Juvenile Nile tilapias (*Oreochromis niloticus*) were purchased from Nile tilapia farm available in Thailand. The weights of the juvenile fish were between 30 - 40 g. Fish was pooled together in an indoor blue tank at Department of Aquaculture, Faculty of Fisheries, Kasetsart University. Tilapias were daily fed ad libitum with commercial feed twice a day. Prior to start the experiment, fish was acclimatized at least 7 days at room temperature in experimental blue tank containing 150 liters tap water and continuously aerated by using air stone. During the experimental period, excretion and feces were removed every day and water was changed every two days. Photoperiod was maintained at a 12:12 h light-dark cycle. One hundred and forty fish were divided into 4 groups, 35 fish per group. Group I was intraperitoneally injected with 0.1 ml normal saline (0.85% NaCl). Group II, III and IV were exposed to 1, 10 and 100 mg Ag NPs/kg fish *via* the same route of

administration in control. Blood samples from 3 fish in each group were withdrawn from caudal vein with 1 ml heparinized syringe at weeks 1, 2, 4, 6 and 8 after administration to examine phagocytic activity, red blood cell count, white blood cell count and percent hematocrit.

4.6.1.2 Phagocytic activity assay

One milliliter of blood was carefully withdrawn from caudal vein and diluted with 2 ml of RPMI medium (Sigma Aldrich, USA). Three milliliter of diluted blood was transferred into a new 15 ml tube containing 3 ml of Histopaque® solution (Sigma Aldrich, USA), which was used for separating blood components into three phases based on gradient centrifugal force at 400 xg for 30 min. The upper phase was diluted plasma. The intermediate phase was Histopaque® solution. The lower phase was red blood cells. Phagocytic cells were buoyancy between the upper and intermediate phase. They were carefully removed into a new tube and washed twice with phosphate buffer saline (PBS) pH 7.4 followed by centrifugation at 250 xg for 15 min. Afterwards, the numbers of phagocytic cells were calculated using hemocytometer, adjusting the cells to 5×10^6 cells per milliliter. The phagocytic cells were loaded onto the surface of the cover slip, incubating for two hours at room temperature to allow cells tightly adherent. The cells on cover slip were washed thrice with PBS pH 7.4. The prepared latex beads (Sigma Aldrich, USA), at concentration of 2×10^7 latex beads per milliliter, were added onto the cover slip, incubating for one and a half hours at room temperature to permit the beads completely phagocytosed by the cells. After finishing the incubation, the cover slips were washed thrice with PBS pH 7.4 followed by fixing with methanol and staining with eosin and methylene blue, for 10 s each, respectively. The phagocytic cells were finally observed by light microscope. Phagocytic activity (PA), phagocytic index (PI) and phagocytic efficiency (PE) were calculated as described by Puangkaew *et al.* (2004) and Koenigsknecht and Landreth (2004) below.

Phagocytic activity (%PA) = Number of phagocytic cells containing latex beads X 100 / 300 adherent cells observed under microscope

Phagocytic index (PI) = Average number of latex beads ingested by phagocytic cell / Total phagocytic cells

Phagocytic efficiency (PE) = Number of latex beads appeared in cytoplasm / Total phagocytic cells

4.6.1.3 Hematological parameters

Blood was collected from 3 fish in each group in 6.1.1. To determine the number of red blood cells (RBC) and white blood cells (WBC), 5 µl of blood was added into 995 µl Natt and Herrick staining solution (Natt and Herrick, 1952) providing 1:200 ratio, mixed together, dropped into the hemocytometer and determined under light microscope. Hematocrit (Hct) was determined by filling blood into the heparinized glass capillary tube (Modulohm A/S, Denmark). At least three-fourths height of capillary tube was filled with blood, sealed the tube with modeling clay and centrifuged in the microhematocrit centrifuge (Suranaree Medical Equipment, Thailand) at 12,000 rpm for 5 min. %Hct level was measured with hematocrit reader.

4.6.2 Effect of Ag NPs on specific immune response

4.6.2.1 Preparation of formalin-killed antigen *Streptococcus agalactiae* (FASA)

Streptococcus agalactiae SAAQH001 which is a pathogenic bacterium causing a systemic infection and a causative agent of streptococcosis for Nile tilapia. It was provided by Laboratory of Aquatic Animal Health Management, Department of Aquaculture, Kasetsart University. The bacteria were cultured in tryptic soy broth (TSB) for 18 h at 33 ºC. The bacterial suspension was centrifuged at 800 xg for 5 min, washed twice with 0.85% sodium chloride (NaCl) solution and treated with 1% formalin in 0.85% NaCl. Afterwards, the suspension was kept in 4 ºC for 24 h. After overnight incubation, the bacterial suspension was next centrifuged at 800 xg for

5 min to discard the supernatant and 0.1% formalin in 0.85% NaCl was added and kept in 4ºC until used. The suspension was centrifuged at 800 xg for 5 min to expel the supernatant, wash for 2 times by 0.85% NaCl. The proper turbidity was adjusted with 0.85% NaCl to get optical density (OD) at 0.6, which was equal to $1x10^9$ colony forming unit (CFU)/ml, by spectrophotometer (Spectronic Bio Mate 3, UK) with the specific wavelength at 560 nm (Samrongpan et al., 2010).

4.6.2.2 Experimental animal design

Two hundred and twenty fish which prepared in 6.1.1 were randomly set into 5 groups, 44 fish per group. Fish in each group were maintained and acclimatized as described above. After 7 days, fish in group I was intrapertoneally injected with 0.1 ml normal saline (0.85% NaCl). Group II was vaccinated by injecting with previously prepared 0.1 ml of formalin-killed antigen *Streptococcus agalactiae* (FASA). Fish group III, IV and V were immunized with FASA and exposed to Ag NPs at 1, 10 and 100 mg/kg *via* the same route of administration in control and vaccinated groups. Blood samples from 3 fish in each group were weekly withdrawn from caudal vein with the method as previously described to examine levels of antibody titer. At week 3 after the antibody level during the first period was declined, the 2nd immunization was conducted in groups II, III, IV and V by the similar method above, while fish in group I was injected with normal saline with the same protocol.

4.6.2.3 Antibody titer assay

One milliliter of blood was withdrawn from caudal vein of 3 fish in each group and transferred into 1.5 ml Eppendorf tube. The tube was inclined and incubated for 2 h at room temperature to allow serum separated. The tube was centrifuged at 600 xg for 15 min. The serum was removed and transferred into a new Eppendorf tube. The antibody titer was conducted by varying the concentrations of fish serum in the U-shape 96-well plate by serial two-fold dilution method using 50 µl of serum and 50 µl of normal saline. The 50 µl solution containing antigen (FASA) at concentration of $1x10^8$ CFU/ml obtained from previous step, was loaded into each well. After incubation at 37 ºC for 24 h, the result was interpreted as the positive result when sufficient antibody in fish serum formed a mat of antigen-antibody complex

showing clear liquid, while the negative result demonstrated as a white button-like spot at the bottom of the well (Kwon et al., 2006).

4.7 Effect of Ag NPs on bacterial resistance in Nile tilapia

4.7.1 Preparation of bacterial suspension of *Streptococcus agalactiae*

Streptococcus agalactiae SAAQH001 virulent strain was enriched in generalized media, tryptic soy broth (TSB). After incubation at 33 ºC for 18 h, the bacterial cells were precipitated by centrifugation at 800 xg for 5 min, washed twice with 0.85% NaCl and the OD was adjusted to 0.6, equal to $1x10^9$ CFU/ml, by spectrophotometer with the specific wavelength at 560 nm (Samrongpan *et al.*, 2010).

4.7.2 Bacterial resistant analysis in normal Nile tilapia exposed to Ag NPs

At the termination of phagocytic activity and hematological analyses in 6.1, 20 remaining fish from each group were randomly selected and transferred to two 150 L fiberglass tanks containing 100 L freshwater (10 fish each). Fish were further acclimatized for 2 days. Challenge test was conducted by intraperitoneal injecting to all fish in every group with 0.1 ml of previously prepared *S. agalactiae* suspension ($1x10^9$ CFU/ml). Cumulative mortality in each group was recorded daily until day 7 of experiment.

4.7.3 Bacterial resistant analysis in FASA-vaccinated Nile tilapia exposed to Ag NPs

At the termination of antibody titer assay in 6.2, fish were continuously challenged with 0.1 ml of *S. agalactiae* suspension by the similar method as described above. Cumulative mortality in each group was recorded daily until day 7 of experiment.

4.8 Statistical analysis

Relative expression ratio of cellular stress response-related genes in each organ at different time points, PA, PI, PE, hematological parameters, antibody titer levels and cumulative mortality rates in each treatment group at different time courses of each experiment were used to statistically determine by using one-way analysis of variance (ANOVA) and Duncan's new multiple range test (DMRT) was used to compare the differences of means of all observation values at 95% confidence interval ($P<0.05$).

CHAPTER V
RESULTS

5.1 Cloning and characterization of full-length cDNAs encoding *On*-TXNIP and *On*-SEPP genes

5.1.1 Cloning and characterization of *On*-TXNIP full-length cDNA

The full-length cDNA encoding the Nile tilapia *On*-TXNIP gene was 2,260 bp (Accession no. KF601827) and consisted of 116 bp of 5’ untranslated region (UTR), 1,188 bp of open reading frame (ORF) (equal to 396 amino acid residues), 925 bp of 3’ UTR and 28 bp of a poly A tail (Fig. 2). The calculated theoretical isoelectric point (p*I*) and molecular weight (MW) of *On*-TXNIP were 7.81 and 43.95 kDa, respectively. Amino acid sequence analysis revealed that it contained an arrestin-like N domain (residues 11–127), an arrestin-like C domain (residues 178–301) and two conserved cysteine residues at Cys-65 and Cys-250. In addition, two PPXY motifs (residues 334–337 and 376–379) were found near the tail region (Fig. 3).

5.1.2 Cloning and characterization of *On*-SEPP full-length cDNA

The full length cDNA encoding the Nile tilapia SEPP gene (*On*-SEPP) was 2,427 bp (Accession no. KF601828) and consisted of 99 bp of 5’UTR, 1,239 bp of ORF (equal to 413 amino acid residues), 1,086 bp of 3’UTR, and 29 bp of a poly A tail (Fig. 4). The calculated p*I* and MW of *On-SEPP* were 6.27 and 46.36 kDa, respectively. A selenocysteine insertion sequence (SECIS) search in the 3’UTR revealed that there were two SECIS elements (nucleotides 1,618–1,713 and 2,256–2,346) (Fig. 4 and 5). The amino acid sequence alignment revealed 17 selenocysteines (U) encoded by TGA codons that appear within the coding region of the ORF (Fig. 6). The configuration patterns of the selenocysteine codons were either UXU or UXUXU, where U represents selenocysteine and X represents any amino acid.

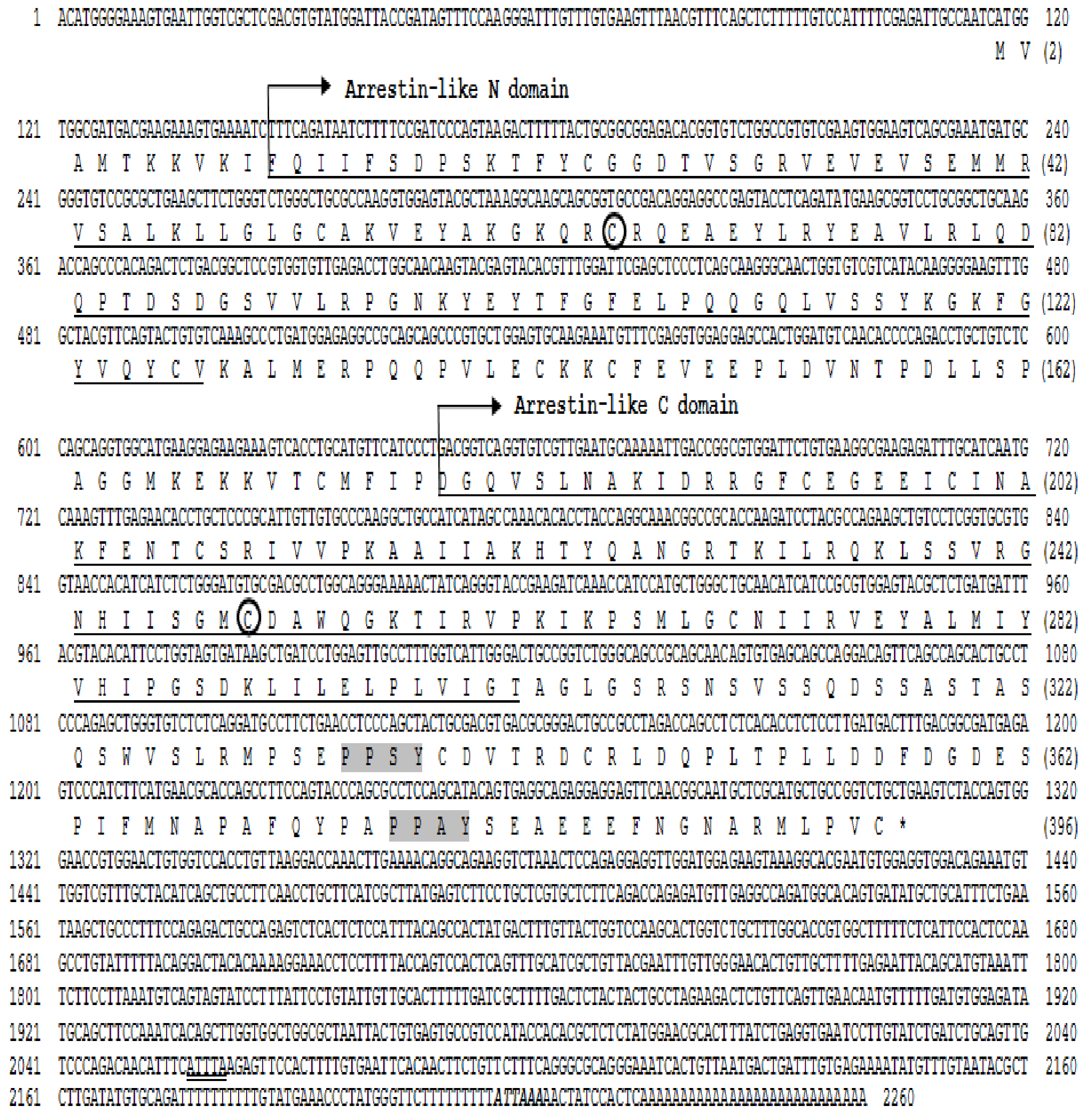

Figure 5.1 The full-length cDNA encoding the Nile tilapia *On*-TXNIP gene. Arrestin-like N and C domains are underlined. Conserved cysteine residues are in circles. PPXY motifs are shown with gray highlighting. Instability motifs (ATTTA) are double-underlined. Bold and italic alphabets indicate the polyadenylation signal (ATTAAA).

```
                         <----------------------------   Arrestin-like N domain ---------------------------------------------
Human          1:-MVMFKKIKSFEVVFNDPEKV-YGSGEKVAGRVIVEVCEVTRVKAVRILACGVAKVLWMQGSQQCKQTSEYLRYEDTLLLEDQPTGENEMVIMRPGNKYEYKFGFELP-QGPLGTSFKGK 117
Rat            1:-MVMFKKIKSFEVVFNDPEKV-YGSGEKVAGRVTVEVCEVTRVKAVRILACGVAKVLWMQGSQQCKQTLDYLRYEDTLLLEDQPTGENEMVIMRPGNKYEYKFGFELP-QGPLGTSFKGK 117
Frog           1:-MVVFKKVKCFEVSFTDPQKV-YCGGDKVTGKVLVEVAEVTRVTAVRLLACGVAKVLW---SQNCKQEMEYLRYEDTLQVDDQPLDSDGSVLMRPGNCYEYKFGLELP-QGPLGSIFKGK 114
Nile tilapia   1:MVAMTKKVKIFQIIFSDPSKTFYCGGDTVSGRVEVEVSEMMRVSALKLLGLGCAKVEYAKGKQRCRQEAEYLRYEAVLRLQDQPTDSDGSVVLRPGNKYEYTFGFELPQQGQLVSSYKGK 120
Zebrafish      1:MVAMTKRVKVFEIAFNDPSKTFYCSGDKVAGKVLVEVSEVTRVMAMKVLGVGCAKVEYAKGKQRCREEVDYLKYEDVVQLDEHPTDNDGSVILRPGNKYEYSFGFELPAQGQLVSSYKGK 120
Salmon         1:MVAMPKRVKTFEVIFSDASKAFYCSGDKVAGRIVVEVAEVTRVSAMRVLGVGCAKVEYAKGKQKCREENEYLRYEEVVQLVDQPADHDGSVVLRPGNKYEYMFGFELPQQGQIVSSYKGK 120
                   .*..* *.. * *. *  *..*..*.*.. *** *..** * . *  * ***   . * * .. .**.**. . . ..*.. ...*  ****.*** **.*** ** . .. ***

                 ------>                                           <--------------  Arrestin-like C domain -------------
Human        118:YGCVDYWVKAFLDRPSQPTQETKKNFEVVDLVDVNTPDLMAPVSAKKEKKVSCMFIPDGRVSVSARIDRKGFCEGDEISIHADFENTCSRIVVPKAAIVARHTYLANGQTKVLTQKLSSV 237
Rat          118:YGCVDYWVKAFLDRPSQPTQEAKKNFEVMDLVDVNTPDLMAPVSAKKEKKVSCMFIPDGRVSVSARIDRKGFCEGDDISIHADFENTCSRIVVPKAAIVARHTYLANGQTKVLTQKLSSV 237
Frog         115:YGSVKYWVKAFLDRPSHPPQEVKKSFQVEDSVNVDTPDLMSPVSGKKYKKMTCMFIPDGHISMSACINRKGFCEGDDICISADFENTCSRIVVPKAAIIGKHTYLANGQTKVFTQKLCSV 234
Nile tilapia 121:FGYVQYCVKALMERPQQPVLECKKCFEVEEPLDVNTPDLLSPAGGMKEKKVTCMFIPDGQVSLNAKIDRRGFCEGEEICINAKFENTCSRIVVPKAAIIAKHTYQANGRTKILRQKLSSV 240
Zebrafish    121:FGFVQYYVKALMERPCQPALECKKHFEVEEPLDVNTPDLLSPTGGMKEKKVTCMFIPDGQVSLNAKIDRRGFCEGEEICIDAKFENTCSRIVVPKAAIVAKQTYQANGRTKVFRQKLSSV 240
Salmon       121:FGYVQYFVKALMERPAQPALECKKHFEVEEPLDVNTPDLLSPTGGMKEKKVTCMFIPDGQVSLNAKIDRKGFCEGEDICICAKFENTCSRIVVPKAAIISKHTYQANGRTKVFRQKLSSV 240
                   * * * ***   ** .*  * ** *.*.   .*.**** .*  . *.**..******* .*  * *.*.*****  *.* * ****************  ...** *** **.   ***.**

                 ------------------------------------------------------->
Human        238:RGNHIISGTCASWRGKSLRVQKIRPSILGCNILRVEYSLLIYVSVPGSKKVILDLPLVIG--SRSGLSSRTSSMASRTSS----EMSWVDLNIPDTPEAPPCYMDVIPEDHRLESPTTPL 351
Rat          238:RGNHIISGTCASWRGKSLRVQKIRPSILGCNILRVEYSLLIYVSVPGSKKVILDLPLVIG--SRSGLSSRTSSMASRTSS----EMSWIDLNIPDTPEAPPCYMDVIPEDHRLESPTTPL 351
Frog         235:RGNHIISGMTDSWRGKSIRIPKIKPSILGCNILRVEYSLLVYVSVPGAKKIILDLPLVIG-CSSSGMSSRTSSMASQASS----EMSWLELNIPGTPEAPPGYLDIVPEDHRLESPTTPL 349
Nile tilapia 241:RGNHIISGMCDAWQGKTIRVPKIKPSMLGCNIIRVEYALMIYVHIPGSDKLILELPLVIGT---AGLGSRSNSVSSQDSSASTASQSWVSLRMP--S-EPPSYCDVTR-DCRLDQPLTPL 353
Zebrafish    241:RGNHIISGMCDAWQGKSIRVPKIKPSILGCNIIRVEYALMIYMHIPGSEKLILELPLVIGTVPYNGFGSRTNSMSSQDGSISNASNSWVSLRMP--SSAPPSYCDITR-DCCIDQPLTPL 357
Salmon       241:RGNHIISGMCDAWQGKTIRVPKIKPSMLGCNIIRVEYALMIYMHIPGSEKLILELPLVIGT---AGLGSRTNSVSSTDGSVSNASASWVSLRMP--S-APPSYCDVTR-DCRLDQPLTPL 353
                 ********... * **..*..**.**.***** **** * .*.  **. * ** ******    *. **. *. *  .*      **. *  *    .** * *.   * .. * ***

Human        352:LDDMDGSQDSPIFMYAPEFKFMPPPTYTEVDPCILNNNVQ----                                                                            391
Rat          352:LDDVDDSQDSPIFMYAPEFQFMPPPTYTEVDPCVLNNNNNNVQ-                                                                            394
Frog         350:LDDLDGACDSPIFMYAPEFKYMPPPTYTEVDVNKNTCVQ-----                                                                           388
Nile tilapia 354:LDDFDGD-ESPIFMNAPAFQYPAPPAYSEAEEEFNGNARMLPVC                                                                            396
Zebrafish    358:LDDYDGG-DSPIFMNAAQFQFPPLPAYSEVEEEFNANARMLPVC                                                                            400
Salmon       354:LNDYDGD-DSPIFMHPSAFQFPPLPAYSEVDEEFNGNTRMLQVC                                                                            396
                 *.* *.  .***** .. *.. ..* * *..   . .
```

Figure 5.2 Amino acid sequence alignment of TXNIP of Nile tilapia and other vertebrates. Asterisks (*) indicate residues conserved in all sequences. Colon (:) and dot (.) mark residue in high and low conserved in most sequences, respectively. The conserved cysteine residues are highlighted in gray. PPXY motifs are shown as bold and italic alphabets.

```
   1  ACATGGGGGTTTTTGTTCAGAGTTGCAGCCACGCAGAGCTGAACAGAGGGTAGGAAACTTTTTAACCAAAGGTTGGGGCAGTAAGGAGGCGCCACAGAGATGTGGGCAGGCCTCAGTCTG  120
                                                                                                            M W A G L S L   (7)
 121  CTCCTCACTCTCTGCCTGCTCCATGGGGGTGGGGCAGAGAGTGAGGGGGGAGGGCCCCGCTGTCAGCTACCTTCAGATTGGAGGATAGGAGACGTGGAGCCTATGAAGGGGTCAGTAGGT  240
      L L T L C L L H G G G A E S E G G G P R C Q L P S D W R I G D V E P M K G S V G   (47)
 241  CGAGTGACGGTGGTGGCCCTTTTACAGGCCAGCTGACTGTTCTGCTTGGTGCAGGCTTCCAGACTAGATGGCCTGCAGCAGAAGCTGGAACGTCAAGGTCTTAAGAATGTGGTCTACATG  360
      R V T V V A L L Q A S U L F C L V Q A S R L D G L Q Q K L E R Q G L K N V V Y M   (87)
 361  GTCGTTAACCACCAGGGGGAGCAATCACGACATCTGCACCCTTTGCTGGAGGCCAAACTTTCCAAGAACATCATACTCTACAAGCAGGACGGGCATCAGCCTGATGTTTGGCAGACACTG  480
      V V N H Q G E Q S R H L H P L L E A K L S K N I I L Y K Q D G H Q P D V W Q T L   (127)
 481  GCTGGAGAGAAAGACGACTTTTTCATTTATGACAGGTGTGGCCGTCTAACCCACCGTATTTCACTTCCATACTCCATCATTGGAGAGGGCCATATTGAGAAGGCAATCAAAGACACCTAC  600
      A G E K D D F F I Y D R C G R L T H R I S L P Y S I I G E G H I E K A I K D T Y   (167)
 601  TGCAAACGCCTATGTGGAGACTGCACACATGAGAGTGCTGAGATCCCAGAGGAGTGCAAAGATAATGCAGGTGTCCAGCCTGATGTCCCAGCTGAACAGGACGACACCAGACATGATCAT  720
      C K R L C G D C T H E S A E I P E E C K D N A G V Q P D V P A E Q D D T R H D H   (207)
 721  CACCATGGTCATGGTCATGGTCATCACCATGGTCACGGTCATCACCATGGTCACGGTCACGGCCACCATGGGGATAATCAGGATGTTCATCCTCATGGCCATGGCAGTGATCACAACAAT  840
      H H G H G H G H H H G H G H H H G H G H G H H G D N Q D V H P H G H G S D H N N   (247)
 841  GGCCATCATCATAGAAATCACGATGGTGCCGACCAGACACAGCATGGTGTTAGACCACATGGGCATTTCCACGAAGGGGACATGCCTCAGACCCAGCATCATTTTGATTTAGGCCAGATT  960
      G H H H R N H D G A D Q T Q H G V R P H G H F H E G D M P Q T Q H H F D L G Q I   (287)
 961  CCCCAGGAAGTGCACAATCACCAGGTGGCACAGGAGGCTCATGCAGTCATTGAAAGGCCTTGATTATCTAGGAAGAATAGGTGAAAGTTGAAGTACAACTGACAGGGTCTGACAGGCTCT  1080
      P Q E V H N H Q V A Q E A H A V I E R P U L S R K N R U K L K Y N U Q G L T G S   (327)
1081  GACAATGAAATTAAGAGCAGCTGATGCTGACACTGACGCCAGCTGTTTGGCGAGGCAGGGAGTGAGCAGTCAGTCGGTCTCTGACACTGTGATGAGGCGTTACCAACCTCCTGACGGTGA  1200
      D N E I K S S U C U H U R Q L F G E A G S E Q S V G L U H C D E A L P T S U R U   (367)
1201  CACGGGCTGATAGGCGATGCAGTCAATGACGTTAGGGAGACCTGACAGTGACGCTTGCCTCACGCTGCCTGACAGGAGCCTCAGCCAGCCCAGTGAGCCTGACCCCCAGGTGTTGTTAGC  1320
      H G L I G D A V N D V R E T U Q U R L P H A A U Q E P Q P A Q U A U P P G V V S   (407)
1321  TGAGGGTGAGAGCAGCTGTAAGCTGATACTGATATCAGCTTCATATTAAAACCAGTCAGCAACACCGGGGCCAAATGCTCTTTTTACCAACAAGTATTACCCTGAGAGAGGAGGGTGGGC  1440
      U G U E Q L *                                                                                                   (413)
1441  ATTCCTGACAGTAGAGCAATGGGTTAAATTAGAGTCCAGCAGCTATTGTTAGCTAATCATCGAAGTAATTTGAATATGATTTAAAGAATATCACAATTTTAACTATCTCAATTTATCTGT  1560
1561  CAGTGGGTTGCCCTCCTCCTTTCTCAGGAGTGTATGTGATTGTCATGTTTTCAAATTACCCCCACCATTATGTTTTTTCCATCCTTTATGAAGGCAGGTGCAGAAACTATGATCTAGTTG  1680
1681  CATCTGTCTGATGTTTGGCCATACAGAGCAGAGAGGGTTGTTCAAAACATGATAAACGCTTCAAAGCTTTCAAAGTTCAGAAGTAGAAATGACACAAAAAACTGCAAATAAAAATAAACG  1800
1801  TAATTTAGAAGGAATAGTAGACAAGAGCATCACAGTTTATGAATTAGATAAAAATATGTCTTAAGGTTGAATTTACCTTTTTATTAGGGAAGCCCAACAGCACTGATCCATTACTGAGGA  1920
1921  AAACCCCCAAAAGACATTTAAATTTAAATCTAAAAACTATAGACCAATCATGTTTTTTTTATTGGGCATTCTATCGATTATCATTCTTAAGTACATATATAACAGCAGTAACTAGCAGAT  2040
2041  GTTTTGTGCACAAGCTTTAACATATCTCAGGTGTGACTTTTTCTATAAGAGAAAATCTTTAAATTTTGAGTACTAACAGTTCAATTTATTCTTTCACTTTTCTTTTTACCAAATTTGGTA  2160
2161  TTTAGCCTTCAAAGTTCACACATTGCTACAAGGCTTTTTTCTTTTTTTGAAGTTCAGGAAGACAGCAAGGAAAAACGAGAGTAAAGCAATGAATGACTGTTTGAATACGCTCTGTTCTTA  2280
2281  ATGAAGGTTTCAGTGGGAAGACATCAGTCACCCTGAAAACCCGAGGATCGGACAGAGAACTGTATTGGAATTGTATGCAAGGTGAAAAATGAGAGTTGAATAAAGATGGCTCAATTACAA  2400
2401  AAAAAAAAAAAAAAAAAAAAAAAAAAA  2427
```

Figure 5.3 The full-length cDNA encoding the Nile tilapia *On*-SEPP gene. Selenocysteines (U) are circled. Histidine-rich motifs are shown with gray highlighting. Instability motifs (ATTTA) are double-underlined. Predicted SECIS elements are underlined. Polyadenylation signal (AATAAA) is shown in bold and italic.

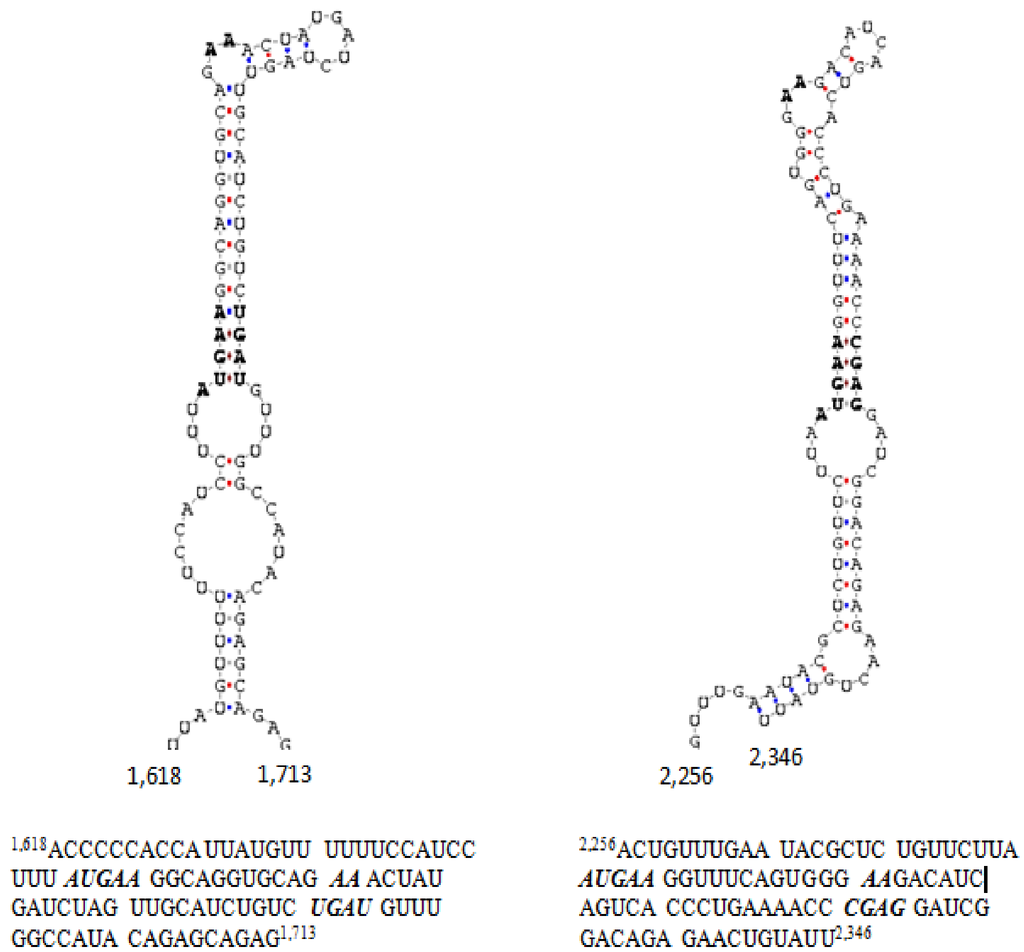

Figure 5.4 Selenocysteine insertion sequence (SECIS) elements of the *On*-SEPP gene. SECIS elements were predicted with the SECISearch 2.19 program.

```
Human         MWRSLGLALALCLLPSGGTESQDQSSLCKQPPAWSIRDQDPMLNSNGSVTVVALLQASUYLCIIEASKLEDLRVKLKKEGYSNISYIVVNHQGISSRLKYTHLKNKVSEHIPVYQQEENQ 120
Rat           MWRSLGLALALCLLPYGGAESQGQSPACKQAPPWNIGDQNPMLNSEGTVTVVALLQASUYLCLLQASRLEDLRIKLENQGYFNISYIVVNHQGSPSQLKHAHLKKQVSDHIAVYRQDEHQ 120
Chicken       MWAGLGLALVLCLLPGGGTESQR----CQEPPEWHIGEESPMLNARGSVAVVALLQASUYLCLLQASRLEDLRVKLENEGLVNISYVVVNHQSPHSQKKFHLLQESVSDHITVYQQDDHQ 116
Frog          MWKGFALALALCLLPWGGAESQGHRSRCKQPPDWSIGDQNPMIQSAGKVTVVALLQASUYLCLLQASRLEDLRLKLEKEKLVGISYVVVNHQGRHSRAKYDLLKSKVSEHIPVYQQEENQ 120
Nile tilapia  MWAGLSLLLTLCLLHGGGAESEGGGPRCQLPSDWRIGDVEPMKGSVGRVTVVALLQASULFCLVQASRLDGLQQKLERQGLKNVVYMVVNHQGEQSRHLHPLLEAKLSKNIILYKQDGHQ 120
Zebrafish     MWKALSLTLALCLLVGCSAESETEGARCKLPPEWKVGDVEPMKNALGQVTVVAYLQASULFCLEQASKLNDLLLKLENQGYPNIAYMVVNNREERSQRLHHLLQERLLN-ITLYAQDLSQ 119
              ** .:.* *.****   .:**:    *: .. * : : .**  : * *:*** ***** :*: :**:*:.*  **:.:   .: *:***::   *:  .  *:  : . * :* *:  *

Human         TDVWTLLNGSKDDFLIYDRCGRLVYHLGLPFSFLTFPYVEEAIKIAYCEKKCGNCSLTTLKDEDFCKRVS------LATVDKTVETP---SPHYHHEHHHNHGHQHLGSSELSENQQPGA 231
Rat           TDVWTLLNGNKDDFLIYDRCGRLVYHLGLPYSFLTFPYVEEAIKIAYCEKRCGNCSFTSLEDEAFCKNVS------SATASKTTEPS---EEHNHHKHHDKHGHEHLGSSKPSENQQPGA 231
Chicken       ADVWTTLNGNKDDFLIYDRCGRLVYHLGLPYSFLSFQYVEEAIKIAYCENNCGNCSYTEPDIDNICENITKKEDENLAGIEPEPEPSGQHSHHHQLHRHRHHHHREGGRHSKTQNHQA 236
Frog          PDVWSLLKGDKDDFFIYDRCGRLVQHLELPYSLLHFPYVEEAVRIAYCGDKCGECEHKIPDAD-VCKKPEE-----QPEQEKPVEEK--VERPRPHRNHHRHHRPKHSGHRHRHHNNEGG 232
Nile tilapia  PDVWQTLAGEKDDFFIYDRCGRLTHRISLPYSIIGEGHIEKAIKDTYCKRLCGDCTHESAEIPEECKDNAG------VQPDVPAEQD---DTRHDHHHGHGHGHHHGHGHHHGHGHGHHG 231
Zebrafish     PDAWQAVNAEKDDILVYDRCGRLTYHLSLPYTILSHPHVEEAIKHTYCDRICGECSLESSAQLEECKK-AT------EEVNKPVE-E---EPRQDHGH-HEHGHHEHQGEAERHRHGHH- 226
              .*.*  : ..***:::*******. :: **::::   ::*:*:: :**   **:*          *:            .   *     .    : : . * : .  .    . :

Human         PNAPTHPAPP-----GLHHHH---KHKGQH---------------RQGHPENRDMPASEDLQD-------LQKKLCRKR---CINQLLCKLPTDSELAPRS-UCCHCRHLIFEK-TGSA 315
Rat           LDVETSLPPS-----GLHHHHHHHKHKGQH---------------RQGHLESUDMGASEGLQLSL-----AQRKLURRG---CINQLLCKLSEESGAATSS-CCCHCRHLIFEK-SGSA 320
Chicken       PSESQRRHPH-----NGRRHRVFNHNRHDQ---------------IGSHEQVETLPPGEGVENLP-----RVTKLUKKGKTICKNQLTUNWQTASDSTTSS-UCCHCRHLLFEE-LGNS 328
Frog          QAAEVDAFQT-----NNRAGS--HNGQGQS---------------VVPQSEVVFVPQREADVPVL-----ALQPUKKAKSU--KKQYLUEWREDAGKAFNS-UCUHURQLSFEI-AQNE 320
Nile tilapia  DNQDVHPHGHGSDHNNGHHHRNHDGADQTQHGVRPHGHFHEGDMPQTQHHFDLGQIPQEVHNHQVAQEAHAVIERPULSRKNRUKLKYNUQGLTGSDNEIKSSUCUHURQLFGEAGSEQS 351
Zebrafish     -----HPHHH------HHHHRGQQQVDVDQ--------------QVLSQVDFGQVAVETP----------MMKRPU-AKHSRUKVQYSU--QQGADSPVAS-UCUHURQLFGGEGYGRV 306
                                 :                          : :  :                           :       :     *  * * *:*

Human         IT-UQCKENLPSLCSU---QGLRAEENITESCQURLPPAAUQISQQLIP------TEASASURUKNQAKKUEUPSN----- 381
Rat           IT-UQCAENLPSLCSU---QGLFAEEKVIESCQCRSPPAAU-HSQHVSP------TEASPNUSUNNKTKKUKUNLN----- 385
Chicken       IT-UQCRGALPNSCRU---HGQLLAEDITESUQURLLTAAU-ESAAGGG------SETSDTUQUQERAGNUAUKTN----- 393
Frog          VA-URCQEALPASUQU----QELLSDSLSESUQURLSAAAU-HSHSTGLPELDTESETNAPUAUPQEAENUQUKELURFLM 395
Nile tilapia  VGLUHCDEALPTSURUH-GLIGDAVNDVRETUQURLPHAAU-QEPQPAQUAU---PPGVVSUGUEQL------------- 413
Zebrafish     AGLUHCDEPLPASUPUQ-GLK-EQDNHIKETUQURPAPPAEUELSQPT-UVU---PAGDATUGURKK------------- 367
                *:*   **    *        : : *: * *   .*             .     * * :
```

Figure 5.5 Amino acid sequence alignment of Nile tilapia SEPP and other vertebrates. Asterisks (*) indicate residues conserved in all sequences. Colon (:) and dot (.) mark residue in high and low conserved in most sequences, respectively. The conserved selenocysteine residues are highlighted in gray. Histidine-rich residues are shown in italics.

5.2 Evolutionary analyses of the *On*-TXNIP and *On*-SEPP genes

5.2.1 Evolutionary analysis of *On*-TXNIP gene

Nucleotide and amino acid sequence comparisons of the Nile tilapia TXNIP gene with the TXNIP genes of other living organisms revealed the highest identity scores, 78.5% for nucleotides and 84.1% for amino acids, with salmon. Amino acid sequence similarity was 91.8% and 91.7% with zebrafish and salmon, respectively (Table. 3). The phylogenetic tree of the *On*-TXNIP genes clearly shows that these genes are divided into two major clusters: the first group includes the TXNIP genes of higher vertebrates, while the second group, which includes the Nile tilapia *On*-TXNIP gene, contains the teleost TXNIP genes of the closely related salmon and zebrafish (Fig. 7).

Table 5.1 Comparison of the nucleotide and amino acid sequences of the Nile tilapia *On*-TXNIP gene and other vertebrate TXNIP genes.

TXNIP	Accession number	Nucleotide identity (%)	Amino acid	
			Identity (%)	Similarity (%)
Mammals				
Human (*Homo sapiens*)	NM 006472	62.2	58.7	76.8
Dog (*Canis lupus*)	XM 533037	61.2	58.0	76.8
Cow (*Bos taurus*)	NM 001101875	60.6	58.6	76.3
Mouse (*Mus musculus*)	NM 023719	62.4	56.9	76.3
Rat (*Rattus norvigicus*)	NM 001008767	61.6	58.1	76.8
Amphibian				
Frog (*Xenopus laevis*)	NM 001093153	62.1	58.6	78.3
Teleost fish				
Salmon (*Salmo sarlar*)	NM 001140431	78.5	84.1	91.7
Zebrafish (*Denio rerio*)	NM 200087	75.0	82.3	91.8

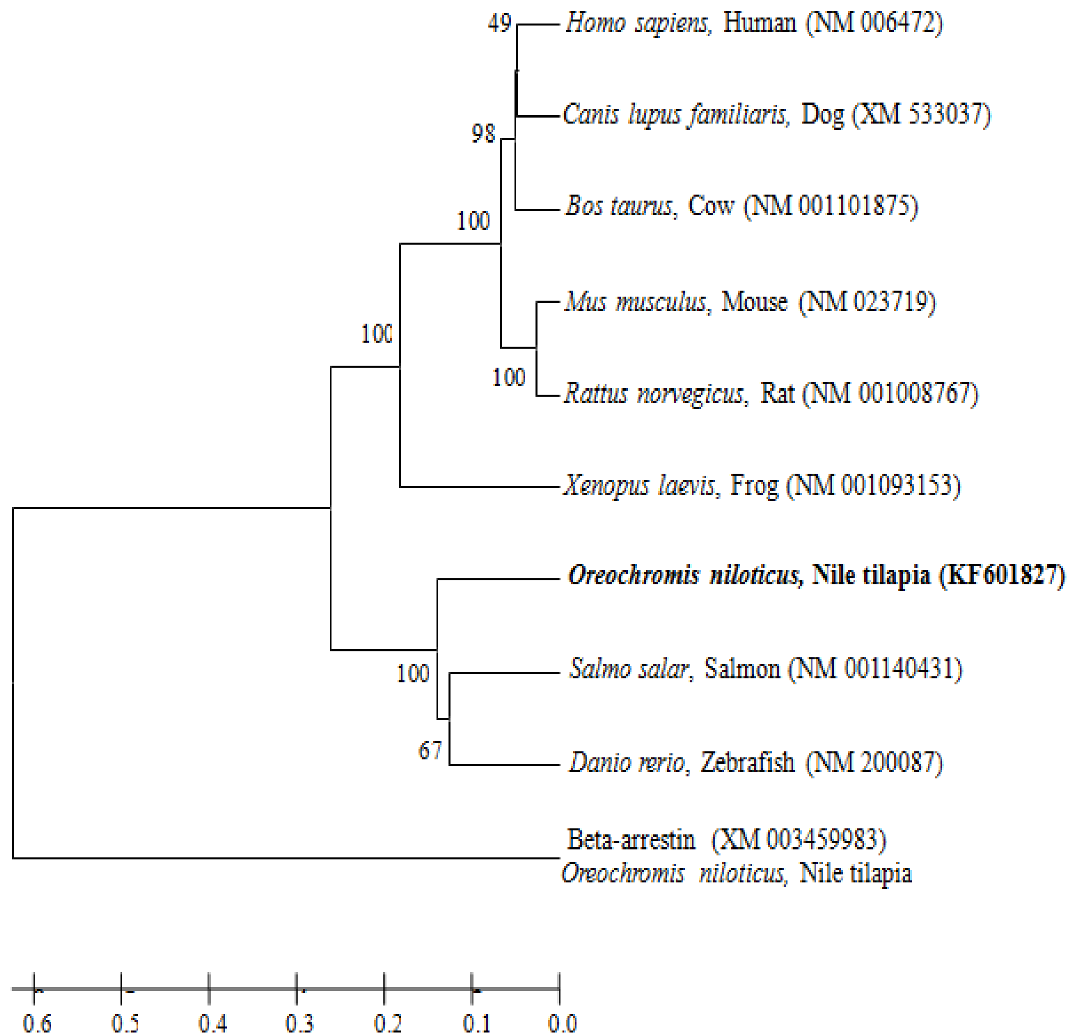

Figure 5.6 Phylogenetic tree of the Nile tilapia *On*-TXNIP gene. The GenBank accession numbers of the TXNIP genes of each species are indicated in parentheses following their scientific and common names. Beta-arrestin of Nile tilapia is used as an out-group.

5.2.2 Evolutionary analysis of *On*-SEPP gene

Nucleotide and amino acid sequence comparisons of the Nile tilapia SEPP gene with the SEPP genes of other living organisms revealed the highest nucleotide identity score of 59.9% with the closely related zebrafish. The highest amino acid identity and similarity scores were determined to be 46.6% and 61.9%, respectively, also with zebrafish (Table. 4). The phylogenetic tree of the *On*-SEPP genes was again clearly divided into two major clusters. The first group comprised the vertebrate SEPP genes, while the second one comprised the invertebrate SEPP genes. The Nile tilapia SEPP gene was closely related to the zebrafish SEPP gene in the minor subgroup of lower vertebrate SEPP genes (Fig. 8).

Table 5.2 Comparison of the nucleotide and amino acid sequences of the Nile tilapia *On*-SEPP gene and SEPP genes from other organisms.

SEPP	Accession number	Nucleotide identity (%)	Amino acid	
			Identity (%)	Similarity (%)
Mammals				
Human (*Homo sapiens*)	CAA77836	53.8	36.5	54.3
Dog (*Canis lupus*)	NP001108590	55.9	39.0	55.1
Cow (*Bos taurus*)	BAA84781	55.2	36.5	54.0
Mouse (*Mus musculus*)	CAA68140	53.2	37.5	52.5
Rat (*Rattus norvigicus*)	AAA42129	53.5	38.0	53.5
Amphibian				
Frog (*Xenopus laevis*)	NP001186825	55.3	38.3	57.6
Aves				
Chicken (*Gallus gallus*)	NP001026780	55.5	37.9	55.8
Teleost fish				
Zebrafish (*Denio rerio)*	AAI59241	59.9	46.6	61.9
Invertebrates				
Deer tick (*Ixodes scapularis*)	XP002410766	44.8	21.8	34.1
Acorn worms (*Saccoglossus kowalevskii*)	ADB22637	47.4	21.8	38.1
Urchin (*Strongylocentrotus purpuratus*)	XP001199348	35.8	19.2	29.0

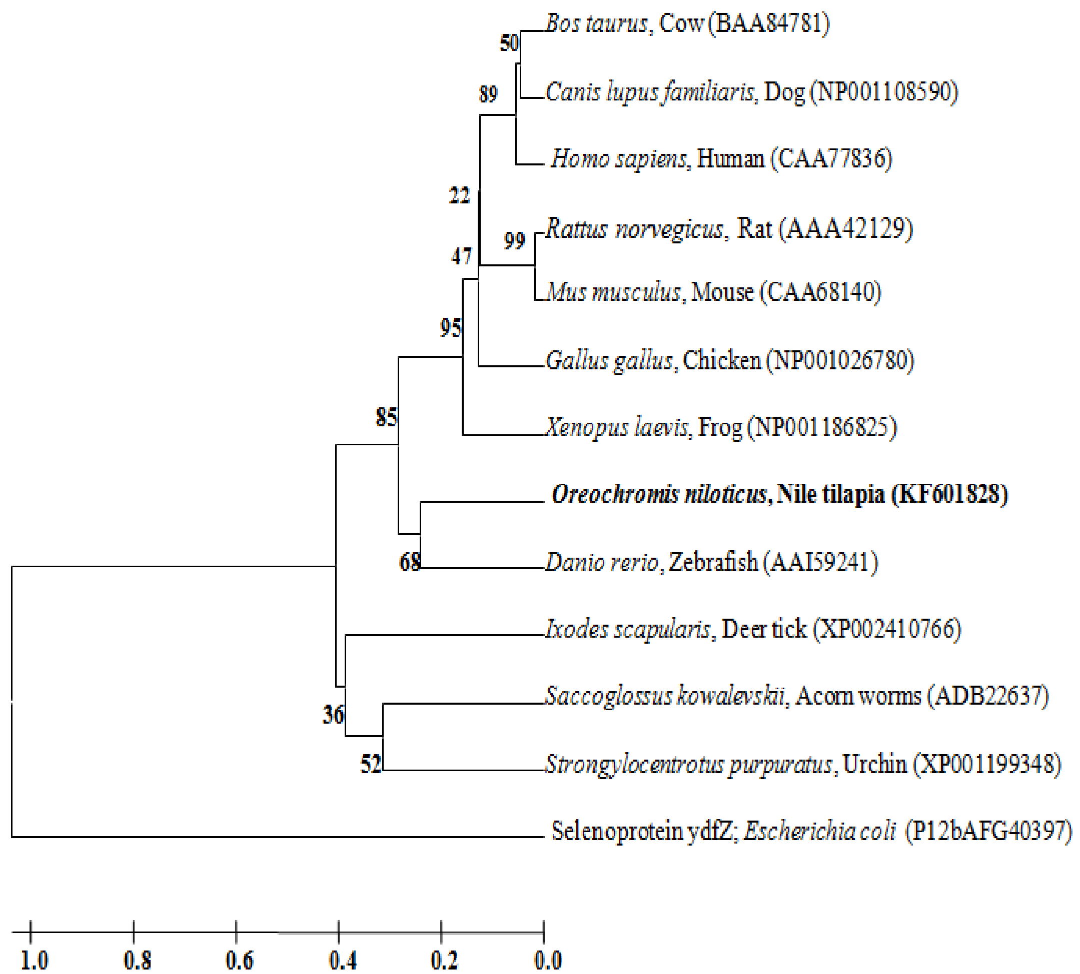

Figure 5.7 Phylogenetic tree of the Nile tilapia *On*-SEPP gene. The GenBank accession numbers of SEPP genes of each species are indicated in parentheses following their scientific and common names. Selenoprotein ydfZ of *Escherichia coli* (P12bAFG40397) is used as an out-group.

5.3 Normal tissue distribution analyses of the *On*-TXNIP and *On*-SEPP mRNA transcripts

Real-time RT-PCR was carried out to analyze the levels of the *On*-TXNIP and *On*-SEPP mRNA transcripts in various normal tissues. The analysis showed that all thirteen tissues expressed the *On*-TXNIP mRNA. The highest *On*-TXNIP expression level was detected in the PBLs, followed consecutively by the posterior kidney, anterior kidney, gills, liver and spleen (Fig. 9). Expression analysis of the *On*-SEPP mRNA transcripts revealed the highest expression level in the posterior kidney, followed by the liver and intestine; the other tissues only displayed low expression (Fig. 10).

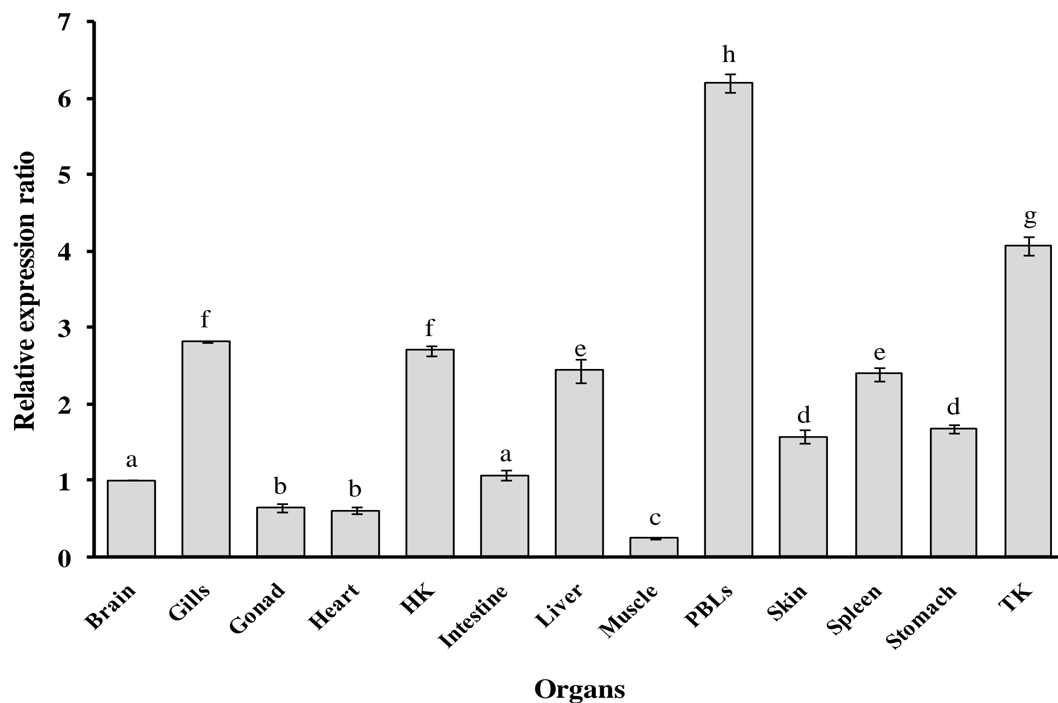

Figure 5.8 Normal tissue expression of *On*-TXNIP transcripts. Each bar indicates an organ. The examined organs are: brain, gills, gonad, heart, head kidney (HK), intestine, liver, muscle, peripheral blood leukocytes (PBLs), skin, spleen, stomach and trunk kidney (TK). $P < 0.05$ is considered significant.

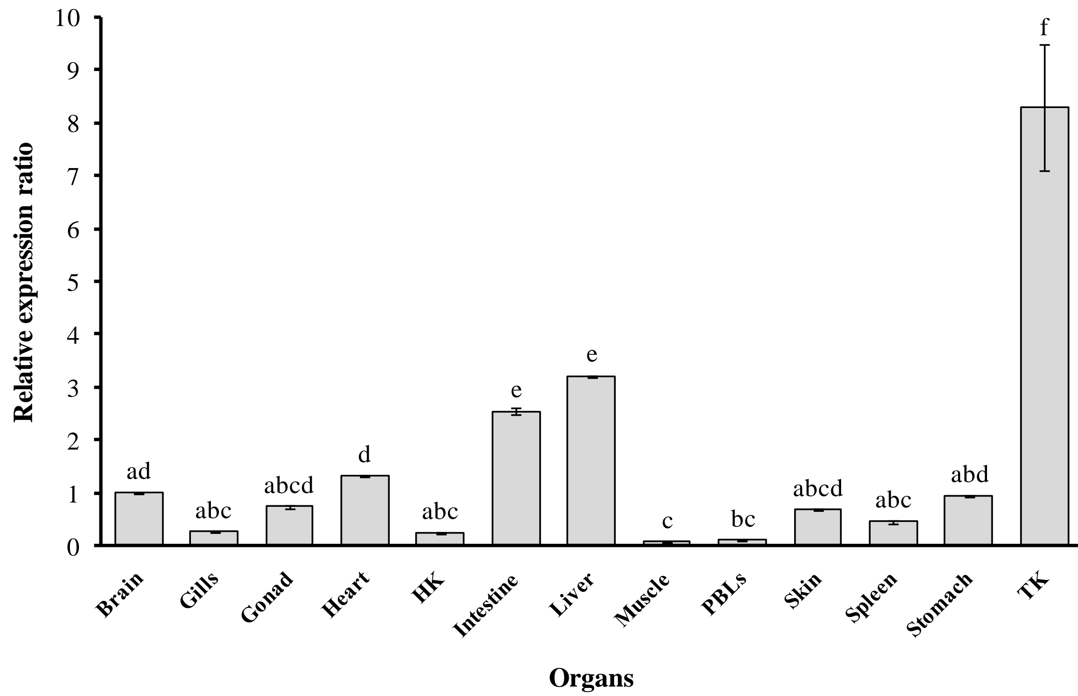

Figure 5.9 Normal tissue expression of *On*-SEPP transcripts. Each bar indicates an organ. The examined organs are: brain, gills, gonad, heart, head kidney (HK), intestine, liver, muscle, peripheral blood leukocytes (PBLs), skin, spleen, stomach and trunk kidney (TK). $P < 0.05$ is considered significant.

5.4 Effect of Ag NPs on the expression levels of cellular stress response genes

5.4.1 Expression levels of the *On*-TXNIP transcripts

Expression analysis of the *On*-TXNIP gene in fish exposed to 1, 10 or 100 mg of Ag NPs/kg fish revealed that *On*-TXNIP transcripts were up-regulated in a dose-dependent manner in treated groups at hours 6, 12, and 48 in the liver and spleen and at hours 12 and 48 in the head kidney (Fig. 11). During the early phase of exposure (6-48 hpi), expression of the *On*-TXNIP gene was dramatically changed in all examined tissues compared to later times (1st-4th wpi). In the group receiving 10 mg Ag NPs/kg, the highest expression of *On*-TXNIP transcripts was found at 24 hpi (10.48-fold induction; Fig. 11a), while in the group receiving 100 mg of Ag NPs/kg, the highest up-regulation was found at 12 hpi (9.67-fold induction; Fig. 11a). In the spleen, *On*-TXNIP mRNA expression was obviously up-regulated by 9.26-fold at 12 hpi in fish exposed to 100 mg of Ag NPs/kg; after this time point, no high expression in any treated groups was observed until the end of the experiment (Fig. 11b). In the anterior kidney, significant differences in *On*-TXNIP expression were observed at almost all time intervals, especially at 6 to 48 hpi, at which times fish groups injected with 10 and 100 mg Ag NPs/kg exhibited strong induction. Slightly differences in expression were identified from the 1st to 4th wpi (Fig. 11c).

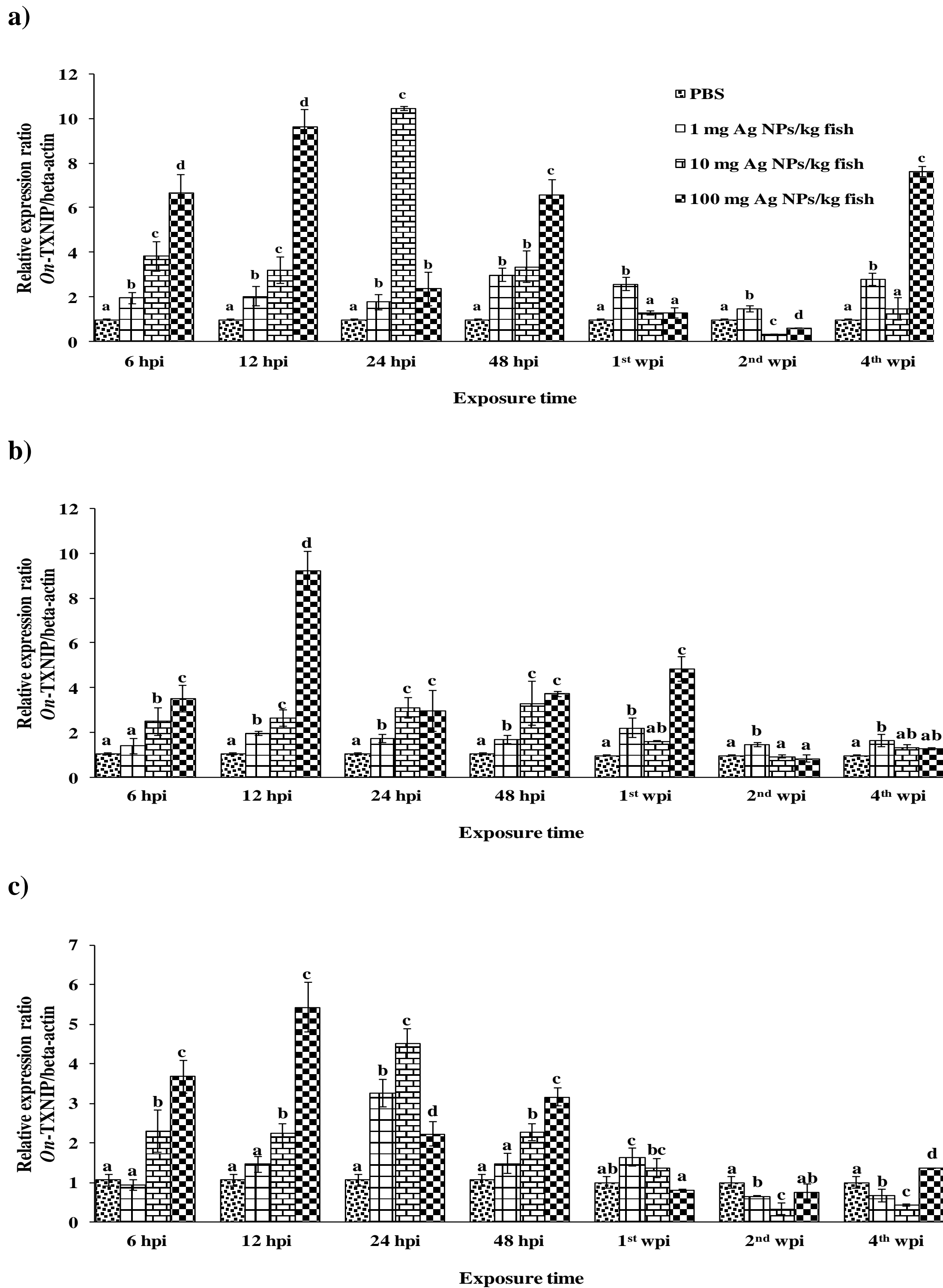

Figure 5.10 Relative expression ratio of *On*-TXNIP gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. *P*< 0.05 is significant consideration.

5.4.2 Expression levels of the *On*-SEPP transcripts

The *On*-SEPP mRNA transcript analysis demonstrated that the expression levels of *On*-SEPP in the liver were significantly up-regulated in all treated groups starting at hours 6 through 48 and down-regulated in a dose-dependent manner in all treatment groups at weeks 1, 2 and 4 after administration compared to the control (Fig. 12a). The expression levels of *On*-SEPP in the spleen were up-regulated at hours 6, 12 and 24 and later fluctuated independently from hour 48 through the end of exposure (Fig. 12b). In the anterior kidney, the expression levels of *On*-SEPP were up-regulated immediately after induction to 24 hpi and slightly different after hour 48 through the first week of exposure (Fig. 12c). However, the expression levels in all of the treated groups were significantly up-regulated compared to the control at weeks 2 and 4 after treatment ($P<0.05$) (Fig. 12c).

5.4.3 Expression levels of the *On*-HSP40B9 transcripts

The expression of *On*-HSP40B9 transcripts in fish liver, spleen, and head kidney in all Ag NPs-treated groups were significantly up-regulated ($P<0.05$) at hours 6 and 12 (Fig. 13a, b, c). At hour 48, the *On*-HSP40B9 mRNA in liver and head kidney in all doses-exposed fish was statistically down-regulated ($P<0.05$) (Fig. 13a, c). At 1st-wpi the expression of the *On*-HSP40B9 gene in fish liver was clearly up-regulated in dose-response relationship by 1.83-, 3.45- and 5.23-fold, respectively (Fig. 13a), and in spleen, the *On*-HSP40B9 mRNA was also found to high induction, especially in high dose (100 mg/kg) by 3.91-fold induction (Fig. 13b).

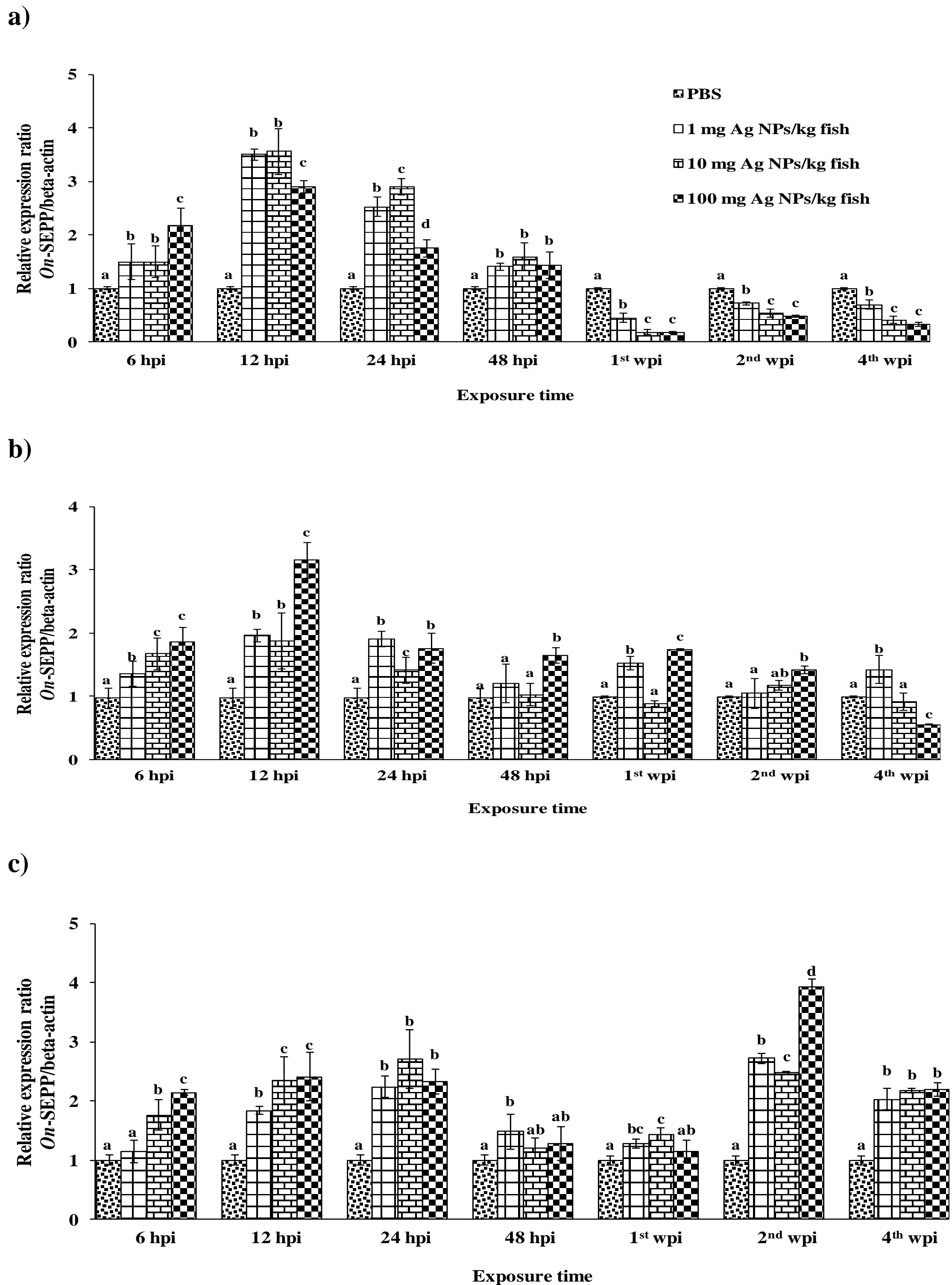

Figure 5.11 Relative expression ratio of *On*-SEPP gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. *P*< 0.05 is significant consideration.

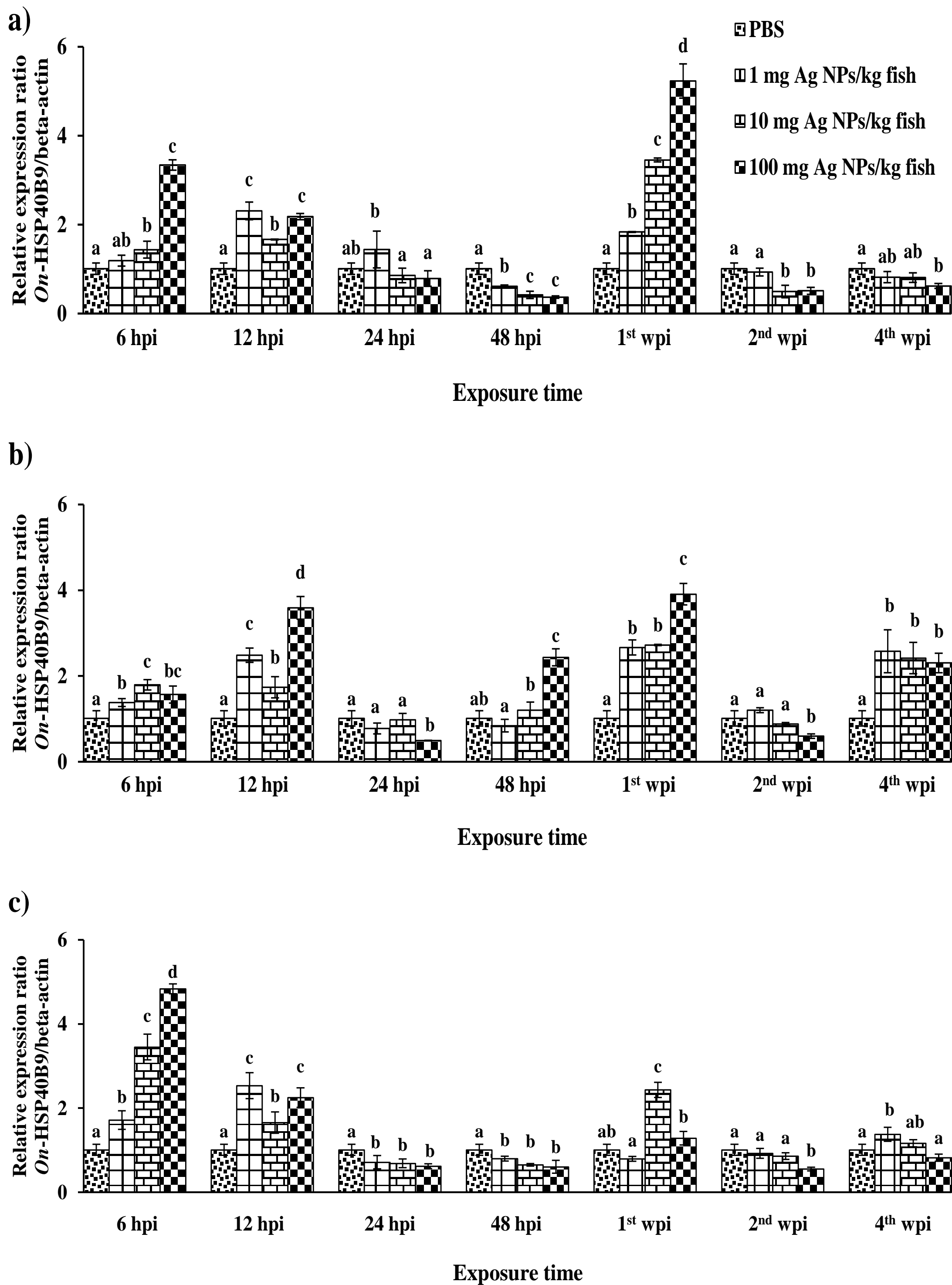

Figure 5.12 Relative expression ratio of *On*-HSP40B9 gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. $P< 0.05$ is significant consideration.

5.4.4 Expression levels of the *On*-HSP40C3 transcripts

For *On*-HSP40C3 gene, its expression in liver was observed to highly elevated in groups 10 and 100 mg Ag NPs/kg fish at only hour 6 (Fig. 14a) and significantly up-regulated *On*-HSP40C3 transcripts ($P<0.05$) in liver and spleen in all Ag NPs-exposed groups were observed at hour 12 (Fig. 14a, b). In the spleen, up-regulated expression of the *On*-HSP40C3 mRNA in dose-dependent pattern was found at 12 hpi with the highest induction by 18.42-fold in group of 100 mg Ag NPs/kg fish (Fig. 14b). Noticeably, the expression of the *On*-HSP40C3 gene in liver, spleen, and head kidney in all treatment groups was found to statistically up-regulated ($P<0.05$) at the 1st wpi (Fig. 14a, b, c). In contrast, the expression of the *On*-HSP40C3 gene in head kidney in all Ag NPs-treated groups was found to down regulation at the beginning of the monitoring times (6 hpi) continuously until hour 24 and only in groups of 10 and 100 mg Ag NPs/kg fish at hour 48 (Fig. 14c).

5.4.5 Expression levels of the *On*-HSP70 transcripts

The *On*-HSP70 transcripts in fish liver in all Ag NPs-exposed groups were dramatically suppressed at the beginning of observation (6 hpi) continuously until hour 48 (Fig. 15a). In contrast, the *On*-HSP70 mRNA in spleen and head kidney was found to inductive expression (Fig. 3b, c). In the spleen, the significantly up-regulated *On*-HSP70 transcripts ($P<0.05$) in Ag NPs-exposed groups were clearly observed at hours 6 through 48 (Fig. 15b). In head kidney, the statistically elevated *On*-HSP70 mRNA ($P<0.05$) in Ag NPs-treated fish was found at hours 6, 24, 48, weeks 1 and 2 after administration (Fig. 15c).

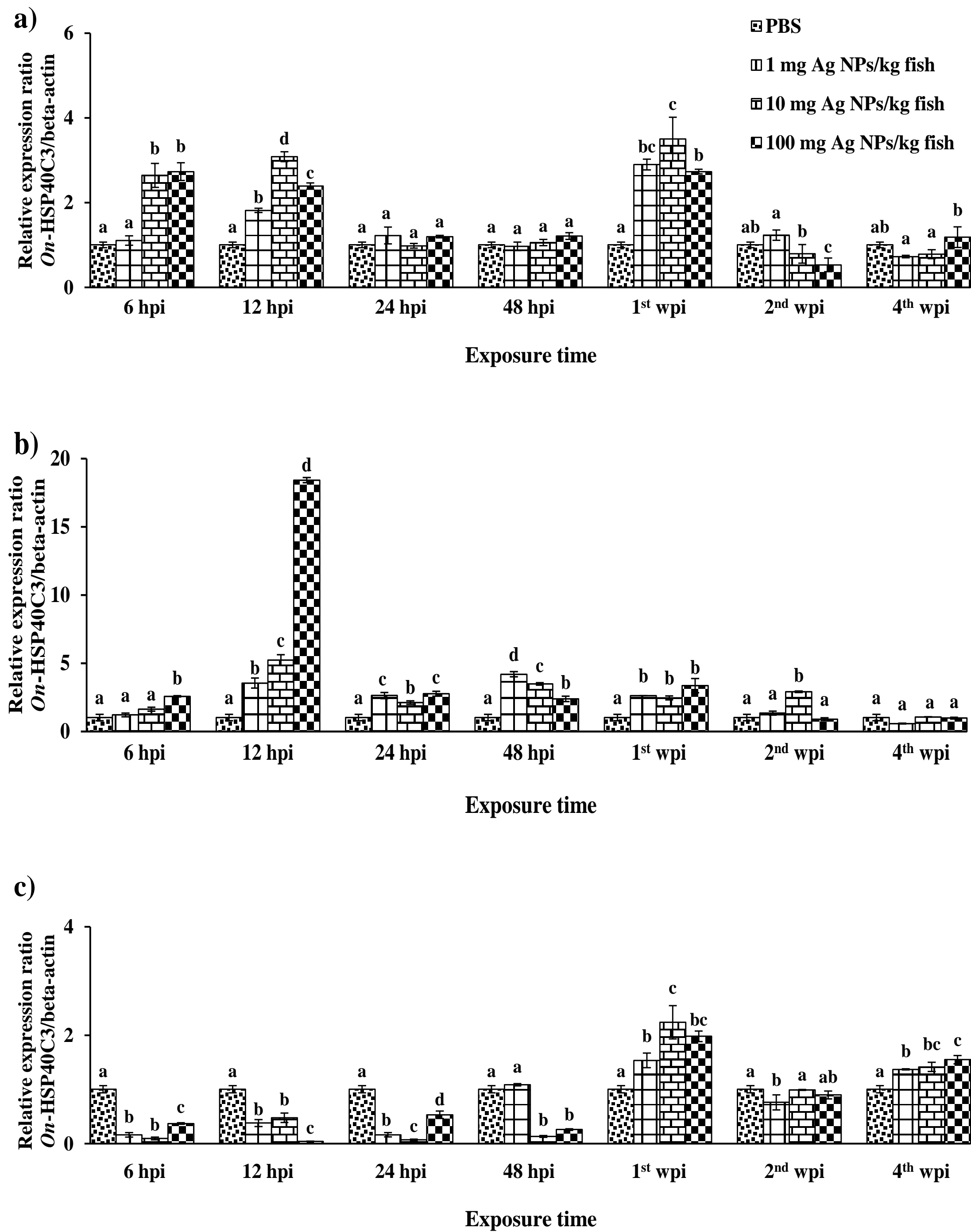

Figure 5.13 Relative expression ratio of *On*-HSP40C3 gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. *P*< 0.05 is significant consideration.

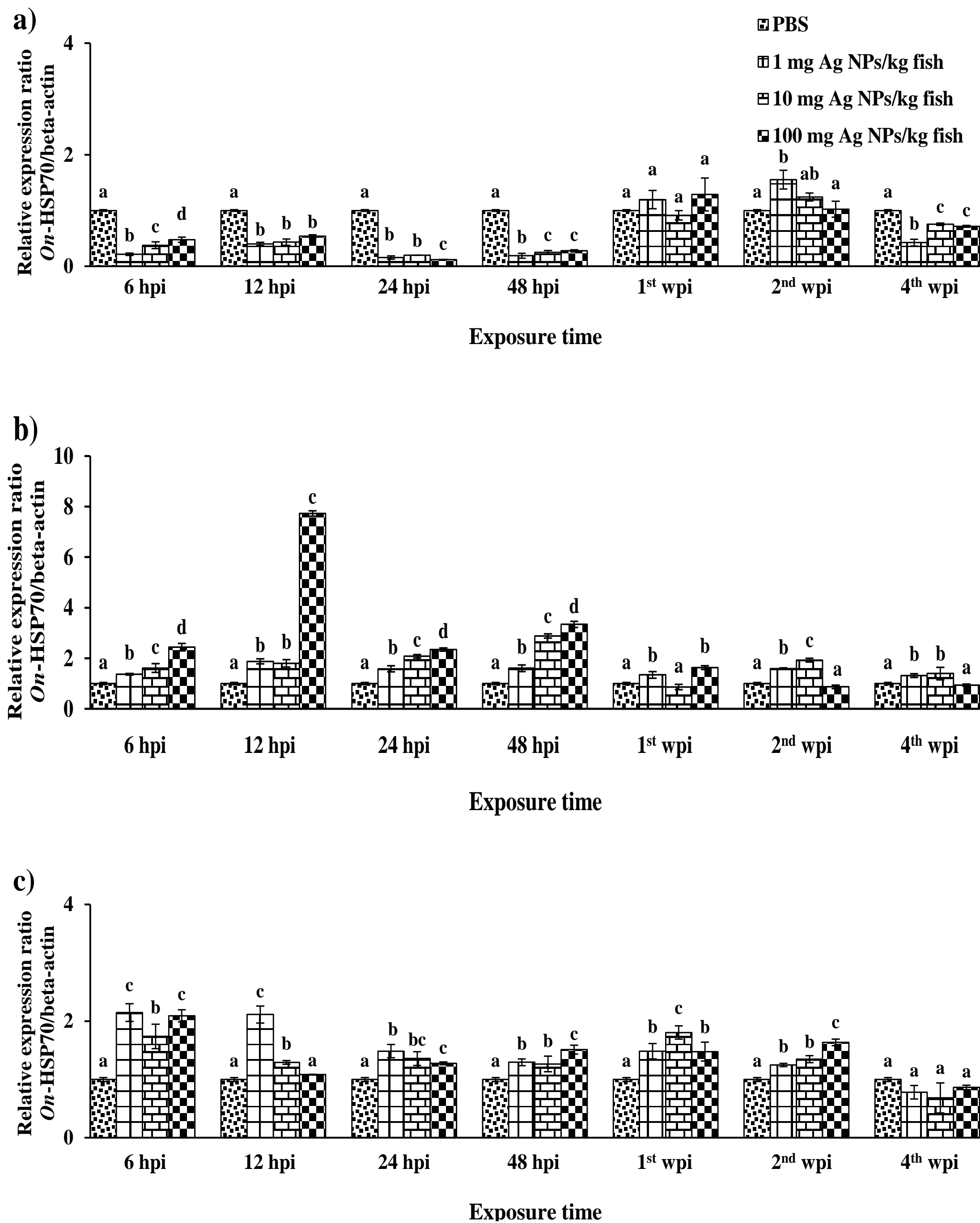

Figure 5.14 Relative expression ratio of *On*-HSP70 gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. $P< 0.05$ is significant consideration.

5.4.6 Expression levels of the *On*-HSP90α transcripts

The expression of *On*-HSP90α transcripts in fish liver and spleen in all groups of Ag NPs exposure was highly significant up-regulation ($P<0.05$) at 6 and 12 hpi (Fig. 16a, b). Dose-dependent response of *On*-HSP90α expression was obviously observed in spleen at hour 6 (Fig. 16b). In head kidney, the *On*-HSP90α transcripts at 100 mg Ag NPs/kg fish were highly expressed at 6 hpi and later suppressed at hours 12 to 48 (Fig. 16c). At 48 hpi and the 2nd wpi for liver and at 24 and 48 hpi for head kidney, the *On*-HSP90α mRNA in Ag NPs-exposed fish was observed to statistically repressive expression ($P<0.05$) (Fig. 16a, c).

5.4.7 Expression levels of the *On*-HSP90β transcripts

The up-regulation of *On*-HSP90β gene in fish exposed to Ag NPs was clearly observed in liver, spleen, and head kidney at early phase of exposure (hours 6 and 12) (Fig. 17a, b, c). In the liver, the expression of the *On*-HSP90β mRNA in Ag NPs-exposed groups, which displayed dose-dependent relationship, was dramatically noticed at 6 and 12 hpi (Fig. 17a). The *On*-HSP90β transcripts in spleen in all groups of Ag NPs exposure were initially suppressed at hour 24 and the 1st wpi (Fig. 17b). In head kidney, dose-dependent response of expression of the *On*-HSP90β gene was only observed at week 2 after exposure (Fig. 17c).

5.4.8 Expression levels of the *On*-MT transcripts

The significantly suppressed *On*-MT mRNA ($P<0.05$) was found in fish liver in all Ag NPs-exposed groups at the beginning of the monitoring times at hours 6 to 48 (Fig. 18a). In spleen, the *On*-MT gene in all groups of Ag NPs exposure was statistically down-regulated ($P<0.05$) at hours 6 and 24 (Fig. 18b). The *On*-MT transcripts in head kidney in fish exposed to Ag NPs were clearly observed to significantly elevated ($P<0.05$), respecting to dose-dependent manner, at hour 12 and continuously expressed later at 24 hpi until the 4th wpi, except week 2 that only 100 mg Ag NPs/kg fish showed statistical significance ($P<0.05$) (Fig. 18c). At week 4, in head kidney, *On*-MT expression was appealingly inverted in dose-dependent manner (Fig. 18c)

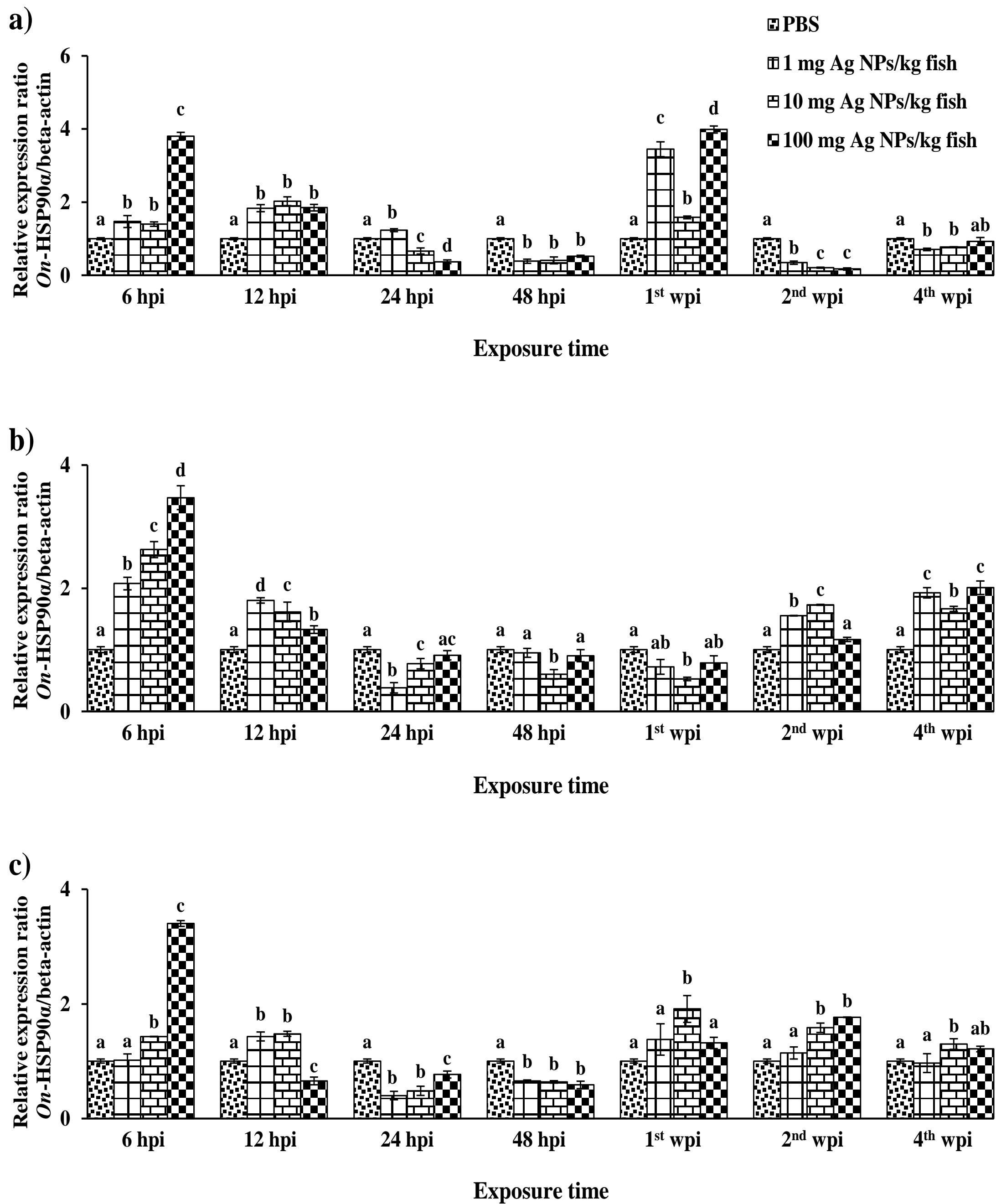

Figure 5.15 Relative expression ratio of *On*-HSP90α gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. $P< 0.05$ is significant consideration.

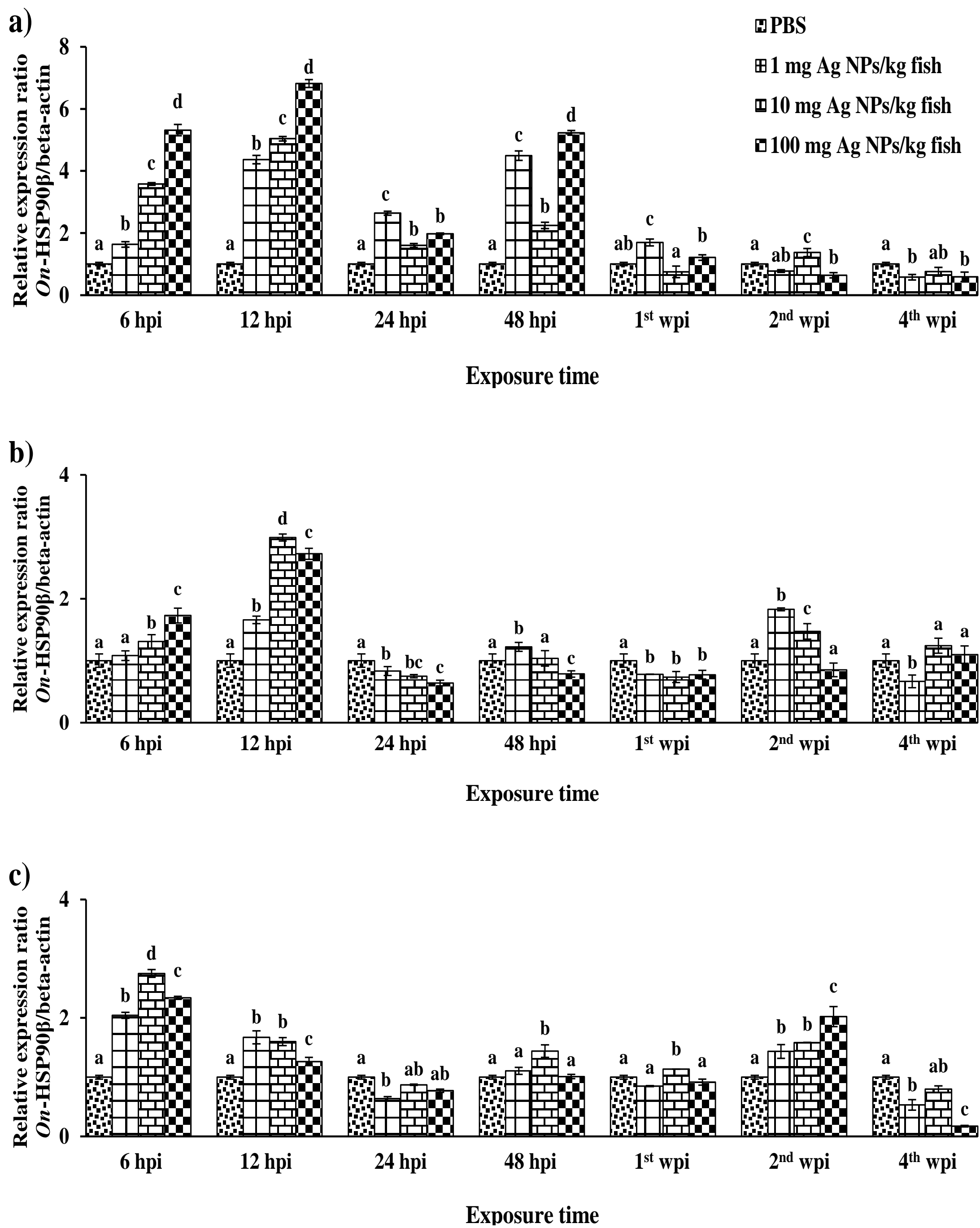

Figure 5.16 Relative expression ratio of *On*-HSP90β gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. $P < 0.05$ is significant consideration.

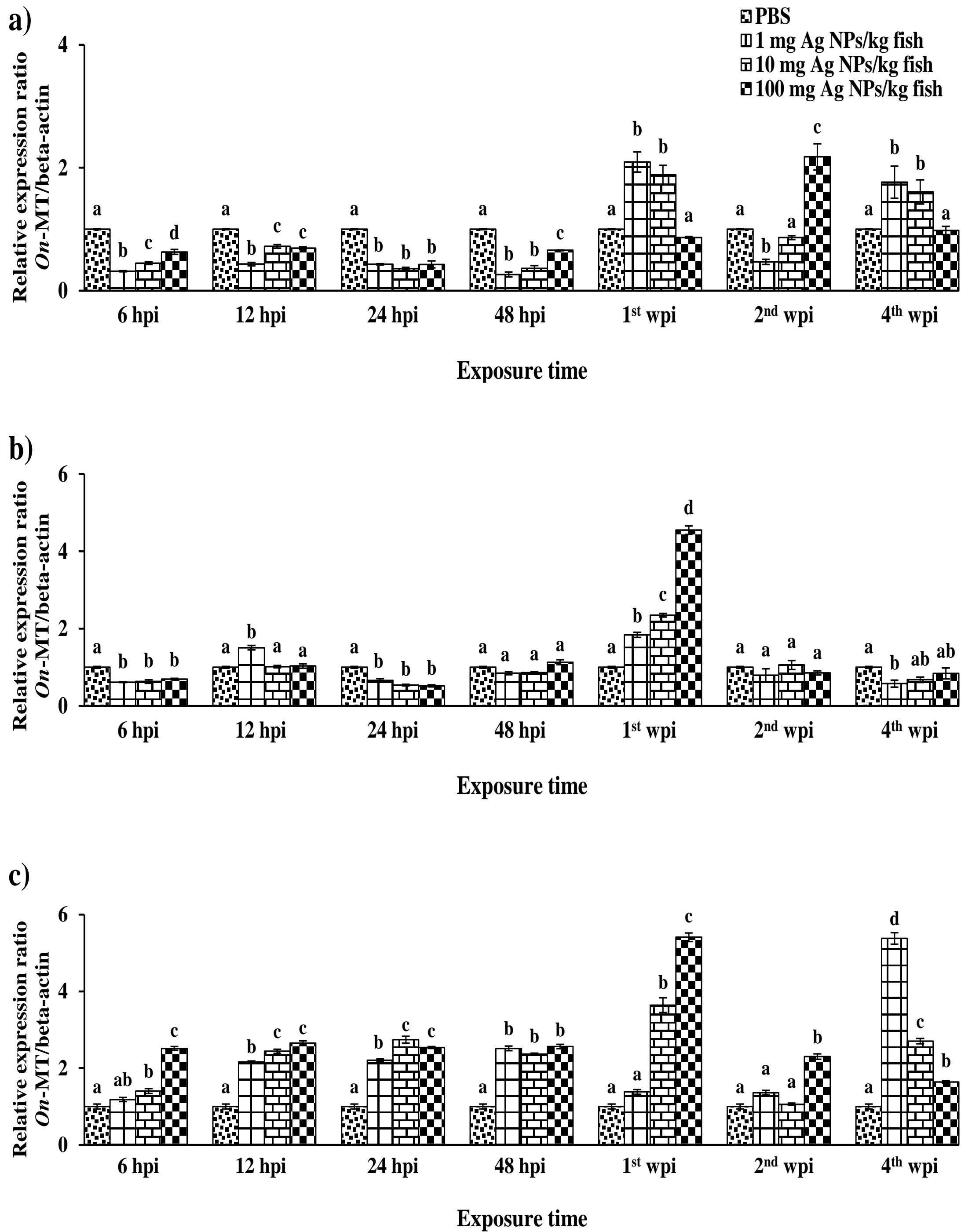

Figure 5.17 Relative expression ratio of *On*-MT gene in Nile tilapia liver (a), spleen (b) and anterior kidney (c) exposed to 1, 10 and 100 mg Ag NPs/kg fish at 6, 12, 24, 48 hpi, 1st, 2nd and 4th wpi. Different English alphabets in each bar indicate significant differences. $P< 0.05$ is significant consideration.

5.5 Histopathological changes induced by Ag NPs in the liver and spleen

Liver histopathology of Nile tilapia exposed to various concentrations of Ag NPs revealed that increased doses of Ag NPs correlate with increased liver abnormalities (Fig. 19). Liver tissues from the PBS-treated group (Fig. 19a, e, i) appeared homogeneously flattened, which is typical of normal fish liver cells. Abnormal changes were observed in the 1, 10 and 100 mg/kg Ag NP-treated groups, especially at the highest dose (100 mg Ag NPs/kg fish). The liver tissue damage became more severe over time and included enlargement of the hepatocytes, increased blood congestion along the sinusoidal area and central vein, fatty degeneration, Kupffer cell infiltration, bi-nucleated cells and necrosis (Fig. 19h, l). At the lowest dose (1 mg/kg) (Fig. 19b, f, j), hepatocyte swelling and increased Kupffer cell infiltration were observed at the 1st and 2nd wpi. Focal necrosis and liver exudates were obviously found in all fish treated with 10 or 100 mg Ag NPs/kg fish at all time-points. At the 2nd and 4th wpi (Fig. 19h, l), the liver tissues of fish exposed to 100 mg Ag NPs/kg fish displayed enlarged sinusoids and more congestion; this congestion occurred not only in the sinusoids but also in the central vein. Histopathological observation of the spleen revealed little change. Spleen histology in the control group revealed normal white and red pulps (Fig. 20a, e, i). The spleen tissues of all Ag NP-exposed fish revealed a dose-dependent thickening of the capsule layer, especially in fish exposed to 100 mg Ag NPs/kg fish (Fig. 20j, k, l).

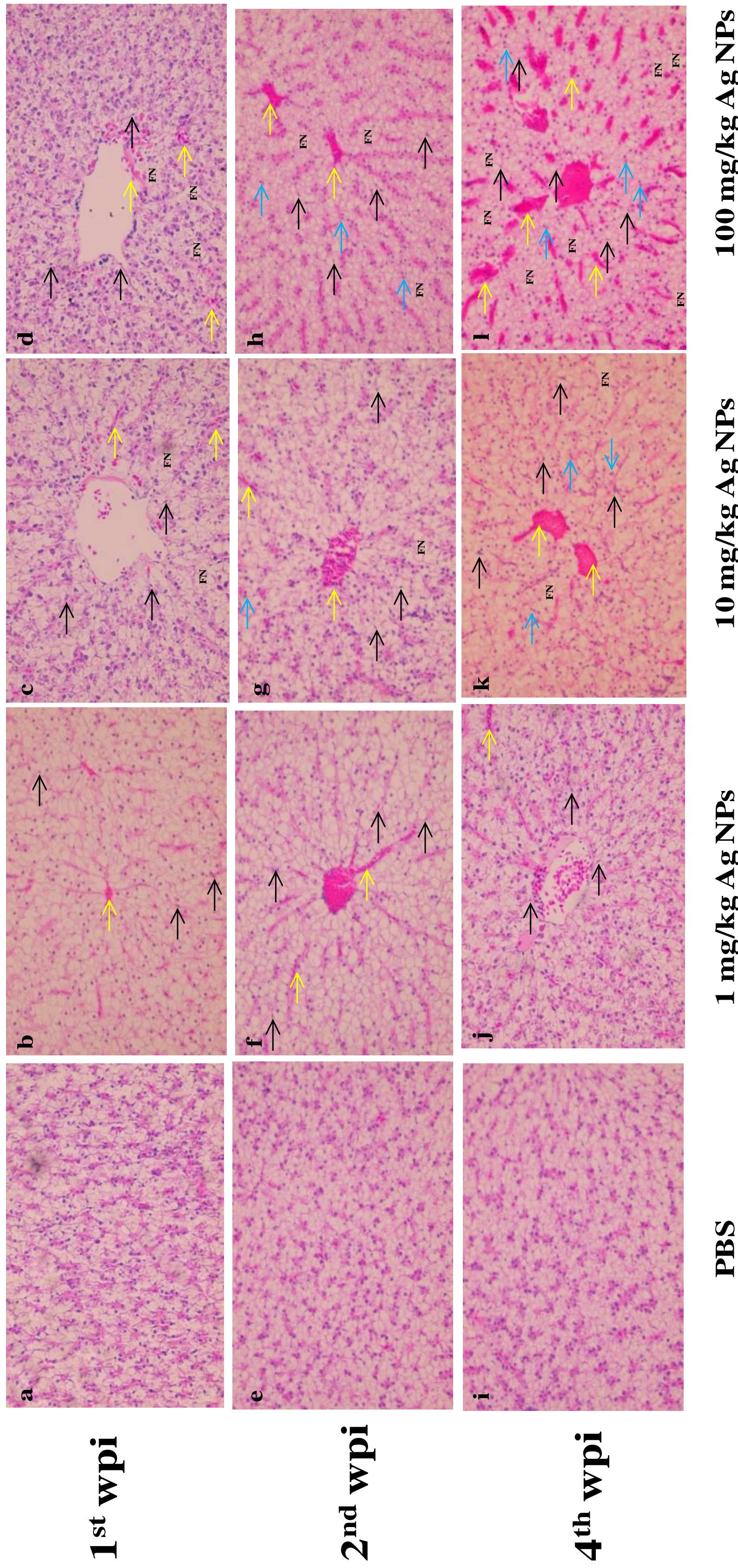

Figure 5.18 Histopathological abnormalities of liver (100x) in Nile tilapia exposed to various concentrations of Ag NPs at various time points [1st, 2nd and 4th week post injection (wpi)]. Normal liver tissues in fish injected with PBS are indicated as controls (a, e, i). Fish liver tissues were exposed to 1 mg/kg (b, f, j), 10 mg/kg (c, g, k) and 100 mg/kg (d, h, l) Ag NPs. Black arrow indicates Kupffer cell infiltration. Yellow arrow points to blood congestion. Blue arrow indicates bi-nucleated cell. FN indicates focal necrosis.

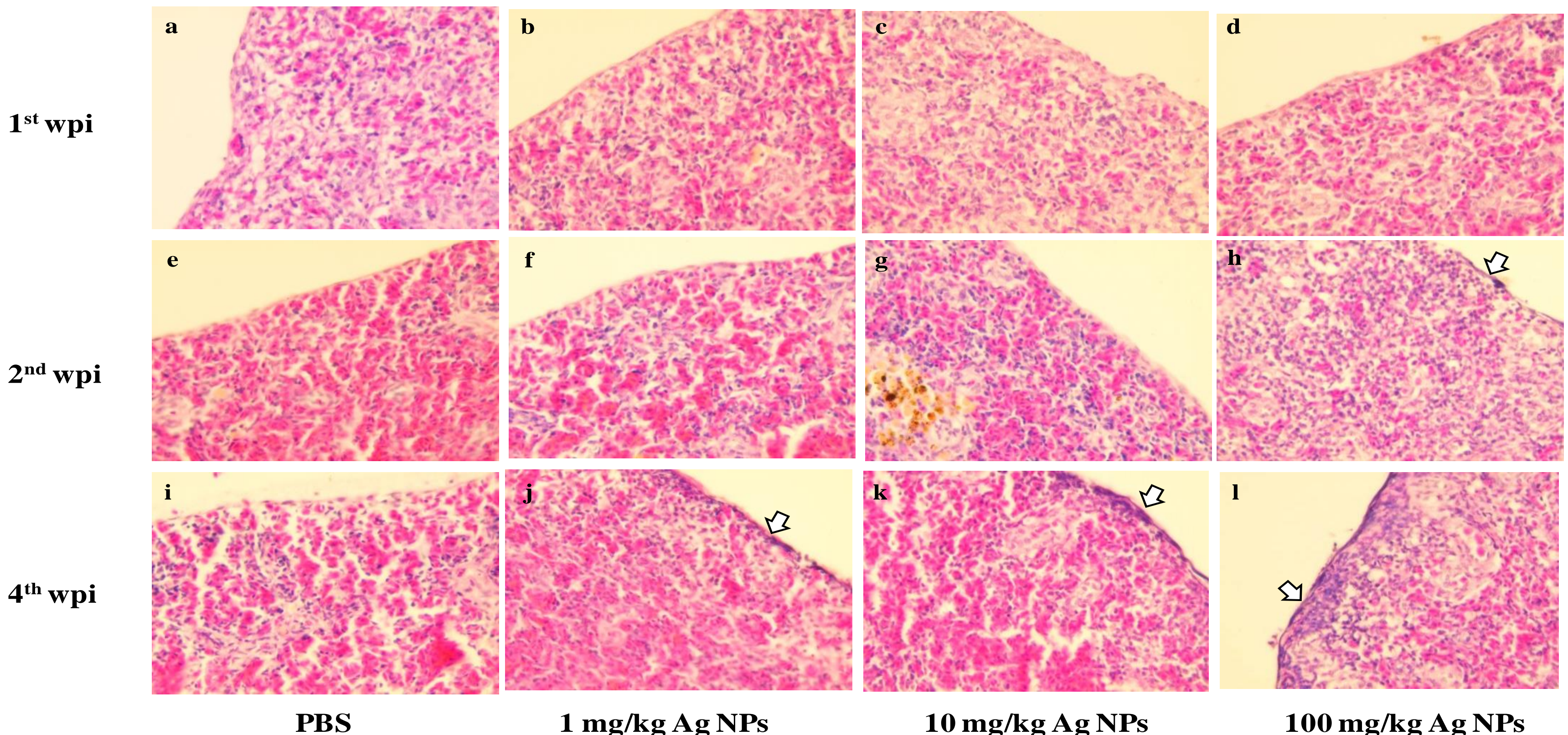

Figure 5.19 Histopathological abnormalities of spleen (100x) in Nile tilapia exposed to various concentrations of Ag NPs at various time points [1st, 2nd and 4th week post injection (wpi)]. Normal spleen tissues in fish injected with PBS are indicated as control (a, e, i). Fish spleen tissues were exposed to 1 mg/kg (b, f, j), 10 mg/kg (c, g, k) and 100 mg/kg (d, h, l) Ag NPs. White arrow indicates the thickness of the capsule layer.

5.6 Effects of Ag NPs on Nile tilapia hematology

The result of %Hct levels revealed that at 1st wpi, 10 and 100 mg/kg Ag NPs-exposed groups were found to be significantly decreased in %Hct ratio and continuously declined at weeks 2, 6 and 8 compared to control. The %Hct values in fish exposed to 1 mg Ag NPs/kg fish initiated decrease at week 2 and were found to be statistically prolonged diminished until week 8 (Table. 5). The result of amounts of RBCs revealed that fish in Ag NPs-exposed groups (1, 10 and 100 mg/kg) showed significantly continuous decrease (P<0.05) in number of RBCs compared to control at weeks 1 until 8 (Table. 6). Total white blood cell (WBC) count showed that only at 1st-wpi, amounts of WBCs of fish in Ag NPs-treated groups (1, 10 and 100 mg/kg) were statistically decreased (Table. 7). However, at weeks 2 until 8 the number of WBCs in each group was not significantly different (P>0.05).

Table 5.3 Percent of hematocrit (%Hct) in Nile tilapia exposed to 1, 10 and 100 mg Ag NPs/kg fish at 1st, 2nd, 4th, 6th and 8th wpi. Significantly different English alphabets on means±SD in each column indicate significant differences. P< 0.05 is significant consideration.

Experimental group	%Hematocrit				
	1st wpi	2nd wpi	4th wpi	6th wpi	8th wpi
0.85% NaCl	28.30 ± 1.53^{a}	26.70 ± 1.53^{a}	26.70 ± 1.53^{a}	28.70 ± 1.15^{a}	28.00 ± 1.00^{a}
1 mg Ag NPs/kg fish	26.70 ± 2.08^{a}	19.30 ± 1.15^{c}	16.00 ± 1.00^{c}	19.30 ± 1.15^{d}	19.7 ± 0.58^{c}
10 mg Ag NPs/kg fish	24.30 ± 1.53^{b}	20.30 ± 2.08^{bc}	28.30 ± 1.53^{a}	22.30 ± 2.08^{c}	17.7 ± 1.15^{d}
100 mg Ag NPs/kg fish	19.30 ± 1.15^{c}	23.00 ± 2.00^{b}	21.00 ± 2.00^{b}	25.30 ± 0.58^{b}	22.00 ± 1.00^{b}

Table 5.4 Red blood cells (RBCs) count in Nile tilapia exposed to 1, 10 and 100 mg Ag NPs/kg fish at 1st, 2nd, 4th, 6th and 8th wpi. Significantly different English alphabets on means±SD in each column indicate significant differences. *P*< 0.05 is significant consideration.

Experimental group	Red blood cell count ($x10^6/mm^3$)				
	1st wpi	**2nd wpi**	**4th wpi**	**6th wpi**	**8th wpi**
0.85% NaCl	1.68 ± 0.28[a]	1.60 ± 0.20[ab]	1.65 ± 0.14[a]	1.69 ± 0.06[a]	1.66 ± 0.12[a]
1 mg Ag NPs/kg fish	0.96 ± 0.30[bc]	1.75 ± 0.13[a]	1.50 ± 0.27[ab]	1.45 ± 0.10[b]	1.45 ± 0.10[b]
10 mg Ag NPs/kg fish	1.48 ± 0.28[ab]	1.46 ± 0.17[b]	1.51 ± 0.16[ab]	1.52 ± 0.09[b]	1.50 ± 0.12[ab]
100 mg Ag NPs/kg fish	0.57 ± 0.33[c]	1.02 ± 0.11[c]	1.27 ± 0.05[b]	1.49 ± 0.11[b]	1.49 ± 0.14[ab]

Table 5.5 Total white blood cells (WBCs) count in Nile tilapia exposed to 1, 10 and 100 mg Ag NPs/kg fish at 1st, 2nd, 4th, 6th and 8th wpi. Significantly different English alphabets on means±SD in each column indicate significant differences. *P*< 0.05 is significant consideration.

Experimental group	Total white blood cell count ($x10^4/mm^3$)				
	1st wpi	**2nd wpi**	**4th wpi**	**6th wpi**	**8th wpi**
0.85% NaCl	14.67 ± 0.15[a]	12.25 ± 0.25[a]	11.25 ± 0.17[a]	11.25 ± 0.35[a]	11.50 ± 0.19[a]
1 mg Ag NPs/kg fish	13.33 ± 0.12[a]	10.25 ± 0.24[a]	10.00 ± 0.23[a]	10.25 ± 0.15[a]	10.75 ± 0.34[a]
10 mg Ag NPs/kg fish	10.50 ± 0.21[b]	12.75 ± 0.17[a]	11.00 ± 0.08[a]	9.75 ± 0.17[a]	11.00 ± 0.08[a]
100 mg Ag NPs/kg fish	10.25 ± 0.10[b]	12.25 ± 0.25[a]	10.75 ± 0.13[a]	10.25 ± 0.19[a]	10.25 ± 0.26[a]

5.7 Immunological responses in Ag NPs-exposed Nile tilapia

5.7.1 Innate immunity

The activity of phagocytic cells revealed that at high doses (10 and 100 mg Ag NPs/kg fish), exposed fish showed obviously decrease in PA at week 1 until the end of the experiment (Fig. 21a). At low dose (1 mg Ag NPs/kg fish) PA was declined at week 2 until week 6 (Fig. 21a). At week 1, PI in all doses of Ag NPs-exposed fish was not different among groups ($P>0.05$). However, at weeks 2 to 8, PI was statistically decreased in dose-dependent relationship (Fig. 21b). PE in fish exposed to Ag NPs was dramatically diminished at week 1 and continuously decreased until the end of the experiment (Fig. 21c).

5.7.2 Specific immunity

Antibody titer against formalin-killed antigen *Streptococcus agalatiae* (FASA) was conducted to investigate the effect of Ag NPs on fish specific immune response. The result revealed that the positive control (FASA immunization) showed normal pattern of the assay by the 1st highest peak was found at week 1 with antibody titer of 21.33 ± 9.24 and later declined to the lowest level at week 3. The antibody titer reached the 2nd highest peak at week 6 after the 2nd immunization was administrated (Fig. 22). The antibody titer in group of 1 mg/kg Ag NPs-injected fish was found to be sharply the 1st peak (170.67 ± 73.9) at week 2 and immediately low (6.67 ± 2.31) at week 3. After 2nd immunization, the antibody level of fish in group 1 mg/kg was slightly changed. At 10 mg/kg Ag NPs-exposed fish, the antibody titer was insensitive in the 1st immunization by no antibody level detected. When the 2nd immunization was performed the serum antibodies showed slightly response. Abnormally, the antibody titer of fish exposed to 100 mg Ag NPs/kg fish could not be detected in the 1st immunization. After 2nd immunization was given, the serum antibodies were gradually increased every week until reached to the highest peak (341.33± 147.80) at week 7.

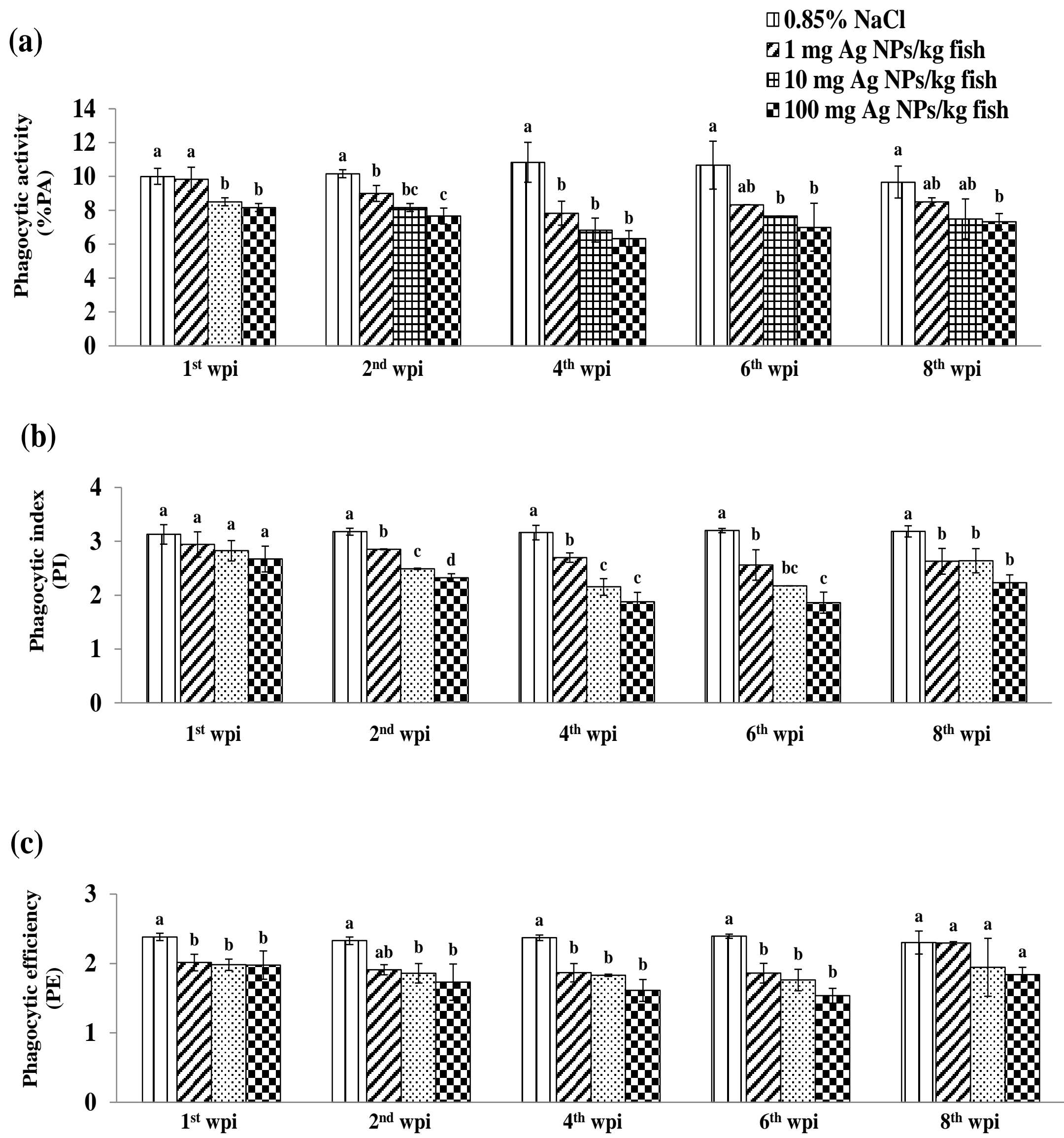

Figure 5.20 Phagocytic activity (PA) (a), phagocytic index (PI) (b) and phagocytic efficiency (PE) (c) of peripheral blood leukocytes in Nile tilapia exposed to 1, 10 and 100 mg Ag NPs/kg fish at 1st, 2nd, 4th, 6th and 8th wpi.

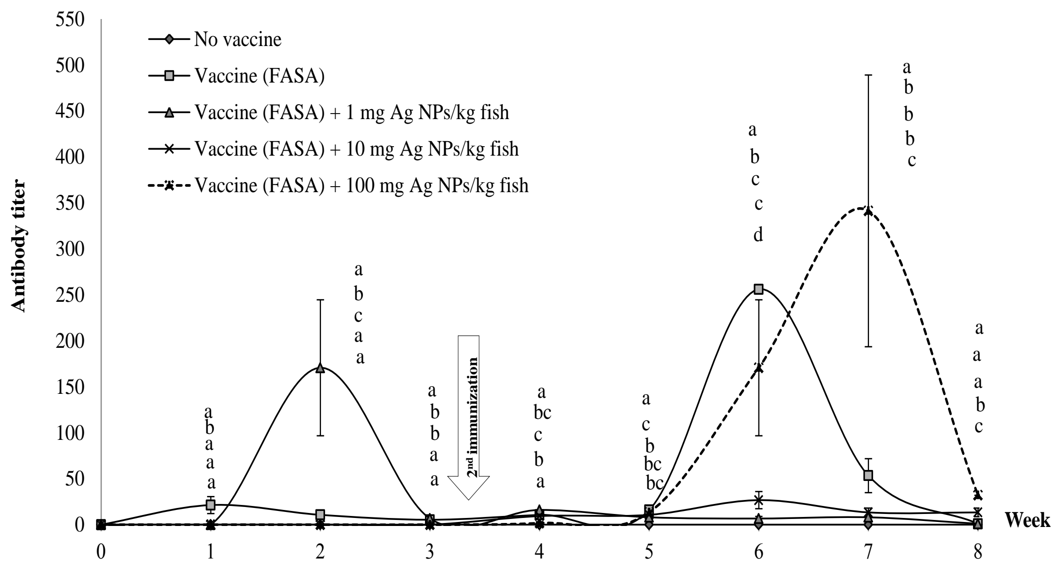

Figure 5.21 Antibody titer levels in Nile tilapia immunized with vaccine (FASA) and exposed to various doses of 1, 10 and 100 mg Ag NPs/kg fish. English letters at each time point indicate significant different (*P*<0.05). This description is used through Fig.8-10.

5.8 Bacterial resistance in different exposure conditions

5.8.1 Normal Nile tilapia exposed to Ag NPs

Significant differences of mortality among groups (*P*<0.05) were observed daily. In days 1 and 2 post infection, the result revealed that fish exposed to the highest dose of Ag NPs (100 mg/kg) showed significantly mortality with 75±7.07% at day 1 post challenge (Fig. 23), while 45±7.07%, 35±7.07% and 50±14.14% mortality were observed in control, 1 and 10 mg/kg Ag NPs-exposed groups, respectively. In day 2, mortality of control and all treated groups was 60±0.00%, 45±7.07%, 50±14.14% and 80±7.07%, respectively. Insignificant difference between control and treated groups was observed at days 3 until 7 with 70-80% mortality in all groups challenged.

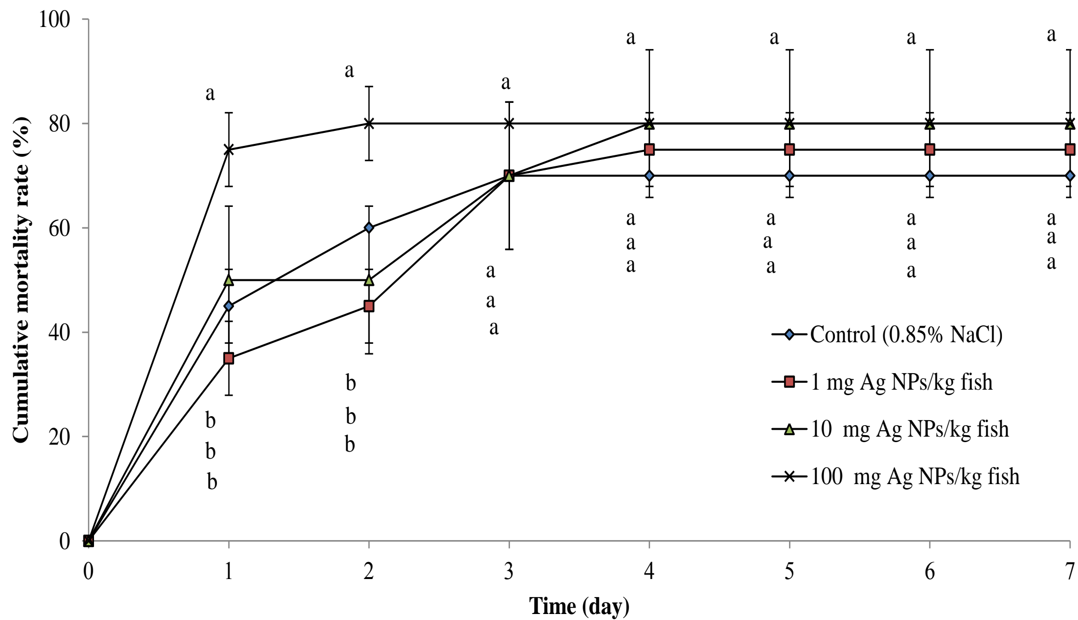

Figure 5.22 Cumulative mortality (%) in Nile tilapia challenged with 1×10^8 CFU/ml *S. agalactiae* in each group of fish exposed to 1, 10 and 100 mg Ag NPs/kg fish.

5.8.2 Nile tilapia immunized with FASA and exposed to Ag NPs

At the end of antibody titer experiment, fish in all groups were continuously challenged with virulent *S. agalatiae*. The cumulative mortality in each group revealed that at days 1 until 7 post challenge, FASA-vaccinated fish and FASA-vaccinated fish exposed to all doses of Ag NPs (1, 10 and 100 mg/kg) showed insignificantly different mortality (10-15%) among groups at day 1 post challenge until the end of the experiment (7 days) (Fig. 24). However, in control group (0.85% NaCl) showed about 45%, 60% and 70% mortality at day 1, 2 and 3-7 post injection, respectively. At day 7, significant differences of mortality (P<0.05) between control and test groups were recorded with the values of 70±7.07%, 10±14.14%, 15±21.21%, 10±14.14% and 15±7.07%, respectively.

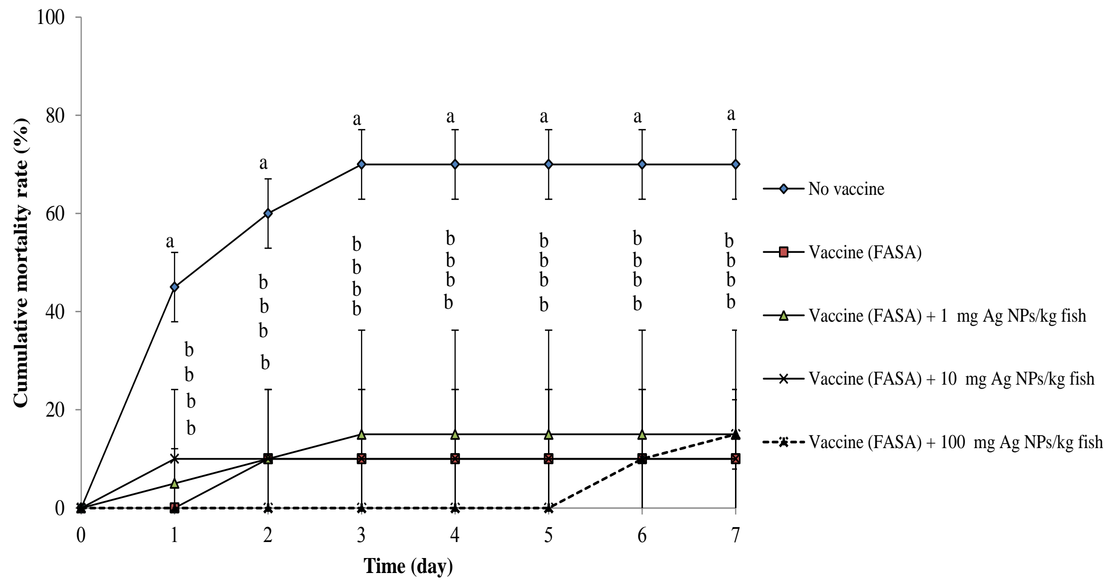

Figure 5.23 Cumulative mortality (%) in Nile tilapia challenged with $1x10^8$ CFU/ml *S. agalactiae* in each group of fish immunized with FASA and simultaneously exposed to Ag NPs at various doses of 1, 10 and 100 mg/kg.

CHAPTER VI
DISCUSSION

A great number of Ag NPs' products have been abundantly available in different product markets worldwide (Wijnhoven *et al.*, 2009; Shaw and Handy, 2011; Gunasekaran *et al.*, 2012; Hadrup and Lam, 2014). Intensively used Ag NPs take high opportunity in contamination to environments, especially in aquatic habitation. Consequently, aquatic animals are unavoidable to exposure to this toxic chemical. The effects of Ag NPs in many higher vertebrates have been previously reported (Korani *et al.*, 2011; Loghman *et al.*, 2012; Sardari *et al.*, 2012; Platonova *et al.*, 2013; De Jong *et al.*, 2013). However, such information in aquatic animals remains limited, and only 3 model fish species (zebrafish, medaka and fathead minnow) have been investigated (Choi *et al.*, 2010; Laban *et al.*, 2010; Wu *et al.*, 2010).

Direct contact, i.e. gills, mucus and fins, should be the main target to be firstly considered. However, when Ag NPs are released into aquatic resources, water chemistry is the major parameter that directly affects to the Ag NPs resulting in changes in physicochemistry of Ag NPs, including solubility, aggregation and surface charge (Fabrega *et al.*, 2011; Shaw and Handy, 2011). The water chemistry may interrupt the degree of toxicity to aquatic animals. Experimentally, we selected intra-peritoneal injection as the main route of Ag NPs' administration in order to reduce the impact of environmental factors from water on these particles.

Based on previous data, a large number of scientific reports have stated that Ag NPs have the potential to induce cytotoxicity through the generation of reactive oxygen species (ROS), resulting in the deprivation of antioxidant molecules (Chairuangkitti *et al*., 2013; Foldbjerg *et al*., 2011; Hussain *et al*., 2005; Wise *et al*., 2010). The generated intracellular ROS consequently disturbed mitochondrial membrane permeability, leading to destructive membrane potential, finally cell mortality taking place *via* mitochondrial-dependent apoptotic pathway (Hussain *et al.*, 2005; Hsin *et al.*, 2008; Teodoro *et al.*, 2011).

Chairuangkitti *et al.* (2013) declared that the toxic effects of Ag NPs on human lung cells were mediated by (1) a ROS-dependent pathway leading to cytotoxicity and (2) a ROS-independent pathway resulting in cell cycle arrest. Hsin *et al.* (2008) demonstrated that Ag NPs induced programmed cell death *via* ROS and JNK-mediated mitochondrial apoptosis pathway in a mouse fibroblast cell line. Mitochondria have been suggested to be the main target of Ag NPs. In a rat liver cell line, Ag NPs have been proposed to cause the destruction of the mitochondria as evidenced by disruption of the membrane potential, shrinkage of the liver cells, ROS elevation, and decreased glutathione (GSH) levels (Hussain *et al.*, 2005).

These data suggest that Ag NPs generate cellular oxidative stress by inducing intracellular ROS, resulting in oxidative damage to cellular compartments, especially in mitochondria, powerhouses of cells. Consequently affect to unavoidable animals in the environments, especially in aquatic animals that are directly exposed to toxic chemicals in aquatic milieu. However, it is difficult to relatively determine the toxicity of Ag NPs in reported animal species because the effects may depend on chemical compositions, atomic arrangements and particle sizes of the Ag NPs (Sardari *et al.*, 2012).

Ideally, molecular biomarkers are important tools that are used to monitor the toxic effects that occur in tested organisms to manipulate certain strategies to control contaminated harmful chemicals and/or be used to set warning parameters that can be applied for risk assessment. The biomarkers are defined as the indicators of biological events and can be used as tools to clarify the relationship between exposed units and exogenous substances (Mussali-Galante *et al.*, 2013).

In this study, we used Nile tilapia as a representative for experimental aquatic animal and we selected 8 genes responsible for cellular stress response as the representative for biomarker genes. We attempted to clone and characterize 2 novel cDNAs responsible for cellular stress responses in Nile tilapia; (1) thioredoxin interacting protein (*On*-TXNIP) and (2) selenoprotein P (*On*-SEPP) genes. The remaining selected genes are composed of 5 members of HSPs' families including *On*-HSP40B9, *On*-HSP40C3, *On*-HSP70, *On*-HSP90α and *On*-HSP90β and 1 member of metal exposure-indicating gene, metallothionein (*On*-MT).

TXNIP is an alpha-arrestin protein (Masutani *et al.*, 2012). Its expression is induced by exposure to various stressful stimuli, including H_2O_2, irradiation, UV, heat shock, serum deprivation, and growth-inhibitory factors such as transforming growth factor-1 (Kim *et al.*, 2007). Characterization of *On*-TXNIP revealed that it is similar to other vertebrate TXNIPs, containing 2 arrestin domains and 2 PPXY consensus motifs (Masutani *et al.*, 2012) (Fig. 2). These motifs are believed to permit the negative regulation of TXNIP by binding to the E3 ubiquitin ligase, which promotes TXNIP proteasomal degradation (Zhang *et al.*, 2010). In addition, the two cysteine residues at amino acids 65 and 250 are crucial for the interaction with thioredoxin (TRX) and inhibition of TRX antioxidant activity (Nishiyama *et al.*, 1999; Junn *et al.*, 2000). TXNIP can also stimulate a proapoptotic signal by abolishing the TRX-mediated inhibition of apoptosis signal-regulating kinase-1 (ASK1), further activating the downstream JNK/p38 MAPK pathway (Zhou *et al.*, 2011).

Analyzing the evolutionary relationship of the TXNIP genes in the phylogenetic tree demonstrated that the *On*-TXNIP gene is closely related to zebrafish TXNIP (Fig. 7). Amino acid sequence homology and evolutionary analysis revealed that the *On*-TXNIP gene has high homology scores with other vertebrate TXNIPs (76–78%) and shows the highest similarity to zebrafish and salmon (92% and 92%, respectively), suggesting that this gene may perform a similar fundamental function in both the ray-finned fishes and other vertebrates.

Analyzing the normal tissue distribution of *On*-TXNIP transcripts by quantitative real-time RT-PCR revealed that the *On*-TXNIP mRNA is expressed in all tissues (Fig. 9), suggesting that it performs an important biological function in teleost fish cells. The highest level of *On*-TXNIP mRNA expression was observed in PBLs, followed by the posterior kidney, anterior kidney, gills, liver and spleen (Fig. 9), indicating that these vital organs, which all have a high rate of blood perfusion, may functionally respond to cellular stress conditions. The induction of stress could lead to the generation of different levels of ROS dependent upon the degree of stress. Therefore, the different expression levels of the *On*-TXNIP gene in each organ may proportionally indicate the strength of the stress generated.

Another stress effector gene that has not yet been characterized in Nile tilapia is the selenoprotein P (SEPP) gene. The characterization of cDNA encoding the *On*-SEPP gene revealed that the coding region contained 17 selenocysteines (Sec), which contain selenium in place of the thiol group and are encoded by UGA codons (Fig. 4). UGA normally encodes a stop or termination signal, except in the case of selenoproteins. The key element that ensures Sec incorporation is a consensus motif called the SECIS element, which is located in the 3' UTR (Kryukov *et al.*, 2003). The *On*-SEPP cDNA was found to contain 2 SECIS elements (nucleotides 1,618-1,713 and 2,256-2,346) (Fig. 5), which were similar to those reported in zebrafish SEPP cDNA but had different nucleotide positions and configuration patterns (Tujebajeva *et al.*, 2000).

The numbers of UGA codons and SECIS elements are important characteristics of fish SEPP genes that differ from those of mammals. The fish SEPP genes contain 17 Sec codons and 2 SECIS elements, while mammals have fewer Sec codons (10–12) and only 1 SECIS element in the 3'UTR (Tujebajeva *et al.*, 2000). Additionally, evolutionary analysis of the *On*-SEPP gene indicated that it belongs to the vertebrate lineage (Fig. 8) and is closely related to zebrafish, sharing 61.9% amino acid similarity. Because of the limited characterization studies of the SEPP gene in teleost fish and other aquatic animals, the evolutionary correlations among fish species are not yet clear.

The normal tissue distribution analysis of *On*-SEPP mRNA revealed that *On*-SEPP mRNA was expressed in all tissues (Fig. 10), supporting the hypothesis that SEPP functions as a selenium-sequestering protein and can deliver selenium to the peripheral tissues. The highest *On*-SEPP mRNA levels were found in the posterior kidney, followed by the liver and intestine, suggesting that the posterior kidney, which functions as a sieve for controlling electrolytes and ions homeostasis, may re-circulate selenium back into the body. All vertebrates must obtain selenium *via* dietary consumption. Therefore, the posterior kidney, intestine and liver are likely to be the major organs that encounter and retain selenium for blood circulation and thus display the highest expression levels of the *On*-SEPP gene.

This study also identifies these cellular stress response genes (*On*-TXNIP and *On*-SEPP) as molecular indicators for monitoring the consequences of chemical-induced cellular stress, especially metal exposure. Many manufacturers worldwide use Ag NPs as bactericidal agents in many products, leading to the release of Ag NPs directly into the environment, including aquatic environments, which harbor many invertebrate and vertebrate aquatic animals.

In a previous study, an acute toxicity test for Ag NPs in fry Nile tilapia was performed to determine the 50% lethal concentration after 96 h (96 h-LC_{50}). The concentration of Ag NPs was serially graded from 0, 5, 10, 25, 50, 100, to 500 mg/L. However, the trial failed because the experimental fish did not die even after exposure to the highest concentration (500 mg/L) (data not shown). Previously, Choi *et al.*, (2010) reported acute Ag NP toxicity studies in fish. They established that the 24 h-LC_{50} of Ag NPs in zebrafish is 250 mg/L. Accordingly; these results suggest that Ag NPs may have less acute toxicity in Nile tilapia.

Griffitt *et al.*, (2009) demonstrated that exposing zebrafish to 1000 μg/ml of Ag NPs did not affect the gill filaments. In contrast, zebrafish treated with the same concentration of Ag ions dissociated from Ag NPs showed significantly thicker gill filaments and elevated silver concentrations in the fish body. These data correlate with the experimentally determined toxicity of silver nitrate-induced branchial Na^+/K^+ ATPase enzyme inhibition in rainbow trout gill cells (Morgan *et al.*, 1997). Together, these data suggest that Ag NPs are less toxic than silver ions in acute testing.

Based on this finding, we adjusted the experimental protocol to test the effects of chronic exposure by changing the route of exposure. Although the experimental fish were intraperitoneally exposed to high doses of Ag NPs (100 mg/kg), none of the injected fish died. However, some fish in this group displayed significant physiological changes including loss of appetite, lethargy, darkened stripes along the body, and increased opacity of the eyes at 1 week after the injection, suggesting that high concentrations of Ag NPs may cause chronic effects rather than acute toxicity.

The degree of Ag NP-induced toxicity may depend on the specific form of the synthesized Ag NPs, the types of dispersant agents added, the exposure time, the type of animal exposed, the route of administration and the targeted organs. The

species, ages and sizes of experimental animals are not excluded. Additionally, the effects of the physico-chemical properties of water on Ag NP toxicity should be considered. As the adverse effects of Ag NPs on the environment have not been well studied, this experiment was designed to measure the chronic adverse outcomes of Ag NP on Nile tilapia after administration *via* intraperitoneal injection. To document the responses of the fish, the expression levels of cellular stress genes in the liver, spleen and anterior kidney were monitored, accompanied by histopathology of the liver and spleen. These major organs are responsible for many metabolic reactions that control fish homeostasis and immunity (Reynaud *et al.*, 2008; Uribe *et al.*, 2011). Previously, Platonova *et al.*, (2013) demonstrated that induction of Ag NPs *via* the gastrointestinal tract of rats resulted in the accumulation of Ag NPs in the liver and spleen. Transmission electron microscopy (TEM) observation revealed that Ag NPs preferentially accumulated in the capillary lumen, blood cells, hepatocyte microvilli and hepatocytes of hepatic lobules and in the blood vessel lumen and the red pulp reticular cells of the spleen.

In this study, *On*-TXNIP mRNA expression was clearly up-regulated in the liver, spleen, and head kidney, suggesting that these organs may be targeted by Ag NPs. The up-regulation of *On*-TXNIP expression levels in a dose-dependent manner was observed in all examined tissues of fish at an early phase of Ag NP exposure (at 6 to 48 hpi), suggesting that Ag NPs acutely induce the expression of *On*-TXNIP in experimental fish. Up-regulated *On*-TXNIP mRNAs could be immediately detected at hour 6, suggesting that TXNIP, a vital intracellular signaling molecule, suddenly changed under cellular stress conditions. This result agreed with Mei *et al.* (2012) and Rahman *et al.* (2009). The TXNIP transcripts have been reported to be up-regulated by 1.9-fold in response to acute Ag NP exposure at 4 hours in a mouse lymphoma cell line (5 µg/mL Ag NPs) (Mei *et al.*, 2012) and up-regulated in mouse brain at 24 hours in adult male mice (100-1,000 mg/kg Ag NPs, i.p.) (Rahman *et al.*, 2009). The results from the TXNIP gene expression analysis indicate that this gene is sensitive to acute Ag NP exposure rather than chronic intoxication. ROS induction by Ag NPs was proposed by Hussain *et al.* (2005), Hsin *et al.* (2008) and Foldbjerg *et al.* (2009). It can be suggested that ROS generated by Ag NPs may be the primary inducer of TXNIP up-regulation.

SEPP is believed to be an antioxidant protein (Higuchi *et al.*, 2010). Beer *et al.*, (2012) suggested that silver ions dissociated from Ag NP suspension play a major role in the cytotoxic effects of Ag NPs in the A549 human lung carcinoma epithelial-like cell line by generating ROS. Therefore, Ag NPs could exert toxicity by promoting ROS production and suppressing key antioxidant proteins, including thioredoxin (TRX), as observed by the up-regulation of *On*-TXNIP and *On*-SEPP at an early phase of Ag NP exposure and the down-regulation of *On*-SEPP in the liver at weeks 1 to 4; this event may lead to the accumulation of intracellular ROS. The accumulated ROS may cause macromolecular damage and stimulate multiple signal transduction cascades, including cell death signals, ultimately leading to a loss of homeostasis.

In this study, *On*-SEPP transcripts were also found to be significantly up-regulated in the early phase of exposure in all tested tissues and markedly down-regulated at weeks 1 through 4 in the liver, suggesting that Ag NPs could have potentially suppressive effects on the *On*-SEPP gene following chronic exposure in this organ. Consequently, selenoproteins may be easily disturbed by Ag NPs and likely lost in the regulatory function of cellular selenium homeostasis, leading to bodily selenium deprivation. Selenium has recently been reported elsewhere to be involved in the reproductive system in terrestrial animals (Burk & Hill, 2009). Disturbance of the selenium balance may affect the sexual integrity of animals. SEPP and other selenoproteins have also been suggested to be related to the antioxidant system (Burk *et al.*, 1995; Atkinson *et al.*, 2001). The *On*-SEPP gene in Ag NP-exposed fish was found to be persistently suppressed at weeks 1 through 4 in fish liver, especially in the 10 and 100 mg of Ag NPs/kg groups, suggesting that Ag NPs may interfere with fish antioxidant proteins in this organ. Although noticeable down-regulation of the *On*-SEPP gene in the liver was observed at the 2nd and 4th weeks post-injection, anterior kidney tissue exhibited significantly higher expression levels of *On*-SEPP than the control at all Ag NP concentrations. This result suggests that this organ may play a crucial role in balancing or compensating for the expression mechanisms in other impaired organs to maintain continuous function of *On*-SEPP and normal homeostasis in the fish.

Li *et al.* (2010) found that SEPP mRNA was significantly down-regulated in MRC-5 human fetal lung fibroblast cells treated with 1 nM gold nanoparticles at 72 hours after exposure. Ciofani *et al.* (2014) observed down-regulated SEPP transcripts in the acute response of Ag NP-treated rat pheochromocytoma PC12 cells at 72 hours after exposure. This indicates that suppressed SEPP mRNA can be observed in response to cellular stress and persist in the chronic response based on the observation of continuously down-regulated *On*-SEPP in the liver across all weeks during Ag NP exposure. Selenoprotein W (SEPW) was also found to be significantly down-regulated in zebrafish exposed to nanocopper (Griffitt *et al.*, 2009). It was suggested that nanomaterials can suppress the expression of antioxidant molecules, particularly the SEP protein families.

Heat shock proteins (HSPs) have been well documented in many relevant scientific research articles as stress proteins that regulate cellular protein stabilization and homeostasis (Feder and Hofmann, 1999; Iwama *et al*., 1998; Roberts *et al*., 2010). Expression of HSP genes has been a popular bio-indicator of cellular and physiological changes under stress conditions (Gupta *et al*., 2010). In this study, the expression patterns of several HSP genes in Nile tilapia under toxic chemical-induced cellular stress were determined to elucidate the adverse effects of Ag NPs on the cellular stress response in an economic teleost freshwater fish.

Based on this experiment, at the early phase of response (hours 6 and 12) to Ag NPs in all treated groups, the *On*-HSP40B9 transcripts were significantly up-regulated in fish liver, spleen and head kidney. This result suggests the vital functions of HSPs in these organs in response to chemical-induced cellular stress. A large number of studies in mammalian cells have revealed the important role of HSP40B9 (DnaJB9 or ERdj4) in assisting protein folding in the endoplasmic reticulum (ER) by working as a co-chaperone with its partner “binding immunoglobulin protein (BiP)” or “glucose-regulated protein (GRP) 78”, which is a member of the HSP70 family in the ER compartment (Dong *et al*., 2008; Lai *et al*., 2012; Shen *et al*., 2002).

Interestingly, the *On*-HSP40B9 gene was up-regulated in Nile tilapia exposed to Ag NPs, suggesting that under chemical-induced stress conditions, adverse effects may have a substantial influence on ER integrity, leading to ER stress. This possibility could be supported by the report of Lafleur *et al*. (2012), who revealed that many drugs and chemicals can generate toxicity by disturbing ER integrity and homeostasis. The HSP40B9 has been attributed to mediating the activation of misfolded protein deconstruction through the ER associated degradation (ERAD) mechanism (Kaufman *et al*., 2010; Lai *et al*., 2012; Otero *et al*., 2010). It can be suggested that Ag NP-exposed fish may use this mechanism to eliminate a large number of misfolded proteins overloaded in the ER lumen under stress conditions. Additionally, the *On*-HSP40B9 mRNA in Ag NP-treated groups was highly up-regulated at 1st wpi in fish liver and at weeks 1 and 4 in the spleen, suggesting that impaired proteins could be generated in the chronic toxic response to Ag NPs.

The HSP40C3 (p58-kDa inhibitor of the RNA-activated protein kinase or $P58^{IPK}$) has been documented to play a variety of roles in many biological events, such as cell cycle regulation, transcriptional processes, protein folding and neurogenesis (D'Andrea and Regan, 2003; Korth *et al*., 1996; Melville *et al*., 2000). The function of HSP40C3 is to maintain protein homeostasis by promoting protein refolding in the ER (Otero *et al*., 2010; Tao *et al*., 2010). In this study, the *On*-HSP40C3 transcripts in fish exposed to Ag NPs were clearly up-regulated in the liver (hours 6, 12 and week 1) and the spleen (hours 12, 48 and week 1). It can be suggested that fish cells attempt to counterbalance the overloading of impaired proteins in the ER lumen by promoting the refolding of misfolded proteins to their proper conformation. Interestingly, the *On*-HSP40C3 transcripts in the head kidney were significantly down-regulated, suggesting that Ag NPs may suppress HSP40C3 gene expression in this organ, leading to dysfunction of the refolding processes of misfolded overloads in the ER, finally resulting in ER stress.

Another HSP member is *On*-HSP70, which has been abundantly studied in various animal species as a cellular stress response bio-indicator (Gupta *et al*., 2010; He *et al*., 2010; Iwama *et al*., 1999; Yamashita *et al*., 2010). In 2009, Chae and co-workers performed an Ag NP acute toxicity test in Japanese medaka (*Oryzias latipes*) and noticed that the *On*-HSP70 gene was significantly up-regulated at day 1 in fish liver and was gradually down-regulated later at day 2 until day 10. In this experiment, the *On*-HSP 70 gene in fish liver in the Ag NP-exposed groups was obviously down-regulated at the early phase of exposure until 48 hpi. It can be suggested that Ag NPs may exert suppressive effects on *On*-HSP 70 gene expression, leading to insufficient reserved protein chaperones to help correct the folding of misfolded client proteins under cellular stress conditions. At the 4th wpi in Nile tilapia liver, slightly down-regulated *On*-HSP70 mRNA was observed in all Ag NP-exposed groups ($P<0.05$). This interesting phenomenon correlated with another result in a previous study showing that HSP70 gene expression was significantly down-regulated in male medaka exposed to chronically Ag NPs at day 28 at all concentrations (1 and 25 µg/l) in Ag NP-treated fish (Pham *et al*., 2012). This result suggests that acute and chronic Ag NP exposure induced the down-regulation of HSP70 gene expression in fish liver. In contrast, the *On*-HSP70 transcript in the spleen and head kidney was up-regulated, starting at the beginning of exposure and continuing until hour 48 for the spleen and until week 2 for the head kidney. This up-regulation can be suggested to be a compensatory mechanism for fish to use instead of impaired functional organs to maintain the homeostasis of certain effective proteins.

The other 2 HSP members examined in this experiment were *On*-HSP90α (inducible form) and *On*-HSP90β (constitutive form). The HSP90α has been reported to be involved in cytoprotection against toxic chemicals and oxidative stress (Ali *et al*., 1996; Padmini and Usha Rani, 2009). Choi *et al*. (2008) demonstrated that cadmium-exposed Pacific oyster (*Crassostrea gigas*) exhibited significantly increased HSP90 mRNA in the digestive gland and gills with dose- and time-dependent relationships. Padmini and Usha Rani (2011) found that HSP90α expression was obviously induced in the hepatocytes of gray mullet fish (*Mugil cephalus*) that tolerantly lived in a pollutant-contaminated estuary. This result suggests that, in both invertebrate and vertebrate animals, HSP90 is a vital molecule protecting cells against

stress induction. In higher vertebrates, healthy female donkeys (*Equus africanus asinus*) were supplemented with selenium nanoparticles (Se NPs) at 0.5 mg/kg for 10 days, and, at 24 and 72 hours post-treatment, HSP90 transcripts were inductively expressed in gluteal muscle (Kojouri *et al*., 2013). This result indicated that HSP90 could potentially protect cells from stress after exposure to nanoparticles. In the current study, *On*-HSP90α transcript expression in the liver of Ag NP-exposed Nile tilapia was markedly up-regulated at the early phase of exposure (hours 6 and 12) but down-regulated at 48 hpi and 2 and 4 wpi, while continuous up-regulation at 2 and 4 wpi was observed in the spleen, suggesting the fundamental function of *On*-HSP90α in stress-induced cytoprotection caused by Ag NPs. In the head kidney, *On*-HSP90α mRNA was down-regulated at hours 24 and 48, suggesting that the acutely suppressive effects of Ag NPs were also noted in this organ.

The HSP90β plays a major role in many cellular processes including early embryonic development, germ cell maturation, cytoskeletal stabilization, cellular transformation, signal transduction, and long-term cell adaptation (Manchado *et al*., 2008; Sreedhar *et al*., 2004). Significantly increased expression of the HSP90β gene ($P<0.05$) in the liver of the white cloud mountain minnow (*Tanichthys albonubes*) exposed to copper and cadmium was observed at an early phase of exposure (hours 24 and 48), as reported by Liu *et al*. (2012). In the current study, the expression patterns of *On*-HSP90β transcripts in liver, spleen and head kidney in Nile tilapia exposed to various doses of Ag NPs were clearly up-regulated at the beginning of observation (hours 6 and 12) and continuously expressed until 48 hpi for the liver. It is likely that *On*-HSP90β may be inductively responsible for fish exposed to heavy metals and Ag NPs at the early phase response.

The last candidate gene in this study is metallothionein (MT). Generally, it is employed as a biomarker molecule to monitor environmental pollutant exposure in many experimental animals (Cheung *et al*., 2005; Lam *et al*., 1998; Thirumoorthy *et al*., 2007). Choi *et al*. (2008) found that significantly increased MT mRNA levels in cadmium-exposed oysters showed dose- and time-dependent responses in both the digestive gland and gills. Experimentally, Ag NP-exposed Nile tilapia revealed prominently up-regulated *On*-MT mRNA in the head kidney at hour 12 that was

continuously expressed until week 4, with the exception of week 2. These results can support the use of the MT gene as a bio-indicator in aquatic animals under acute and chronic toxic chemical exposure. Pham *et al*. (2012) demonstrated that the MT gene in the liver was significantly down-regulated at day 7 in 25 μg/l Ag NP-exposed male medaka and highly up-regulated later at day 21 in both 1 and 25 μg/l Ag NP-treated groups. This finding agreed with the results observed in Nile tilapia exposed to Ag NPs, where the expression of the *On*-MT gene in the liver was suppressed at hour 6 continuously until hour 48 and inductively expressed later at week 1. Based on its suppressive effects after Ag NP exposure, it can be suggested that the *On*-MT gene in fish liver is a negative effect biomarker in the acute phase response, while up-regulated *On*-MT transcripts may be indicators for chronic exposure.

Platonova *et al*. (2013) observed the accumulation of Ag NPs in the liver and spleen of rats after injecting Ag NP solution into an isolated small intestinal loop. In addition, in adult zebrafish exposed to Ag NPs, the accumulation of Ag NPs was observed in fish liver (Choi *et al*., 2010). It can be suggested that Ag NPs may also accumulate in Nile tilapia exposed to Ag NPs by intraperitoneal injection. The suppressive effects of Ag NPs on the *On*-MT gene in fish liver suggest that Ag NPs may disturb the sequestering processes that fish use to eliminate metals from the liver, leading to the additional accumulation of Ag NPs in that organ. Notably, *On*-MT transcripts in fish liver were suppressed, while in the head kidney, they were continuously up-regulated, suggesting the compensatory mechanism that fish use to maintain *On*-MT levels in the blood circulation to sequester Ag NPs out of the fish body.

Based on the results of expression analysis, fish could respond to Ag NPs during the early phase of exposure. Sensitive biomarker genes were purposed to be used in this study. The *On*-TXNIP, *On*-SEPP, *On*-HSP40B9, *On*-HSP40C3, *On*-HSP90α, and *On*-HSP90β genes in most examined organs were found to highly up-regulated during the beginning of exposure, while at the same time, suppressive expression of *On*-HSP70 mRNA in liver and *On*-MT transcripts in liver and spleen were obviously observed. It indicates that induction and suppression of bio-indicator genes for Ag NPs in Nile tilapia should be parallel considered in the assessment.

Generally, the liver is classified as a major organ involved directly in the elimination of toxicants *via* biotransformation and detoxification. Therefore, this organ must be a good candidate to study the effects of Ag NP exposure in toxicology studies. Histopathological analysis clearly revealed a dose-dependent increase in blood congestion in the overcrowded sinusoidal area, binucleated cells and hepatic necrosis of the Nile tilapia liver. Similarly, a study examining the effects of Ag NPs in drinking water on the livers of broiler chickens revealed hepatocyte swelling, liver cell necrosis, inflammatory cell recruitment and the incretion of fibroplasias (Loghman *et al.*, 2012). Heydarnejad *et al.* (2014) also demonstrated that the histopathological changes in the livers of mice treated with Ag NPs included central venous hyperemia, cell swelling, and an in increase in Kupffer and inflammatory cells.

In addition, many studies reported the effects of Ag NPs that strongly induced degeneration in several types of cells, resulting in apoptotic and necrotic phenomena (Foldbjerg *et al.*, 2009; Orlowski *et al.*, 2012; Ciftci *et al.*, 2013). At weeks 1 and 2 after exposure to 1 mg Ag NPs/kg fish, the livers of the fish exhibited hepatocyte swelling. However, this alteration was not detected in Nile tilapia livers exposed to 10 or 100 mg Ag NPs/kg fish, suggesting that the swelling process may be an early response to high doses of Ag NPs and may have already been terminated in a favor of other changes after 1 week. Although programmed cell death evaluation was not performed in this study, Nile tilapia exposed to high concentrations of Ag NPs exhibited necrotic regions in large areas of the liver, and some observed cells were similarly prone to apoptotic conditions, suggesting that liver cells of Nile tilapia are also degenerated *via* an apoptosis pathway similar to other vertebrates in previous reports.

Based on the apoptotic phenomena observed in the liver cells of experimental animals, TXNIP has recently been reported to mediate the apoptosis factor signal-regulating kinase-1 (ASK1), which further activates the downstream JNK/p38 MAPK pathway (Zhou *et al.*, 2011). In addition, in adult zebrafish exposed to Ag NPs, hepatotoxicity was observed via the induction of malondialdehyde (MDA) levels, the formation of pyknotic nuclei, a decrease in oxyradical-scavenging enzymes, an increase in DNA damage and the up-regulation of apoptotis-related genes (Choi *et*

al., 2010). Thus, Ag NPs may be able to activate programmed cell death *via* these signal mediators.

One important indicator noted in the liver histopathology of Nile tilapia is the occurrence of binucleated cells. This alteration is known to be caused by the genotoxic effects of Ag NPs and has been clearly supported by several studies (Kawata *et al.*, 2009; Uygur *et al.*, 2009; Kim *et al.*, 2011; Apoorva *et al.*, 2013). The recruitment of Kupffer cells in fish livers was reminiscent of a study on Wistar albino rats (*Rattus norvegicus*) that were intraperitoneally injected with Ag NPs (Yousef *et al.*, 2012). Additionally, our study demonstrated dramatically increased sinusoidal congestion with erythrocytes and an increased number of Kupffer cells in the sinusoidal walls, indicating that Ag NPs may stimulate the inflammatory processes in this organ.

TXNIP has also been reported to bind to NLRP3 (nucleotide binding domain and leucine-rich repeat containing family, pyrin domain containing 3), resulting in inflammasome activation followed by inflammatory responses (Davis *et al.*, 2011). Following TXNIP-induced inflammation, Ag NPs may mediate immunological responses by activating the Kupffer cells lining the walls of the sinusoids. The activated Kupffer cells may then secrete chemokines to communicate with and recruit other immune cells to the liver sinusoids, resulting in initiation of the inflammatory response, which is observed as sinusoidal blood congestion.

Liver histopathology also revealed mild cytoplasmic vacuolation that appeared in the hepatocytes of fish treated with 10 or 100 mg Ag NPs/kg. Vacuolation increased in a dose-dependent manner upon Ag NP exposure. Liver vacuolation normally occurs when organisms are exposed to xenobiotics or toxic chemicals (Nayak *et al.*, 1996). These data are supported by a study from Yousef *et al.*, (2012), who found that Wistar albino rats exposed intraperitoneally to Ag NPs demonstrated the enlargement of hepatocytes and vacuole-like spaces in the cytoplasm. Therefore, Ag NPs may induce liver vacuolation, which is a detoxification process, suggesting that Nile tilapia could utilize this mechanism to detoxify Ag NPs and trap these chemicals inside the fat vacuoles.

Histopathological abnormalities, including the dose-dependent thickening of the capsule layer, were also observed in the spleen, especially at 4 weeks after injections. These data are consistent with those of a previous study by Sardari *et al.*, (2012), suggesting that the thickening of the capsule layer of the spleen could be used as a biophysical indicator of chronic Ag NP exposure in tilapia.

Hematological parameters are important factors that generally refer to fish health status (Cheraghi *et al*., 2013; Imani *et al*., 2014). Shaluei *et al*. (2013) conducted subacute toxicity studies of Ag NPs on silver carp (*Hypophthalmichthys molitrix*) and found that RBCs and %Hct levels in exposed groups were significantly lower at days 7 until 14. Imani *et al*. (2014) reported a decrease in %Hct levels in rainbow trout (*Oncorhynchus mykiss*) after 8 days of Ag NP exposure. Likewise, %Hct and the amount of RBCs following all doses of Ag NP exposure in Nile tilapia showed significant decreases at weeks 1 until 8, supporting the notion that Ag NPs may disrupt RBC integrity, resulting in a reduced number of RBCs and %Hct levels.

The toxic effects of Ag NPs on hematology have been reported not only in lower vertebrates but also in higher vertebrates such as rodents, which also showed severely abnormal hematology. Cheraghi *et al*. (2013) conducted a 15-day oral administration of an Ag NP solution in rats and found that RBCs and %Hct levels were significantly decreased in treated rats. This finding suggests that RBCs are the main target of Ag NP insult. Ag NPs exert toxicity by inducing ROS (Chairuangkitti *et al*., 2013; Foldbjerg *et al*., 2011; Hussain *et al*., 2005; Wise *et al*., 2010;). Generally, ROS can destroy RBC integrity and impair their function (Mohanty *et al*., 2014). It can be suggested that the ROS generated by Ag NPs may affect RBCs in circulation, leading to interference in oxygen carrying and cellular respiration.

Cheraghi *et al*. (2013) found that both male and female rats administered an Ag NP solution by gavage for 15 days showed a decreased amount of WBCs. This result suggests the suppressive effects of Ag NPs on hematopoietic tissues. Experimentally, the amount of WBCs in Ag NP-exposed Nile tilapia in each group was significantly reduced at week 1 and non-significantly altered at weeks 2 until 8. These findings suggest that fish WBCs may suffer during the early phase of exposure and are then able to recover and maintain balance in the hematopoietic system.

Generally, phagocytic activity is a popular immunological parameter used in the evaluation of immunotoxic chemicals (Fournier *et al*., 2000). In healthy Nile tilapia, the normal range of phagocytic activity is between 15-18% (El-Hawarry, 2011; Suwannasang *et al*., 2014). In this study, the phagocytic activity of normal Nile tilapia in the control group was less than the normal range compared with other reports. The weights of the experimental Nile tilapia used in this study were only 30 g per individual; therefore, reduced amounts and integrity of the extracted peripheral blood monocytes were observed.

Greulich *et al*. (2011) demonstrated that the agglomeration and cytotoxicity of Ag NPs, which were cell-specific responses, demonstrated dose-dependent patterns in human monocytes, but these responses were not found in T-cells. The cytotoxic effects of Ag NPs on mouse RAW 264.7 monocytes revealed that Ag NPs could induce necrosis and caspase-1 activity (Orlowski *et al*., 2012). Liu *et al*. (2013) found that phagocytic activity of Wistar rat (*Rattus norvegicus*) alveolar macrophages exposed to Ag NPs significantly declined in a concentration-dependent manner, and its viability was dramatically reduced. This result suggests that Ag NPs are toxic to phagocytic cells, especially for circulating monocytes.

Aboud (2010) studied the impact of heavy metals on Nile tilapia immunity and found that phagocytic activity was dramatically declined in metal-exposed groups, and the mortality rate was clearly increased in Nile tilapia infected with *Pseudomonas fluorescens*. Correspondingly, Nile tilapia exposed to Ag NPs also exhibited a decrease in phagocytic activity, and the mortality rate at days 1 and 2 in fish exposed to 100 mg Ag NPs/kg fish was significantly higher than for the other groups. These findings suggest that, under metal-induced stress conditions, phagocytic function is vulnerable and inactive, which leads to the compromise of innate immunity in experimental animals and makes them easily infected and disease intolerant.

Rault *et al*. (2013) observed the effects of various heavy metals (cadmium, lead, mercury and silver) on phagocytic activity in the marine bivalve *Mytilus edulis*. The results revealed that silver ions induced a decrease in phagocytosis. Likewise, a significant decrease in phagocytic activity indicated by PA, PI and PE was clearly observed in Ag NP-exposed Nile tilapia. This finding suggests that both Ag ions and Ag NPs may lower phagocytic activity in experimental animals.

In this study, the specific immune response in fish exposed to various doses of Ag NPs was also determined by the detection of serum antibody titer levels. Different levels of serum antibodies were detectable at different times in each group of Nile tilapia immunized with FASA and simultaneously exposed to Ag NPs. This result indicates that different patterns of antibody production may be affected by different doses of Ag NPs. De Jong *et al*. (2013) performed a 28-day intravenous injection of Ag NPs to male and female Wistar rats and found that the IgM and IgE titers of the rats were significantly increased at benchmark doses (BMD) of 0.15 and 0.02 mg of Ag NPs/kg, respectively. The elevated rat IgM and IgE suggest that proliferation of B-cells might be increased, consistent with the increase in rat spleen weight. Based on the antibody response study in Nile tilapia, although monocytes isolated from peripheral blood leukocytes showed a decrease in phagocytic activity, the fish were still able to produce serum antibodies. It can be suggested that 1) B- and T-cells may retain the potential to play crucial roles in antigen processing and epitope recognizing/B-cell triggering, respectively, and 2) tissue macrophages, especially in the spleen and other organs may retain phagocytosis and antigen presentation activity. Therefore, the processes of proliferation, differentiation of B-cells to plasma B-cells and intact antibody production are not disturbed.

Van de Zande *et al*. (2012) conducted a 28-day oral administration of 90 mg of Ag NPs/kg in rats and found that serum IgG and IgM were not significantly changed, and B- and T-cell proliferation rates were not significantly altered. It can be suggested that different administration routes of Ag NPs may affect different antibody responses. In our study, Nile tilapia first immunized with FASA and exposed to 1 mg of Ag NPs/kg could respond to FASA antigen by producing serum antibodies that were highly detectable at week 2, while at 10 and 100 mg of Ag NPs/kg, the fish could not produce antibodies against FASA. This result indicates that, at a lower dose, Ag NPs may be less immunotoxic to Nile tilapia. In contrast, Ag NPs at higher doses may have an immunosuppressive effect, leading to disease intolerance in Nile tilapia infected with bacterial pathogens.

After the fish were administered the 2nd immunization with the same antigen (FASA) but not a repeated dose of Ag NPs, the fish in groups exposed to 1 and 10 mg Ag NPs/kg fish showed slight increases in serum antibodies but not sharp increases, while in the group receiving 100 mg Ag NPs/kg fish, serum antibodies were detected at a high level. It can be suggested that Nile tilapia has the potential to recover antibody production. Additionally, fish in the Ag NP exposure groups demonstrated a significantly lower mortality rate (10-15%) when challenged with the virulent pathogen *Streptococcus agalactiae*, while fish in the control group exhibited approximately 70% mortality at days 3 until 7. This finding indicates that after Ag NP exposure for 2 months, fish immunity can completely recover and function properly against bacterial infection.

Based on the results of this study, even Nile tilapia administered a high dose of Ag NPs (100 mg/kg) did not die, and the immune system was able to recover, showing the potential to protect against pathogenic bacterial infection. It can be suggested that Nile tilapia and its immunological components are tolerant to Ag NPs. Moreover, Ag NPs have been widely documented to have toxic effects on bacteria, fungi, algae, and microcrustaceans, some of which are the primitive live-feeds for aquatic larvae in the food chain system (Becaro *et al.*, 2015; Wijnhoven *et al.*, 2009). Becaro *et al.* (2015) revealed the high toxicity of Ag NPs in certain microcrustaceans, ranging from 3.42×10^{-4}- 1.09 mg/l. Ringwood *et al.* (2010) also found that oyster embryos (*Crassostrea virginica*) exposed to 1.6 µg/l Ag NPs were found exhibit clear drops in normal embryonic development. However, Ag NPs have been reported to be less toxic to teleost fish (Bilberg *et al.*, 2010; Choi *et al.*, 2010; Yeo and Kang, 2008), including the Nile tilapia in this study. This result suggests that Ag NPs may be more toxic to microorganisms and to aquatic animals in the larval stage than animals at older stages. Previously, Benn and Westerhoff (2008) reported that the amount of Ag NPs leaching from sock fabrics into water was 1.3 ppm. This concentration may generate direct and indirect effects on smaller aquatic fauna. Therefore, Ag NPs may consequently affect aquatic ecosystems, which is currently a high environmental concern.

CHAPTER VII
CONCLUSION

1. The TXNIP and SEPP cDNAs were completely cloned and characterized for the first time in Nile tilapia. The full length cDNA of *On*-TXNIP was 2,260 bp and consisted of 116 bp of 5’ UTR, 1,188 bp of ORF (equal to 396 amino acid residues), 925 bp of 3’UTR and 28 bp of a poly A tail. The calculated theoretical isoelectric point (p*I*) and molecular weight (MW) of *On*-TXNIP were 7.81 and 43.95 kDa, respectively. The full length cDNA of *On*-SEPP was 2,427 bp and consisted of 99 bp of 5’UTR, 1,239 bp of ORF (equal to 413 amino acid residues), 1,086 bp of 3’UTR, and 29 bp of a poly A tail. The calculated p*I* and MW of *On*-SEPP were 6.27 and 46.36 kDa, respectively.

2. Comparison analysis by nucleotide and amino acid sequence alignments indicated that *On*-TXNIP and *On*-SEPP genes were similarly conserved with zebrafish. Correlately, the evolutionary relationship of the *On*-TXNIP and *On*-SEPP genes in the phylogenetic tree also demonstrated that these 2 genes were closely related to zebrafish.

3. The normal tissue distribution of *On*-TXNIP and *On*-SEPP transcripts by quantitative real-time RT-PCR revealed that these 2 mRNAs were expressed in all tested tissues. The highest level of the *On*-TXNIP mRNA expression was observed in PBLs, followed by the posterior kidney, anterior kidney, gills, liver and spleen, respectively. While, the highest *On*-SEPP mRNA levels were respectively found in the posterior kidney, followed by the liver and intestine. It indicated that these vital organs may functionally respond to cellular stress conditions. Both genes were expressed in all normal tissues tested, indicating their crucial roles in the mediation of physiological homeostasis and the cellular stress response.

4. The adverse effects of exposure to Ag NPs could be observed through abnormalities in liver and spleen histology. Higher doses of Ag NPs did not acutely affect the fish but instead chronically increased Kupffer cell infiltration, formation of bi-nucleated hepatocytes, and sinusoidal blood congestion in the liver. Thickening of the capsule layer of the spleen was a predominant indicator of chronic Ag NP exposure.

5. Under early phase of exposure to Ag NPs-induced cellular stress in Nile tilapia, significantly up-regulated *On*-TXNIP, *On*-SEPP, *On*-HSP40B9, *On*-HSP90α and *On*-HSP90β genes were obviously observed in liver, spleen and head kidney at hours 6 and 12, and noticeably down-regulated *On*-HSP70 and *On*-MT transcripts were clearly found in liver at hours 6 to 48. All of these genes may possibly be applied to bio-indicators for acutely Ag NPs-exposed Nile tilapia. While the *On*-SEPP transcripts were also found to be markedly down-regulated at weeks 1 through 4 in the liver, indicating that the *On*-SEPP gene could be a biomarker for the chronic exposure.

6. Hematological parameters of Nile tilapia exposed to Ag NPs revealed that a prolonged decrease in %Hct and amount of RBCs was observed at weeks 1 until 8. However, amounts of WBCs were not significantly changed at all-time exposure. It indicated that Ag NPs may disrupt RBC integrity, resulting in a reduced number of RBCs and %Hct levels.

7. Effects of Ag NPs on innate and adaptive immune responses were intensively investigated. It showed that Nile tilapia exposed to Ag NPs clearly exhibited a decrease in phagocytic activity. It indicated that the phagocytic function may be vulnerable and inactive, which leads to the compromise of innate immunity in experimental animals and makes them easily infected and disease intolerant. Fish immunized with *Streptococcus agalactiae* vaccine and simultaneously exposed to Ag NPs at 10 and 100 mg/kg were found to effectively decrease in antibody titer of fish at only early phase. Finally in challenge test, the vaccinated fish on Ag NPs-exposed groups were still protected against *S. agalactiae* infection. This finding indicated that after Ag NP exposure for 2 months, fish specific immunity could completely recover and function properly against bacterial infection.

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APPENDIX

COMPOSITION OF MEDIA AND REAGENTS

Tryptic Soy Broth

Tryptone	17.00 g
Phytone	3.00 g
Glucose	2.50 g
NaCl	5.00 g
K_2HPO_4	2.50 g

Add distilled water to total volume of 1 liter

The following ingredients are dissolved under gentle heat and then autoclaved for 15 minutes at 121 °C.

Luria Bertani (LB) Broth

Tryptone	10.00 g
Yeast Extract	5.00 g
NaCl	5.00 g

Add distilled water to total volume of 1 liter

The following ingredients are dissolved under gentle heat and then autoclaved for 15 minutes at 121 °C.

Super Optimal broth with Catabolite repression (SOC)

Tryptone	20.00 g
Yeast extract	5.00 g
NaCl	0.50 g
1M KCl	2.50 ml

Add distilled water to total volume of 1 liter. Adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, add 20 ml of sterile 1 M glucose immediately before use.

Natt-Herrick's stain solution

NaCl	3.88 g
$NaSO_4$	2.50 g
Na_2HPO_4	1.74 g
KH_2PO_4	0.25 g
Formalin (37%)	7.50 ml
Methyl violet	0.10 g

Add distilled water to total volume of 1 liter and filter through Whatman # 10 medium filter paper.

1X Phosphate-buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g

Start with 800 ml of distilled water to dissolve all salts. Adjust the pH to 7.4 with HCl. Add distilled water to total volume of 1 liter

The following ingredients are dissolved under gentle heat and then autoclaved for 15 minutes at 121 °C.

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