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Research Article

The effects of copper-based nanoparticles on the immune system of the white shrimp (*Penaeus vannamei*)

Sadralsadati H.[1]; Mashinchian Moradi A.[1*]; Afsharnasab M.[2]; Ghavam Mostafavi P.[1]; Ramezanifard E.[1]

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Abstract

The application and development of nanotechnology is increasingly expanded in many areas. However, considering this expansion, several concerns regarding their potential toxicity in marine biology have been addressed. Indeed, the overuse of these materials can adversely influence marine ecosystems and living organisms. Herein, the potential impact of copper-based nanoparticles on the expression level of three genes, i.e. prophenoloxidase, serine protein and glutathione peroxidase genes functioning in shrimp immune response, were assessed. For this end, the shrimps were exposed to three semi-acute toxicity treatments, including 0.25, 0.5 and 1 mg/L of CuNPs. The qRT-PCR results indicated negative effect of supplied nanoparticle on the expression level of these genes. Additionally, histopathological alterations in the hepatopancreas and lymphoid organs were observed in the shrimps after exposure in different concentrations of CuNPs. Overall, we showed that toxic concentration of CuNPs can damage shrimp immune system as well as some internal organs. These results open up novel insights into innate immunity of shrimps subjected to copper-based nanoparticles.

Keywords: White shrimps, CuNPs, Immune system, Hepatopancreas, Lymphoid organs

1- Department of Marine Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
2- Department of Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
* Corresponding author's Email: ali2m @yahoo.com

Introduction

In recent years, marine shrimp farming is expanding throughout the world particularly in Asian countries. However, this cultivation industry, likewise some others, has widely been faced with unfavorable conditions (Valderrama and Anderson, 2011). White shrimp, *Penaeus vannamei,* is extensively cultured in Asian and American countries, and is among the most prominent and valuable species in aquaculture. This species, with a total global production of 3,500,000 metric tons in 2018, is currently one of three major cultivated shrimps. Nanoparticles (NPs) have become a major part of human kind daily life, mostly in the forms of drug delivery system, biosensors, cosmetics and therapeutics (Can *et al.,* 2011; Corsi *et al*. 2014). Accordingly, due to their fast-growing application in aforementioned industries, as well as potential nanotoxicity, NPs has drawn serious attention among scientists and consumers. Nanotechnology is widely applied in different areas; from agriculture to industry. The synthesized and engineered NPs are broadly exploited in medical science, energy, biotechnology, environment, aquaculture and agriculture (Zhang *et al.,* 2008; Rather *et al*., 2011; Sekhon, 2014). Copper (Cu) NPs the broadly synthesized applied materials are manufactured in nanotechnology. The delivery rate of Cu NPs into the aquatic ecosystems in 2011 stood at 11 tons. Therefore, the toxicity of Cu NPs to organisms needs to be investigated (Kahru *et al.,* 2008).

Although copper in higher amount leads to dangerous consequences in biological ecosystems (Chen *et al.,* 2013), for proper biological activities in organisms, this element is vital. To clarify, Cu is incorporated into structural backbones of a number of determinative enzymes such as cytochrome c oxidase and superoxide dismutase (Arockiaraj *et al.,* 2013). In lower concentrations, it is also required for normal metabolism and biologically active reactions of marine organisms (Ashraf, 2005). Moreover, in shrimps, hemocyanin is formed of a number of subunit proteins, each of which has two Cu atoms. These Cu-based proteins bind to Oxygen molecules (O2) (Waxman, 1975). One genuine way to investigate the influence of Cu on shrimp biology is to study functionally important genes in molecular level. The hepatopancreas, or so-called mid-gut gland, is an extremely principle organ in crustaceans (Bhavan and Geraldine, 2000, Ahearn *et al.,* 2004). This organ plays an important role in detoxifying many types of substances, sequestering, and biotransformation. Indeed, hepatopancreas is responsible in activation of a number of detoxifying proteins (Wu *et al.,* 2008). It is also well established that function and structure of this important organ in crustaceans can be affected by different levels of certain xenobiotics (Bautista *et al.,* 1994, Bhavan and Geraldine, 2000).

Nowadays, analyzing transcript abundance is considered as a reliable and cost-effective approach in understanding cell biology. Prophenoloxidase, glutathione peroxidase and serine protein, are three important gene functioning in shrimp immune system, and fluctuations in their expression can result in different immune responses. By controlling and rapid response to pathogen infection, prophenoloxidase activation cascade plays a significant role in invertebrate immune system (Kakoolaki *et al.*, 2011, Amparyup *et al.*, 2013). Moreover, serine protein is triggered by the activation of prophenoloxidase lead to suitable reaction against applied pathogen (Amparyup *et al.*, 2013). Glutathione peroxidase has an anti-oxidant activity, preventing RNA and DNA damages (Cerenius and Söderhäll, 2004). The expression of these genes changes due to stressful conditions such as invasive pathogens, toxicity with toxic materials. Thus, by measuring mRNA level of these genes in exposed shrimps, a proper decision can be made. The expression level of above mentioned genes in white shrimp, subjected to higher concentration of copper, is still elusive. The study presented here is aimed to investigate the potential impact of copper-based nanoparticles on the expression level of prophenoloxidase, glutathione peroxidase and serine protein genes in white shrimp. Furthermore, we analyzed the histopathological alterations in the hepatopancreas and lymphoid organs of *P. vannamei* after exposure to semi-toxic CuNPs.

Material and methods

Biological material and treatments

The project was carried out in Shrimp Research Center in Bushehr Province. Twelve 50×50×60cm aquariums were filled with 33°C seawater. A total number of 240 shrimps (with average weight of 9 to 11 gr) were prepared form a shrimp proliferation and cultivation Company in Shif Island. The shrimps were divided into 12 aquariums (each of which contain 20 shrimps), and were adapted for one week. The shrimps were also exposed twice in a day.

The treatments were prepared and applied based on the research conducted by Frías-Espericueta *et al*. (2003). They reported that the lethal concentration 50 (LC50) of copper particles for *P. vannamei* is 4.2 mg/L; additionally, Ghorbani *et al*. (2006) determined 4 mg/L of ionic copper as LC50 dosage for this shrimp. Accordingly, three concentrations of sub-acute toxicity including 0.25, 0.5 and 1 mg/L of CuNPs were considered for this study. Moreover, a control treatment with no exposure to CuNPs was cultured to evaluate the potential impact of applied concentrations on shrimp's immune system. It should be noted that three replications were performed for all studied treatments, including control treatment.

RNA extraction cDNA synthesis

In order to study the transcript abundance of prophenoloxidase, serine protein and glutathione peroxidase genes, total RNA was extracted from cephalothorax tissue. For this purpose, three shrimps from each aquarium were picked and after truncating their head, the samples were transformed into liquid nitrogen and immediately stored at 80°C. Total RNA from 100 mg of homogenized tissue was extracted using TRIzol reagent kit. The quality and quantity of extracted RNA were assessed via NanoPhotometer NP80-Implen and gel electrophoresis, respectively. The specificity of each primer was assessed via RT-PCR assay, and the results demonstrated the exact length of amplicons.

Afterwards, the first strand cDNA was synthesized using reverse transcriptase enzyme (RevertAid M-MuLV reverse transcriptase; Fermentas, Lithuania). Oligo (dt) 18 primer was used to amplify all mRNAs. To study the expression of the aforementioned genes, we designed specific primers by aligning multiple sequences. To confirm the specificity of each primer, RT-PCR assay was conducted with synthesized cDNA as a template, the results demonstrated exact length of amplicons. The primers sequences are listed in Table 1.

Table 1: List of primer sequences used in qRT-PCR assay.

Primer no.	Primer name	Use	Primer sequence
1	$Pro_{forward}$	qRT-PCR	5'- GCCTTGGCAACGCTTTCA-3'
2	$Pro_{reverse}$	qRT-PCR	5'- CGCGCATCAGTTCAGTTTGT-3'
3	$Gpx_{forward}$	qRT-PCR	5'- TCGGCAAAGTCGACGTCAA-3'
4	$Gpx_{reverse}$	qRT-PCR	5'- GCAGTCGCTCCTTCAGGTACTTA-3'
5	$SP_{forward}$	qRT-PCR	5'- CGTCGTTAGGTTAAGTGCGTTCT-3'
6	$SP_{reverse}$	qRT-PCR	5'- TTTCAGCGCATTAAGACGTGTT-3'
7	$ACTIN_{forward}$	Reference gene	5'- GAGCAACACGGAGTTCGTTGT -3'
8	$ACTIN_{reverse}$	Reference gene	5'- CATCACCAACTGGGACGACATGGA-3'

Expression analysis using qRT-PCR assay

The qRT-PCR was carried out using a Bio-Rad MiniOpticon system with the fluorescent dye SYBR®Green. The reaction for each sample was set up with 2.5 unit Taq DNA polymerase (Fermentas, Lithuania), 10 pmol of each primer, 10 mM dNTP, 1.5 mM MgCl2 and 1X PCR buffer. The program was run with 94°C for 10 min as an initial denaturation, and followed by 40 cycles of 95°C (15 Sec), 52°C (30 Sec) and 72°C (30 Sec). When each run was completed, to inspect the specificity of the primer sets, the dissociation stage was accomplished by slowly ramping up the temperature from 60°C to 95°C (1°C increase per ten seconds). The results of obtained melting curve and peak clearly indicated the specific amplicons.

For quantifying transcription levels, β-actin gene was selected as an internal

control. Eventually, Cycle Threshold (CT) was analyzed using Livak method (2 –delta delta Ct) on REST software. It should be noted that for each sample, three replications were considered.

Histopathological analysis of hepatopancreas and lymphoid organs

In order to evaluate histopathological changes in hepatopancreas and lymphoid organs, a total of 36 shrimps were selected from aquariums (three shrimps from each aquarium). To prevent post-mortem changes, Davidson's fixative solution was injected into the samples. The samples were larger than 2 cm, so Davidson's fixative was initially injected to hepatopancreas and then injected in both sides of carapace and ventral segments. Shrimps were divided into two parts from dorsal to ventral section using one sterilized scissors, and were placed into tubes containing Davidson's fixative solution for 48 hrs. Afterwards, the samples were transferred into a tube containing 70% ethanol, and kept at 25^{o}C until preparation time. Impregnation and processing of the samples was carried out using Tissue Processor equipment. After impregnation, sections were embedded in paraffin and truncated with rotary microtome at 5 µm. The obtained samples were mounted on slides identifying with specific cods and incubated at 40 °C for one hr. Staining was performed according to Lightner method using hematoxylin, eosin and phloxine; and histopathological changes were observed using a light microscope (Lightner and Redman, 1992).

Results

CuNPs down-regulate the expression of immune responsive genes

The quality and quantity of extracted RNA was confirmed. Then, in order to test the specificity of designed primers and determine the best melting temperature, touchdown PCR assay was carried out. The results confirmed the specificity of the primers and identified 58^{o}C for Actin, Pro and SP, and 52^{o}C for GPx gene.

Furthermore, the expression level of all studied genes was assessed using real-time PCR assay. Figure 1 demonstrates the mRNA level of Pro in comparison with the control treatment. According to the results of qRT-PCR, application of CuNPs down-regulated the expression of this gene; however, various concentrations led to different expression profile. Application of 0.25 and 1 mg/L of CuNPs significantly decreased ($p< 0.05$) the mRNA level of Pro gene compared to untreated samples; however, 0.5 mg/L had no significant impact on expression profile (Fig. 1a).

Transcript abundance of GPx was considerably decreased when treating with different concentrations of CuNPs (Fig. 1b). However, different concentrations resulted in various relative expressions in this gene. For instance, treating shrimps with 0.25, 0.5 and 1 mg/L of CuNPs caused 3, 4.2 and 3 folds lower than control samples.

Likewise the Pro gene, it can be seen that lowest and highest concentrations of this element up-regulated the expression of this gene; whereas, the concentration of 0.5 mg/L had lower effects on transcript level. Eventually, the expression rates of SP down-regulated all three concentrations. Nonetheless, comparing treated samples with controls demonstrated no significant difference in each one of the studied levels (Fig. 1c).

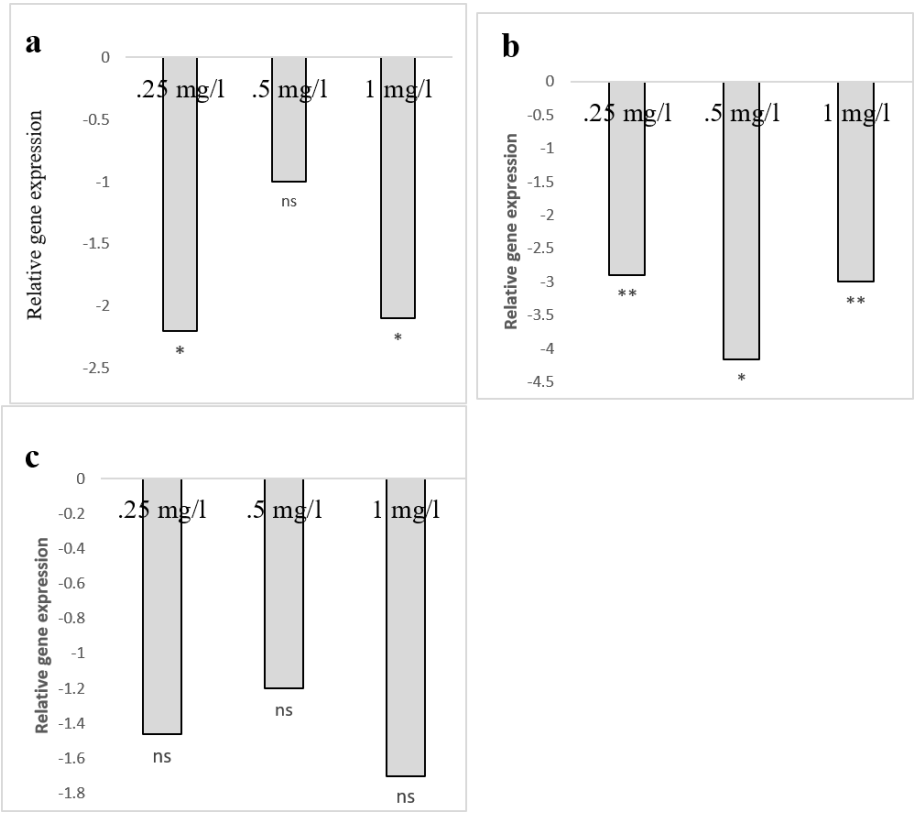

Figure 1: Transcript abundance of prophenoloxidase (a), glutathione peroxidase (b) and serine protein genes (c) in *Penaeus vannamei* subjected to different concentration of CuNPs. The expression rates of all studied genes were down-regulated in applied dosage of CuNPs. Results are expressed as fold-change relative to untreated sample (control). Data were normalized to β-actin endogenous reference gene. Significance was at $p<0.05$, (*); highly significant was at $p<0.01$ (**); ns was at $p>0.05$, no significant difference.

Histological studies on hepatopancreas of P. vannamei

According to histological results, application of CuNPs caused some disorders such as vacuolation, degeneration of the central cell and enlargement of E-cells (Embryonic cells). In 1 mg/L treatment, the amount of vacuoles was more than the two other doses (Fig. 2d). However, in the 0.5 mg/L treatment, abnormalities in R-cells (Resorptive cells), E-cells and F-cells (Fibrous cells) were observed. Furthermore, the degeneration of hepatopancreas central cells in 0.5 doses was visible (Fig. 2c). In 0.25

mg/L of CuNPs, the tissue between hepatopancreatic tubules (CT) was destroyed, which is due to the enlargement of the E-cells (Fig. 2b). Nonetheless, the healthy tissue of the hepatopancreas in untreated samples was observed (Fig. 2a).

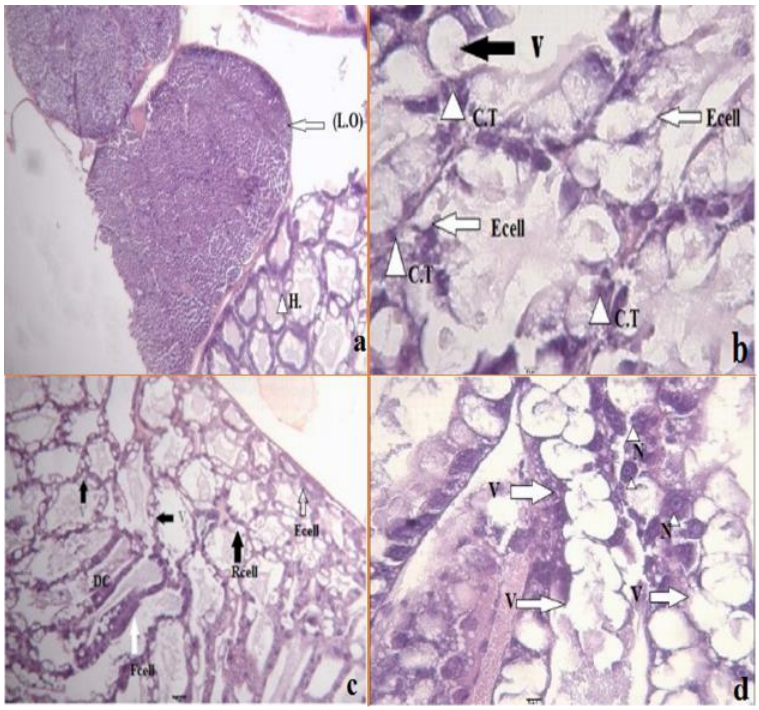

Figure 2: Photomicrographs of hepatopancreas from a control (a) and treated with different concentrations of CuNPs including 0.25 mg/L (b), 0.5 mg/L (c) (H&E X400) and 1 mg/L (d) in *Penaeus vannamei*. Different alterations stemmed from application of copper-based nanomaterials are identified in the slides; V: (vacuole), C.T: (connective tissue), N: (Nucleus), DC: (Degeneration), H: (Hepatopancreas), L.O: (Lymphoid Organ) E-cells: Embryonic (Embryonalzellen) Cells, F-cells: Fibrous (Fibrillenzellen) Cells, R-cells: Resorptive Cells (H&E X1000).

Analyzing lymphoid organs in treated and untreated shrimps also demonstrated some changes such as hypertrophy of lymphoid cells, cell necrosis, and tissue compression. In 1 mg/L treatment, separation of the tissue lobes (Fig. 3H Arrow) was observed which is probably due to higher concentration of the nanoparticle. In addition, treating with 1 mg/L of CuNPs led to increased compression in some areas (white arrows) than others, as well as interaction among connective tissue of tubules. The muscles surrounding the tissue were also lysed and the muscular tendons were separated (identified with stars), and the space between epithelial and muscular tissues was increased (Fig. 3G). In the treatment of 0.25 mg/L, we also

observed some disorders such as lymphoid cell hypertrophy (Fig. 3F, marked with arrow), and cell lysis (Fig. 3F, head of arrow). In contrast, as we expected, no change occurred in control samples (Fig. 3E), indicting the potential impact of applied concentrations of CuNPs on shrimp health.

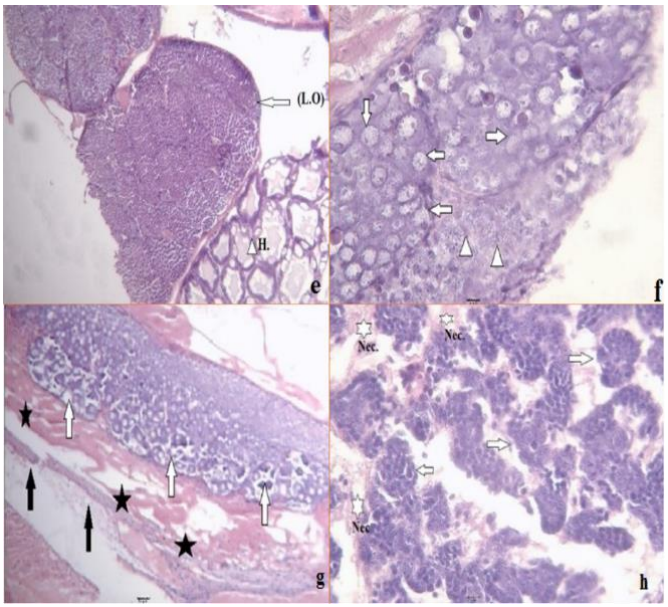

Figure 3: Lymphoid organ from treated and untreated white shrimp (*Penaeus vannamei*) with three levels of sub-toxic CuNPs, i.e. 0.25 mg/L (f), 0.5 mg/L (g) (H&E X400) and 1 mg/L (h). Comparing control (e) and exposed samples (f-h) indicate the adverse impact of concentrations of CuNPs on the shrimp lymphoid organ (H&E X1000).

Discussion

Nowadays, due to increased application of nano-materials in industrial sector, the negative consequences of these materials on both agriculture and aquaculture are not neglectable (Can *et al.*, 2011; Corsi *et al.*, 2014). The sensitivity to pollutants varies among different species, and is chiefly depends on size, age, species and environmental conditions (Hedayati and Safahieh, 2011). In the past few decades, scientists have considerably inclined to investigate the potential impact of NPs on marine organisms, particularly by taking advantage of molecular biomarkers, biochemically and enzymology approaches and so forth (Schlenk *et al.*, 2006). Studying the expression of responsible genes functioning in innate immune system can provides novel insights into biological metabolism. In shrimps, it is well-established that the activity of prophenoloxidase, serine protein and glutathione peroxidase genes play crucial roles in defending and lowering

occurred damages (Amparyup *et al.*, 2013).

Arthropods possess a primitive and slow developed immune system, comparing with vertebrates. Among them, shrimps mostly depend on non-specific immunity to cope with pathogenic agents. The proper activity of phenol oxidase is probably the most important level of defending, thereby attracting scientist's attentions. It is reported that the lower activity of this enzyme would result in the lower shrimp resistance (Li *et al.*, 2005; Wang and Chen, 2006).

Over the past few decades, the activity and function of Pro has been fairly studied, and the results confirmed its importance in invertebrate immune system. The activation of this system is regulated by a multistep pathway (Cerenius and Söderhäll, 2004, Arockiaraj *et al.*, 2013, Bautista-coavrrubias *et al.*, 2015). Overall, Pro system recognizes pathogen and stimulates the humoral immune responses. The enzyme also oxidases phenolic compounds such as tyrosine, which successively lead to melanin production. It should also be noted that the cellular melanotic encapsulation is the most efficient mechanism against intruder materials. Therefore, the activity of Pro enzyme has a great influence on the strong immune response. Herein, we found that the expression of this gene is considerably decreased when exposed to CuNPs.

Bautista-covarrubias *et al*. (2015) reported that subjected white shrimp to copper caused lower immunity in this organism, which is due to the decreased activation of Pro enzyme. Moreover, the research conducted on penaeid shrimp by Burge *et al*. (2007) revealed that most immune cells concentrate on the infected organ, resulting in weakened innate immune system. Furthermore, relation of nanomaterials and free oxygen species is well-established, which ultimately results in oxidative stress and fluctuations in gene expression. This reaction can adversely disturb normal metabolism, lower immunity, and finally lead to cell death. As a consequent, we assume that CuNPs caused an oxidative stress on the studied shrimps, and decreased the expression rate of genes responsible in response in immune system. It is also interesting to note that some other parameters, including concentration and time of exposure, may have an impact on the mRNA level and immunity of the shrimps. Herein, we realized important factors as more concentrations and time exposure, the lower expression of the genes; and consequently, the lower immune response in shrimps.

Histopathological indices opened new avenues to assess toxicological impact of contaminated waters in marine biology. In this project, we evaluated the influence of different concentrations of CuNPs on hepatopancreas and lymphoid organs in white shrimp. Hepatopancreas is among the main organs in shrimp and its proper function for shrimp health is

vital (Bhavan and Geraldine, 2000; Ahearn *et al.*, 2004; Wu *et al.*, 2008). The results presented here indicate the negative impact of CuNPs on this organ; vacuolation, degeneration of the central cell and abnormalities in R-cell, E-cell and F-cell. Abad-Rosales *et al*. (2010) showed that after 10 days exposure of white shrimp with copper, the shape of R-cells and E-cells change, and epithelial cells are destroyed. Additionally, Li *et al*. (2007) showed that exposing to copper in the range of 0.01 to 0.4 mg/L can result in structural alterations in hemocytes, lymphoid cell enlargement and hepatopancreatic necrosis. Consequently, it is highly likely that the more dosage of toxic CuNP, result in the more cell vacuolation; therefore, the higher damage to hepatopancreas. Moreover, applying CuNPs resulted in degenerated hepatopancreas central cells in comparison with control sample. These results are in consistence with the research conducted by Zilli *et al*. (2003). Likewise, analysis of lymphoid organ revealed that the shrimps subjected to CuNPs had some disorders such as hypertrophy of lymphoid cells, cell necrosis, and tissue compression compared to untreated samples. It is also observed that by increasing CuNP concentration, the level of cell hypertrophy and necrosis increases. In addition, the lymphoid organ of treated shrimps showed more changes, which are probably due to attempts to neutralize the potential effects of these particles in the body.

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