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Sub lethal effects of copper oxide (CuO) nanoparticles on blood parameters of common carp (*Cyprinus Carpio*)

Mohadeseh Miri[1*], Abdolali Rahdari[2]

[1]*Department of Natural Ecosystems Mananegmen, International Center of Hamoon Wetland, University of Zabol, Zabol, Iran*

[2]*Department of Fisheries, International Center of Hamoon Wetland, University of Zabol, Zabol, Iran*

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Abstract

Nanotechnology is identifying and controlling materials in dimensions between 1-100 nanometers as physical, chemical and biological properties of material are unusual in these dimensions. The concerns about probable dangers of releasing nanoparticle materials to the environment are increasing with increasing development of this technology. The objective of this study is to examine the effect of nanoparticle copper oxide and AST and ALT enzymes in the blood serum of common carp. In this research, six groups of sixteen carp (*Cyprinus carpio*) consisting of a control group and five groups are exposed to nanoparticles concentrations of 5, 10, 50, 100 and 150 $\mu g.l^{-1}$ after which bleeding was done after 24, 48, 72 and 96 hours. AST and ALT enzymes, protein, urea, and glucose in the blood serum were measured by auto-analyzer and cortisol by commercial kits. The results showed that the amount of AST and ALT enzymes, glucose, cortisol and urea significantly ($p<0/05$) increased in the blood of carps exposed to nanoparticles compared to control fish. Also, significant differences ($p<0/05$) were observed between quoted and basic factors at various periods of time, but protein did not show significant differences ($p>0/05$) in comparison with the control and 5 other. Groups Carps reacted to nanoparticle copper in the environment and it has been possible to use measured factors such as AST and ALT enzymes as the pollution index of an environment with nanoparticle copper.

***Corresponding Author:** Mohadeseh Miri ✉ mirimohadeseh@uoz.ac.ir

Introduction

Nanotechnology development has been rapid due to its application in industries such as health, cosmetics and sanitary, provisions, production of antibacterial materials and toy manufacturing (Gong *et al.,* 2007; Wijnhoven *et al.,* 2009; Saber and John, 2009). Also, the stability of these particles against optical and chemical corrosion has made them suitable materials for application in potential semiconductor in solar energy industry. Other applications of nanoparticles are in gas-fired sensors, resonators of big sound waves, catalysis, electrodes and electrical and optical equipments. Therefore, it is not surprising that many nanoparticle products are currently available to consumers (Saber and John, 2009).

Concerns about probable risks of nanoparticles entering the environment are increasing due growing interest and use (Blaise *et al.,* 2008; Asharani *et al.,* 2008). Nanoparticles such as metal oxides (ZnO, CuO and TiO_2) are used in industrial and commercial productions extensively; therefore it is obvious that nano-scale productions enter into aquatic environments.

Metal particles can enter into water bodies and may accumulate d in sediments or bind to nutrients (Griffitt *et al.,* 2008). Inhaling or ingesting is the route by which nanoparticles are absorbed in terrestrial organisms while aquatic animals can absorb nanoparticles through gills and external mucous layers in addition to inhaling or ingesting (Shaw and Handy, 2011). There are many reports regarding pollution of aquatic ecosystems by nanoparticles (Baun *et al.,* 2008; Griffitt *et al.,* 2008; Handy *et al.,* 2008; Shaw and Handy, 2011; Al-Bairuty *et al.,* 2013). Among nanoparticles, copper oxide is mostly used in industries, pharmaceutical industry, production of insecticides (Kiaune and Singhasemanon, 2011) and antibacterial products (Gabbay and Borkow, 2006). Copper is an essential element for living organisms present in cellular metabolisms as a co-factor. A high density of copper in an environment can be toxic to aquatic organisms due to their oxidizing and reducing properties (Grosell *et al.,* 2007). The objective of this study is to examine the effect of nanoparticle copper oxide and AST and ALT enzymes in the blood serum of (*Cyprinus carpio*).

Materials and methods

Copper oxide nanoparticle (CuO-NPs) was used in this experiment and was obtained from US NANO, USA. The size of the nanoparticle was 40 nanometers produced by Stoke nanoparticle with 100 density microgram perliter.*Cyprinus carpio* obtained from Zahak Fish Culture Farm, were transferred to the Hamoun International Wetland Laboratory. Fish were acclimatized to laboratory conditions in glass tanks for one month before exposure. Water temperature was maintained at 25±1◦C. Dissolved oxygen, hardness and pH were 6.5–7.8 mg L^{-1}, 76.4–79.1 mg L^{-1} and 7.1–7.3, respectively. The fish were fed one month with formulated feed and the dead fish were immediately removed to avoid possible water contamination. All procedures used in the present study complied with ethical and practical principles of animal use.

The experiment consisted of 6 groups of carp (control, 5, 10, 50, 100, 150 ($\mu g.l^{-1}$) with each group having 16 carp was carried out in 18 aquariums (40 liter capacity) in three replicates. Fish were maintained in static renewal conditions where water and CuO were completely replaced every 24 h, transferring fish to freshly prepare toxicant solutions (OECD). Bleeding of fish in each group after 24, 48, 72 and 96 hours was done after exposure to copper nanoparticle before being placed in aquariums (basic). At the end of each duration, four fish were removed from the aquaria and used as replicates. Fish were immediately anesthetized with MS222, and blood samples were taken from the caudal vein of each fish. Plasma separation was done by using the centrifuge at 3000 times per minute for 15 minutes by measuring alanine aminotransferase, aspartate aminotransferase, glucose, protein, urea and cortisol. The separated plasma in Eppendorf Micro tube was

kept at -80°C until analysis (Acerete *et al.,* 2004). The levels of alanine aminotransferase, aspartate aminotransferase, glucose, protein, and urea were measured by using an auto analyzer (Model Selectra-PRO, ELISA kits). The level of cortisol in blood of plasma was also measured by purchased commercial kits.

SPSS 16. (Statistical Package Social Science) software was used for statistical analysis. The difference between the 5 groups and control and between various periods of time and basic group were identified by using one-way ANOVA followed by the Duncan test. A p-value < 0.05 was considered statistically significant.

Results

The average comparison of the amount of ALT enzyme showed that there is a significant difference (P<0.05) between the 5 groups of carp and the control group at different concentrations. The least amount of ALT at different concentrations in the control group was approximately 1.5 U.l^{-1} and the highest amount in 24, 48, 72 and 96 hours was respectively 4, 5.5, 6 and 3.5 U.l^{-1} respectively between 100 and 150 concentrations. The amount of ALT enzyme increased in carps exposed to nanoparticle copper in comparison with the control group with consideration to obtained results, but there was no significant difference between the various groups of carp exposed to nanoparticle copper at different concentrations. It was observed that the amount of ALT has a significant difference (P<0.05) with the basic group in different days according to statistical analyses. The least and the highest amount of ALT was 1.62 U.l-1 in the basic group and 4.7 U.l-1 in 100 µg.l^{-1} nanoparticle copper. The comparison among the duration of exposure to nanoparticle copper also showed significant differences (P<0.05) as the amount of ALT increased after 24, 48 and 72 hours but reduced after 96 hours (Fig. 1).

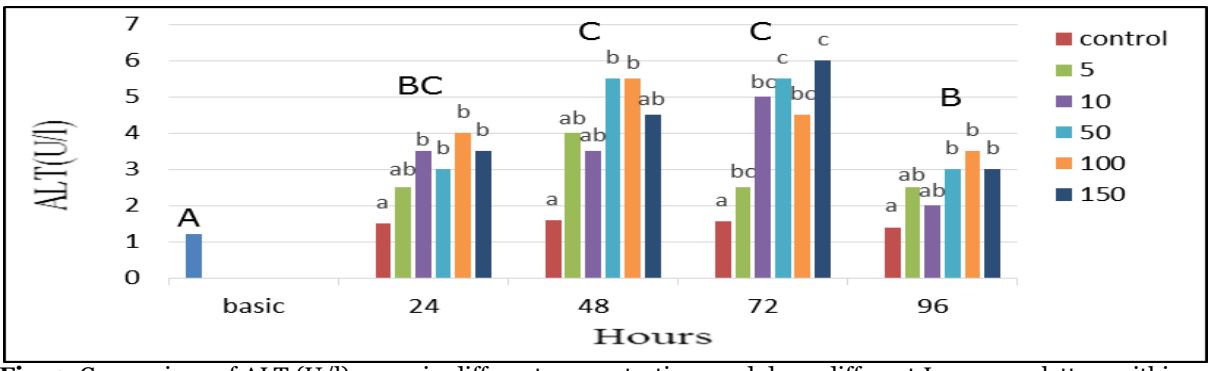

Fig. 1. Comparison of ALT (U/l) mean in different concentrations and days; different Lowercase letters within a time point indicate significant differences between treatments and uppercase letters indicates significant difference to basic with different time, and designate statistically significant values (One-way ANOVA–Duncan test, P < 0.05).

The comparison of statistical tests as regards the amount of aspartate aminotransferase (AST) enzyme showed that the various groups of carp showed significant differences (p<0.05) with the control group of carp at different concentrations. The lowest amount of AST in the control group was approximately 67.3 U.l^{-1} and the highest amount of AST at different time periods (after 24,48, 72, and 96 hours) were 184, 277.5, 234, and 200 U.l^{-1}, respectively at high concentrations of nanoparticle copper as compared to the obtained results. The amount of AST enzyme in carps exposed to the nanoparticle copper also increased in comparison to the control. It was identified that the amount of AST in the groups at different concentrations showed no significant differences (p<0.05) however, significant difference were observed after 72 hours among groups at 10, 50, 100, and 150 µg.l^{-1} concentrations in

comparison to the exposed carp group. The statistical analyses showed that the amount of AST enzyme showed significant differences ($p<0.05$) with the 5 groups of carp at different days. The lowest and highest amount of AST in the basic group was 96 and 216.7 $U.l^{-1}$, respectively on exposure to nanoparticle copper after 48 hours. The amount of AST increased after 24 and 48 hours, with a reduction observed after 72 and 96 hours (Fig. 2).

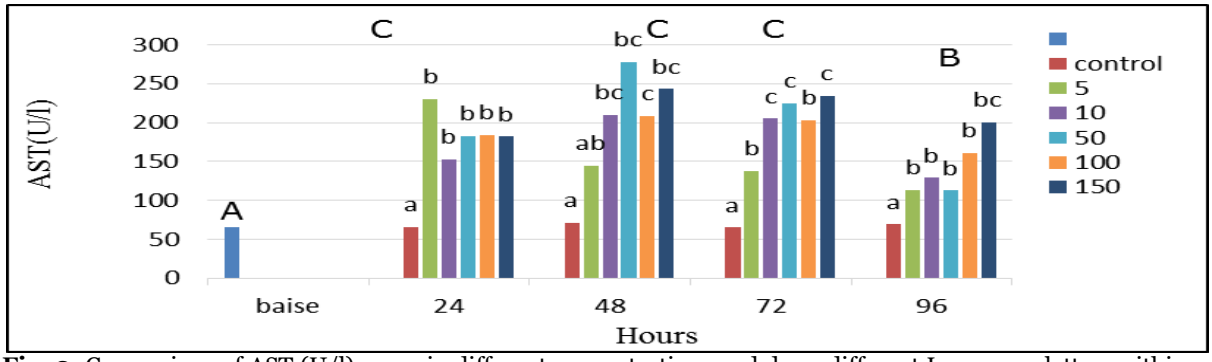

Fig. 2. Comparison of AST (U/l) mean in different concentrations and days; different Lowercase letters within a time point indicate significant differences between treatments and uppercase letters indicates significant difference to basic with different time, and designate statistically significant values (One-way ANOVA–Duncan test, $P < 0.05$).

The average comparison of the amount of cortisol showed that there was a significant difference ($p<0.05$) between the 5 groups of carp and the control group at different concentrations ($p<0.05$). The lowest and the highest amount of cortisol was 7 ($\mu g.dl^{-1}$) in control group of carp at all periods of time and 18.75 $\mu g.dl^{-1}$ on exposure to nanoparticle copper after 72 hours. There was no significant difference ($p<0.05$) among the different groups exposed to the poison ($p>0.05$). It was observed that the amount of cortisol was also significant at $p<0.05$ in the basic group of carp on exposure to nanoparticle copper after 48 hours. With the least and most amount being 6.5 $\mu g.dl^{-1}$ and 17.38 $\mu g.dl^{-1}$ respectively. The comparison of nanoparticle copper exposure showed significant differences ($p<0/05$) at different time periods. The amount of Cortisol increased after 24 hours exposure to nanoparticle copper. The lowest and highest amount of cortisol in carps' blood was 7.2 $mg.dl^{-1}$and 17.38 $mg.dl^{-1,}$ respectively, in the basic group on exposure after 48 hours (Fig. 3).

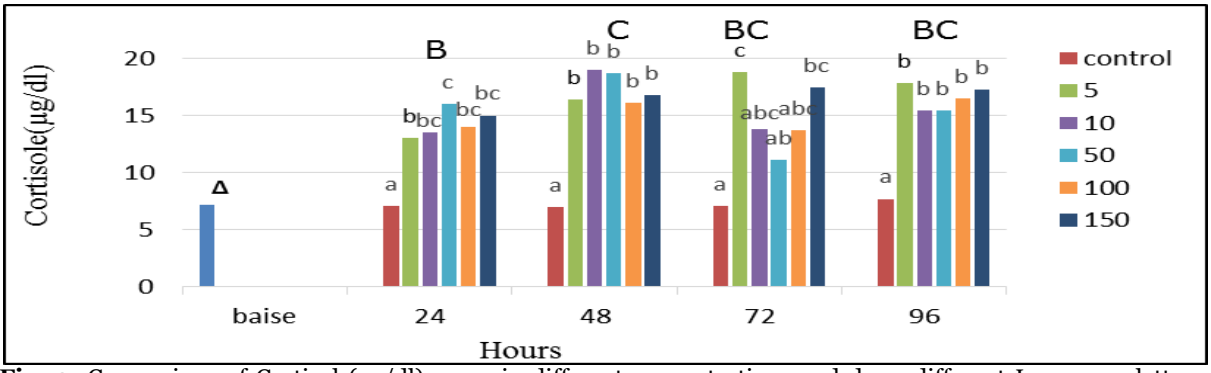

Fig. 3. Comparison of Cortisol (µg/dl) mean in different concentrations and days; different Lowercase letters within a time point indicate significant differences between treatments and uppercase letters indicates significant difference to basic with different time, and designate statistically significant values (One-way ANOVA–Duncan test, $P < 0.05$).

The average comparison of the amount of glucose showed that various groups of carp showed significant differences ($p<0.05$) with the control group on exposure to nanoparticle copper after 24 hours at 5 $\mu g.l^{-1}$and 96 hours at 100 $\mu g.l^{-1}$. The control group did not show any significant difference ($p>0/05$) on exposure after 48 and 72 hours. The lowest and highest amount of glucose in the control group was 38.5 38.5 $mg.dl^{-1}$ at all periods of time and 74.5 $mg.dl^{-1}$ at 100 $\mu g.l^{-1}$ on exposure to nanoparticle copper after 24 hours. There was no significant differences ($p>0.05$), among the various groups exposed to nanoparticle copper. Figure 4 shows that the amount of glucose in carps' blood was significant at $p<0.05$ on exposure to nanoparticle copper in comprise with the control group at different periods of time. The least and the most amount of glucose was 39.5 $mg.dl^{-1}$ and 61 $mg.dl^{-1}$, respectively, on exposure to nanoparticle copper after 72 hours in the basic group (Fig. 4).

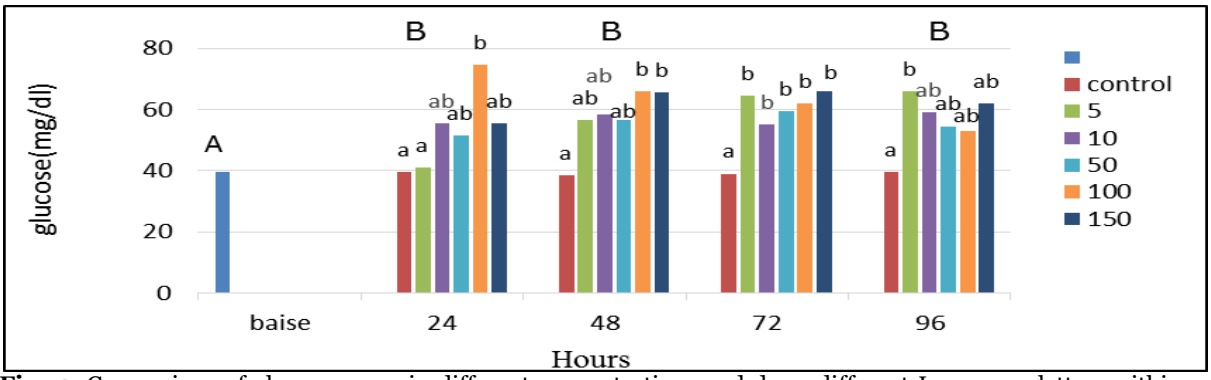

Fig. 4. Comparison of glucose mean in different concentrations and days; different Lowercase letters within a time point indicate significant differences between treatments and uppercase letters indicates significant difference to basic with different time, and designate statistically significant values (One-way ANOVA–Duncan test, $P < 0.05$).

Fig. 5 shows that the comparison of urea levels in carps' blood exposed to nanoparticle copper with control group differ in density. The results show that the amount of urea increased on exposure to nanoparticle after 24 hours, but there was no significant difference with the control group. The amount of urea also increased significantly at $p<0.05$ by 100 and 150 $\mu g.l^{-1}$ on exposure after 48 and 72 hours in the groups and control, respectively. Significant differences were also reported after 96 hours exposure to nanoparticle copper among the groups and the control group (Fig. 5).

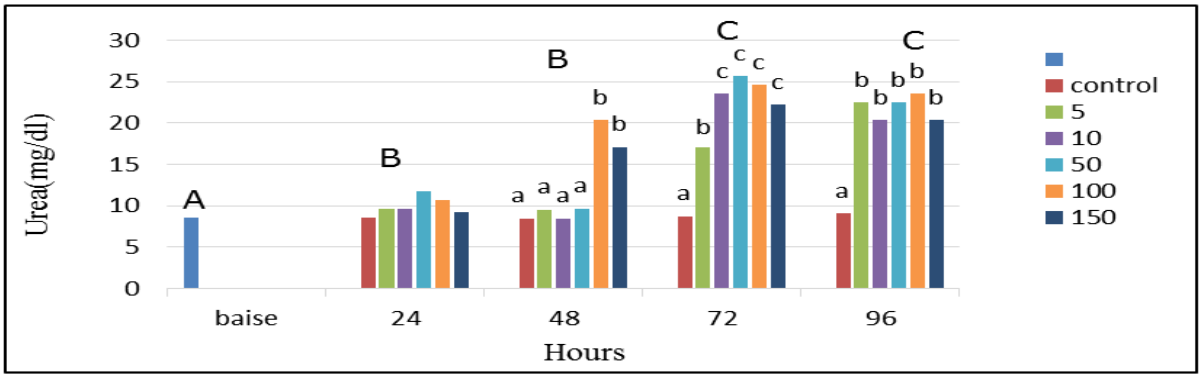

Fig. 5. Comparison of urea mean in different concentrations and days; different Lowercase letters within a time point indicate significant differences between treatments and uppercase letters indicates significant difference to basic with different time, and designate statistically significant values (One-way ANOVA–Duncan test, $P < 0.05$).

The least and the most amount of urea in carps' blood was 8.5 mg.dl^{-1} in the witness group and 24.64 mg.dl^{-1} in 100 µg.l^{-1} concentration exposure after 72 hours. The comparison of urea levels on exposure to nanoparticle copper with the basic group at different time periods was significant at $p<0.05$. Exposure after 24 hours showed no significant differences with the basic group however, other periods of time showed significant differences. Finally, the results showed that there is a significant difference between exposure after 72 and 96 hours with 24 and 48 hours (Fig. 5).

It was identified that the amount of protein in carps' blood had no significant differences in various groups in comparison with control group according to statistical analyses. Also, results show that there were no significant differences among different periods of time on exposure to nanoparticle copper in comparison to basic group (Fig. 6).

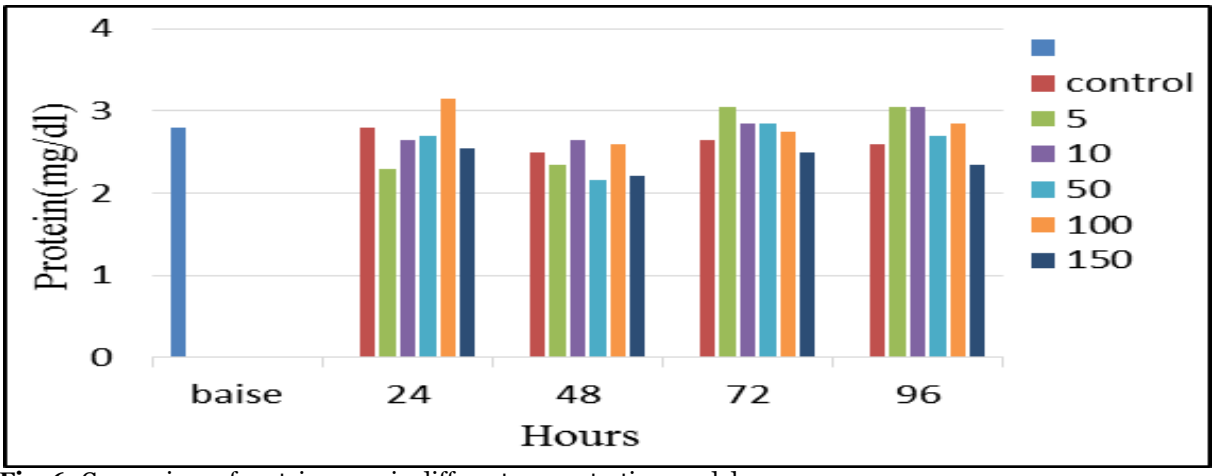

Fig. 6. Comparison of protein mean in different concentrations and days.

Discussion

Many researchers have concluded that blood cells are good indices to study and evaluate the environmental effects of poisoned materials or other kinds of stresses (Kondera, 2011); therefore, the activities of enzymes such as ALT and AST are used to identify diseases and to discover the structural damages caused by environmental pollution on carps (Flart *et al.,* 2011).

An increase in these enzymes indicates sensitivities and cellular damages which is represented by the serum liquid index which also shows stress disorders (Palanivelu *et al.,* 2005). Generally, poisons cause to damages to liver cells and these cellular damages cause the release of cellular enzymes into the blood serum. An increase in these enzymes in the blood serum causes apoptosis of liver tissues (Flart *et al.,* 2011).

In the current study, the amount of AST and ALT enzymes increased in carps' blood serum exposed to nanoparticle copper in comparison with witness group and this increase showed significant differences ($p<0.05$) in comparison with control group. It can thus be concluded that the result of increased enzyme secretion in the blood causes liver necrosis.

Harvey *et al.* (1994) declared that the levels of AST and ALT in blood may increase due to cellular damages in liver and high levels of these enzymes in serum usually indicate liver disorders in animals (Harvey *et al.,* 1994).

Lee *et al.* (2014), studied the effect of ZnO nanoparticle on common carps' ALT and AST enzymes levels for 12 weeks. It was identified that the amount of these enzymes increased in carps' blood serum had significant differences ($p<0.05$) with the control group. They declared that the result of increasing enzyme levels is liver damage and

according to these results, it is possible to use these enzyme levels as a suitable index for nanoparticle zinc oxide pollution. It can thus be concluded that ALT and AST enzymes increase in the blood of carps exposed to nanoparticle copper can be used as a nanoparticle pollution index (Lee *et al.,* 2014).

Changing levels of carps' cortisol serum has been vastly identified as a primitive response to stress resulting from poisoned materials. The internal axis of the hypothalamus-hypophysis (HPI) in carp secretes cortisol and steroid cortisol hormone for protecting disrupted haemostats (Gagnon *et al.,* 2006). Dethloff *et al*., (1999) reported that cortisol is released in blood through stimulation of the axis by poisoned materials (Dethloff *et al.,* 1999). Cortisol is not stored in internal textures but is synthesized on a demand basis (Sumpter, 1997). In the current study, the cause of increasing cortisol in blood circulation can be a result of (HPI) stimulation in response to exposure to nanoparticle. Generally, an increase in the level of cortisol in the serum of carp is a response to environmental stress (Wendelaar Bonga, 1997).

Increased concentration of blood glucose in fishes is considered as second response to environmental stress (Sepici-Dinc *et al.,* 2009). The amount of glucose increased in carp under stress according to Cick and Engin's (2005) study. The cause of the increased blood sugar is probably due to increasing glucose-6-phosphate and glycogen breaking in the liver and or synthesizing glucose from protein out of amino acids in the liver (Almeida *et al.,* 2001).

Raja *et al.* (1992) declared that the cause of increasing glucose levels in carps' blood which is groomed with insecticide is a symptom of disorder in carbohydrate metabolism (Raja *et al.,* 1992). Increasing blood glucose results from increasing liver glycogen which probably occurs from decreased insulin activities (Das and Mukherjee, 2003).

Farmen *et al.* (2012) showed that the amount of urea in the blood had significant effect ($p<0.05$) on increasing the amount of nanoparticle in the water of aquariums which is similar to the results obtained in this study (Farmen *et al.,* 2012).

Protein did not show meaningful change according to statistical analyses in this study. Protein is used as an index of liver disorder in toxicology studies (Yang and Chen, 2003).

Conclusion

Despite the relative high concentrations tested in this study, they do not represent the current reality. The results can contribute to better understand the potential risks of CuO NP in aquatic environments. The results showed that *C. carpio* viability was affected with an increase in CuO NP concentrations. The levels of AST and ALT enzymes, urea and glucose in carp were significantly inhibited by CuO NPs during the 4 day sub-acute toxicity tests. The observed changes in the serum may provide useful information regarding environmental conditions and risk assessments in aquatic organisms.

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