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Toxic Effect of Silver Nanoparticles on Liver, Gill and Muscle Tissues of Zebrafish *Danio rerio*

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Abstract

The present study was aimed to evaluate the toxic effect of silver nanoparticle on the liver, gill and muscle tissues of the Zebrafish, *Danio rerio*. The experiment was designed to understand the chronic toxicity of silver nanoparticles (AgNPs) in adult fish, *Danio rerio.* In the chronic toxicity study adult fish, *Danio rerio* were divided in to two groups. First group was experimental group in which fish were successively treated with a graded series of 0.3, 0.6, 0.9 mg/l an average 60 nm. PVP Coated AgNPs treatment was given for 21 days. At the end of experimental period, Reduced glutathione activity (GSH), Lipid peroxidation activity (LPO), Lactate dehydrogenase (LDH) and Total protein in liver, gill and muscle tissues were assayed. Second group were kept as a control which was free from AgNPs exposure. The levels of Reduced glutathione activity (GSH) and Total protein were found to be decreased were as Lipid peroxidation activity (LPO) and Lactate dehydrogenase activity (LDH) were found to be elevated in liver, gill and muscle tissue of AgNPs treated Zebrafish *Danio rerio*. This study clearly revealed the adverse effects of AgNPs in different concentrations on the functions of the liver, gill and muscle tissues of Zebrafish, *Danio rerio*.

1. Introduction

Nano-sized products have been developed in the fields of drug delivery, diagnostics, tissue engineering, agricultural sciences and environmental remediation[1]. Ag-NPs concentrations in environmental waters is largely unknown, but there is a common belief among scientists that through incidental release or manufacturing discharge, there is an impact of environmental Ag-NPs on aquatic ecosystems[2]. The toxicity of AgNPs was shown to be a result of an interplay of the particle uptake, dissolution and induction of oxidative stress[3].

Toxicity of AgNPs had three possible toxicity mechanisms. Firstly, free Ag^+ uptaken by cells destroy the production of ATP and DNA replication. Furthermore, the initiation of Reactive Oxygen Species (ROS) is promoted on the surface of AgNPs and Ag ions. Thirdly, AgNPs directly damage cell membrane[4]. The tiny nanomaterials can easily penetrate inside the tissues of fish, such as the liver, gills, brain and muscles[5]. The rare information of

AgNPs regarding aquatic toxicity and their linkage with living components has raised concerns in the last decade[5].

Excessive production of Reactive Oxygen Species in cells by direct interaction with particles and or dissolved species is presently accepted as one of the main mechanisms of cellular toxicity of engineered nanoparticles[6]. The low cost, small size and available resources of the zebrafish, *Danio rerio*, make it a good model for toxicological assessment and make it an excellent model organism for measuring environmental fate and toxicity of Ag-NPs[7]. Zebrafish has 80% sequence homology towards humans and share many common biological pathways and biological systems to mammals[8]. The objective of this present study was to evaluate the toxic effect of silver nanoparticles on the liver, gill and muscle tissues of the Zebrafish, *Danio rerio*.

2. Materials and Methods

2.1 AgNPs Suspension, Preparation and Characterization

Silver nanoparticle (AgNP) powder (stated by the manufacturer to be spherical 60 nm particles), coated with Polyvinyl Pyrrolidone (PVP) (Figure 1) and a purity of 99.5%, and was purchased from Indian platinum Pvt. Ltd. Vile Parle West, Mumbai. PVP coated silver nanoparticles AgNPs) were chosen, as they should easily disperse in water. The crystal structure of the AgNP powder was examined by X-ray diffraction analysis using an X-ray powder diffractometer at UICT (University Institute of Chemical Technology) of KBC North Maharashtra University, Jalgaon (Figure 1).

The morphology, average diameters, and dispersion of AgNP were determined with a Field Emission Scanning Electron Microscopy (FE-SEM) system at UICT (University Institute of Chemical Technology) of KBC North Maharashtra University, Jalgaon. FE-SEM analysis powered Sample of AgNP was used for Field Emission scanning electron microscopic Analysis. FE-SEM Technique was employed to see the size and shape of the Silver nanoparticles.

2.2 Electron Dispersion X-ray Spectroscopy (EDX or EDS)

Powered Sample of AgNP was used for Electron Dispersion X-ray Spectroscopy (EDX or EDS) Analysis and XRD study powdered sample of AgNP was used for X-ray diffraction. The coherently diffracting Crystallography domain size of the Silver nanoparticle was calculated from the width of the XRD peaks using Scherer formula, FE-SEM. work was conducted at UICT (University Institute of Chemical Technology) of KBC North Maharashtra University, Jalgaon.

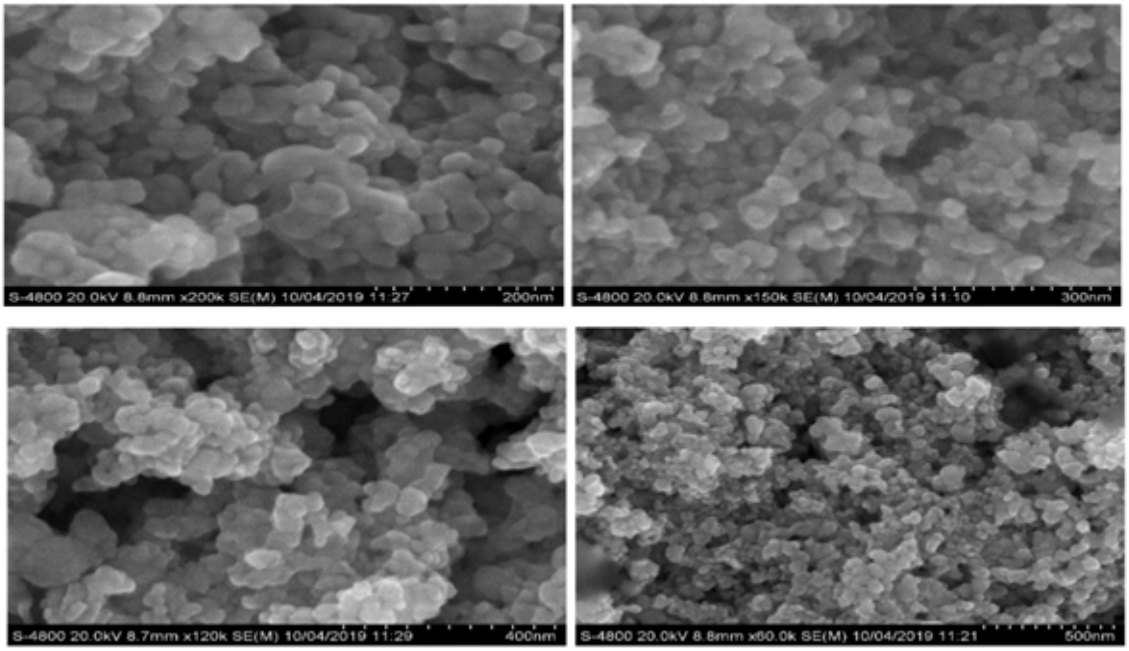


Figure 1. Characterization of silver nanoparticles (AgNPs) By FESEM.

2.3 Fish and Exposure Conditions

In the acute toxicity study adult fish, *Danio rerio* was divided in to two groups Group-I was experimental in which for the 96-h toxicity test, healthy fish, *Danio rerio* (standard length 28.1 ± 0.2 mm weighing 0.42 ± 0.04 g) were chosen for the experiments and divided into different 60 nm PVP coated AgNPs treatment groups (0, 0.4, 0.8, 1.5, 3.5 and 4.5 mg/L), (20%, 40%, and 60%) of 96-h LC value of AgNPs as calculated earlier. Group II-was kept as a control which was free from AgNPs exposure. All fish were kept in 10-L aquaria and fasted for the duration of exposure. The test water was changed each day. In the 96-h static exposure, dead animals were recorded for the calculation of median lethal concentration (LC_{50}) values. Based on the LC_{50} values, 60 nm PVP coated AgNPs concentrations of 0.3, 0.6, 0.9 mg/L were given for a sub-chronic exposure study.

2.4 Animal Groups

Chronic toxicity (LC_{50}) test of silver nanoparticles on adult fish, *Danio rerio* were conducted in a static water renewal experiment, according to the Organization for Economic Cooperation and Development (OECD) guideline for testing of chemicals[9].

In the chronic toxicity study adult fish, *Danio rerio* was divided into two groups.

Group-I was an experimental group in which fish were consecutively treated with a graded series of 0.3, 0.6, 0.9 mg/L an average 60 nm. PVP coated AgNPs treatment were given for 21 days, in each group 10 fish were equally allocated into three 10-L water aquaria.

Group-II were kept as a control which was free from AgNPs exposure.

The fish, *Danio rerio* were not feed 24 hours prior to or during the experiment in order to maintain constant exposure concentrations, given that AgNPs nanoparticles might adhere to food and faeces particles[10].

2.5 Behavioural Study

Indicator of exposure-related stress, the fish behaviour was assessed by a human observer after 0, 3, 6, 12, 24, 27, 30 and 48 hours of 60 nm. PVP coated AgNPs treatments. The behavioural components considered with operculum movements (ventilation rate), surface respiration, jerk movement, circular swimming, bottom resting, aggressive behaviour and loss of equilibrium. Further, excess mucus production was recorded. Mortality was checked continuously and fish were considered dead when operculum movement and response to mechanical stimuli could no longer be detected. At the end of the 21th day, ten Zebrafish, *Danio rerio* from each of the treatments and control were collected. Liver, gill and muscles were dissected and subjected to estimations.

2.6 Biomarkers

Tissue extracts for antioxidant enzymes measurements were obtained from liver, gill and muscle by homogenizing the tissues using 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol by method[11]. Reduced glutathione activity (GSH) was estimated by method[12]. Lipid peroxidation activity (LPO) was estimated by method[13]. Lactate dehydrogenase (LDH) was estimated by method[14]. Total protein estimation by method[15].

2.7 Statistical Analysis

One Way Analysis of Variance (ANOVA) was applied to test the significance of data. All the data are expressed as means (n = 5) ± Standard Deviation (SD) and differences were considered significant at $P<0.05$.

3. Results

3.1 Reduced Glutathione (GSH) Activity in Liver, Gill and Muscle

The reduced glutathione activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 6.06 ± 0.30, 5.13 ± 0.47 and 4.10 ± 0.43 µM/mg protein respectively for 21 days. The reduced glutathione activity of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be decreased in exposed with various subleathal concentrations of silver nanoparticles 0.3 mg/l AgNPs (5.41 ± 0.31, 3.38 ± 0.39 and 3.11 ± 0.68), 0.6 mg/l AgNPs (4.15 ± 0.40, 2.23 ± 0.31, 2.18 ± 0.20 and 0.9 mg/l AgNPs (3.27 ± 0.56, 1.38 ± 0.19 and 1.38 ± 0.27) for 21 days respectively. The decrease in the reduced glutathione activity of the liver gill and muscle was more in 0.9 mg/l.

3.2 Lipid Peroxidation (LPO) Activities in Liver, Gill and Muscle

The Lipid Peroxidation activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 10.27 ± 0.40, 7.09 ± 0.62 and 6.14 ± 0.66 U/MDA/mg protein respectively for

Table 1. GSH-teduced glutathione activity (µM/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days

Concentrations (Mg/L)	Tissues		
	Liver	Gill	Muscle
(00 mg/l) Control	6.06 ± 0.30**	5.13 ± 0.47**	4.10 ± 0.43**
(0.3 mg/l)	5.41 ± 0.31**	3.38 ± 0.39	3.11 ± 0.68
(0.6 mg/l)	4.15 ± 0.40**	2.23 ± 0.31**	2.18 ± 0.20**
(0.9 mg/l)	3.27 ± 0.56**	1.38 ± 0.19**	1.38 ± 0.27**

Values represent mean (n=5), ± standard deviation (SD) of five individual observations. Values are significant at P<0.05**

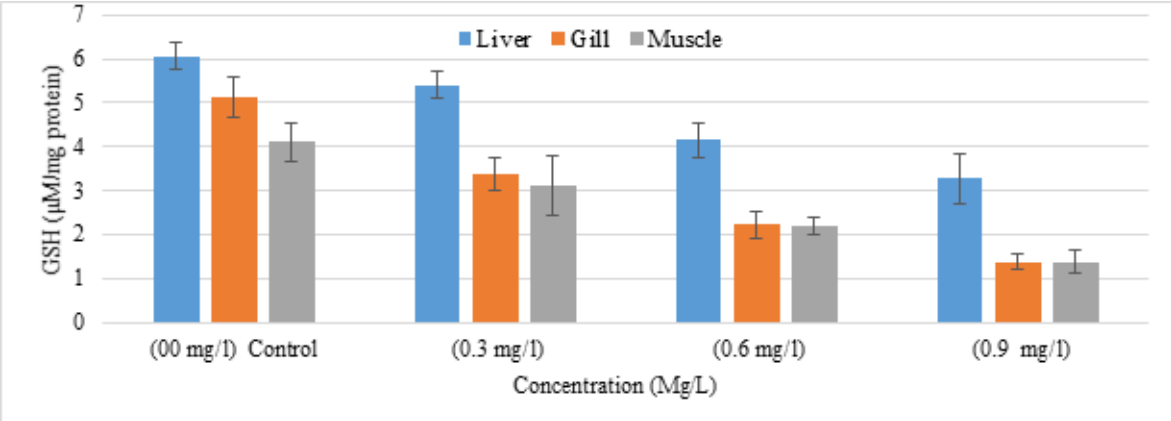


Figure 2. GSH-reduced glutathione activity (µM/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days.

Table 2. LPO-Lipid peroxidation activity (U/MDA/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days

Concentrations (Mg/L)	Tissues		
	Liver	Gill	Muscle
(00 mg/l) Control	10.27 ± 0.40**	7.09 ± 0.62**	6.14 ± 0.66**
(0.3 mg/l)	11.14 ± 0.35**	8.05 ± 0.30**	7.22 ± 0.49**
(0.6 mg/l)	12.02 ± 0.92**	9.11 ± 0.60**	8.13 ± 0.35**
(0.9 mg/l)	13.20 ± 0.55**	11.09 ± 0.57**	9.05 ± 0.14**

Values represent mean (n = 5), ± Standard Deviation (SD) of five individual observations. Values are significant at P<0.05**

21 days. The Lipid Peroxidation activity of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be elevated in exposed with various subleathal concentrations of silver nanoparticles 0.3 mg/l AgNPs (11.14 ± 0.35, 8.05 ± 0.30 and 7.22 ± 0.49), 0.6 mg/l AgNPs (12.02 ± 0.92, 9.11 ± 0.60, 8.13 ± 0.35 and 0.9 mg/l AgNPs (13.20 ± 0.55, 11.09 ± 0.57 and 9.05 ± 0.14) for 21 days respectively. The increase in the Lipid Peroxidation activity of the liver gill and muscle was more in 0.9 mg/l.

3.3 Lactate Dehydrogenase (LDH) activity in liver, gill and muscle

The Lactate Dehydrogenase activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 27.13 ± 0.37, 15.21 ± 0.57 and 31.11 ± 0.40. U/mg protein respectively for 21 days. The Lactate Dehydrogenase activity of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be elevated in exposed with various subleathal concentrations

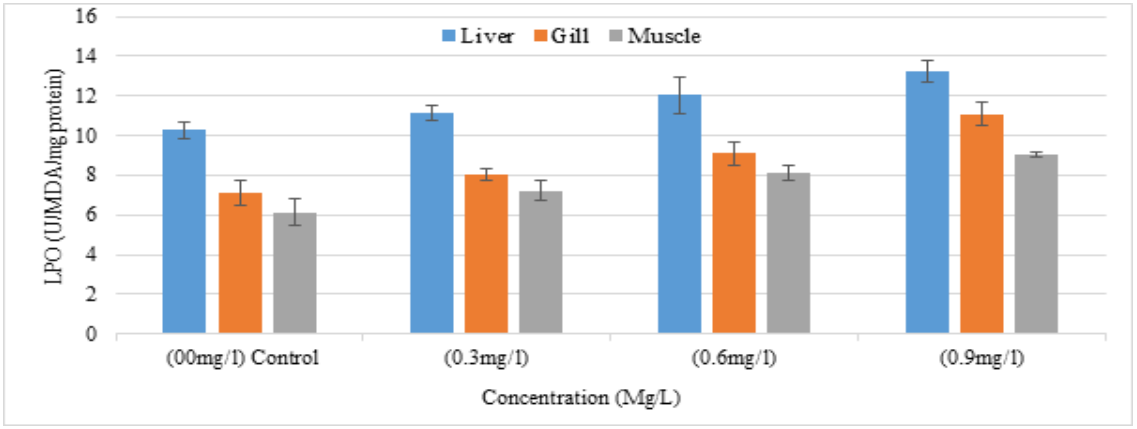


Figure 3. LPO - Lipid peroxidation activity (U/MDA/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days.

Table 3. LDH - Lactate Dehydrogenase activity (U/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days

Concentrations (Mg/L)	Tissues		
	Liver	Gill	Muscle
(00 mg/l) Control	27.13 ± 0.37**	15.21 ± 0.57**	31.11 ± 0.40**
(0.3 mg/l)	31.18 ± 0.64**	16.55 ± 0.80**	36.25 ± 0.33**
(0.6 mg/l)	37.08 ± 0.44**	18.01 ± 0.70**	38.04 ± 0.23**
(0.9 mg/l)	39.05 ± 0.39**	19.16 ± 0.47**	39.07 ± 0.67**

Values represent mean (n=5), ± standard deviation (SD) of five individual observations. Values are significant at P<0.05**

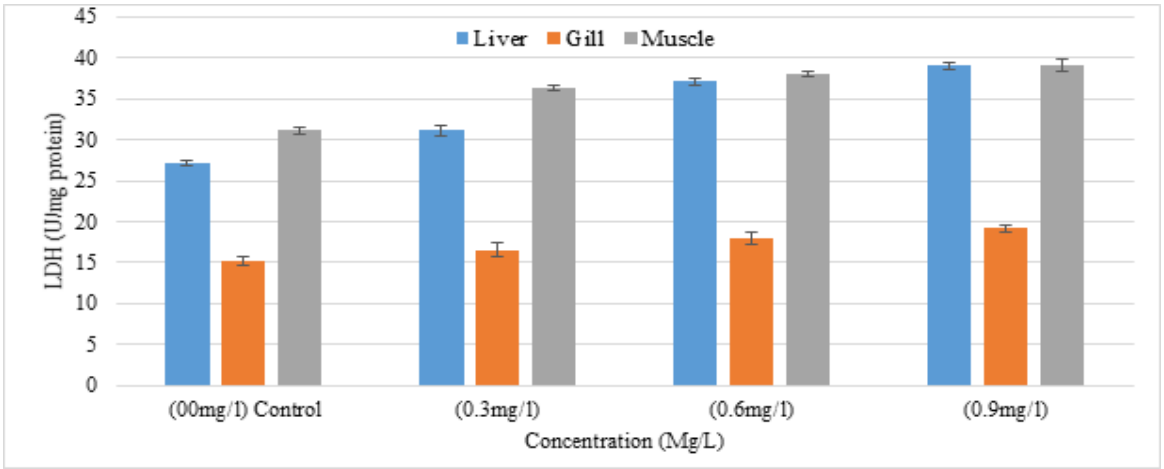


Figure 4. LDH-Lactate Dehydrogenase activity (U/mg protein) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days.

of silver nanoparticles 0.3 mg/l AgNPs (31.18 ± 0.64, 16.55 ± 0.80 and 36.25 ± 0.33), 0.6 mg/l AgNPs (37.08 ± 0.44, 18.01 ± 0.70, 38.04 ± 0.23 and 0.9 mg/l AgNPs (39.05 ± 0.39, 19.16 ± 0.47 and 39.07 ± 0.67) for 21 days respectively. The increase in the Lactate Dehydrogenase activity of the liver gill and muscle was more in 0.9 mg/l.

3.4 Protein Level in Liver, Gill and Muscle

The protein level in control liver, gill and muscle of Zebrafish, *Danio rerio* were 107.06 ± 0.31, 105.11 ± 0.73 and 110.01 ± 0.58 mg g^{-1} wet weight respectively for 21 days. The protein level of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be decreased in exposed with various subleathal concentrations of silver nanoparticles 0.3 mg/l AgNPs (93.12 ± 0.48, 90.03 ± 0.37 and 104.19 ± 0.62), 0.6 mg/l AgNPs (73.29 ± 0.61, 85.06 ± 0.54, 87.21 ± 0.30 and 0.9 mg/l AgNPs (42.12 ± 0.71, 81.14 ± 0.56 and 80.11 ± 0.57) for 21 days respectively. The decrease in the protein level of the liver gill and muscle was more in 0.9 mg/l.

4. Discussion

The GSH levels in the liver, gills and muscle of the AgNP treated Zebrafish, *Danio rerio* significantly differed from the control values after 21 days of exposure. A dose-dependent GSH depletion was also observed in all tissues, increased MDA concentrations in the liver and gill were observed, signifying dose-related responses.

GSH analysis results showed decreasing trends in the liver, gill and muscle of the AgNPs exposed Zebrafish,

Table 4. Protein activity (mg g^{-1} wet weight) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days

Concentrations (Mg/L)	Tissues		
	Liver	Gill	Muscle
(00 mg/l) Control	107.06 ± 0.31**	105.11 ± 0.73**	110.01 ± 0.58**
(0.3 mg/l)	93.12 ± 0.48**	90.03 ± 0.37**	104.19 ± 0.62**
(0.6 mg/l)	73.29 ± 0.61**	85.06 ± 0.54**	87.21 ± 0.30**
(0.9 mg/l)	42.12 ± 0.71**	81.14 ± 0.56**	80.11 ± 0.57**

Values represent mean (n = 5), ± Standard Deviation (SD) of five individual observations. Values are significant at P<0.05**

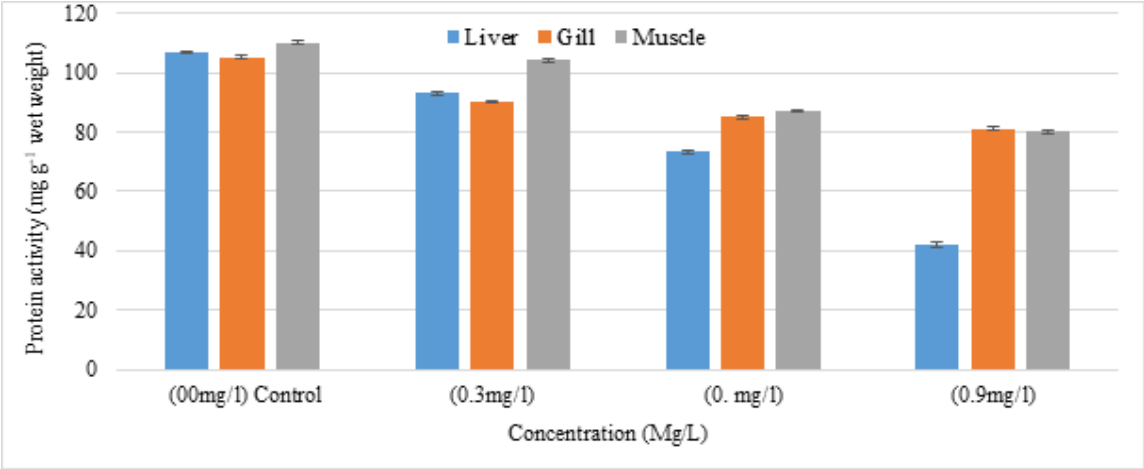


Figure 5. Protein activity (mg g^{-1} wet weight) in Zebrafish, *Danio rerio* exposed to silver nanoparticles (AgNPs) during 21 days.

Danio rerio. Glutathione depletion in cells exposed to AgNPs, accompanied by decreased antioxidant enzymes and increased lipid peroxidation, is reported to be highly correlated with increased ROS levels. A dose dependent enhancement in MDA concentration (a biomarker for lipid peroxidation) in AgNPs-treated liver, gill and muscle was observed in the present study. It suggests that AgNPs exposure caused in functional damage and ROS accumulation in tissues, which was in turn allied with oxidative damage.

The MDA is a key biomarker for monitoring of lipid peroxidation and the health condition of biological membranes that rich in polyunsaturated fatty acids. In the present investigation, liver, gills and muscle of the Zebrafish, *Danio rerio* exposed to Ag-NP concentration shown a significant change in MDA level, the increase of MDA in fish exposed to the highest Ag-NPs could be explained by the depletion of the antioxidant system.

Lactate Dehydrogenase is an anaerobic enzyme intricate in the conversion of pyruvate to lactate in the Embden Meyerhoff pathway. In the present study the level of the Lactate Dehydrogenase in the liver homogenate was found to subsequent significant increased. Increased level of the Lactate Dehydrogenase may be due to an alternate anaerobic glycolytic pathway in conversion of lactate to pyruvate for the production of glucose. Lactate Dehydrogenase in the gill and muscle of the Zebrafish, *Danio rerio* also were found to increase as compared to control.

In the present study decrease in protein content of liver, gill and muscle tissues of Zebrafish, *Danio rerio* may be due to the inhibition of protein synthesis or its increased degradation in the presence of the AgNPs. The decline in the protein content under exposure of AgNPs may be due to the utilization of amino acids in the various catabolic reactions or may be due to increased proteolysis.

Studies have shown that acute AgNP exposure induces oxidative stress to the liver of medaka and zebrafish, by measuring the lipid peroxidation, total GSH levels and gene expression associated to detoxification and radical scavenging action[16]. Levels of reduced glutathione are mainly determined by equilibrium among GSH synthesis, conjugation by glutathione S-transferase (GST), oxidation by non-enzymatic mechanisms or by GPx and reduction of oxidized glutathione (GSSG) to GSH by Glutathione Reductase (GR) [17].

The raised level of lipid peroxidation in the liver of *C. auratus* in reaction to the exposure to copper sulphate suggests that there is increased production of ROS[18]. Observed a significant increase in the total lipids level of *Oreochromis niloticus* and *Chapalichthys pardalis* exposed to cadmium and silver nanoparticles, respectively reported the increase in serum total lipids may be due to disturbance of lipids metabolism[19].

The significant enhanced lactate dehydrogenase (LDH) activity levels in the fish exposed to nanosilver may be reflect the increased rate of conversion of lactate to pyruvate and then to glucose as reported in other species[20]. Lactate Dehydrogenase (LDH) catalyzes the interconversion of lactic and pyruvic acids and thus plays a significant role in regulation of energy metabolism, especially in exposure environmental pollutants[21]. LDH is present in all tissues and determination of this enzyme activity can be used a potential biomarker for assessing the toxicity of chemicals and stress in fish[22]. An apparent cell uptake of TiO_2 NPs through liver, kidney and gills with a small amount in muscle of fish *Trachinotus carolinus* has been reported by[23]. They also reported thatTiO_2 NPs can cross the epithelial surfaces of internal organs. Under stress condition the organisms need more energy to cope with the stress which results alteration in LDH activity[24].

Generation of free radicals in response to AgNPs and temperature stress should be scavenged by the various antioxidant systems to serve as a protecting response to detoxify the ROS generated. In crabs, the defence system is equipped with enzymes to counteract free radicals produced during exposure to stressors[25]. Significant decrease in total proteins, albumin and globulin contents of *O. niloticus* after exposure to copper and zinc oxide nanoparticles, respectively reported by[26]. The decrease in protein content observed in the exposed tissues could be attributed to mitochondrial damage[27]. Silver nanoparticles (AgNPs) are recognized to induce oxidative stress by triggering ROS through the mitochondrial electron transport chain[28]. Also, protein synthesis is generally down-regulated during oxidative stress[29].

5. Conclusion

In the current study the level of Reduced glutathione activity (GSH) and Total protein were found to be decreased were as Lipid peroxidation activity (LPO) and Lactate Dehydrogenase activity (LDH) were found to be elevated in gill, liver and muscle tissue of AgNPs treated Zebrafish *Danio rerio.* This study clearly revealed the adverse effects of AgNPs in different concentrations on the function and

structure of the liver, gill and muscle tissues of Zebrafish, *Danio rerio*. Its exposure induces oxidative stress. In future the applications and use of these silver nanoparticles should be controllable to protect aquatic ecosystem. Moreover, the recovery strategy for metal nanoparticles pollutants is essential for fish species according to their importance in the food web.

6. Acknowledgment

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