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Effect of Silver Nanoparticles on Antioxidant Enzymes of Zebrafish, *Danio rerio*

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Abstract

The objective of the present study was to know the effect of Silver nanoparticle on antioxidant enzymatic activities in selected tissues of the Zebrafish, *Danio rerio.* For the chronic toxicity study adult fish, *Danio rerio* were divided in to two groups. First group was 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 was given for 21 days at the end of experimental period. Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) in gill, liver and muscle tissues were assayed. The levels of Catalase (CAT), was found to be decreased were as the Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) were found to be increased significantly in gill, liver and muscle tissue of AgNPs treated fish.

Keywords: Antioxidant Enzymes, *Danio rerio,* Nanotoxicology, Silver Nanoparticles (AgNPs)

1. Introduction

Nanotoxicology is one of the cutting-edge areas of biological research in past few years, which addresses the adverse effect of nanomaterials in biological systems[1]. The low cost of manufacturing AgNPs has also made them the largest and fastest growing class of nanomaterials in product applications such as plastics, metals, textiles and in medical and veterinary devices[2]. Soaring use of nanoparticles in commercially available products such as paints, car tyres, tennis rackets, textiles and sunscreen triggered the toxicological researchers to investigate the risk of unintended nanomaterials exposure[3].

It is projected that the nanotechnology industry will continue to grow significantly and the specific production of AgNPs is expected to reach approximately 800 t by 2025[4–5]. Silver nanoparticles (AgNPs) are widely used nanomaterials in commercial products, with uses ranging from disinfecting medical products to household items and water treatment[6]. Because their high production increases the probability of AgNPs being discharged through effluents into the aquatic ecosystems[7–8].

In terms of the mechanisms of AgNPs' toxicity, several studies have suggested that the toxicity of AgNPs is associated with the uptake of silver in the exposed organisms[9]. The Ag-NPs can enter the cell through diffusion or endocytosis which causes mitochondrial dysfunction and ROS generation, this leads to protein and nucleic acid destruction inside the cell, and finally inhibition of cell proliferation[10]. An important toxicity mechanism of Ag-NPs is the interaction of both the ionic and nano-form of silver with sulphur containing macromolecules such as proteins due to silver's strong affinity for sulphur[11].

Antioxidant enzymes have been recognized as important modulators in AgNP induced oxidative stress. Two of them, Catalase (CAT) and Superoxide Dismutase (SOD), are prominent for maintaining the level of ROS in organisms and are used as bioindicators of increased ROS production[12]. Previous research demonstrated that AgNPs induce oxidative stress by altering the activity of both enzymes in in vivo and in vitro assays. For example, the activities of SOD and CAT were upregulated when

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Drosophila melanogaster was exposed to 10 nm AgNPs in vivo[13].

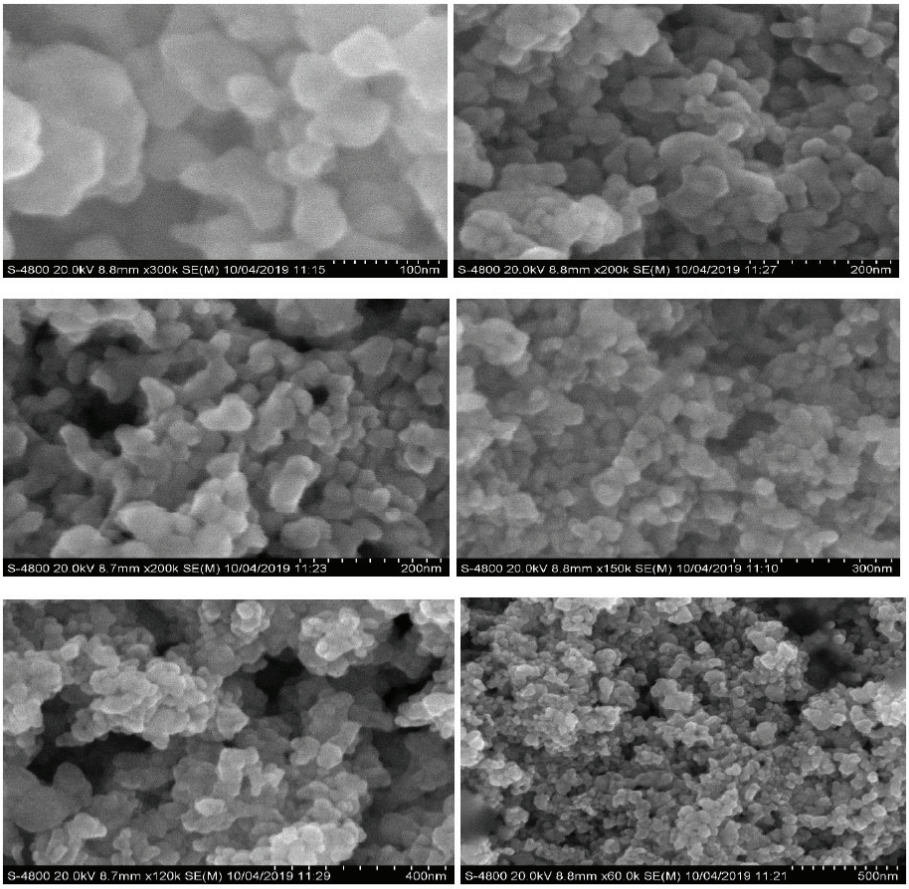
Zebrafish has a lot of physiological and genetic similarities with humans, including the brain, digestive tract, musculature, vasculature and innate immune system[14]. Also 70% of human disease genes have functional similarities with those of zebrafish[15]. The aim of this present study was to assess the effect of silver nanoparticles on antioxidants enzymatic activity in 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 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 electronmicroscopy (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.



Photos: FESEM images of Silver nanoparticles (AgNPs).

Figure 1. Characterization of Silver nanoparticle (AgNP) by FESEM.

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.

2.3 Fish and Exposure Conditions

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. All fish were be kept in 10-L aquaria and fasted for the duration of exposure. The test water was changed each day. During 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 subchronic exposure study.

As 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[16].

For the chronic toxicity study adult fish, *Danio rerio* were divided in to two groups. First group was 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. Second group 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[17].

As an 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 monitored continuously and fish were considered dead when operculum movement and response to mechanical stimuli could no longer be detected. At the end of the 21st day, ten Zebrafish, *Danio rerio* from each of the treatments and control were collected. Gill, liver, muscles were dissected and subjected to enzyme estimations.

2.4 Biomarkers

Tissue extracts for antioxidant enzymes measurements were obtained from gills, liver 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 (DTE) according to Bacchetta et al.[18] Catalase (CAT) was estimated by method prescribed by[19] Superoxide Dismutase (SOD) was estimated by method[20]. Glutathione Peroxidase (GPx) was estimated by method[21]. 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 and Discussion

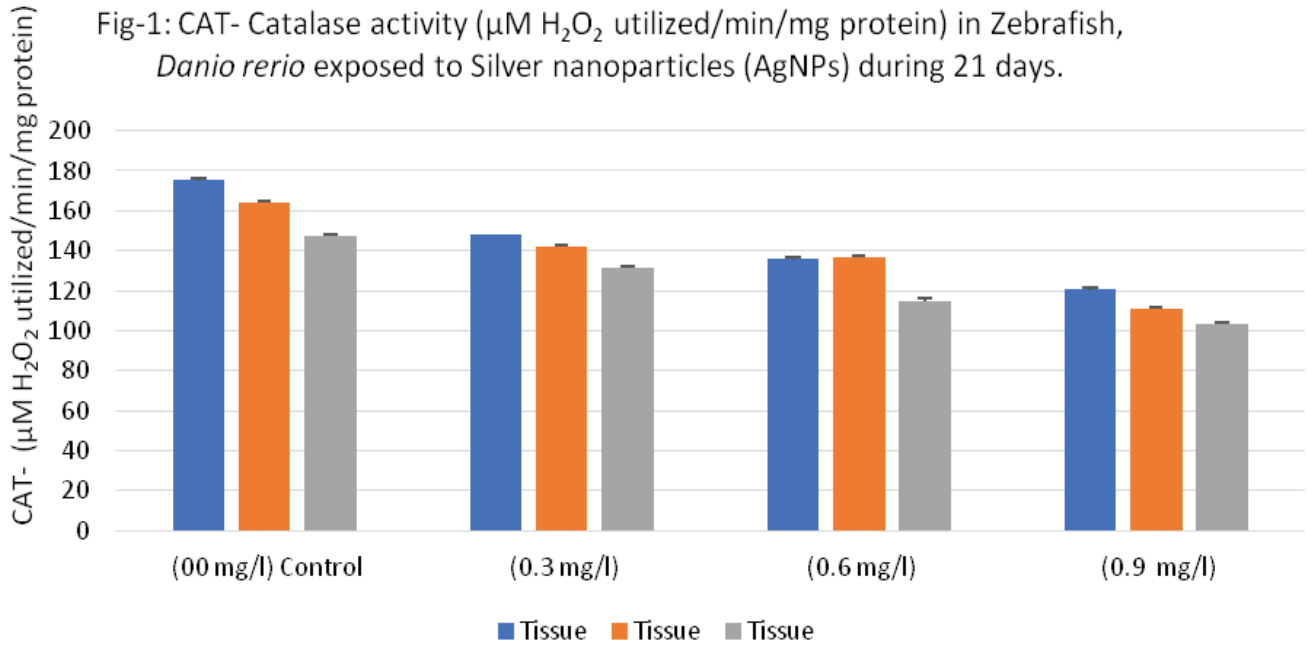
3.1 Catalase (CAT) Activities in Control Liver, Gill and Muscle

The catalase activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 175.77 ± 0.70, 164.17 ± 0.53 and 147.39 ± 0.54 µM H_2O_2 utilized/min/mg protein respectively for 21 days. The catalase activity of liver, gill and muscle of Zebrafish, *Danio rerio* were significantly decreased in exposed with various subleathal concentration of silver nanoparticles 0.3 mg/l AgNPs (147.97 ± 0.38, 142.19 ± 0.50 and 131.49 ± 0.91), 0.6 mg/l AgNPs (136.02 ± 0.67, 136.92 ± 0.47, 115.05 ± 0.89) and 0.9 mg/l AgNPs (120.85 ± 0.48, 111.11 ± 0.41 and 103.17 ± 0.68) for 21 days respectively. The decrease in the catalase activity of the liver gill and muscle was more in 0.9 mg/l. (Table 1, Figure 1).

Table 1. CAT-Catalase activity (µM H_2O_2 utilized/min/mg protein) in Zebrafish, *Danio rerio* exposed to Silver nanoparticles (AgNPs) during 21 days

Concentration (Mg/L)	Organ/Tissue		
	Liver	Gill	Muscle
(00 mg/l) Control	175.77 ± 0.79**	164.17 ± 0.59**	147.39 ± 0.60**
(0.3 mg/l)	147.97 ± 0.42**	142.19 ± 0.55**	131.49 ± 1.02**
(0.6 mg/l)	136.02± 0.75**	136.92 ± 0.53**	115.05 ± 1.00**
(0.9 mg/l)	120.85 ± 0.54**	111.11 ± 0.46**	103.17 ± 0.76**

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



3.2 Superoxide Dismutase (SOD) Activities in Control Liver, Gill and Muscle

The Superoxide Dismutase activity activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 5.40 ± 0.41, 3.07 ± 0.48 and 2.36 ± 0.37 U/mg protein respectively for 21 days. The Superoxide Dismutase activity of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be elevated in exposed with various subleathal concentration of silver nanoparticles 0.3 mg/l AgNPs (7.22 ± 0.49, 4.28 ± 0.27 and 3.17 ± 0.53), 0.6 mg/l AgNPs (9.06 ± 0.64, 5.93 ± 0.52, 4.06 ± 0.55) and 0.9 mg/l AgNPs (12.00 ± 0.67, 6.26 ± 0.43 and 5.11 ± 0.68) for 21 days respectively. The increase in the Superoxide Dismutase activity of the liver gill and muscle was more in 0.9 mg/l. The increased levels were recorded in silver nanoparticle treated group indicating the toxic nature of silver nanoparticle. (Table 2, Figure 2).

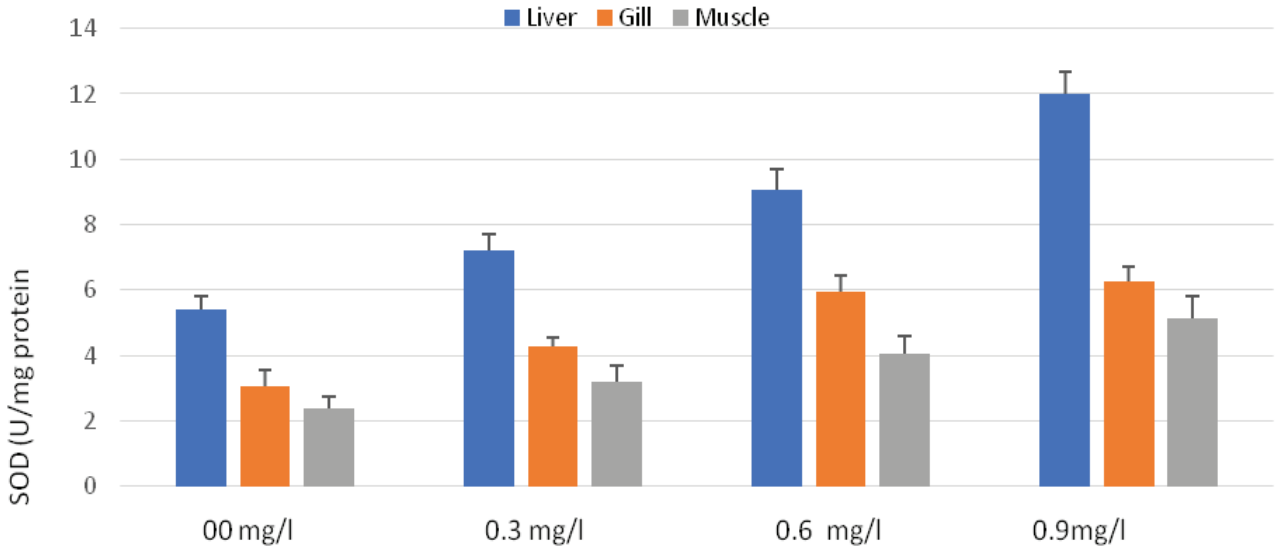
Table 2. SOD- Superoxide Dismutase activity (U/mg protein) in Zebrafish, *Danio rerio* exposed to Silver nanoparticles (AgNPs) during 21 days

Concentration (Mg/L)	Organ/Tissue		
	Liver	Gill	Muscle
(00 mg/l) Control	5.40 ± 0.45**	3.07 ± 0.54**	2.36 ± 0.41**
(0.3 mg/l)	7.22 ± 0.54**	4.28 ± 0.30**	3.17 ± 0.59**
(0.6 mg/l)	9.06 ± 0.71**	5.93 ± 0.59**	4.06 ± 0.61**
(0.9 mg/l)	12.00 ± 0.75**	6.26 ± 0.49**	5.11 ± 0.77**

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

Concentration (Mg/l)

Fig.2: SOD- Superoxide dismutase activity (U/mg protein) in Zebrafish, *Danio rerio* exposed to Silver nanoparticles (AgNPs) during 21 days.



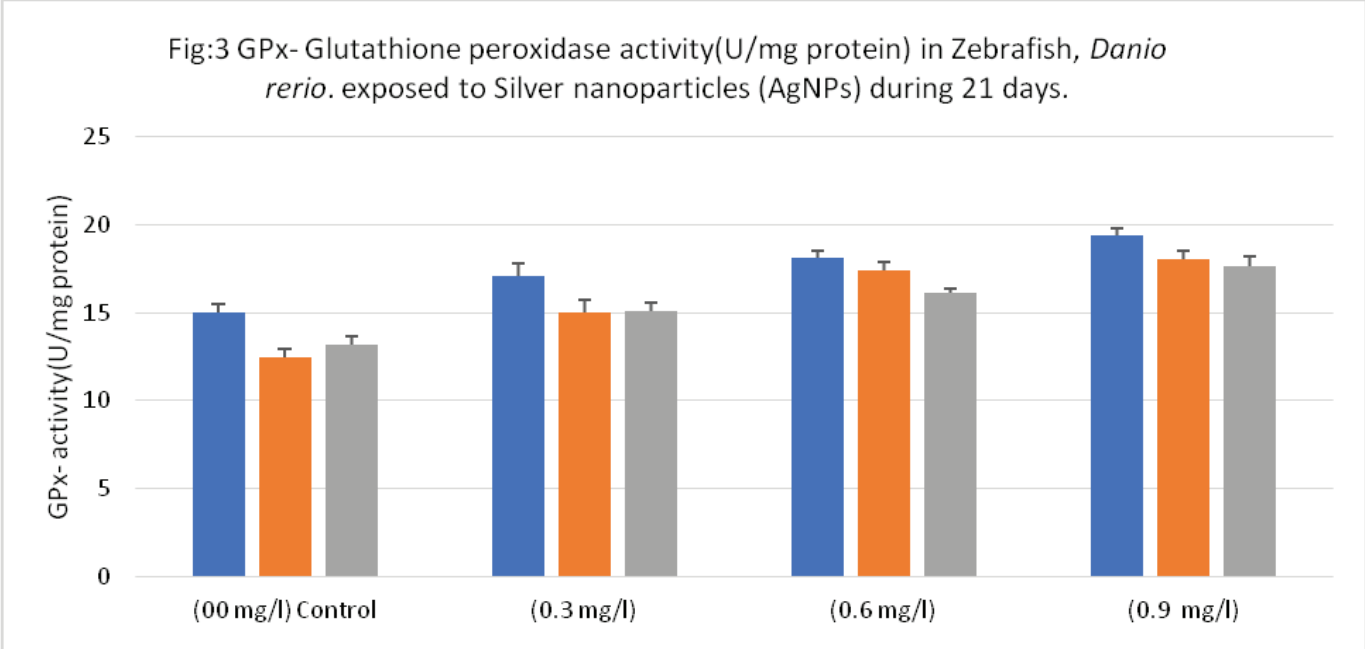
3.3 Glutathione Peroxidase (GPx) Activities in Control Liver, Gill and Muscle

The Glutathione Peroxidase activities in control liver, gill and muscle of Zebrafish, *Danio rerio* were 15.01 ± 0.51, 12.44 ± 0.53 and 13.18 ± 0.51 U/mg protein respectively for 21 days. The Glutathione peroxidase activity of liver, gill and muscle of Zebrafish, *Danio rerio* were found to be elevated in exposed with various subleathal concentration of silver nanoparticles 0.3 mg/l AgNPs (17.11 ± 0.72, 15.00 ± 0.75 and 15.07 ± 0.47), 0.6 mg/l AgNPs (18.14 ± 0.39, 17.36 ± 0.53, 16.14 ± 0.26) and 0.9 mg/l AgNPs (19.42 ± 0.58, 18.01 ± 0.48 and 17.62 ± 0.54) for 21 days respectively. The increase in the Glutathione peroxidase activity of the Liver Gill and Muscle was more in 0.9 mg/l. (Table 3, Figure 3).

Table 3. Glutathione peroxidase activity (U/mg protein) in Zebrafish, *Danio rerio*. exposed to Silver Nanoparticles (AgNPs) during 21 days

Concentration (Mg/L)	Organ/Tissue		
	Liver	Gill	Muscle
(00 mg/l) Control	15.01 ± 0.57**	12.44 ± 0.59**	13.18 ± 0.57**
(0.3 mg/l)	17.11 ± 0.80**	15.00 ± 0.4**	15.07 ± 0.53**
(0.6 mg/l)	18.14 ± 0.44**	17.36 ± 0.59**	16.14 ± 0.29**
(0.9 mg/l)	19.42 ± 0.65**	18.01 ± 0.54**	17.62 ± 0.60**

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



In the present study, the Silver nanoparticle AgNPs treated group showed decreased CAT activity in liver, gill and muscle tissues, while higher activity was noted in the control group. It assumes that the excessive release of superoxide radicals after AgNPs exposure can cause a decrease of CAT activity. The decrease in the CAT activity may be the metallic nature of nanoparticles and the presence of transition metals encourages the production of Reactive Oxygen Species (ROS) leading to oxidativestress. In the present investigation tissue specific responses in the activities of antioxidant enzymes such as SOD and catalase were observed during sublethal AgNPs exposure which may indicate the different rates of free radical generation and different antioxidant potentials of these tissues.

In the present study, the GPx activities in the liver, gill and muscle tissues of Zebrafish, *Danio rerio* fluctuated with a concentration. Exposure AgNPs, SOD activities of different tissues were stimulated and showed a remarkable increase. Present study reveals the increased SOD level in gill, liver and muscle tissue of AgNPs treated fish indicates a detoxifying mechanism against the toxicity. In the present study was found a significant increase in the activity of Glutatione Peroxidase (GPx) in the liver, gills and muscle of Zebrafish, *Danio rerio* exposed to AgNPs. Toxicity of AgNPs in zebrafish increase oxidative substances in cells, which may cause an elevation of antioxidant enzymes as a defence mechanism.

Oxidative stress is a condition characterized by the elevation in the cellular steady-state concentration of Reactive Oxygen Species (ROS) from an imbalance between the production of ROS and the destruction by antioxidant enzymes[22]. Oxidative stress is elevated which results of the three factors (a) an induction in oxidant generation, (b) a reduction in antioxidant protection and (c) failure to repair oxidative damage[23], The gills may also be able to excrete metal ions from the systemic circulation by active efflux on branchial ion transport pathways, but this would not

apply to nanometals. Liver acts as the most likely route of excretion for nanometals[24], High concentration of metals in fish tissues can lead to redox reactions, generating free radicals, especially reactive oxygen species e.g. oxygen superoxides, peroxides, hydroxyl radical and hydrogen peroxides[25]. The highly reactive compounds, molecules or ions formed by the incomplete reduce oxygen, may induce alteration and change some physiological responses[26].

CAT is a tetrameric heme-containing enzyme that catalyzes dismutation reactions of H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stress conditions. CAT is also important in the removal of H_2O_2 generated in peroxisomes during the β-oxidation of fatty acids, photorespiration and purine catabolism[27–28]. Superoxide Dismutase belongs to the group of oxido-reductases. SOD contributes significantly to protecting the organism from the toxic effects of O_2•-. In living systems, O_2•- is capable of reacting with another molecule of O_2•- (dismutation) or is also able to react with another radical, such as NO•. Formation of HO• from O_2•- via the metal-catalyzed Haber-Weiss reaction has a reaction rate 10000 times faster than that of spontaneous dismutation, so SOD provides the first line of defence against ROS[29].

Glutathione peroxidise has eight known isoenzymes that, in active positions, may contain co-factors, such as heme and residues of cysteine or selenocysteine. GPx1-4 and GPx6 are selenoenzymes, which contain the non-metal selenium[30]. Selenocysteine participates directly in the transfer of electrons to a peroxide substrate, thereby oxidizing it. Organisms have both enzymatic and non-enzymatic antioxidant defences against ROS[31]. One of the first enzymes that act in defence against ROS is Superoxide Dismutase (SOD), that catalyses the conversion of reactive superoxide anions (O_2–) to hydrogen peroxide (H_2O_2), which is subsequently detoxified by Catalase (CAT) and Glutathione Dependent Peroxidase (GPx). GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced Glutathione (GSH), one of the most important non-enzymatic antioxidants in the cell. [32]

4. Conclusion

Silver Nanoparticle AgNPs treated group showed decreased CAT activity in liver, gill and muscle tissues, while higher activity was noted in the control group. The decrease in the CAT activity may be the metallic nature of nanoparticles and thepresence of transition metals encourages the production of Reactive Oxygen Species (ROS) leading to oxidative stress. Tissue specific responses in the activities of antioxidant enzymes such as SOD and catalase were observed during sublethal AgNPs exposure which may indicate the different rates of free radical generation and different antioxidant potentials of these tissues. Significant increase in the activity of Glutathione Peroxidase (GPx) in the liver, gills and muscle tissues of Zebrafish, *Danio rerio* exposed to AgNPs. Toxicity of AgNPs in zebrafish increase oxidative substances in cells, which may cause an elevation of antioxidant enzymes as a defence mechanism.

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