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Effect of Silver Nano Particle on Fish (*Labeo Rohita*) Hematology

Muhammad Naveed[1], Ayaz Ahmad[2]
[1] Department of Zoology UVAS Lahore, Pakistan
[2] Assistant Professor, Department of Biology, Government College Block Number 17 D G Khan, Pakistan

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Abstract
The use of silver nanoparticles (Ag-NPs) is rapidly increasing, but there are limited data on their effects on the aquatic environment. The present study aimed to determine the acute toxicity and evaluate the effect of subacute concentrations of Ag-NPs on hematological and plasma biochemical indices of, Roho, *Labeo rohita*. The 24-, 48-, 72- and 96-h median lethal concentration values of silver Nanoparticle for *Labeo rohita* were estimated at 5mg/l, 10 mg/l and 20 mg/L. Red blood cell (RBC) count, hemoglobin (Hb) count and hematocrit (Hct) level were significantly reduced at both concentrations tested ($p < 0.05$). In conclusion, Ag-NPs intoxication resulted in erythrocyte reduction, hematological disturbances, leucocytosis and stress response in *Labeo rohita* and offered a simple tool to evaluate toxicity-derived alterations.

Keywords: Nanotechnology, Fish, AgNO3, Toxicity test

Introduction
Nanoparticles are particles that have one dimension that is 100 nm or less in size. Nanoparticles are increasingly being used, or being evaluated for use, in many fields. Silver (Ag) is one of the most commonly used nanoparticles due to its bactericide effect [1]. The annual production of AgNPs is estimated at around 500 t worldwide and grows systematically [2]. The estimated production of Nano-enabled products has risen from less than 10 tons per year in 2011 [3] to 300 tons per year in 2015 [4]. Products based on Ag nanoparticles (Ag-NPs), such as odor-resistant textiles, food packaging, cosmetics, household appliances and medical devices may release Ag particles (nanoparticles or aggregates) or Ag ions via effluent discharge into the aquatic environment [5]. Fisheries and aquaculture contribute over 40% to the world's reported finfish production from less than 0.01% of the total volume of water on earth. These fisheries provide food for billions and livelihoods for millions of people worldwide [6].

Palmitic acid is the most abundant fatty acid in all species ranging from 32% to 46%. Although these fish contained reasonable amounts of essential PUFA such as docosahexaenoic, eicosapentaenoic and arachidonic acids, *Labeo rohita* contained the highest amounts of PUFA and protein. These fish contained appreciable levels of Omega-6 PUFA suggesting that these fish especially *L. rohita* could be used as a source of healthy diet for humans. These findings may benefit the fishing industry, nutritionists and researchers who are striving to improve the nutritive value, processing and marketing of selected fish species [7]. The present study aimed to determine the acute toxicity and evaluate the effect of subacute concentrations of Ag-NPs on hematological and plasma biochemical indices of, Roho, *Labeo rohita*. The 24-, 48-, 72- and 96-h median lethal concentration values of silver Nanoparticle for *Labeo rohita* .

Material and method
The present research was performed at the research and training center, Department of fisheries and aquaculture, UVAS, Ravi Campus .Block C, Pattoki. For the preparation of silver nanoparticles, plant extract was used. Plant extract was prepared with water and ethanol solution. After filtration, phytochemical analysis was done. Silver metal solution was mixed with that guava plant extract in chemistry Department of UET, Lahore [8]. Nano synthesis was conformed from color variation of nanoparticle solution from light yellow to brown and dark brown. Their absorbance was checked on UV-Vis spectroscopy and different peaks were observed. Particle size was check on particle analyzer. And ranged between 1-100 Nanometer in their size. Their relating functional groups were also checked from FTIR. *Labeo rohita* was used as an experimental animal

Fig 1: Extract Preparation of Guava

Fig 2: UV-Vis Spectroscopy apparatus

Severe toxicity valuation of silver nanoparticle (AgNps)

Severe toxicity influence of silver nanoparticles was examined on (*Labeo rohita*). All experiments performed for a time period of 4 weeks and silver nanoparticles were introduced to fish at three different concentration (5,10 and 20 mg/L) designed as treatment no. 1, treatment no. 2 and treatment no 3. Fourth one is control concentration which is without nanoparticles. Controls are compared with treatments.8 fishes were kept in each group. Control group was carried in tap water with free of any addition of nanoparticles, while treatment groups were revealed to the doses of 5, 10 and 20 mg/L of nano silver solution, for a period of 96- hours.

Water assumed rank parameter

Water assumed parameters like dissolved oxygen, pH, temperature, total hardness and electrical conductivity were measured on daily basis. The procedure of determining these parameter are given below by following the method of (A.P.H.A, 2005). DO meter was calibrated and probe was dipped in water to measure temperature and dissolved oxygen of aquarium water. Reading was noted when needle became stable on screen in mg/L. pH is a -log of H ion concentration in a medium. pH was measured by pH meter. In 50 ml of water sample, 1-2 drops of sodium- potassium titrate solution were added and mix well: 1 ml Nessler's reagent was added for the development of color and kept in stand for 15 minutes. Concentration was measured by spectrophotometer. Standards and sample were run at 420 nm for 1 cm light path. Calibration curve was formed at the same temperature and reaction time used for samples. Concentration readings were measured against a reagent blank and parallel checks were run frequently against standards in the nitrogen range of samples. It is an ability to conduct electric current or ionic concentration in water. It indicates the amount of ionize able substance (phosphate, nitrate etc.) dissolved in water. Calibrate the conductivity meter. Dip the probe of the meter in water and note the reading on the screen. Electrical conductivity is measured in µs/cm. Add 1 ml of ammonia buffer and 5 drops of indicator to 50 ml of water in a conical flask. The color of water sample will turn wine red. Titrate against EDTA solution, until a blue color appears. Note the end point.

Sample Collection

The experimental fish species *Labeo rohita* (100 g ± 10g , 6cm± 2cm) was picked from fisheries research ponds University of Veterinary and Animal Sciences Ravi Campus Pattoki and Fish were transferred to aquaculture and research center of University of Veterinary and Animal sciences and acclimated to laboratory condition for 2 weeks. Fishes were parceled in 4 glass tank (500L). The experimental aquaria were aerated and fish in holding tank equipped with semi static flow by aerated water system for at least 2 week for acclimatization to the lab before accomplishment of nanoparticles trails. The laboratory temperature was in between 29-30 ^{0}C, pH 7.79 + 0.50, dissolved oxygen 7.93 + 0.25 mg/L and total hardness 298+2.35 mg/L as CaCO3 and normal illumination (approx. 12 hours light and 12 hours dark) was conserved during the whole experimental period. Water pH, temperature and dissolved oxygen were determined by Wagtech portable pH/temp meter and oxygen meter. Water total hardness was determined using portable photometer with commercial kits provided by the manufacturer (Wagtech Portable Photometer 7100, Berkshire, UK).

Statistical analysis

Data was subjected to ANOVA for its statistical significance among treatments. Mean values were compared to assess their intensity of significance among treatment groups through Duncan's multiple range test. Probability level was set at <0.05. The SAS (statistical analysis software) version 2005 was used for all statistical analysis due to its wider application.

Result

The experiment was conducted in department of fisheries and aquaculture, University of veterinary and animal sciences. During this research trial, *Labeo rohita* were grown under controlled condition with three different concentration of silver nanoparticles viz. treatment no 1 with 5 mg/L, treatment no 2 with 10 mg/L, treatment no 3 with 20 mg/L and control group without silver nanoparticles (AgNPs) for a period of 4 weeks. There was no statistical difference at the start of experiment between control and experimental groups. After 4 week of trial there was statistical difference in growth parameters, hematological parameter

Preparation of silver nanoparticles

For the preparation of silver nanoparticles, plant extract was prepared with water and ethanol solution. After filtration, photochemical analysis was done. Silver metal solution was mixed with that guava plant extract in chemistry Department of UET, Lahore [8]. Nano synthesis was conformed from color variation of nanoparticle solution from light yellow to brown and dark brown. Their absorbance was checked on UV-Vis spectroscopy and a different peak was observed. Particle size was check on particle analyzer. And range between 1-100 nanometers in their size. Their relating functional groups were also checked from FTIR.

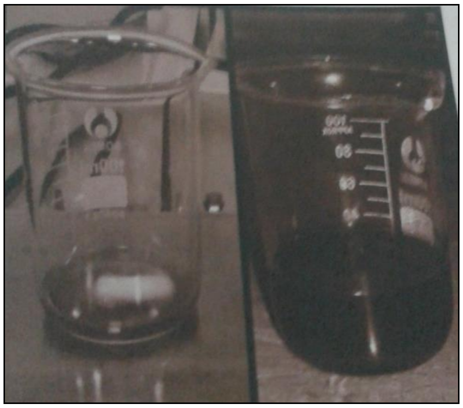

Fig 3: Silver nanoparticle from guava

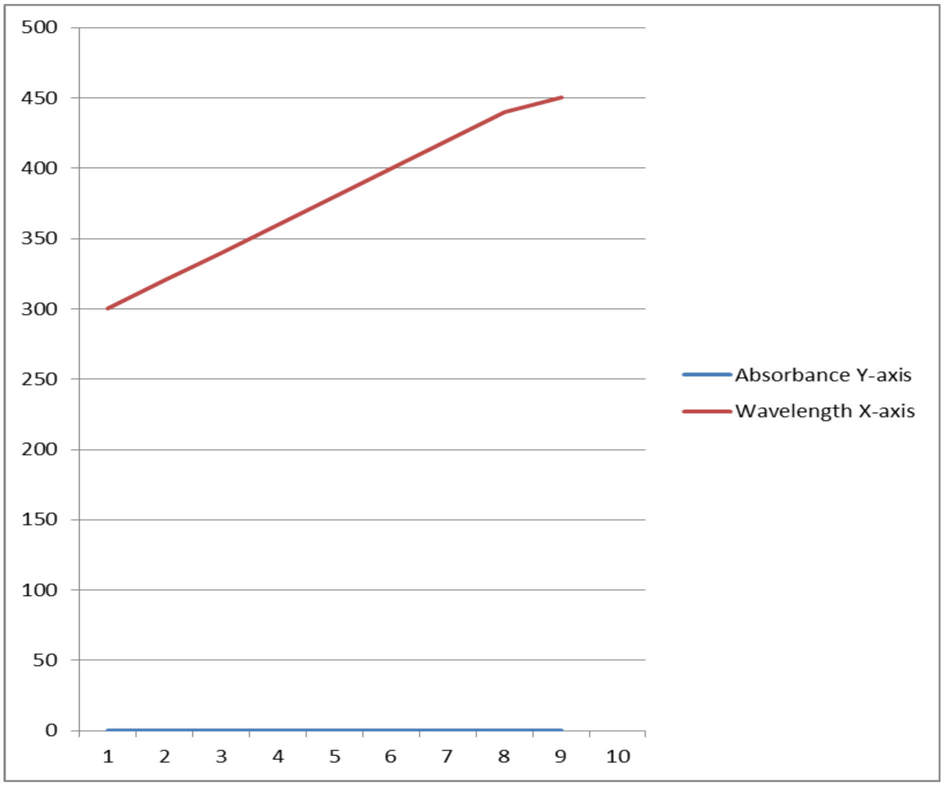

Fig 4: Formation of AgNPs from guava

Physiochemical parameters

Physiochemical parameters of water include temperature, pH, dissolved oxygen (mg/L), electrical conductivity (µs/cm), ammonia (mg/l) and carbon dioxide (mg/L). These water quality parameters helps to standardize the water that is at which fish can grow faster and better .During the experimental period physiochemical analysis of water was checked on daily basis. The data obtained during experiment is shown in Table below. Table 4.1 Show all the physiochemical parameters of water. All the physiochemical parameter provides significant result except ammonia. Ammonia show non-significant results, maximum value recorded in treatment no 3due to light concentration of silver nanoparticles (20mg/L).

Table 1: Physiochemical parameters of water recorded during trial

Parameters	Control	Treatment # 1	Treatment # 2	Treatment # 3
Temperature(^{O}C)	29.63 ±0.03a	30.10±o.95a	29.87±0.85a	30.02±0.95a
Ph	7.50±0.01a	7.43±0.28a	7.39±0.02a	7.50±0.01a
Total hardness (mg/l)	200±0.02a	230±0.03a	250±0.05a	230±0.03a
Ammonia(mg/l)	0.31±0.01c	1.65±0.02b	1.87±0.03b	2.17±0.02a
Dissolved oxygen(mg/l)	5.72±0.04a	5.28±0.86a	5.14±0.46a	5.36±0.13a
Electrical conductivity(µs/cm)	2198.28± 148.37a	2214.86± 247.02a	2218.17± 226.87a	2176.06± 201.76a

Hematology

The number of the WBCs blood cell was higher in treatment no.3 than the control group. Hemoglobin (Hb), Hematocrit (Hct) and RBCs were high in the treatment no 3 as compared to the control group. The MCV in the treatment group's increase as the dose of nanoparticles increased. The values of MCV, MCHC and PLT were also high in treatment no.3 group than control

Table 2: Hematological parameters of fish during trial

Parameter	Units	Control	Treatment 1	Treatment 2	Treatment 3
WBC	µl	110×10^{3}	118×10^{3}	148×10^{3}	150×10^{3}
RBC	µl	2.43×10^{6}	2.77×10^{6}	0.18×10^{6}	2.96×10^{6}
HGB	g/dl	4.1	9.1L	10.4	13.2
HCT	%	43.0	32.5	3.9	40.7
MCV	fl	177H	117H	120H	138H
MCH	pg	28.0	32.9	45.6	44.6
MCHC	g/dl	24.0	28.0	28.2L	32.4
PLT	µl	10×10^{3}	19L	27L	178

WBC: White blood cell count
RBC: Red blood cell count
HGB: Hemoglobin concentration
HCT: Hematocrit (%)
MCV: Mean Corpuscular Volume (IL)
MCH: Mean Corpuscular Hemoglobin (pg)
PLT: Platelet count.
MCHC: Mean corpuscular hemoglobin concentration (g/dl)

Discussion

In the current study, the experiment was conducted in department of fisheries and aquaculture, university of veterinary and animal sciences, Ravi campus, Block C, Pattoki. During this research trial *Labeo rohita* were grown under controlled conditions with three different concentrations of silver nanoparticles viz. Treatment 1 with 5 mg/l, treatment 2 with 10 mg/l and treatment 3 with 20 mg/l and control without silver nanoparticles (AgNPs) for a period of 4 weeks. There was no statistical difference at the start of experiment between control and experimental groups. After 4 weeks of trial there were statistical differences in physiochemical parameters, hematological parameters. The use of Nano silver is growing exponentially because of its strong antimicrobial properties. Ag-NPs may be released into aquatic environments from factory waste discharges, through leaks or spills during transportation and via materials containing Ag-NPs, while only little information is available on their possible eco toxicological effects [9]. The preparation silver of nanoparticle from guava plant extract is linked with green synthesis of nanoparticles. The aim of the present research was work to synthesize nanoparticle from natural components. The *Psidium guajava is* well-known medicinal plant and leaves of it are widely used in the Ayurveda system of medicine as well as in folk medicine [10]. Guava leaf extract is used as astringent, anodyne, febrifuge, antispasmodic, tonic to treat wounds, ulcer, cholera, diarrhea, vomiting, nephritis, toothache, gum inflammation, etc [11]. *Psidium guajava* leaf contains leucocyanidin, flavonoids which includes quercetin, quercetin-3-*O*-α-l-arabinofuranoside, quercetin-3-*O*-β-d-arabinopyranoside, quercetin 3-*O*-β-d-galactoside (hyperin), quercetin-3-*O*-α-l-arabinofuranoside (avicularin), quercetin 3-*O*-β-d-glycoside (isoquercetin), plant sterol, carotenes, tannins, saponins, vitamin C and B6, carbohydrates and diethoxy alkenes as plasma compounds. It is reported that flavonoids [12] present in leaf extract are responsible for reducing Ag^{+} ions and capping for Ag nanoparticles. In this study, some of the flavonoids present in leaf extract may act as reducing and/or capping agent to synthesize Ag nanoparticles.

Physiochemical parameter of water play important role in biology and physiology of fish. All the physiochemical parameters like temperature, dissolved oxygen, pH, total hardness, dissolved solids, ammonia and electrical conductivity were continuously measured on daily basis throughout whole study period of trial. The results of our study are like [13] where they reported that pH values were in between 6.62-7.85 which is optimum for the poly culture pond [14] reported the temperature ranges from (18.3-37.8 ^{o}C) which is larger than variation found in our study.

Silver nanoparticles attract much attention due to expected toxicity. It can cause damage to the brain, liver, and stem cells in the human body. Thus, instead of using human, it is preferable to use animal models in toxicological studies. Among all models, fish is most dominantly used in toxicological studies. This review was published by [15] showed that Ag-NPs pose toxicity to all the life stages of fish. In chronic toxicity studies regarding hematological analysis, level of hemoglobin initially increased significantly and then decreased. Similar trends were recorded in RBC count. It was probably due to elevated levels of metabolic rate under stress condition [16]. Secondly, the flow of RBCs also increases in blood stream due to hypoxia and dehydration. As Ag-NPs produces hypoxic condition and increase the alkalinity, the kidney sensors detect this condition and increase RBCs movement in the blood flow. [17] of our study are like [16] in our study during control the hemoglobin level was 4.1 g/dl. It was normal range of hemoglobin but in treatment no1, treatment no 2 and treatment no 3 its level increase respectively 9.1 g/dl, 10.4 g/dl and 13.2 g/dl. These result shows that the toxicity of silver nanoparticles increase hemoglobin level in fish.

Further, MCH and MCHC values were significantly different from control and decreased with increase in concentrations of Ag-NPs. This lowering of values were due to RBCs counts and hematocrit reduction, which itself was reduced due to deformation or damage to RBCs [18]. Many investigators reported decrease in the level of MCH and MCHC in freshwater fish exposed to metals and nanoparticles [19] the result of [18] and the results of [19] does not match with our result the reason was that they use fresh water. We also use fresh water but here water salinity is higher than normal fresh water due to this our results does not match with both of them. Overall changes in blood parameters were due to the reaction of defense against toxicity through the mechanism of the erythropoiesis [20].

There was also a significant difference in neutrophils of all treatments compared to control. Neutrophils increased in number because Ag-NPs increased the infection and damage in tissues [21]. This leads to neutrophils [22]. The degree of elevation of neutrophils represents the infection severity. Many fluctuations were also seen in a number of monocytes relatively in short response of respiratory burst. This change in monocytes might be due to disease condition or hematological tissue dysfunction [23]. The results of [21, 22] and [23] like as our results according to them number of neutrophil increase due to the effect of silver nanoparticles. Neutrophil is a type of WBC cell. Our result also shows that the number of white blood cell WBC increases with the increase of silver nanoparticles. In control the number of WBC is 110×10^3 which was normal. But in treatment no 1, treatment no 2 and treatment no 3 the number white blood cell increases respectively $118\times10^{3,}$ 148×10^3 and 150×10^3. These result showed that the number of white blood cell increased with the increase of silver nanoparticles. Because silver nanoparticle causes toxicity and white blood cells cause immunity due to this number white blood cell increases with the increase of silver nanoparticles.

Conclusion

In conclusion, our results indicate that inducing stress due to subacute exposure to Ag-NPs (Nanocid) causes erythrocyte reduction, hematological disturbances and leukocytosis in *Labeo rohita*. Based on the results of this study, it is suggested that industrial and commercial applications of Ag-NPs should be more carefully and thoroughly assessed as to their potential toxic effects to the aquatic environment and fish. We concluded that silver nanoparticles affect the histological, biochemical and hematological parameters of fish. And these parameters are most important in every type of living animal's health

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