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ORIGINAL ARTICLE

Toxicity assessment on haemotology, biochemical and histopathological alterations of silver nanoparticles-exposed freshwater fish *Labeo rohita*

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Abstract The increasing use of nano based-products induces the potential hazards from their manufacture, transportation, waste disposal and management processes. In this report, we emphasized the acute toxicity of silver nanoparticles (AgNPs) using freshwater fish *Labeo rohita* as an aquatic animal model. The AgNPs were synthesized using chemical reduction method and the formation of AgNPs was monitored by UV–Visible spectroscopy analysis. The functional groups, crystaline nature and morphological characterizations were carried out by fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and high resolution transmission electron microscopy (HRTEM) analysis. UV-Vis range was observed at 420 nm and XRD pattern showed that the particles are crystalline nature. HRTEM analysis revealed that the morphology of particles was spherical and size ranges between 50 and 100 nm. This investigation was extended to determine the potential acute toxicity, *L. rohita* was treated orally with the lethal concentration (LC_{50}) of AgNPs. The antioxidative responses were studied in the three major tissues such as gill, liver and muscle of *L. rohita*. The results of this investigation showed that increasing the concentration of AgNPs led to bioaccumulation of AgNPs in the major tissues. The haematological parameters showed significant alterations in the treated fish. The histological changes caused by chemically synthesized AgNPs demonstrated the damages in the tissues, primary lamella and blood vessels of *L. rohita*. The histological study also displayed the formation of vacuolation in liver and muscle when compared with untreated tissues (control) of *L. rohita*.

Keywords *Labeo rohita* · Silver nanoparticles · Acute toxicity · Oxidative stress · Histopathology

Introduction

In recent years there is an increasing demand for the nanoparticles in the field of metal industries, and biomedical science etc. Nowadays the nanoparticles are even used in the household appliances. Nanotoxicology is a study of impact of manufactured nanomaterials on living organisms and environment. It also deals with the quantitative evaluation of severity and frequency of nanotoxic effects in relation to the exposure of the organisms. Metal nanoparticles have been used in various fields such as consumer products, industrial applications and health care technology are likely to enter the environment (Masciangioli and Zhang 2003; Nohynek et al. 2007; Benn and Westerhoff 2008) stated that the AgNPs are commonly used in the textiles and these nanoparticles might be released into the environment. The toxicological evidence of AgNPs is still lacking and the safety measurements for these nanoparticles have to be framed. The engineered nanomaterials comprise of numerous different physical forms and some of these materials including carbon nanotubes, carbon spheres called fullerenes (Zhu et al. 2006) and nanoparticles made from metals (Griffitt et al. 2007), metal oxides (Federici et al. 2007) or composites made of several metals (King-Heiden et al. 2009) have adverse effects on fish (Smith et al. 2007). To study the aquatic toxicity, fish species has been widely used as an indicator of pollutant and they strongly respond to stress conditions.

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An earlier report of Ramesh et al. (2013) emphasized the toxicological impact of SiO_2 nanoparticles on antioxidant enzymes and DNA strand break using zebra fish (*Danio rerio*). Earlier literatures have also been discussed about the pathological alterations at morphological level in various severities in different fish organs (Lemaire-Gony and Lemaire 1992; Battaglini et al. 1993).

ROS-mediated oxidative damage to macromolecules namely lipids, proteins and DNA have been implicated in the pathogenicity of major diseases. The oxidative stress implicated in the pathology of a number of disorders, such as atherosclerosis, ischemia–reperfusion injury, cancer, malaria, diabetes, inflammatory joint disease, asthma, cardiovascular diseases, cataracts, immune system decline, play a role in neurodegenerative diseases and aging processes (Young and Woodside 2001). Furthermore, nanoparticles are also shown to cause toxicity by increasing concentration of intracellular ROS and decrease in antioxidant level (Singh et al. 2009; Hussain et al. 2009). The increase in ROS level is also an important indication of predominant mechanism of acute toxicity (Kaewamatawong et al. 2006). In this study, synthesized AgNPs were assessed to explore the acute toxicity effects using *Labeo rohita* as an in vivo model. These findings would provide essential information regarding the potential toxicities and biodistribution of AgNPs in the fish model.

Materials and methods

Synthesis of AgNPs

The AgNPs were synthesized using chemical reduction method of Rashid et al. (2013), with minor modifications. Silver nitrate (0.1 M), 0.8 g of trisodium citrate and 0.1 g of sodium borohydride were freshly prepared. These three solutions were mixed with equal volume (2 mL) and finally made up to 200 mL with distilled water (pH 8.0). The reaction mixture was stirred continuously at room temperature for 3 h and the colour change of the reaction was noticed. The solution was centrifuged at 6000 rpm for 20 min followed by distilled water to purify the reaction mixture. Finally, the pellet was made into powdered form and stored for further studies.

Characterization of AgNPs

UV–Visible spectroscopy

The characterization of synthesized AgNPs was carried out using UV–Visible spectroscopy. The reduction of AgNPs was monitored by measuring the absorbance of reactions mixture at the range of 200–700 nm using synergy HT multi-mode microplate reader (Biotek Instruments, Inc, Winooksi, VT, USA).

X-ray diffraction (XRD)

The AgNPs were subjected to XRD analysis using PAN analytical-XPERT-PRO diffractometer system, Eindhoven, Netherlands and the target was Cu Kα radiation with a wavelength of 1.54 Å, the generator was operated at 40 kV and with a 30 mA current. The average grain size of AgNPs was determined using Scherrer's formula. $D = 0.94\lambda/\beta \cos\theta$, where, D is the average crystal size, k is the Scherer coefficient (0.94), λ is the x-ray wavelength, θ is Bragg's angle and β is the full width at half maximum in radians.

FTIR and HRTEM analysis

FTIR spectroscopy was used to identify the possible functional groups involved in the reduction of silver ions. The FTIR spectra of synthesized AgNPs were analyzed in the range of 400–4000 cm^{-1} and the measurement was carried out by JASCO (FTIR-6200) spectrum. The size and morphological nature of the AgNPs were determined using transmission electron microscope (JEOL-JEM-200 CX).

Experimental animal and acute toxicity assessment of AgNPs

The experimental animal *L. rohita* was collected from local fish pond and maintained in the aquarium separately. The experimental aquaria were aerated and test media were replaced every day. The laboratory temperature was 28 ± 2 °C and normal illumination (approx 12 h light and 12 h dark) was maintained throughout the experimental period. Acute toxicity effect of AgNPs was investigated on *L. rohita*. All experiments were carried out for a period of 7 days and lethal concentration (LC_{50}) was determined with five different concentrations (25, 50, 100, 500, 1000 mg kg^{-1}) of AgNPs. Among these concentrations, mortality rate was found at two concentrations (500 and 1000 mg kg^{-1}). Hence, 100 mg kg^{-1} concentration was used as a maximum value for further experimental studies. For sub-acute toxicity tests, the LC_{50} concentration of AgNPs in water was maintained modestly below value such as 5, 10, 25, 50 and 100 mg kg^{-1} and AgNPs were orally introduced to the fish. After 7 days of sub-lethal exposure, the blood samples were collected and the experimental fishes were sacrificed and muscle, gill and liver were dissected out to assess the toxic impact of AgNPs in fish by analyzing haematological, antioxidant enzymes and histological parameters.

Analysis of haematological parameters

The Haemoglobin content, total protein, total erythrocytes and leukocytes count of the blood was estimated. The blood samples were collected and immediately subjected to haematological analysis. The RBC and WBC were diluted with appropriated diluting fluids and the total content was calculated using improved Neubauer haemocytometer (Blaxhall 1972). The Sahli's haemoglobinometer was used to estimate haemoglobin (HB) content of the AgNPs-treated fish as well as in control.

Effect on tissue-damaging enzymes

Estimation of acid phosphatase (ACP) and alkaline phosphatase (ALP)

Acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were estimated according to Michell et al. (1970) and Estiarte et al. (2008). The reaction medium for ACP contained 0.7 ml sodium acetate buffer (pH 5.0), 0.25 ml p-nitrophenyl phosphate (pNPP, 5 mM) as substrate and 0.05 ml of enzyme totaling to 1 ml was incubated for 30 min at 37 °C. The reaction was stopped by adding 4 ml NaOH (0.1 N) and incubated for another 30 min at 37 °C. The reaction medium for ALP contained 0.5 ml glycine buffer (pH 7.8), 0.2 ml magnesium chloride (10 mM), 0.25 ml p-nitrophenyl phosphate (pNPP, 5 mM) as substrate and 0.05 ml of enzyme totaling to 1 ml was incubated for 30 min at 37 °C. The reaction was stopped by adding 4 ml NaOH (0.02 N) and incubated for another 30 min at 37 °C. The estimation involves measurement of yellow colour of p-nitrophenol in a synergy HT Multi-Mode Microplate Reader, (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Catalase (CAT)

Catalase activity was assayed by the method of Caliborne (1985). The decomposition of H_2O_2 was measured by the decrease in the absorbance at 240 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.2) and 50 mM H_2O_2, the reaction rate was measured at 240 nm. The extinction coefficient of H_2O_2 was 40 M^{-1} cm^{-1}. One unit of catalase was defined as 1 µmol of H_2O_2 degraded min^{-1} mg^{-1} protein.

Glutathione-s-transferase (GST)

The GST activity was determined using the method of Habig et al. (1974). The reaction mixture (3 ml) contained 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After pre-incubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of glutathione as substrate. The absorbance was followed for 3 min at 340 nm and reaction mixture without the enzyme was used as blank. The activity of GST was expressed as µmoles of GSH-CDNB conjugate formed min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD)

SOD activity was analyzed using the method of Marklund and Marklund (1974). In this test, the degree of inhibition of pyrogallol autoxidation by supernatant of the lenticular homogenate was measured. The change in absorbance was read at 470 nm against blank every minute for 3 min on a Microplate Reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). The enzyme activity was expressed as units per milligram protein.

Histopathological analysis

Samples of muscle, gill and liver tissues for histological assessment were fixed in 4 % paraformaldehyde in 0.1 M

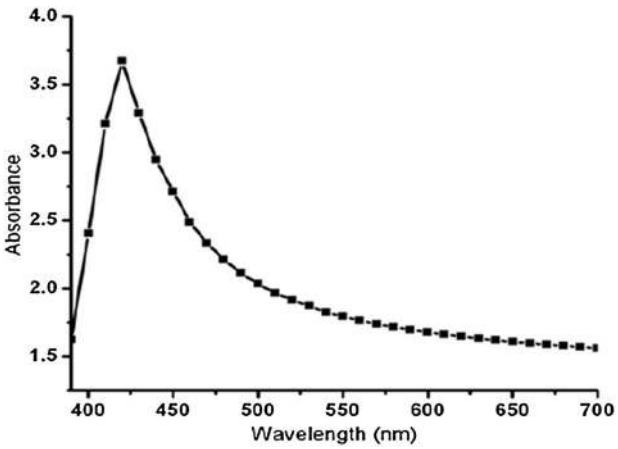

Fig. 1 Shows the UV–Visible spectroscopic analysis of AgNPs

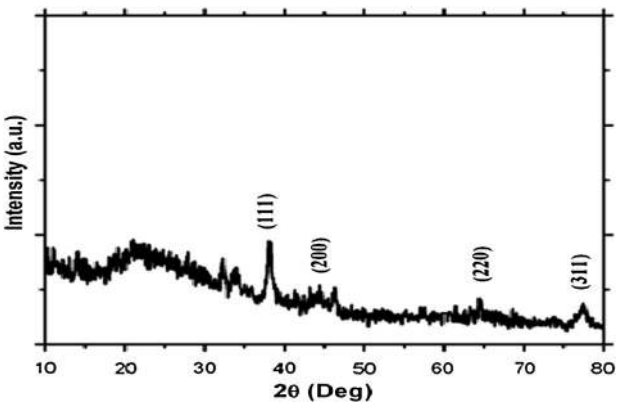

Fig. 2 XRD pattern displayed the crystalline nature of AgNPs

phosphate-buffered solution (pH 7.4) at 4° C, dehydrated in ethanol and embedded in paraplast (Takashima and Hibiya 1995). The histological sections (5 mm thick) were cut with a rotary automatic microtome and sections were mounted on glass slides. Finally, the slides were stained with haematoxylin/eosin to visualize typical morphological features.

Results and discussion

In the present study, AgNPs were synthesized by chemical reduction method and primarily confirmed by the colour change of the reaction mixture from colourless to yellowish brown colour. The appearance of a yellowish brown colour is an indication of the formation of AgNPs (Vignesh et al.

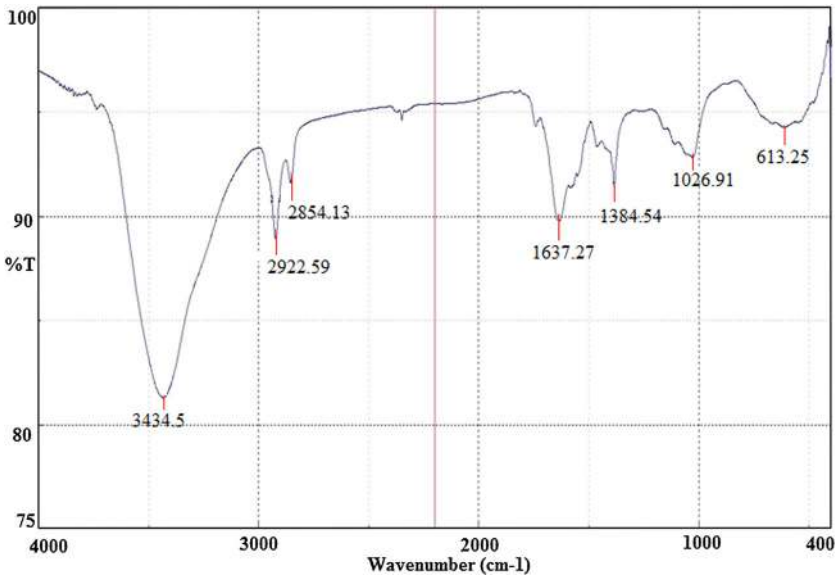

Fig. 3 FTIR analysis of chemically synthesized AgNPs

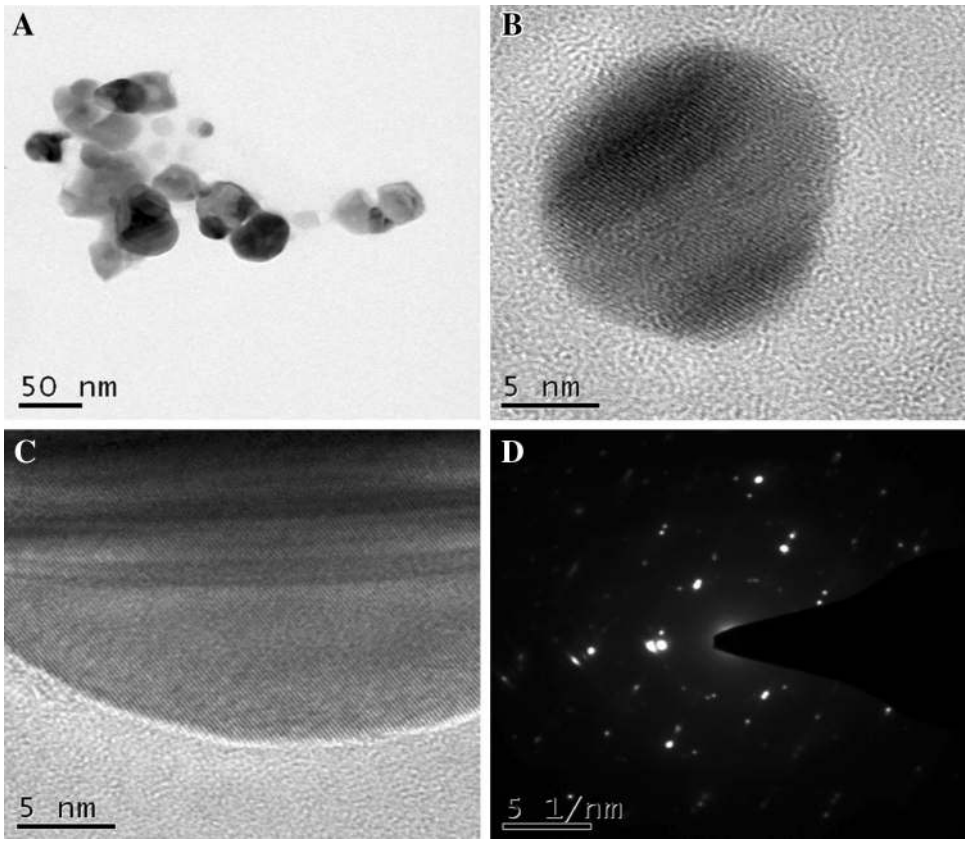

Fig. 4 HRTEM images of AgNPs clearly showed **a** mono-dispersed and **b** and **c** spherical shape of the AgNPs. **d** Showed the SAED pattern of particles in crystalline nature

2013). Here, we have used trisodium citrate and sodium borohydrate as reducing agents. UV–Visible absorption spectra of AgNPs formed in the reaction media was recorded at 420 nm (Fig. 1). Under the UV region, AgNPs give a characteristic absorbance band due to the excitation mode of their surface plasmon which is dependent on the nanoparticle size (Vivek et al. 2012; Kanipandian and Thirumurugan 2014). The earlier reports have concluded that AgNPs production from fungi extract *Rhizopus stolonifer* (Banu et al. 2011) and plant extract *Rhizophora apiculata* (Antony et al. 2011) showed maximum absorbance at 422 and 423 nm, respectively.

X-ray diffraction analysis

The phase purity and crystalline nature of AgNPs were determined by powder XRD study (Fig. 2). The XRD spectrum showed four distinct diffraction peaks at ~38°, 46°, 64.4°, and 77° and these peaks correspond to (1 1 1), (2 0 0), (2 2 2) and (3 1 1) planes of face-centered cubic (fcc) silver phase (Kanipandian et al. 2014). The obtained XRD results were closely associated with the standard card (JCPDS file no. 65-2871). The average size of AgNPs formed in the present investigation was estimated by determining the full width at half maximum (FWHM) of the Bragg's angle and the estimated mean size was 96 nm.

FTIR analysis

FTIR image of chemically synthesized AgNPs is given in Fig. 3 and the FTIR spectrum was analyzed between the ranges of ~400–4000 cm^{-1} and showed seven broad intense bands at ~3434.6, 2922.59, 2854.13, 1637.27, 1384.64, 1026.64 and 613.25 cm^{-1}, respectively. The

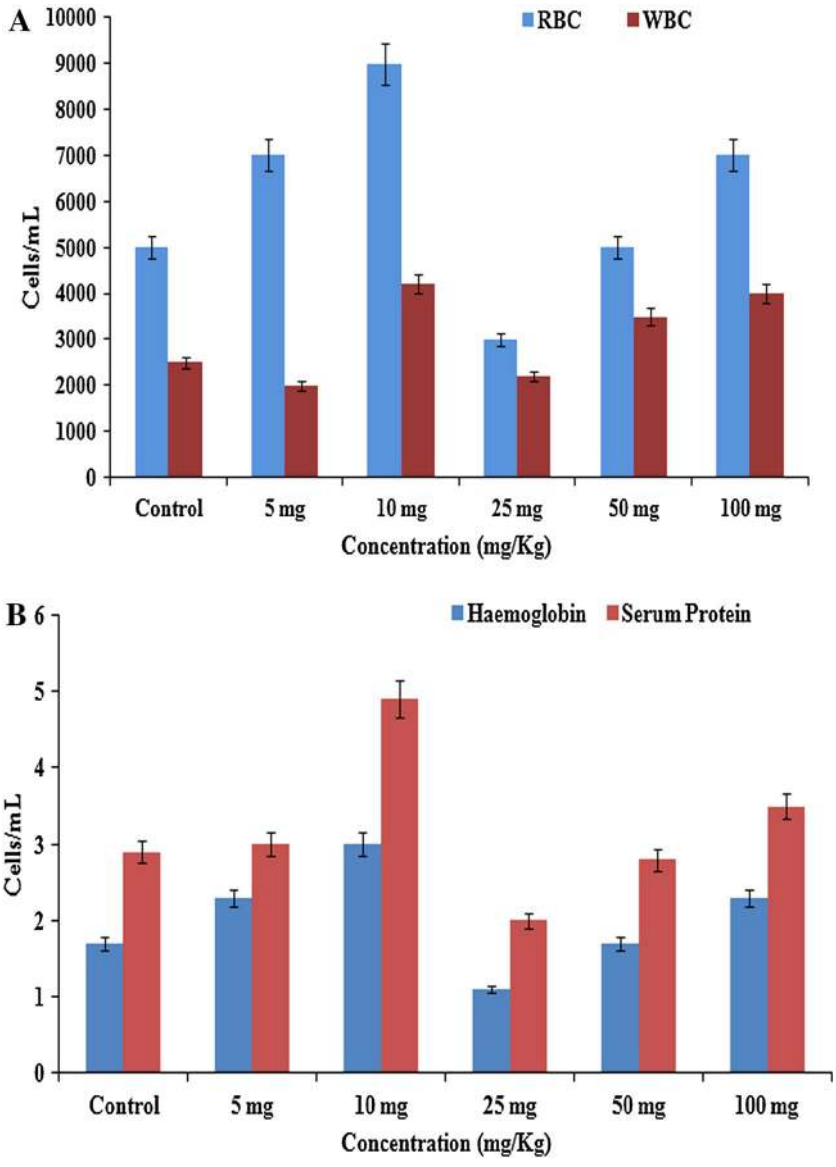

Fig. 5 Illustrates the toxic impact of chemically synthesized AgNPs on Haematological parameters of *Labeo rohita*

bands at ~3434.6 and 2922.59 cm^{-1} attributed to heterocyclic amine, NH stretch and methylene C–H asymmetrical/symmetrical stretch. The band at ~2854.13 cm^{-1} indicated the presence of asymmetric C–H stretch of the methyl and methylene groups. The FTIR peaks at ~1637.27 and 1384.64 cm^{-1} contributed to amide and stretching vibration of NO^{3-} ion present in the nanocomposite (Paulraj et al. 2011). The intense peaks at ~1026.64 and 613.25 cm^{-1} denote the primary amine, CN stretch and alkyne C–H bends.

Electron microscopy studies

The size and morphological structure of synthesized AgNPs were studied by HRTEM (Fig. 4). These analyses revealed the presence of nano-sized materials in the suspension, which showed that the structure of nanoparticles were spherical in nature. The HRTEM clearly demonstrated that the particles were well dispersed and not severely agglomerated. The size distribution of the AgNPs displayed the average particle size ranging from 50 to 100 nm and SAED pattern confirmed the crystalline nature of the particles and agreed to XRD pattern (Fig. 4d).

Acute toxicity of AgNPs

To determine LC_{50} value of AgNPs, the experimental fish (*L. rohita*) was treated orally with different dosage levels (25, 50, 100, 500 and 1000 mg kg^{-1}) of AgNPs. After the treatment period (7 days), the mortality in each group was observed and recorded carefully. The chemically synthesized AgNPs showed a dose dependent activity and mortality was increased with increasing the concentrations of AgNPs. The 100 % of mortality rate was observed at 500 and 1000 mg kg^{-1} concentration and no mortality was noticed in the lowest concentrations such as 25 and 50 mg kg^{-1} of AgNPs. The 50 % of mortality was observed at 100 mg kg^{-1} concentration and no mortality was recorded in control fishes during the tests. Hence, the further studies such as haematological impact, enzymes level

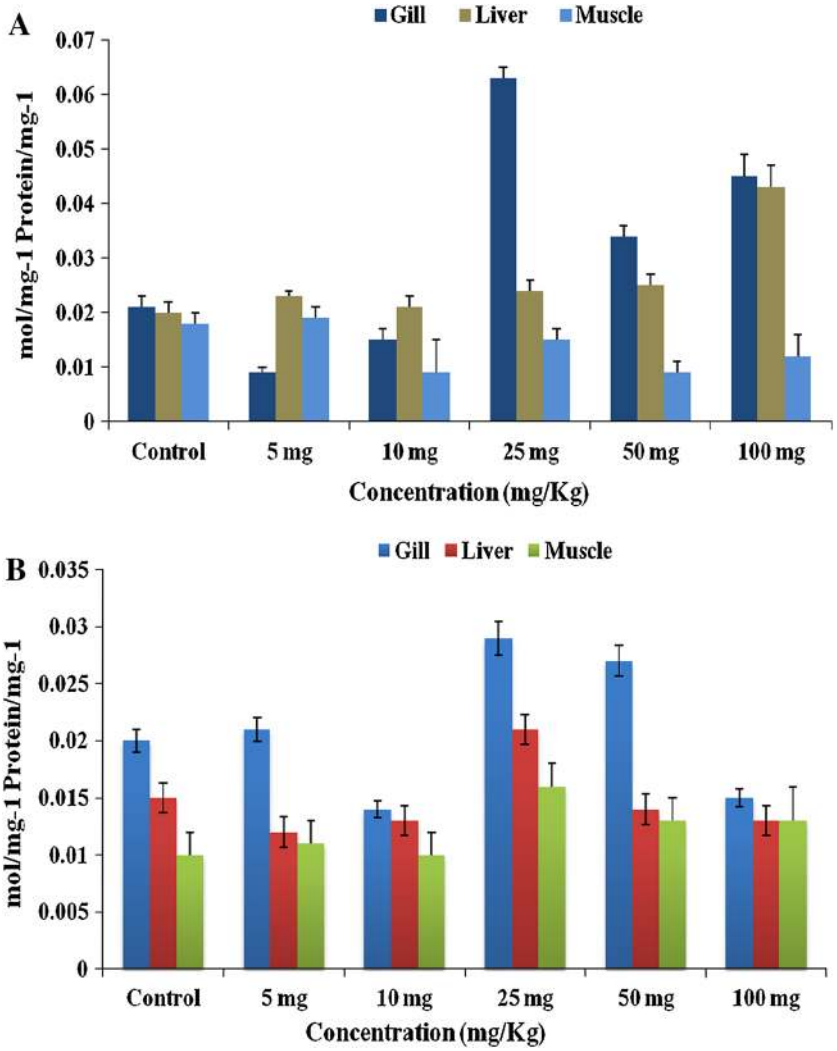

Fig. 6 Analysis of toxicity effect of AgNPs on major tissues of *Labeo rohita* using tissue-damaging enzymes **a** ACP and **b** ALP

and histological study were analyzed below the concentration of LC_{50} value. The similar LC_{50} value 100 mg L^{-1} was estimated in TiO_2-treated *Danio rerio* (Diniz et al. 2013; Xiong et al. 2011).

Haematological parameters

The alterations in RBC, WBC, haemoglobin and total serum protein level were analyzed. From the haematological investigation, we concluded that the levels of all the haematological parameters mentioned above were reduced at 25 mg kg^{-1} concentration when compared with other concentrations and control fish samples (Fig. 5). The changes in the haematological parameters might be a result of stressful conditions which affect the metabolism and normal functioning of the fish physiology (Blaxhall 1972). There were no significant alterations observed at other concentrations and the level was increased with increasing the concentration of AgNPs.

Effect on enzymes activities

The tissue-damaging enzymes such as acid phosphatase (ACP) and alkaline phosphatase (ALP) levels were

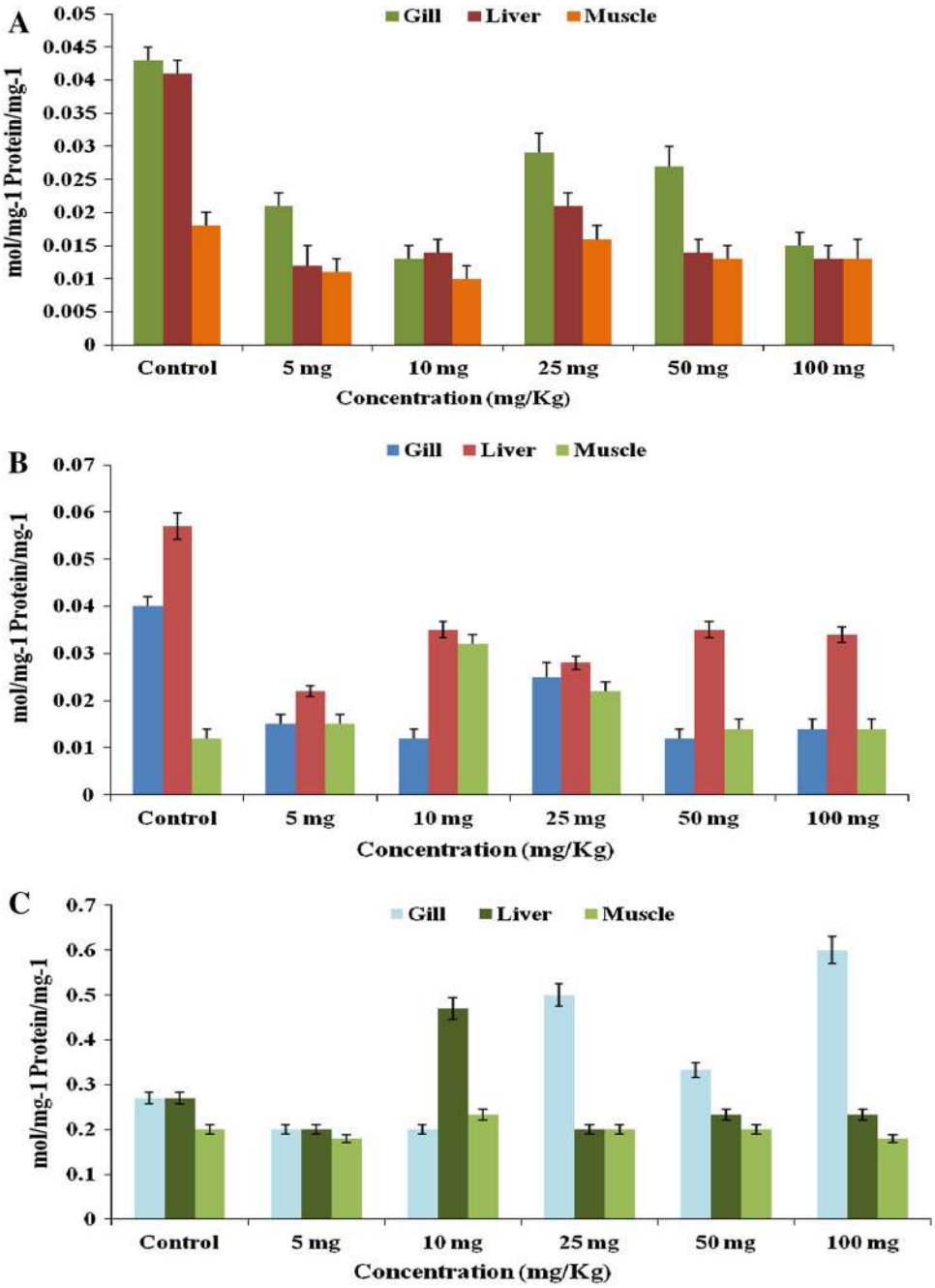

Fig. 7 Depicts the toxicity effect of AgNPs on antioxidant enzymes **a** GST, **b** CAT, **c** SOD

significantly higher in the AgNPs-treated fish tissues of gill, liver and muscle when compared with control tissues (Fig. 6). These results revealed that the tissue-damaging effect was caused by AgNPs. The elevation of these enzymes in the tissues was dose dependent and the higher concentrations of the AgNPs exhibited more enzyme activity.

Oxidative stress is the result of an inequity in the pro-oxidant/antioxidant homeostasis. The activities of antioxidant enzymes in the treated fish tissues such as liver, gill and muscle were assessed. The oral administration of AgNPs caused a significant reduction in the activities of glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD). The treated liver tissue showed the highest changes in antioxidant enzyme activity among the tissues (Fig. 7). The AgNPs can cause the oxidative stress, leading to the depletion of antioxidant enzymes activities. A significant decrease in

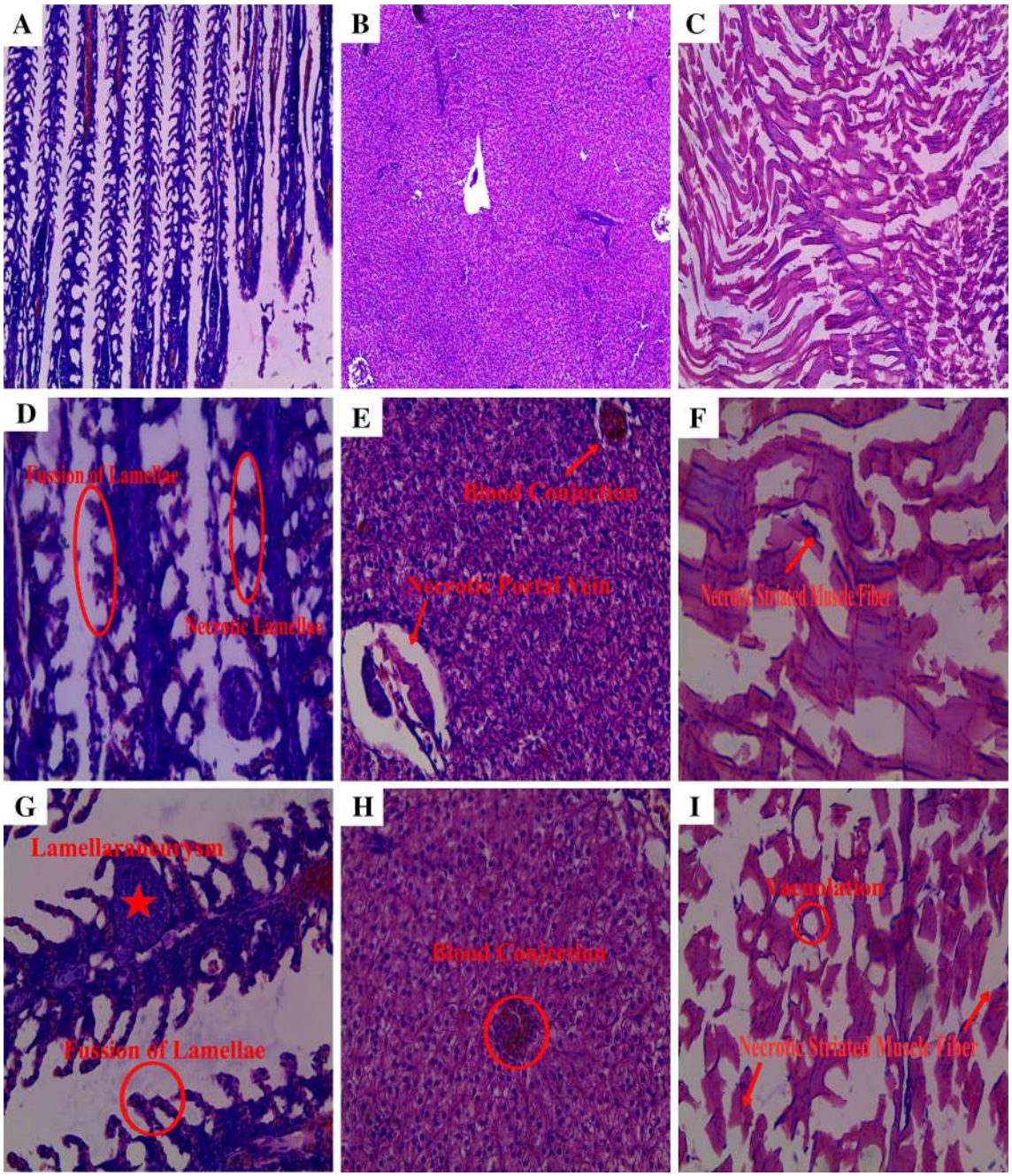

Fig. 8 The images demonstrate the histopathological study of AgNPs-treated gill, liver and muscle tissues of *Labeo rohita*. The control (**a**, **b** and **c**), 5 mg kg^{-1} concentration (**d**, **e** and **f**) and 10 mg kg^{-1} concentration (**g**, **h** and **i**) are displayed

GST, catalase and SOD activity was observed in all treated tissues when compared with control tissues. The mechanism behind this is the metallic nature of nanoparticles and the presence of transition metals encourages the production of reactive oxygen species (ROS) leading to oxidative stress (Mac Nee and Donaldson 2003; Jia et al. 2009).

Histopathological studies

The histopathological changes were observed in gill, liver and muscle tissues of *L. rohita* due to toxicity caused by AgNPs (Figs. 8, 9).

In control, no recognizable changes were observed in gill tissue during the experimental period. It showed

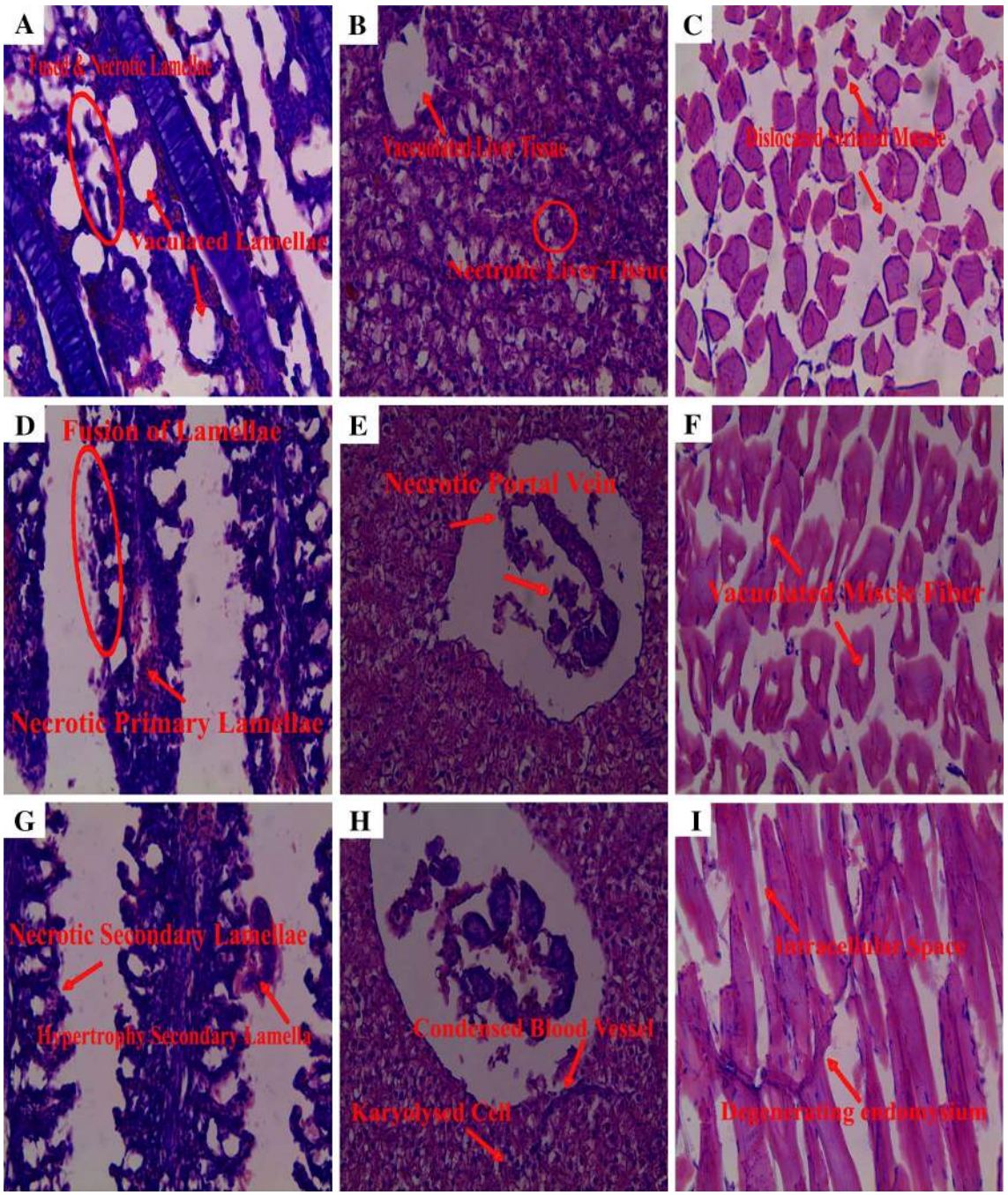

Fig. 9 The histopathological investigation of AgNPs-treated gill, liver and muscle tissues of *Labeo rohita* at 25 mg kg^{-1} concentration (**a**, **b** and **c**), 50 mg kg^{-1} concentration (**d**, **e** and **f**) and 100 mg kg^{-1} concentration (**g**, **h** and **i**) are showed

primary and secondary lamellae with pillar cells and also it consists of central venous sinus, chloride cells. The AgNPs-treated fish groups exhibited proliferation of bronchial chloride cells that leads to lamellae fusion and formation of aneurism. The aneurism was localized, blood-filled balloon-like bulge of a blood vessel. It increases a significant risk of rupture, resulting in severe haemorrhage, other complications or death. These similar results were observed on deltamethrin-exposed freshwater fish *Aphanius dispar* that showed the changes like vacuolization, lifting of the lamellar epithelium and fusion of secondary lamellae (Al-Ghanbousi et al. 2012). The histological responses in the gills of fish were mostly associated with circulatory disturbances and regressive and progressive changes (Van Dyk et al. 2009).

In the liver section, the control tissue showed normal hepatocytes. The AgNPs-treated fish had congestive enlargement of liposomes which lead to vacuolar degenerations in liver. The necrosis were seen at higher level in liver tissues of AgNPs-exposed fish. The section of the control muscle showed normal histological structures such as fiber bundles, connective tissue and arrangement of muscle bundles. The treated fish had an abnormal arrangement of muscle bundles, vacuolization in both liver and muscle. Muscle fibre inflammations were noticed in treated *L. rohita*. The earlier report of Capkin et al. (2010) concluded the similar toxic effect of pesticides on the vital organs of juvenile rainbow trout (*Oncorhynchus mykiss*).

Conclusion

This present study deals with the chemical synthesis of AgNPs and physically characterized by UV–Visible, FTIR, XRD, and HRTEM analysis. This investigation clearly demonstrated the impact of AgNPs on aquatic organisms. These AgNPs caused alterations in the haemotology, enzymes activities and histopathological parameters. From this experimental study, we suggest that before applications of AgNPs in various industrial sectors, the toxic potential must be carefully assessed and the effluents are also to be processed before it gets entered into the environment to protect the aquatic eco-systems as well as human lives.

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