



Ameliorative role of nano-ceria against amine coated Ag-NP induced toxicity in *Labeo rohita*

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Abstract

Silver nanoparticles (Ag-NPs) and its byproducts can spread pollution in aquatic habitat. Liver and gills are key target for toxicity. Oxidative stress, tissue alterations, and hemotoxicity are assumed to be associated with Ag-NPs in target animals. Cerium oxide nanoparticles (nano-ceria) show antioxidant potential in scavenging the free radicals generated in Ag-NP-induced oxidative stress. We determined ameliorated role of nano-ceria against Ag-NP-induced toxicity in fresh water *Labeo rohita* (*L. rohita*). Four groups were used in study including control, nano-ceria, Ag-NPs, and Ag-NPs + nano-ceria. Ag-NPs (30 mg l⁻¹) and nano-ceria (50 µg kg⁻¹) were given through water and prepared feed, respectively. The samples were taken after 28 days. Results demonstrated that pre-treatment of nano-ceria recovered *L. rohita* from Ag-NP-induced toxicity and oxidative stress. Nano-ceria pre-treatment actively mimics the activity of GST, GSH, CAT, and SOD. Furthermore, Ag-NPs' treatment caused severe inflammation and necrosis in hepatic parenchyma which led to congestion of blood in hepatic tissues. Accumulation of a yellow pigment in hepatic tissue was also seen due to necrosis of affected cells. In nano-ceria pre-treatment, there was no congestion in hepatic tissue. Vacuolization of cells and necrosis in some area was recorded in nano-ceria pre-treated group, but the gill and hepatic tissue showed improvement against Ag-NP-induced damage. Nano-ceria pre-treatment also improved hematological parameters in Ag-NP-treated fish. This study concluded that Ag-NP-induced toxicity in treated fish and pre-treatment of nano-ceria show ameliorative role.

Keywords Amelioration · Ceria · Nanoparticle · Oxidative stress · Toxicity · Silver

Introduction

Ag-NPs are much rapidly growing class with 438 commercially available nanoproducts in international markets (Khan et al. 2015a; Wilson 2016). It is being used in batteries, photography, filters (Li et al. 2008), fabrics (Perelshtein et al. 2008), catalysts (Kumar et al. 2008), sensors (Schrand et al. 2008), textiles (Cohen et al. 2007; Ju-Nam and Lead 2008; Sondi and Salopek-Sondi 2004), medicine (Kirsner et al. 2001), in treatment of burns infections (Tredget et al. 1998), anticancer (Vergaro et al. 2011), antimicrobial (Du et al. 2018; Maillard and Hartemann 2013), and antifungal agent (Wright et al. 1999). Furthermore, the silver could

also combine with other elements or compounds for various purposes. Excessive uses of these particles also increase discharge into aquatic habitats and exist in colloidal form. These particles persuade toxicity to invertebrate or vertebrate cell lines by production of reactive oxygen species (Ali 2014), apoptosis (Piao et al. 2011), reduced mitochondrial function (Ahamed et al. 2010), lipid peroxidation of membranes (Khan et al. 2017b; Zhornik et al. 2014), and oxidative stress marker depletion (Arora et al. 2009). Furthermore, Ag-NPs generate oxidative stress in target organisms (Devi et al. 2015; Khan et al. 2017b).

Labeo rohita belongs to widely cultured Indian major carps in south Asia including Bangladesh, India, Myanmar, Nepal, and Pakistan (Dahanukar 2010; Froese et al. 2016). This fish species inhabits freshwater rivers under a depth of 550 M and feed mainly on plankton (Wahab et al. 1995). As fish is the most important component of human food chain, therefore, it was recommended to use as animal model in toxicological studies. Gills are key organs for absorption and histo-pathological changes arise

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very quickly (Hawkins et al. 2015). When Ag-NPs enter in the animal's body, they reach the liver through blood circulation and induce alterations (Khan et al. 2015a). Furthermore, this fish also shows oxidative stress (Khan et al. 2017b), alterations in blood hematology (Zutshi et al. 2010), genetic material (Khan et al. 2017a), and histology (Khan et al. 2017c) when exposed to toxic or environmental contaminates. These parameters can be used in studying the nature of new or novel compound (Khan et al. 2017a, b, c).

To cope with prooxidant level and maintain the healthy life, antioxidants are always necessary in diets (Asghar et al. 2018; Khan et al. 2016a; Zhang 2015). Nano-cerium is one of important antioxidant with long history of use as animal's feed additive (Khan et al. 2015a, b). Historically, Chinese community used cerium for increasing weight in cattle and egg production in breeders (Spivak et al. 2012). Currently, nano-ceria is extensively used in fuel as additive for better performance, polishing agent, radiation protecting, and antioxidant agent (Baker 2011; Karakoti et al. 2010; Park et al. 2007). Extensive application of nano-ceria raised concerned in researchers about long-term effects of exposures (Kumari et al. 2014). Still, insufficient information is available explaining the activities of nano-ceria in biological applications. Little attention was shifted towards unpredicted behavior of nano-ceria in aqueous and biological fluids (Shcherbakov et al. 2011). Review of published literature conformed the role of nano-ceria as antioxidant (Hosseini et al. 2015) and work as superoxide dismutase (SOD) (Korsvik et al. 2007), mimic catalase (CAT) (Pirmohamed et al. 2010), scavenging the hydroxyl radical (Xue et al. 2011), and nitric oxide free radicals (Dowding et al. 2012). In this study, the antioxidant potential of nano-ceria was tested against Ag-NP-induced toxicity and oxidative stress in 28 day treated *L. rohita*.

Materials and methods

Reagents

All reagents or chemicals of analytical grade were purchased from Merck and sigma Aldrich. Nano-ceria was in powder form with average size of 15.78 ± 5.56 nm. Ag-NPs' particles were 16.59 ± 7.61 nm in size and prepared in the previous study by Khan et al. (2017b). The particles were characterized through SEM, TEM, XRD, EDS, and FT-IR analysis. The hydrodynamic size of Ag-NPs and nano-ceria (DLS measurements) was measured through Malvern Zetasizer using back-scattering detector. For DLS, stock solution was diluted with distilled water tenfold than original. Size was represented in intensity (%) of particles.

Experimental animal and laboratory conditions

The *L. rohita* (100 ± 5 g) as experimental model was obtained Punjab fishy department. The fishes were maintained in 40 l separate aquaria at room temperature (below 30 °C) and natural photoperiod. The dose for treatment for Ag-NPs was selected according to the previous study Khan et al. (2017b), where dose for nano-ceria was selected according to Estevez and Erlichman (2014). They used 30 mg kg⁻¹ nano-ceria in diet of mice, but in our study, 50 µg kg⁻¹ was used as higher concentration might produce toxicity in fish.

Two types of diets were formulated for study. Type 1 diet was consisted of only nutritional ingredients. Type 2 diet was consisted of 50 µg kg⁻¹ nano-ceria along with nutritional ingredients. Fishes were divided into four treatments and three replicates, five fishes in each replicate. First treatment served as control and fed with type one diet. Type 2 diet was provided to second treatment. The treatment 3 was exposed 30 mg l⁻¹ Ag-NPs along with feeding of type 1 diet. The treatment 4 was fed with type 2 diets and also exposed to 30 mg l⁻¹ Ag-NPs after 1 h of feeding. All treatments were fed twice a day times with specific diet for a period of 28 days. Water in each aquarium was replaced after 48 h.

Sampling

All sampling was done on the same day from randomly selected fishes of each replicate. Blood was taken via cardiac puncture with heparinized needle in EDTA tube. The gills and liver samples were preserved in 10% formalin solution.

Hematological analysis

Blood samples were analysed with M-20GP (MEDONIC Sweden) automatic hematology analyser. The activities of alanine amino transferase (ALT) and aspartate aminotransferase (AST) were measured with kinetic enzyme assay and expressed in IU/l.

The frequency of micronuclei was calculated through Giemsa stain smear formation as mentioned by Khan et al. (2017a) using formula:

$$\text{MN}(\%) = \frac{\text{No. of cells with micronuclei}}{\text{Total No. of analysed cells}} \times 100.$$

Frequency of comet was measured with methodology of Singh et al. (1988). Damage was measured with tailed DNA migration (comet tail) in µm and frequency of comet. 100 cells per sample were analysed for damage.

Enzymatic analysis

The liver and gill tissue was washed separately with 1.15% KCl (ice cold), weighted 1 g, and homogenised with buffer (50 mM Tris–HCl, pH 7.4 and 1.15% KCl). The homogenised content was centrifuged (Sigma 2–16 k) for 20 min at 10,000 rpm. The supernatant was stored at -20°C after decantation.

The activity of catalase (CAT) was monitored by Bergmeyer (1965) using 5 mM hydrogen peroxide as substrate. Reaction solution was prepared by 50 mM peroxide with 50 mM potassium phosphate buffer. The H_2O_2 decomposition done by CAT was estimated through absorbance of UV spectrophotometer at 240 nm. Values were represented in in mol/mg sample protein.

Activity of superoxide dismutase (SOD) was estimated as U/mg protein with protocol of Payá et al. (1992) with some modification made by Peixoto and Pereira-Moura (2008). Activity assay was started in the presence of 10 mM nitrotriazolium blue chloride (NBT) and 100 mM potassium phosphate buffer (pH 7.0). NBT acted as a detecting agent. About 0.023 U/mol of xanthine oxidase initiated the reaction when added to gill and liver extract at 25°C . Then, enzyme activity was inhibited by 50% NBT.

The Glutathione S-transferase (GST) activity of was determined by Habig et al. (1974) with some modification. Reaction mixture was made with combination of 100 mM potassium phosphate buffer (2 ml), 10%, 1-chloro-2, 4 dinitrobenzene (100 ml), triton X-100, and 100 mM GST. The sample was added to start the reaction and absorbance was recorded at 340 nm. The activity of GST was expressed in mol/mg protein.

The Glutathione (GSH) activity was recorded using protocol of Jollow et al. (1974). In brief methodology, tissue extract was mixed with 500 μl 4% sulphosalicylic acid, incubated at 4°C for 1 h, and centrifuged at 12,000 rpm for 15 min at 4°C . About 0.4 ml of supernatant was initially mixed with 0.1 M potassium buffer (pH 7.4) and then with 0.4 ml DTNB. Colour of mixture was changed to yellow when GSH react with DTNB. Optical absorbance was recorded at 412 nm and expressed as $\mu\text{mol/g}$ protein.

MDA (malondialdehyde) content was estimated for membrane lipid peroxidation (LPO). The methodology of Wilchek and Bayer (1990) was followed for this estimation. About 1 ml of sample extract was mixed with 2 ml of TBA–TCA–HCl, heated for 15 min in boiling water bath, and cooled at room temperature. The mixture was then centrifuged for 15 min at 4000 rpm for recording the absorbance at 535 nm against standard. The standard contained all the contents except sample. The molar concentration coefficient $1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was used for MDA content and expressed in $\mu\text{mol/g}$ protein.

Histological analysis was carried out through haematoxylin–eosin staining as mentioned by Khan et al. (2017c).

Statistical analysis

Data of three replicates were represented in the form of mean \pm SD. ANOVA in general linear model was performed with IBM statistics (v. 20) to estimate the effects of treatment. Tukey's test was used to compare the means. Purpose of performing this test was to compare the control, nano-ceria, Ag-NP-treated, and Ag-NPs + nano-ceria-treated group. The values which were significantly different marked with different letters.

Results

Characterization of particles

SEM image of Ag-NPs represents, and particles were spherical, grain like with agglomeration (Fig. 1). SEM image of CeO_2 -NPs also showed agglomeration of fine nanopowder. The histogram of Ag-NPs represents that most of particles were in 10–20 nm range with average size of 16.59 ± 7.61 nm. The histogram of CeO_2 -NPs shows maximum frequency between the ranges of 10–15 nm with an average size of 15.78 ± 5.56 nm (Fig. 2). TEM image represents spherical nature of sol and separated Ag-NPs. In electron diffraction analysis, ring patterns of electron diffraction shows (110), (200), (220), and (311) along growth direction which actually revealed crystalline, spherical, and face-centered cubic nature of synthesized Ag-NPs (Fig. 3). The hydrodynamic size was also measured through a Malvern Zeta sizer (nano-ZS) with back-scattering detector. Maximum particles were between 10 and 45 nm in case of Ag-NPs and 5–40 in nano-ceria samples. The DLS also presented that sampling dilutions with distilled water reduced agglomerations (Fig. 4). The FT-IR (Thermo Nicolet Avatar 380) spectra revealed coating and associated molecules with Ag-NPs. The peak spectrum at 3431.92 cm^{-1} was due to N-H and O-H stretches. The second absorption band at 2842.03 cm^{-1} was due to C-H group vibrations. The third absorption band at 2382.15 cm^{-1} was due to absorbed or free water molecules vibrations. The fourth band at 1631.24 cm^{-1} represents C=C stretch. The absorption spectrum at 1392.12 cm^{-1} represents the attachment of amine group (C-N). The absorption bands at 1235.96 and 1054.82 cm^{-1} reveal C-N stretch vibrations. Over all the FT-IR spectra confirmed presence of amine interaction with Ag-NPs (Fig. 5). Distinct peaks in XRD spectra at 38.20θ , 44.40θ , 64.81θ , and 77.90θ confirm crystalline, face-centered, and cubic nature of Ag-NPs (Fig. 6). In Fig. 5, the characteristics peaks of XRD (111) at 29.5° , (200) at

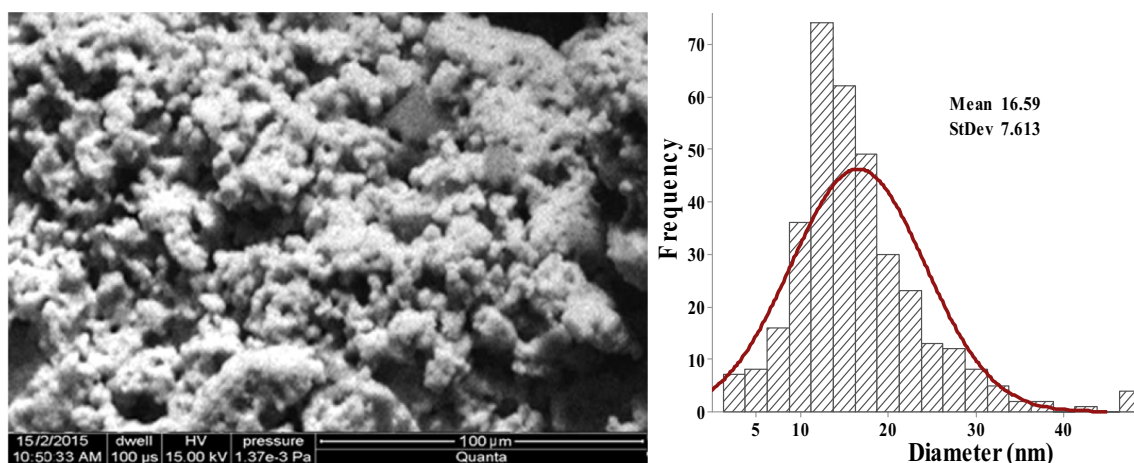


Fig. 1 SEM image of amine-coated and spherical Ag-NPs along with histogram

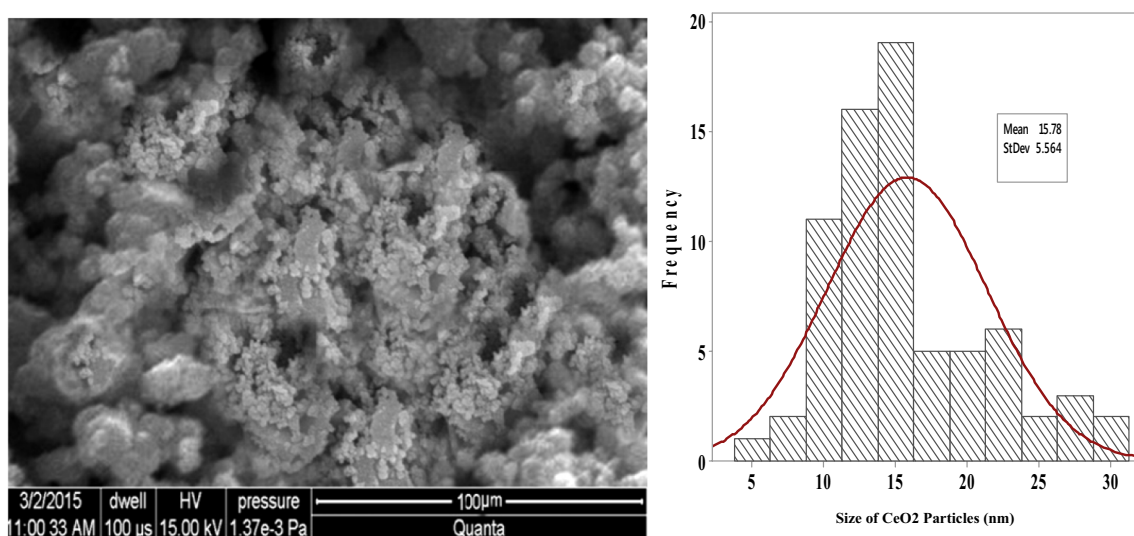


Fig. 2 SEM image of spherical and grain like CeO₂-NPs along with histogram

33.3°, (220) at 47.6°, (311) at 57.5°, (222) at 59.1°, (400) at 64.4°, (331) at 76.6°, (420) at 79.3° and (422) at 88.5° of 2θ indicating the small, crystalline nature of particles and high purity of nano-ceria sample. High sample purity of both types of particles was also confirmed with EDX spectrum analysis. Spectra of both samples were only consisted of silver, cerium, and oxygen peaks. Impurity was not found in both recorded spectra (Fig. 7).

Ameliorative role in oxidative stress

Treatment of Ag-NPs negatively affected the activity of CAT in gill and liver tissue. A substantial increase in activity of CAT and SOD was indication of oxidative stress. The animal receiving pre-treatment of nano-ceria showed significant

protection against CAT and SOD changes (at $p < 0.05$) due to active free radical scavenging and restored the activity of these enzymes (Fig. 8). Activity of GST increased with Ag-NPs' treatment this might be due to conjugation of GSH with Ag⁺ ions. The treatment of nano-ceria reduced the conjugation of Ag⁺ and restored the activity of GST. Ag-NPs also increased the MDA contents in both tissues indicated the broken balance between antioxidant system and oxidative stress. Thus, radicals created increased lipid peroxidation of gill and liver tissue and hence increase the MDA content. Similar trend was in case of GSH. Activity of GSH was increased with increase of MDA content which indicated defensive mechanism of gill and liver against oxyradicals. Pre-treatment of nano-ceria reduced the MDA contents by scavenging oxyradicals and preventing the lipid

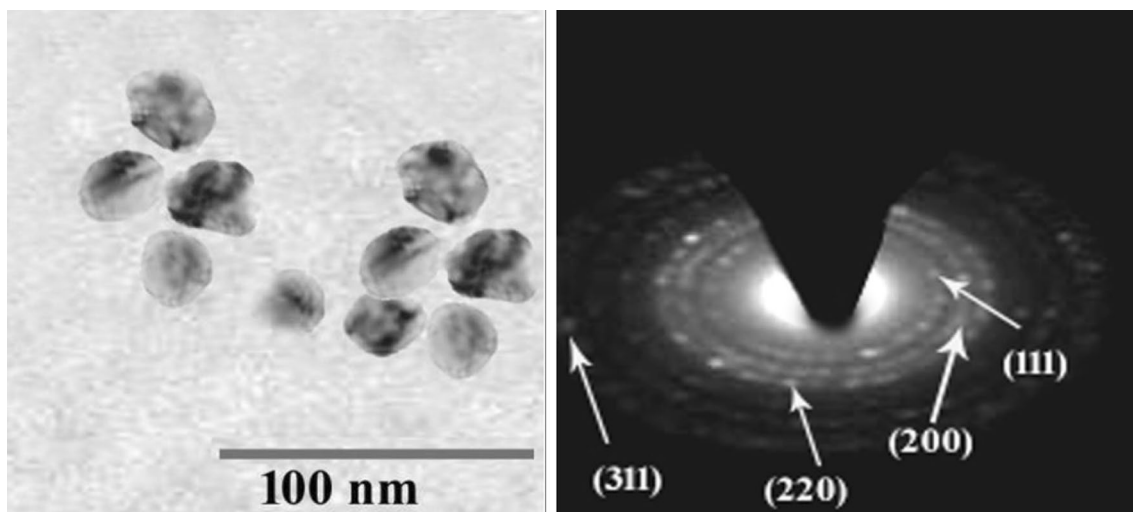


Fig. 3 Optimizing the morphology of Ag-NPs through TEM and bright field image

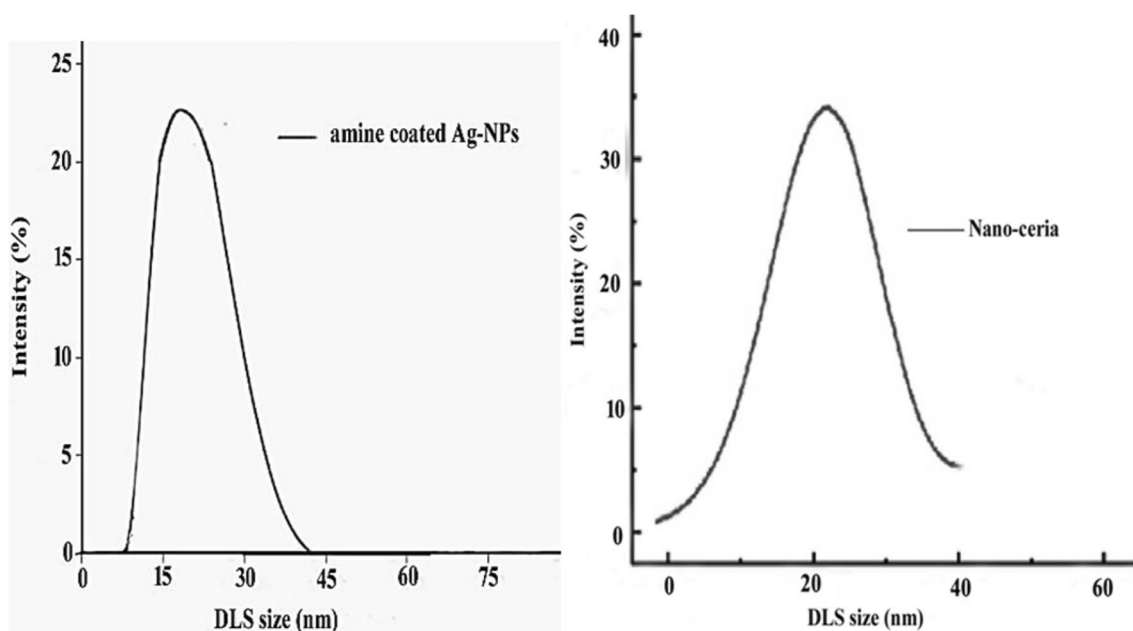


Fig. 4 Hydrodynamic size (DLS) of Ag-NPs and nano-ceria

peroxidation. Nano-ceria also restored activity of GSH. Non-significant difference of values between control and nano-ceria represents that nano-ceria alone posed no harmful effects and significant difference in values between Ag-NPs and Ag-NPs + nano-ceria showed antioxidant behavior of nano-ceria.

Ameliorative role in histopathology

Histological study revealed histo-protective effect of nano-ceria against Ag-NP-induced histopathology. Control and

nano-ceria-treated liver section showed no visible alterations. Ag-NPs' treatment caused severe inflammation and necrosis of hepatic parenchyma which led to congestion of blood in hepatic cells (black arrow). Furthermore, a yellow pigment was accumulated in hepatic tissue (bent arrow) which might be due to necrosis of affected cells (Fig. 9c). In case of nano-ceria pre-treatment, there was no congestion in hepatic tissue. Only vacuolization of cells (Black arrow) and accumulation of a yellow pigment (bent arrow) was recorded in hepatic tissue of nano-ceria pre-treated and Ag-NP-treated fish liver (Fig. 9d).

Fig. 5 FT-IR spectra of capped or associated molecules with Ag-NPs

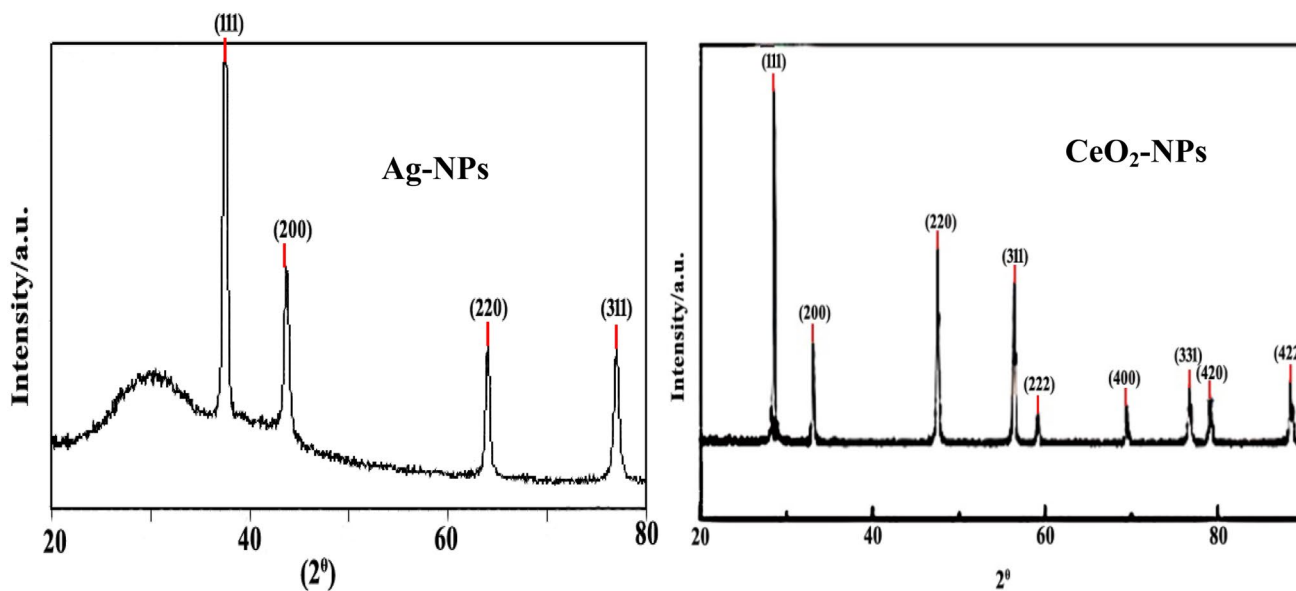
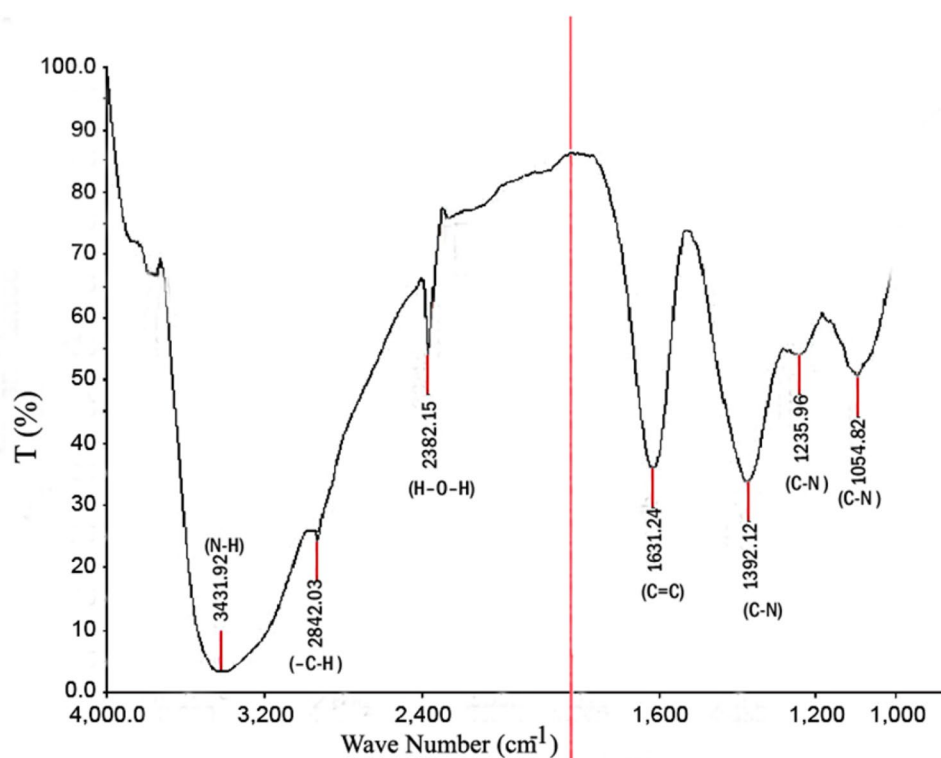


Fig. 6 XRD diffraction spectra of Ag-NPs CeO₂-NPs indicating nature of particles

In gill section, inflammation of cartilage filament, separation of epithelial layer (white arrow), deformation of filament cartilage (black arrow and bent), and swelling and collapsing of gill cartilage (bent arrow) were seen in Ag-NP-treated fish (Fig. 10c, d). Nano-ceria-treated fish

showed improvement in gill tissue. However, some alterations still occurred in the form of fused secondary lamellae (Black arrow), separated epithelial layer, cartilage filament inflammation (black bent arrow), and accumulation of macrophages (white arrow) in gill sections (Fig. 11a, b).

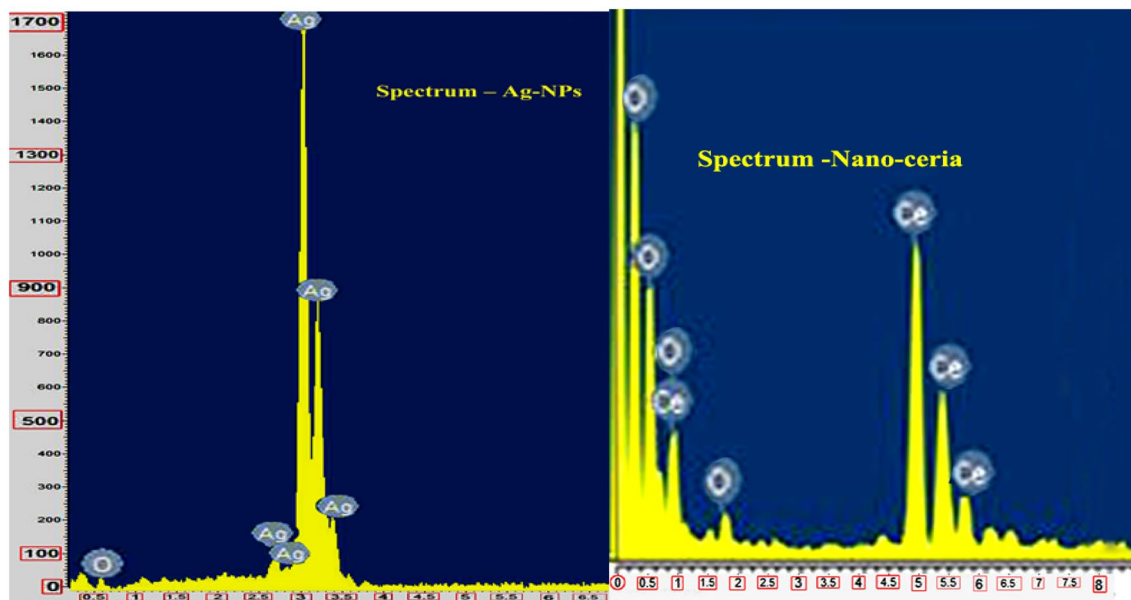


Fig. 7 EDX spectra recorded confirming the sample purity of both Ag-NPs and CeO_2 with out impurity peaks

Ameliorative role in genotoxicity

Genoprotective nature of nano-ceria was confirmed through this study. There was non-significant difference of micronuclei between control and nano-ceria. Ag-NPs increased incidence of micronuclei in erythrocytes and nano-ceria pre-treatment reduced it in Ag-NP-treated fish. Furthermore, Ag-NP also increased the number of comet cells and DNA tail migration. However, nano-ceria significantly reduced the comet cells and DNA tail in Ag-NP-treated fishes (Table 1).

Ameliorated role in hematology

It was revealed that PCV, MCV, and MCH values were significantly reduced in Ag-NP-treated fish. However, the value of MCHC was significantly increased compared to control (Table 2). Furthermore, Ag-NPs' treatment also reduced the level of HB, RBC, and increased WBCs and platelet count. Nano-ceria showed ameliorated role in Ag-NP-treated fish (Table 3). High value of ALT in Ag-NP-treated group represents onset of defensive mechanism of liver against Ag-NPs' toxicity. ALT activity was significantly decreased in nano-ceria pre-treated fish. Similar trend was in AST activity (Table 4).

Discussion

Ag-NPs attracted much attention in toxicological studies. These particles can damage brain cell, liver, and stem cells. Some studies revealed that Ag-NPs can initiate oxidative

stress (Asgar et al. 2016; Devi et al. 2015; Khan et al. 2017b) which is one of most important factor in toxicity (Khan et al. 2016b; Nel et al. 2006). This stress role in induction of apoptosis and damage to DNA (Simonian and Coyle 1996). This study recorded oxidative stress and changes in enzymatic level of gill and liver tissues in Ag-NP-treated *L. rohita*.

The nano-ceria is an evident antioxidant and ameliorative agent due to ability of redox potential (Heckert et al. 2008) and inactivation of ROS through scavenging (Spivak et al. 2012). It has Ce^{+3} and Ce^{+4} oxidation states with the ability to recycle. Oxygen valency is always created when oxidation state Ce^{+4} reduce to Ce^{+3} and forming Ce_2O_3 from CeO_2 (Baalousha et al. 2010). This ability assisted ceria as an important antioxidant at physiological pH (Perez et al. 2008). Non-ceria directly act as antioxidant and block production of ROS by inhibiting program cell death or it activates the ROS defence system and reduce the level of ROS (Ciofani et al. 2014; Schubert et al. 2006). Multi-enzymatic mimetic properties of nano-ceria are supported by recent literature in biological systems (Buettner 2011; Jiao et al. 2012; Pirmohamed et al. 2010).

SOD deals with oxyradicals in antioxidant system and dismutase superoxide radicals into H_2O_2 and molecular oxygen. It is very sensitive in nature and responds quickly to environmental pollutants, therefore, extensively used as indicator of environmental pollutants. Increase level of SOD indicates onset of oxidative stress. However, reduced level indicates overwhelmed of antioxidant system due to excess ROS production (Rocca et al. 2015; Van der Oost et al. 2003). In this study, level of SOD was increased in

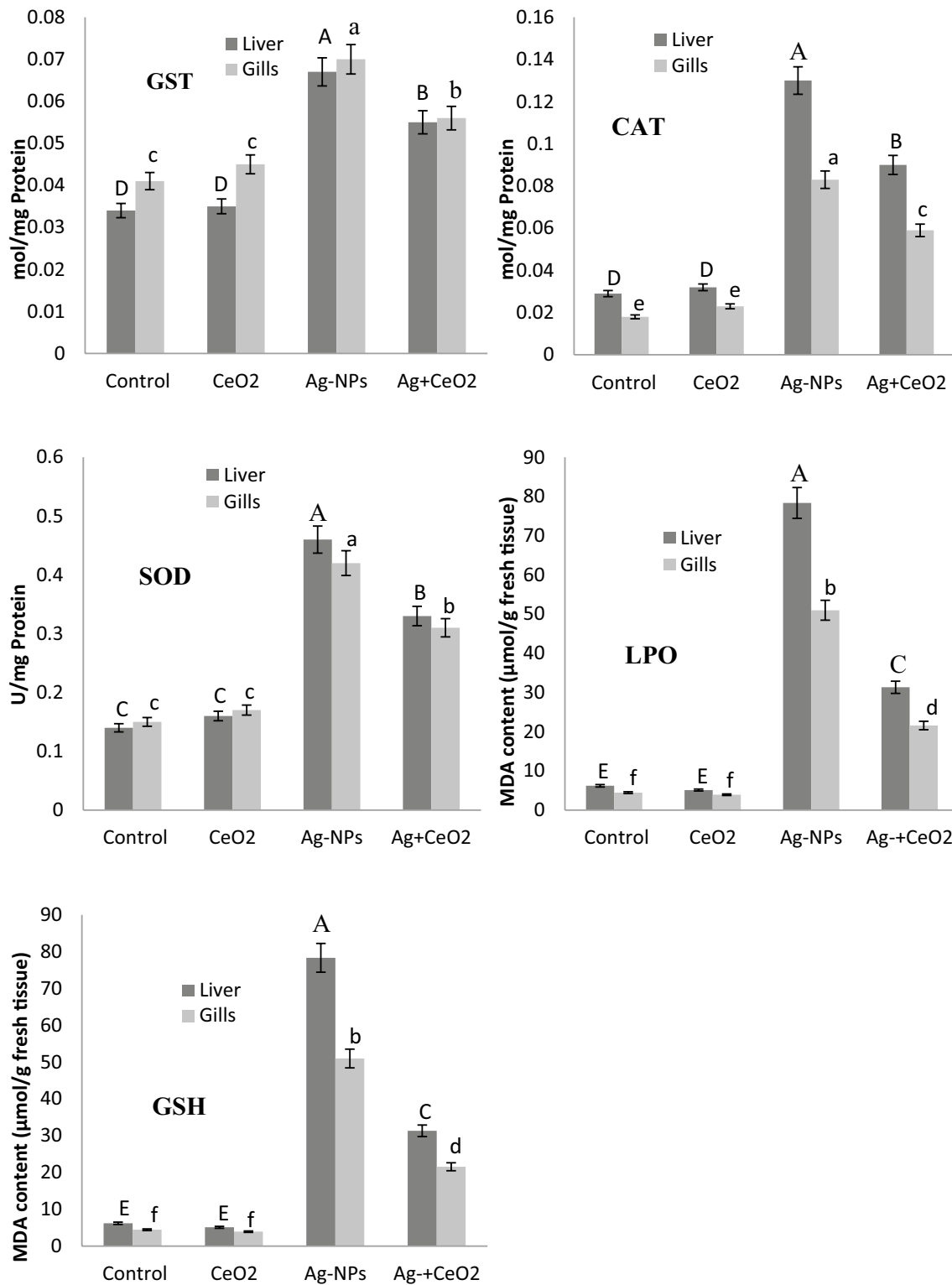


Fig. 8 Ameliorated role of Nano-ceria on activity of GST, CAT, SOD, LPO, and GSH in Ag-NP-treated *L. rohita* (small and caps alphabets represent significantly different value in liver and gill)

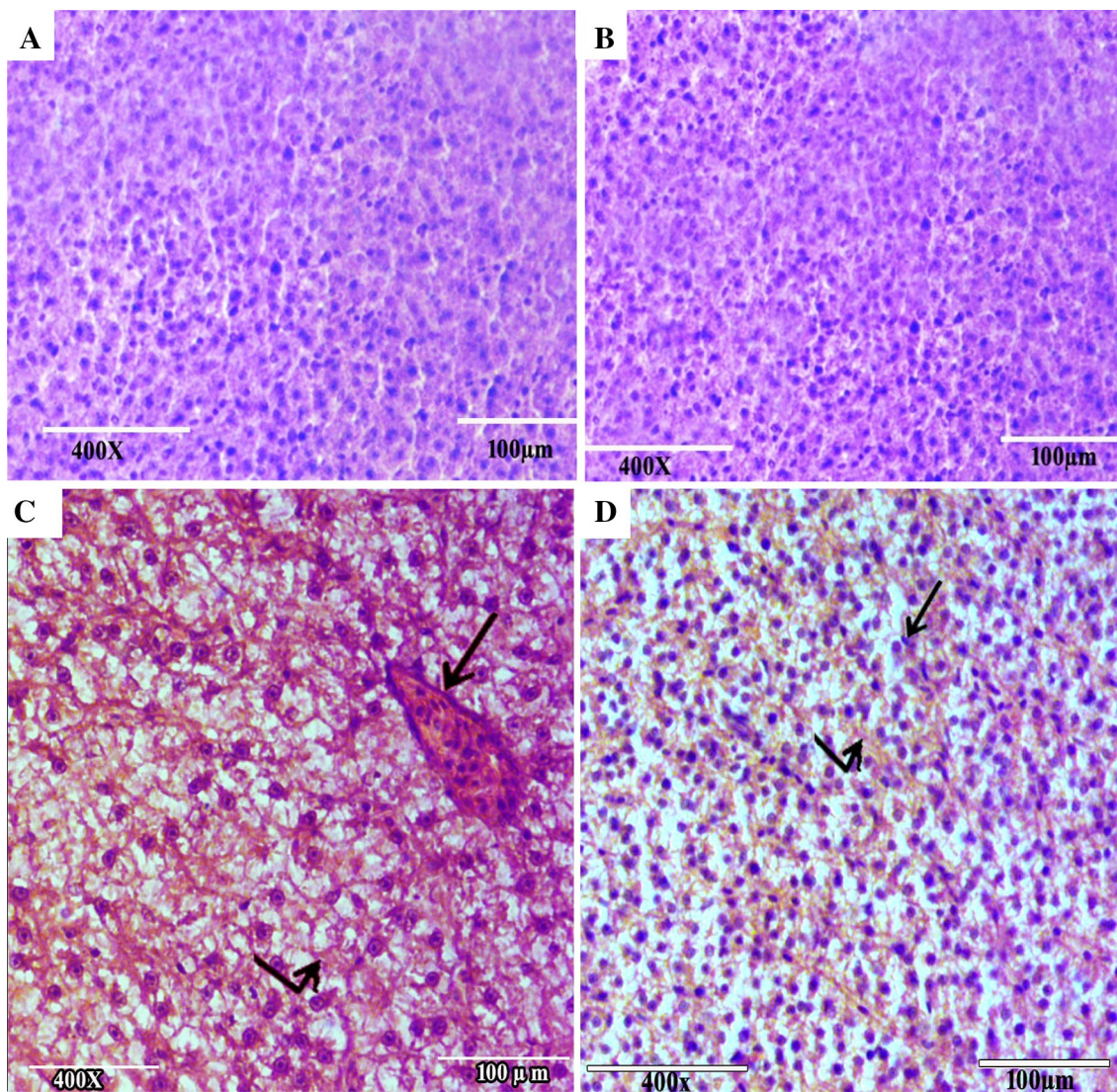


Fig. 9 Ameliorative effective of nano-ceria in histopathology of liver in Ag-NPs treated *L. rohita*. Control (a) and nano-ceria (b) treated liver section showing no visible alteration. The 30 mg l⁻¹ treatment of

Ag-NPs produced alterations (c) marked with arrows, and nano-ceria pre-treatment showed improvement of gill tissue (d)

Ag-NP-treated fishes. Pre-treatment of nano-ceria restored activity of SOD. Oxidation states Ce⁺³ and Ce⁺⁴ of nano-ceria are responsible to mimic SOD enzyme (Celardo et al. 2011). First ever evidence about this ability was found in study of Korsvik et al. (2007) who recorded SOD mimetic activity in relation of Ce⁺³ and Ce⁺⁴ ratio (Korsvik et al. 2007; Kuchma et al. 2010).

CAT is another enzyme of prime importance in antioxidant system due to ability of converting H₂O₂ free radical into molecular oxygen and water. Significantly increased level of CAT was indication of oxidative stress. Pre-treatment of nano-ceria restored CAT activity. Pirmohamed et al. (2010) conducted amplex red assay and recorded CAT like activity of Ce⁺⁴ in redox state. Furthermore, H₂O₂ was generated when nano-ceria acted as SOD. Luckily,

nano-ceria acted as both CAT and SOD. H₂O₂ produces when body enters into dismutation process initiated by nano-ceria. This ability makes nano-ceria a strong antioxidant. Ce⁺³ and Ce⁺⁴ ratio, particles size, and pH are responsible for enzymatic properties of nano-ceria (Alili et al. 2011; Xue et al. 2011). Buffer species can also affect enzymatic properties of nano-ceria. The phosphate buffer can reduce SOD-mimetic activity, but increase CAT-mimetic activities (Alili et al. 2011).

GST is cytosolic in nature and has ability to catalyse conjugation of electrophilic substance with GSH. As GST detoxify exogenous substances, it is major target of toxic substances (Cheeke 1988). Ag-NPs' treatment significantly changed the activity of GST compared to control and nano-ceria-treated group. This might be due to excess production

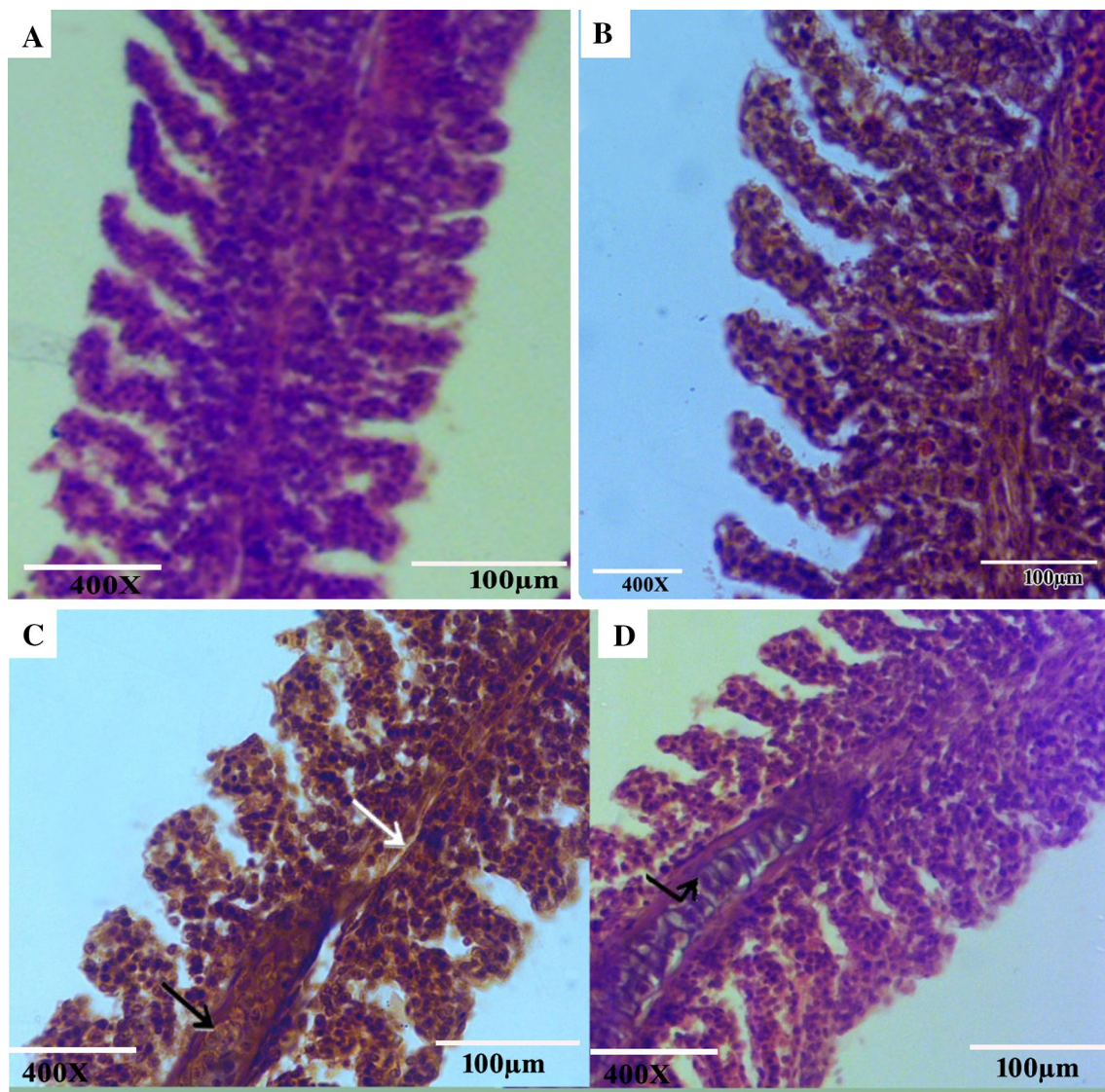


Fig. 10 Control (a) and nano-ceria (b) treated gill section show no markable alteration, where 30 mg l^{-1} treatment (c, d) shows tissue alterations represented by arrows

of ROS (Khan et al. 2017b; Liu et al. 2014). Treatment of nano-ceria increased activity of GST compared to Ag-NP-treated group. Amin et al. (2011) showed similar increase in activity of GST with cerium oxide in monocrotaline-treated Sprague–dawley rats. Furthermore, level of MDA was increased in treated group. This increased level indicated that Ag-NPs induced oxyradicals' production in liver and gills. Treatment of nano-ceria reduced lipid peroxidation of Ag-NP-treated group. It actually mimics the peroxidases activity and reduces the peroxidation of gill and liver similar to the Fenton reactions (Gao et al. 2007).

Retention of GSH is very essential for cellular homeostasis. Nano-ceria has GSH mimetic activity and could be explained on basis of scavenging of free radicals and direct exchange of electrons between ROS and electrons present on

surface area of particles (Hochella et al. 2008). This capacity enables nano-ceria as highly reactive material in free radical scavenging and decreases the level of ROS creation and sustained GSH contents to homeostasis in biological systems (Niu et al. 2011).

In addition, nano-ceria also showed histo-protective role against Ag-NP-induced alterations in liver and gill tissues. Ag-NPs' treatment caused severe inflammation and necrosis in hepatic parenchyma which led to congestion of blood in hepatic tissues. Furthermore, accumulation of yellow pigment in hepatic tissue was also seen due to necrosis of affected cells. In nano-ceria pre-treatment, there was no congestion in hepatic tissue. The vacuolization of cells and necrosis in some area was also occurred, but the hepatic tissue showed improvement against the Ag-NP-induced

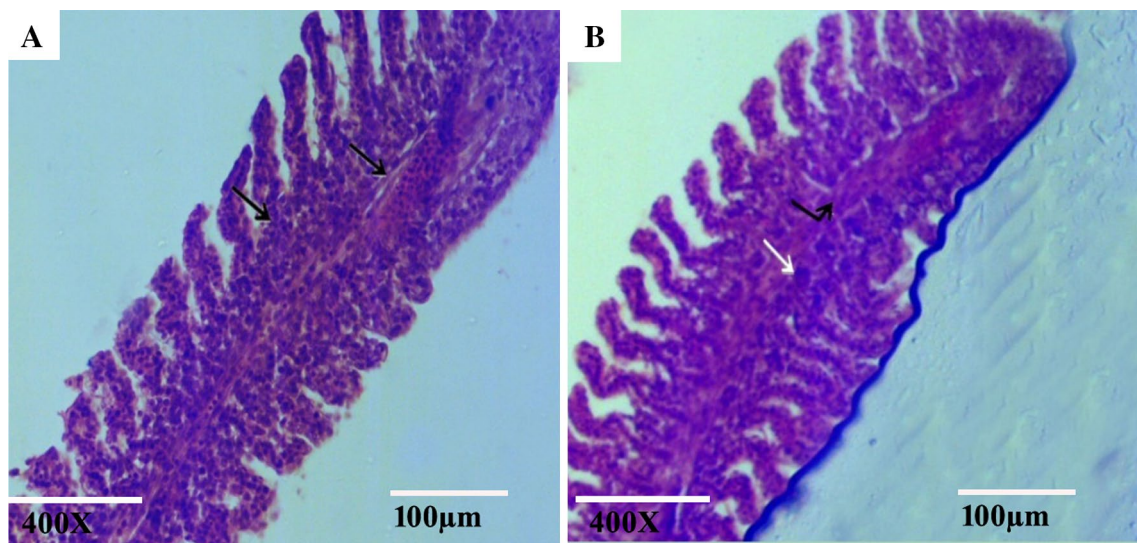


Fig. 11 Ameliorative effective of nano-ceria in histopathology of gill in Ag-NP-treated *L. rohita*. Pre-treatment of nano-ceria (a, b) showing improvement in the gill structure in Ag-NPs treated *L. rohita*

Table 1 Ameliorated effect of nano-ceria on frequency of micronuclei and comet in Ag-NP-treated fish

Treatments	MN (%)	Comet (%)	DNA tail migration (µm)
Control	0.13 ± 0.07 ^C	4.35 ± 2.54 ^C	5.71 ± 2.68 ^D
Nano-ceria	0.36 ± 0.13 ^C	4.33 ± 2.51 ^C	5.01 ± 2.11 ^D
Ag-NPs	6.13 ± 0.55 ^A	17.33 ± 2.08 ^{AB}	33.0 ± 5.30 ^{AB}
Ag-NPs + ceria	4.63 ± 0.33 ^B	12.65 ± 6.66 ^A	26.0 ± 3.46 ^A

Frequency and tail migration was recorded per 100 cells
 Values with different letters in the same column significantly different 5% level

damage in the liver. Similar histo-protective effect was found in case of gill tissue. Tarnuzzer et al. (2005) demonstrated protective effect of nano-ceria against radiation-based cell death in human breast cell line. Furthermore, Niu et al. (2007) showed inhibitory effect of nano-ceria on development of cardiac dysfunction by removal of oxidative or ER stress and inhibition of inflammation.

Detection and level of aminotransferases in blood show damage to liver. These enzymes normally reside in hepatic cells and, however, spill into blood upon damage. Most common aminotransferases are ALT and AST which level increase in blood due to drug toxicity and cell death (Gontijo

Table 2 Ameliorated role of nano-ceria on hematological parameters

Treatments	PCV (%)	MCV (fl)	MCH (Pg)	MCHC (g/dl)
Control	22.96 ± 4.60 ^B	120.57 ± 16.65 ^B	63.54 ± 5.38 ^B	53.03 ± 4.23 ^C
Nano-ceria	29.26 ± 3.58 ^A	147.60 ± 15.50 ^A	74.47 ± 6.57 ^A	50.52 ± 6.91 ^C
Ag-NPs	10.20 ± 1.83 ^D	69.70 ± 12.13 ^D	47.62 ± 8.13 ^C	68.36 ± 4.44 ^A
Ag-NPs + ceria	15.36 ± 1.97 ^C	94.17 ± 11.02 ^C	57.74 ± 6.29 ^B	61.37 ± 1.51 ^B

Values with different letters in the same column significantly different 5% level

Table 3 Ameliorated effect of nano-ceria on the Hb, RBC, WBCs, and platelets counts of Ag-NP-treated fish

Treatments	Hb (g/dl)	RBC count (× 10 ⁶ /µl)	WBC counts (10 ³ /µl)	Platelets (10 ³ /µl)
Control	12.06 ± 1.67 ^A	1.89 ± 0.15 ^B	175.94 ± 4.74 ^C	455.79 ± 11.67 ^C
Nano-ceria	14.76 ± 1.55 ^A	1.98 ± 0.04 ^A	162.74 ± 3.88 ^D	486.87 ± 8.94 ^D
Ag-NPs	6.97 ± 1.21 ^C	1.46 ± 0.031 ^D	213.59 ± 4.30 ^A	677.4 ± 25.3 ^A
Ag-NPs + ceria	9.42 ± 1.10 ^B	1.63 ± 0.04 ^C	197.48 ± 4.44 ^B	516.26 ± 5.89 ^B

Values with different letters in the same column significantly different 5% level

Table 4 Ameliorated effect of nano-ceria on level of AST and ALT in blood

Treatments	ALT (IU/l)	AST (IU/l)
Control	8.90 ± 1.97 ^C	26.22 ± 0.96 ^D
Nano-ceria	11.86 ± 1.22 ^C	38.88 ± 2.07 ^{BC}
Ag-NPs	45.12 ± 3.77 ^A	54.35 ± 2.57 ^A
Ag-NPs + ceria	21.39 ± 2.55 ^B	42.45 ± 1.95 ^B

Values with different letters in the same column significantly different 5% level

et al. 2003). Various chemical, biological, and physiological factors alter the activities of these enzymes producing disturbance in Kerb's cycle. Diminished level of Kerb's cycle is also responsible for reduce level of intermediates which is then compensated by ALT and AST providing α ketoglutarate. This phenomenon increases blood serum level of both enzymes. In this study, a significantly increase in level of ALT and AST was recorded in Ag-NP-treated fish most probably due to damage in hepatic cells and inflammatory responses. This damage is initiated due to irritation of liver oxidant system which increases the level of free radicals (Wijnhoven et al. 2009). These radicals release ALT from hepatic cells into blood streams (Braydich-Stolle et al. 2005). The hepatic cells continuously detoxify toxic compounds. Any change in structure and metabolism changes animal physiological conditions. The present study confirms ameliorated role of nano-ceria in liver damage of Ag-NP-treated fish. El Shaer et al. (2017) found similar ameliorative role against isoproterenol-induced toxicity in male Wistar rats. Treatment of nano-ceria showed promising amelioration and decreased the level of ALT compared to Captopril drug. Like ALT, AST is also related to liver damage and its level increases in blood serum in case of toxicity to liver. However, AST is also found in skeletal muscle, kidney, heart muscle, red blood cells, and brain (Gaze 2007). In the present study, Ag-NPs increased the level of AST in treated fish. However, pre-treatment of nano-ceria significantly reduced the level. Bölükbaşı et al. (2016) used different doses to see the effect of dietary cerium oxide on egg lying performance of hen and found significant increase in egg quality parameters. They further found decreased level of AST in dose-dependent manner.

Ag-NPs produce significant genotoxicity in treated animals due to inflammation and oxidative stress (Asharani et al. 2009). Review of the literature advocated that these particles produce DNA damage in the form of double strands breaks, fusion, and fragmentation (Asharani et al. 2008). This increased frequency of micronuclei, comet, and DNA tail migration. Nano-ceria pre-treatment significantly reduced frequency of micronuclei, comet, and DNA tail migration in Ag-NP-treated fish (Khan et al. 2017a).

Rubio et al. (2016) found anti-genotoxic effect of CeO₂-NPs in human epithelial lung cell line against oxidative stress inducing agent. Zhang et al. (2009) found inhibitory effect of cerium nitrate on tumour cell line. The 0.08 mm/l cerium nitrate shows less or no toxicity to normal cells, but inhibits the proliferation and growth of tumour cell line.

In Ag-NP-treated group, animal becomes anaemic and level of hemoglobin was decreased (Khan et al. 2017a). Furthermore, values of MCHC and MCH were different from control. Significantly, low level of these parameters was consequences of reduction in RBCs' counts and haematocrit due to bleeding, deformation, damage to RBCs (Witeska and Kościuk 2003), haemolysis, or decrease in generation of RBCs (Di Giulio and Hinton 2008; Witeska and Kościuk 2003). Many researchers recorded decrease in level of MCH and MCHC due to exposure of metals in fresh water fishes (Vutukuru 2005). In general, fluctuations in studied blood parameters were due to defensive mechanism of body via erythropoiesis against toxicity (Vinodhini and Narayanan 2008). Decrease in level of MCHC and MCH was indication of toxicity of Ag-NPs in *L. rohita*. Variations in number of WBCs count was also recorded due to non-specific reaction of immune system against stress conditions (Stoskopf 1993; Vandebriel et al. 2014). Decrease in number of WBCs was suppression of immune response due to malfunctioning of hematopoietic system of treated fish (Adams 2002). In this study, WBCs count was increased due to non-specific immune system in response of low dose of particles. Pre-treatment of nano-ceria reduced alterations in hematological parameters due to reduction of oxidative stress. Adua et al. (2015) found dose-dependent increase in growth rate in black rabbits. Dietary 50–100 mg cerium oxide improved weight gain without any deleterious effect on blood parameters and blood serum.

Conclusions

Ag-NPs induce oxidative stress, hematological, and tissue alteration in *L. rohita*. The 28 day treatment produced fluctuations in antioxidant enzymes including GST, GSH, CAT, and SOD. These particles also produced severe inflammation of cartilage filament, separation of epithelial layer, deformation of filament cartilage, and swelling and collapsing of gill cartilage in treated fish. In liver section, Ag-NPs' treatment caused severe inflammation and necrosis of hepatic parenchyma which led to congestion of blood in hepatic cells. Furthermore, a yellow pigment was accumulated in hepatic tissue which might be due to necrosis of affected effected cells. Ag-NPs treatment also produces fluctuations in blood parameters like reduction in PCV, MCV, MCH, Hb, and RBCs' counts and increase in MCHC, WBC counts, and platelets' counts. However, nano-ceria has ameliorative role

due to multiple enzymes mimicking ability in response to higher oxidant level. This study confirmed nano-ceria as a novel antioxidant against oxidative stress, histological alterations and aided in recovery of antioxidant system.

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Compliance with ethical standards

Conflict of interest The authors have declared that they have no conflict of interest in any form.

Ethical approval All adopted procedures and use of fishes in this study were in agreement with ethical standards in research of university, Government College University Faisalabad, Pakistan.

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