



# In vivo toxicity evaluation of biologically synthesized silver nanoparticles and gold nanoparticles on adult zebrafish: a comparative study

Rajan Ramachandran<sup>1</sup> · Chandran Krishnaraj<sup>2,3</sup> · V. K. Abhay Kumar<sup>3</sup> · Stacey L. Harper<sup>4</sup> · Thangavelu P. Kalaiichelvan<sup>1</sup> · Soon-Il Yun<sup>2</sup>

Received: 29 June 2018 / Accepted: 24 September 2018 / Published online: 5 October 2018  
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

## Abstract

In this study, toxicity of biologically synthesized silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) was compared using zebrafish as a model organism. At 96 h, LC<sub>50</sub> of AgNPs and AuNPs was found to be 24.5 µg/L and 41 mg/L, respectively. Following the LC<sub>50</sub> determination, half of the LC<sub>50</sub> of AgNPs (12.25 µg/L) and AuNPs (20.5 mg/L) was exposed to adult zebrafishes for 14 days. Morphological changes, liver marker enzymes, reactive oxygen species (ROS) generation, genotoxic effects and mRNA expression levels of oxidative stress and innate immune response related genes were studied using nanoparticle treated gill, liver and blood cells. In this study, AgNP-treated gill and liver tissues showed a number of morphological changes such as cell membrane damage, irregular cell outlines, pyknotic nuclei and complete disruption of gill and liver cells; on the contrary, AuNPs treated liver tissues alone showed such changes. The levels of liver marker enzymes such as alanine aminotransferase and aspartate aminotransferase were increased after AgNPs treatment when compared to AuNPs treatment. AgNP-treated liver cells showed higher levels of ROS generation than the control; on the other hand, AuNPs treatment exhibited lower levels of ROS generation than the control. Interestingly, AgNP-treated blood cells showed micronuclei formation and nuclear abnormalities, while AuNPs treatment did not show such effects. Based on these observations, it is clear that AgNPs may cause oxidative stress and immunotoxicity to adult zebrafish than the AuNPs. However, these results clearly reveal the significance of relatively safe and less toxic bionanomaterials for possible biomedical applications.

**Keywords** Toxicity · Biologically synthesized nanoparticles · Zebrafish · Liver marker enzymes · Reactive oxygen species · qPCR

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s13205-018-1457-y>) contains supplementary material, which is available to authorized users.

✉ Soon-Il Yun  
siyun@jbnu.ac.kr

- <sup>1</sup> Centre for Advanced Studies in Botany, School of Life Sciences, University of Madras, Guindy Campus, Chennai, Tamil Nadu 600 025, India
- <sup>2</sup> Department of Food Science and Technology, College of Agriculture and Life Sciences, Chonbuk National University, Jeonju 561-756, Republic of Korea
- <sup>3</sup> R&D Centre, Eureka Forbes Ltd, Kudlu, Bangalore 560 068, India
- <sup>4</sup> Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR 97331, USA

## Introduction

As of today, 1827 nano based consumer products have been available in the global market; of which, 442 products represented silver nanoparticles (AgNPs) and 25 products were based on gold nanoparticles (AuNPs) (Project on Emerging Nanotechnologies 2018). This data shows an increase of almost 200 products compared to the previous access made by us (Krishnaraj et al. 2014). Increasing use of these nanoparticles (NPs) in consumer products has resulted in leaching them into the environment and their interaction with ecological populations has led to pose a threat to the environment (Nowack and Bucheli 2007; Impellitteri et al. 2009). Owing to their smaller size, NPs could penetrate biological/ecological systems through novel ways (Haynes 2010). Using zebrafish as a model system, several research groups have recently evaluated the toxicity of metal NPs in

general and AgNPs in particular (Asharani et al. 2008, 2011; Choi et al. 2010; Bilberg et al. 2012; Aerle et al. 2013; Massarsky et al. 2013; Olasagasti et al. 2014; Rajan et al. 2017). Mounting evidences have claimed that chemically synthesized AgNPs were more toxic to adult zebrafish or embryo (Asharani et al. 2008, 2011; Choi et al. 2010; Bilberg et al. 2012; Aerle et al. 2013; Massarsky et al. 2013).

Cytotoxic and genotoxic effects of glycolipid-reduced and -capped AgNPs and AuNPs were tested on HepG2 cells. Interestingly, AuNPs were found to be more cytocompatible compared to AgNPs of similar concentrations, whereas AgNPs caused more DNA damage than the same concentrations of AuNPs (Singh et al. 2010). Curcumin conjugated AgNPs showed good anticancer activity against human epidermoid carcinoma cell line at 60 µg/mL concentration (Shah et al. 2018). Using keratinocyte and lung cell models, Srivastava et al. (2012) have demonstrated that exposure to AgNPs inhibited selenoprotein synthesis and the activity of thioredoxin reductase (TrxR), a key selenoenzyme. Interestingly, AgNPs and AuNPs synthesized via biological routes (microbes and plant extract mediated methods) have been shown to have better antimicrobial, antioxidant and anticancer activities than the chemically synthesized ones (Balakumaran et al. 2015; Rajan et al. 2015). These biological materials were found to be more biocompatible and exhibited least toxic effects (Girilal et al. 2015; Rajan et al. 2015). However, prior to their judicial utilization into biotechnological applications including biomedicine, it is, therefore, imperative to assess the ecofriendly status and safety level of these NPs at least to some extent (Oberdörster et al. 2005; Nel et al. 2006; Sarkar et al. 2014; Rajan et al. 2015).

To the best of our knowledge, very few reports have evaluated the toxicity of plant extract derived AgNPs on adult zebrafish (Sarkar et al. 2014; Krishnaraj et al. 2016). There was no report exist on the toxicity of plant extract derived AuNPs on adult zebrafish. In an interesting study, Sarkar et al. (2014) have compared the toxicity of *Psidium guajava* leaves extract synthesized AgNPs with that of chemically synthesized AgNPs. In their study, 96 h LC<sub>50</sub> for guava extract derived AgNPs was 400 µg/L, while for chemically synthesized AgNPs, it was 80 µg/L. Thus, the chemically synthesized AgNPs were found to have five times more toxic effects than the biologically synthesized AgNPs (Sarkar et al. 2014). In another study, 96 h LC<sub>50</sub> for *Malva crispa* leaves extract synthesized AgNPs was found to be 142.2 µg/L (Krishnaraj et al. 2016). When half of the LC<sub>50</sub> of AgNPs, i.e., 71.1 µg/L was exposed to adult zebrafish, both gill and liver tissues showed significant morphological changes such as cell membrane damage, irregular cell outlines and pyknotic nuclei. In addition, genotoxic effects and elevated levels of liver marker enzymes (ALT and AST) were also observed. Thus, the plant extract derived AgNPs

led to oxidative stress and immunotoxicity in adult zebrafish (Krishnaraj et al. 2016). In our earlier study, *Acalypha indica* leaves extract synthesized AgNPs and AuNPs have shown excellent anticancer activities against MDA-MB-231, human breast cancer cell line (Krishnaraj et al. 2014). Therefore, in this study, we aimed to test whether these NPs could induce toxicity on adult zebrafish.

## Materials and methods

### Chemicals

All chemicals used in this study were reagent grade or higher purchased from Sigma-Aldrich, South Korea.

### Biological synthesis and characterization of AgNPs and AuNPs

In our earlier study, AgNPs and AuNPs were synthesized using the aqueous leaves extract of *A. indica* (Krishnaraj et al. 2012, 2014). Briefly, 10 g of *A. indica* leaves were surface cleaned with running tap water followed by distilled water. Then the leaves were boiled with 100 mL of distilled water at 65 °C for 5 min and the resulting extract was filtered. Twelve millilitres of this extract was mixed with 1 mM AgNO<sub>3</sub> and 1 mM HAuCl<sub>4</sub> solution separately and the mixture was incubated in dark at 37 °C under static condition. *A. indica* leaves extract, silver nitrate and gold chloride solutions were run as the controls throughout the experiment. In addition, the reaction parameters such as leaves extract concentration, metal ions concentration, pH and time were optimized to synthesize monodispersed nanoparticles with uniform size and shape. The color change was observed for 24 h and the synthesized nanoparticles were characterized using UV–Vis spectrophotometer, transmission electron microscopy (TEM) and X-ray diffraction (XRD). The first evidence for nanoparticle synthesis is color change; brown and pinkish violet color formed in the optimized medium indicated the synthesis of AgNPs and AuNPs, respectively. Both these nanoparticles were formed within 30 min under optimized reaction conditions. The absorption peak recorded at 420 and 540 nm in the UV–Vis spectra further confirmed the formation of AgNPs and AuNPs, respectively. Using *Azadirachta indica* leaf broth, Shankar et al. (2004a) have rapidly synthesized metallic AgNPs, AuNPs and bimetallic gold core–silver shell nanoparticles. TEM images showed spherical shaped nanoparticles with less than 30 nm in size. Biogenic gold nanotriangles with a size of 200–500 nm were synthesized using lemongrass extract (Shankar et al. 2004b). XRD spectra showed face-centred cubic (fcc) silver and gold and the data were matched well with the respective JCPDS file number (Krishnaraj et al. 2012, 2014).

## Experimental setup

Adult zebrafishes with an average weight of  $0.24 \pm 0.02$  g and average length of  $30 \pm 0.2$  mm were selected and fed daily with the commercially purchased artemia. The fishes were maintained in an aquarium at a temperature of  $28 \pm 1$  °C with 14 h:10 h light–dark cycle for 3 weeks. Later, the fishes were removed from the recirculating groups, placed in static groups and fasted 24 h prior to experimentation. The water pH and dissolved oxygen (DO) content were maintained at 6.8–7.3 and 5.4 mg/L, respectively.

## Toxicity test

For achieving lethal concentration ( $LC_{50}$ ) of NPs, we followed the guidelines of Organisation for Economic Cooperation and Development (OECD) for testing the chemicals (OECD 1992). Based on the inductively coupled plasma mass spectrometry (ICP-MS) analysis, different concentration of AgNPs (15.5, 18.6, 21.7, 24.8, 27.9 and 31 µg/L) and AuNPs (9.7, 19.4, 29.1, 38.8, 43.65, 48.5 and 58.2 mg/L) suspensions were prepared separately and exposed to the treatment group containing seven fishes for 96 h in a 2 L tank containing 1 L of test solution. In addition, a group of seven fishes was also maintained as the control. Each treatment was run in triplicates under the same conditions with a natural light/dark cycle. The fishes were not fed 24 h prior to or during the experiment to maintain constant exposure concentrations. Similarly, the control group was also starved. The number of dead fishes from each treatment group was recorded at every 12 h and they were removed from the treatment group immediately to avoid contamination. At the end of experiment, the remaining survived fishes were anaesthetized, dissected and observed for possible changes.

In addition to acute toxicity study, sub-acute toxicity study was also carried out. The fishes were exposed to half of the  $LC_{50}$  of AgNPs (12.25 µg/L) and AuNPs (20.5 mg/L) for 14 days in a 2 L semi-static tank containing 1 L of test solution. To keep up the constant concentration of NPs, the test solution was refreshed once in every 24 h.

## Intracellular changes and localization of nanoparticles

After 14 days of sub-acute toxicity study, the fishes were anaesthetized. The gill and liver tissues of control, AgNPs and AuNPs treated zebrafishes were subsequently dissected following the standard procedure (Krishnaraj et al. 2016). Briefly, the gill and liver tissues were fixed separately in modified Karnovsky's fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2), washed thrice in cacodylate buffer at 4 °C for 10 min and post fixed in 1% osmium tetroxide at 4 °C for 90 min.

Then the tissues were washed twice at room temperature using distilled water and enbloc staining was done using 0.5% uranyl acetate and left overnight. Furthermore, the tissues were dehydrated using graded ethanol series 30, 40, 50, 60, 70, 80, 90% and three changes of 100% ethanol for 10 min each. The tissues were embedded in propylene oxide: Embed 812 resin mixture and allowed for polymerization at 60 °C for 48 h. Then the tissues were sectioned using ultramicrotome (Leica Mikrosysteme GmbH, Austria) and were stained with 2% uranyl acetate for 7 min followed by Reynolds lead citrate for 7 min and were examined under biological transmission electron microscopy (Bio-TEM) (Hitachi H7650, Japan).

## Liver marker enzyme assays

Aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) levels from the liver tissues of control, AgNPs and AuNPs treated zebrafishes were assayed using standard colorimetric kit following the manufacturer's protocol (Biovision, Mountain View, CA) (Marcolin et al. 2012).

## Measurement of reactive oxygen species

To understand AgNPs and AuNPs mediated toxic mechanisms, the generation of intracellular reactive oxygen species (ROS) was monitored using the fluorescent marker 2',7' dichlorodihydrofluorescein diacetate (H2DCF-DA-D6883, Sigma-Aldrich, Korea) (Hu et al. 2011). The liver tissues of AgNPs and AuNPs treated zebrafishes were dissected separately and homogenized in 2 mL of phosphate buffered saline (PBS) at 25 °C, centrifuged at 400 rpm for 10 min at 4 °C and the supernatant was removed. Then the pellet was resuspended in 1 mL of PBS at 4 °C and cell counting was done using haemocytometer with the addition of trypan blue. Furthermore, the known quantities of cells ( $1 \times 10^6$  cells/mL) were incubated in dark for 20 min with the addition of 10 µM H2DCF-DA at 28.5 °C. After the incubation, the cells were washed twice with PBS and were centrifuged at 500 rpm for 3–4 min at 25 °C. Finally the cells were carefully transferred into fluorescence-activated cell sorting (FACS) tubes and were analyzed using FACScan flow cytometry (Becton Dickinson, San Jose, CA). At least 10,000 events were collected and the analysis was performed using Cell Quest Software (Becton Dickinson).

## Micronuclei and nuclear abnormalities test

Peripheral blood samples of 3 µL each from control, AgNPs and AuNPs treated zebrafishes were obtained by caudal vein puncture using 0.2% EDTA as anticoagulant in syringe and pooled together ( $n = 3$ ). The blood was immediately smeared

on clean grease free microscope slides, air dried and fixed in methanol for 5 min. The slides were gently rinsed in running tap water for 1 min followed by immersing in periodic acid solution for 5 min at room temperature. Then the slides were rinsed using distilled water and immersed in Schiff's reagent for 15 min at room temperature and were gently washed using running tap water for 5 min. Finally counter staining was done using hematoxylin solution for 90 s and rinsed in running tap water for 30 s. Then the slides were allowed to air dry and were examined under light microscopy using mineral oil (100×) (Hotchkiss 1948; Kiernan 1999).

### Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Control, AgNPs and AuNPs treated liver tissues were homogenized in TRIzol reagent and total RNA was extracted using RNase minikit (Qiagen) following the manufacturer's protocol. The RNA samples were quantitated using a NanoDrop spectrophotometer (Epoch Microplate spectrophotometer). One hundred nanograms of total RNA was used for cDNA synthesis for all the primers (Table S1) using Topscript™ One-step RT-PCR kit according to the manufacturer's instructions (Enzymomics) and the reaction mixture was applied in RT-PCR (Corbett research, Korea). RT-qPCR was performed with SYBR Green in C1000 Touch™ thermal cycler (CFX 96, BioRad) (Krishnaraj et al. 2016).  $\beta$ -actin (ACTB) was used as the internal control to normalize the amount of cDNA added to each reaction ( $\Delta C_T$ ) and the mean  $\Delta C_T$  of control samples was used as the calibrator to calculate the  $\Delta\Delta C_T$ . Quantitation of each transcript was carried out using the comparative  $C_T$  method. In this method, the relative quantity of target mRNA, normalized to the internal control and relative to the calibrator, is equal to  $2\Delta\Delta C_T$  (Schmittgen and Livak 2008). Each experiment was carried out in triplicate.

### Statistical analysis

All experiments were performed in triplicates and the data were presented as mean  $\pm$  standard deviation (SD) of three different experiments.

## Results and discussion

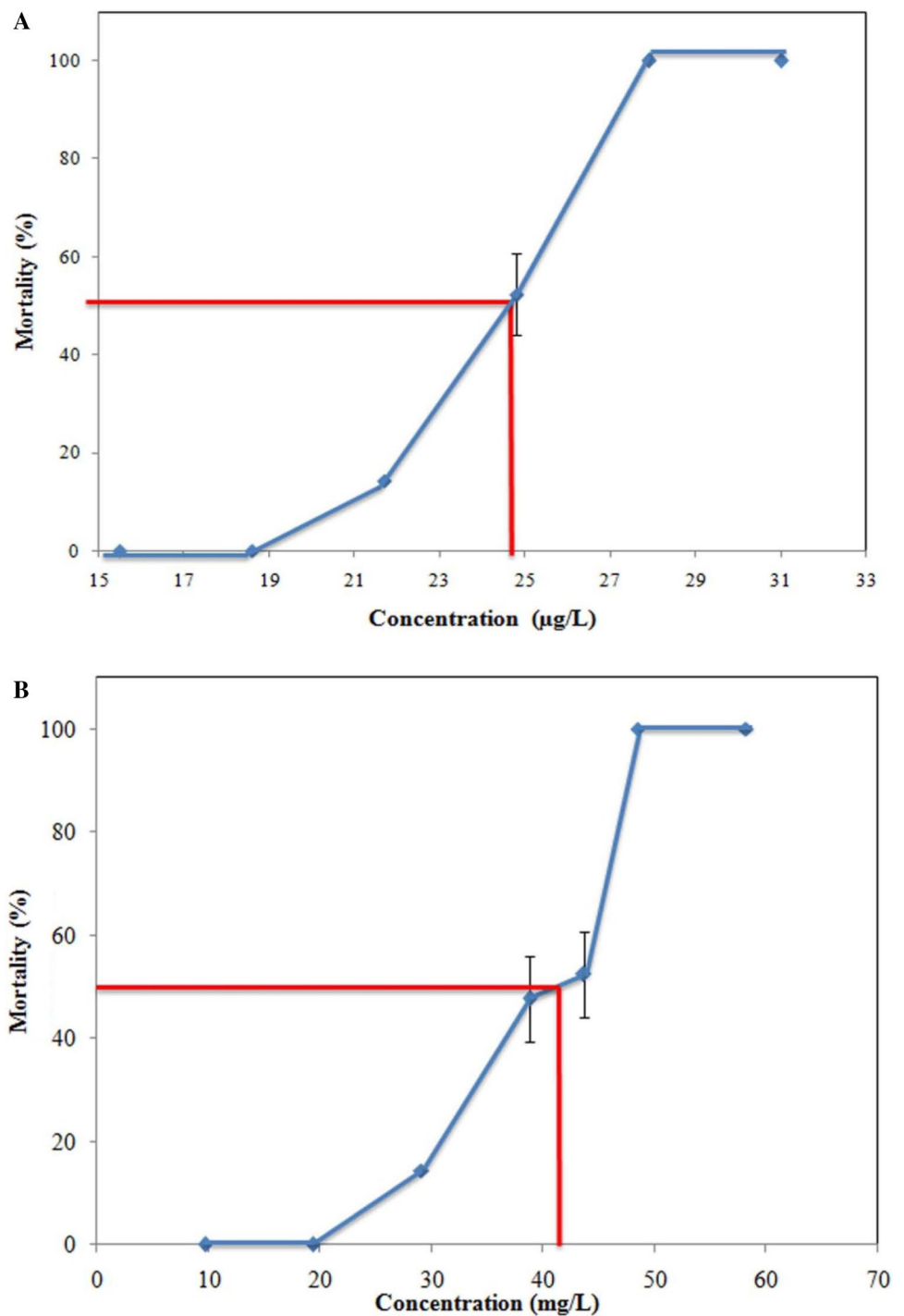
### Toxicity test

Both AgNPs and AuNPs were found to be acutely toxic to adult zebrafishes at the  $LC_{50}$  of 24.5  $\mu\text{g/L}$  and 41 mg/L, respectively, at 96 h exposure (Fig. 1a, b). In this study, the toxicity was increased with increasing concentrations of NPs. No fish mortality was observed

in the control and lower concentrations of AgNPs (15.5 and 18.6  $\mu\text{g/L}$ ) and AuNPs (9.7 and 19.4 mg/L) treated groups; the fishes seemed to be healthy throughout the treatment period. However, at the maximum tested concentration, AgNPs (31  $\mu\text{g/L}$ ) caused 100% mortality within 12 h, while AuNPs (58.2 mg/L) showed similar phenomenon within 24 h. At 96 h, 100% mortality was recorded at 27.9  $\mu\text{g/L}$  of AgNPs and 48.5 mg/L of AuNPs treated groups (Fig. 1a, b). Thus, it is evident from this study that AgNPs synthesized from *A. indica* were more toxic to adult zebrafishes than the AuNPs. In general, different reports on fish toxicity exist in the literature. In our earlier study, 96 h  $LC_{50}$  for *M. crista* leaves extract synthesized AgNPs was 142.2  $\mu\text{g/L}$  (Krishnaraj et al. 2016), indicating less toxicity than the present study. In another study, 96 h  $LC_{50}$  for guava extract synthesized AgNPs was found to be 400  $\mu\text{g/L}$  (Sarkar et al. 2014), which was far less toxic than the  $LC_{50}$  value shown by our earlier study (Krishnaraj et al. 2016) and in the present study. From these discrepancies, it is undoubtedly clear that the toxicity of NPs relied not only on the administered dose but also the size, shape, capping agent, particle stability and quality of the aquatic medium (Bilberg et al. 2012; Massarsky et al. 2013; Olsagasti et al. 2014; Sarkar et al. 2014; Girilal et al. 2015).

In addition, aggressive behavior was noticed within 6 h and 12 h after treatment with AgNPs and AuNPs, respectively. The signs for toxicity were observed at higher concentrations of NPs, at which zebrafish was lying on the bottom of the tank and its respiratory rate was found to be increased. Subsequently, surface respiration took place; however, the fishes stood in the middle of the water and lost equilibrium. Hence, the fishes sank to the bottom of the tank (Figs. S1–S3). In the end, the fishes showed jerky movements before the death (Bilberg et al. 2012; Krishnaraj et al. 2016). After AgNPs treatment, extravasations of blood were seen in the anterior ventral surface of the body, i.e., behind the head of the dead fish; but, this was not found in AuNPs exposed zebrafish, indicating less toxicity (Figs. S2 and S3). At lower concentrations of AgNPs (15.5 and 18.6  $\mu\text{g/L}$ ) and AuNPs (9.7 and 19.4 mg/L) treated groups, no signs of behavioral changes were observed. In addition, no extravasations of blood were observed and the body color was found to be normal in all the fishes. Following the  $LC_{50}$  determination, 14 days sub-acute toxicity study was also carried out. When half of the  $LC_{50}$  of AgNPs (12.25  $\mu\text{g/L}$ ) and AuNPs (20.5 mg/L) was used as the highest concentration, no fish mortality was observed; in addition, no behavioral changes between control and NPs treated groups were also seen throughout the treatment period. These results were in good agreement with the earlier studies shown by Bilberg et al. (2012) and Krishnaraj et al. (2016).

**Fig. 1** Zebrafish mortality during acute exposure to biogenic **a** AgNPs and **b** AuNPs. The fishes were exposed to different concentrations of AgNPs and AuNPs for 96 h. Values are presented as mean  $\pm$  standard deviation of three independent experiments

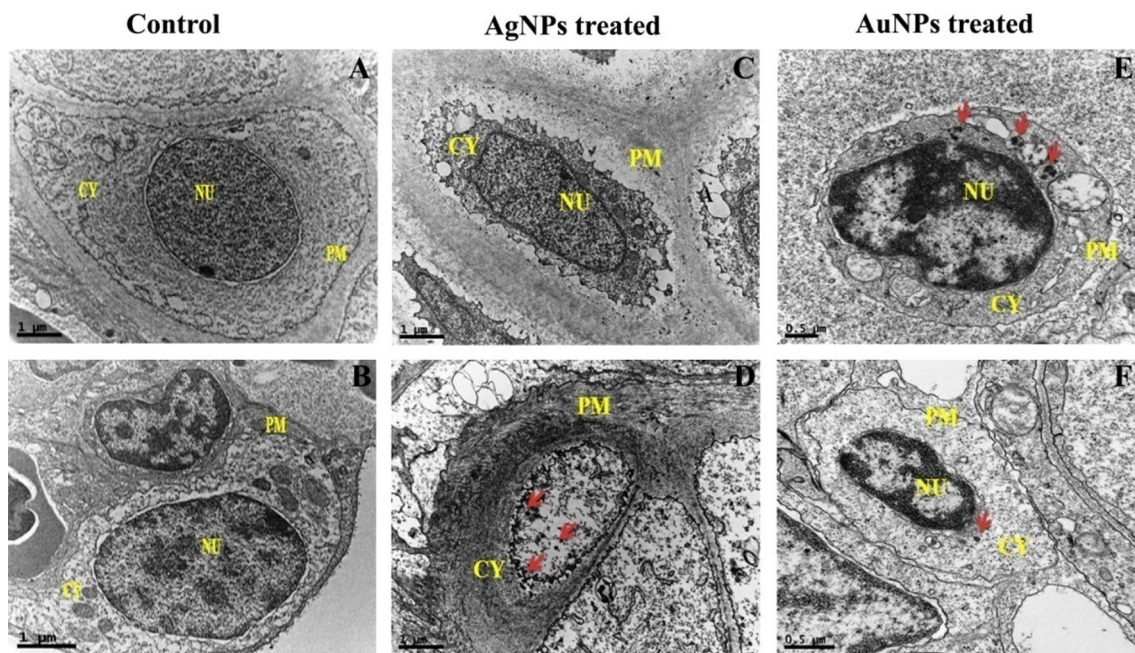


### Intracellular changes and localization of nanoparticles

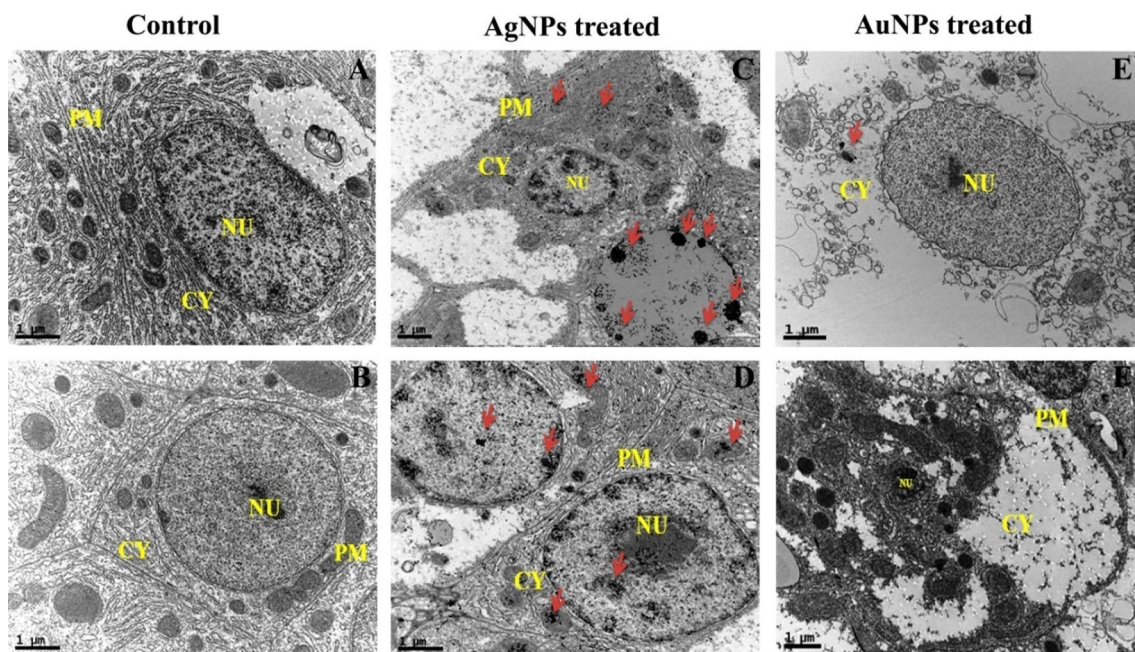
Cytological changes in the gill and liver tissues of control, AgNPs and AuNPs treated zebrafishes were observed under Bio-TEM. As shown in Fig. 2, control and AuNPs treated gill cells showed normal cell architecture with intact nucleus, cell organelles and cell membranes; the cytoplasm was also

appeared to be normal. On the other hand, AgNP-treated gill cells showed a number of morphological changes including cell membrane damage, irregular cell outlines, pyknotic nuclei and complete disruption of gills (Fig. 2). Besides, presence of NPs was also observed in the gills of both the NPs treated groups (Fig. 2). These results complemented our recent work in which *M. crista* synthesized AgNPs caused similar changes in the gills of adult zebrafish (Krishnaraj





**Fig. 2** Bio-TEM images of gill tissues of zebrafishes after exposure to AgNPs and AuNPs. Arrows indicate the presence of nanoparticles. *PM* plasma membrane, *CY* cytoplasm, *NU* nucleus



**Fig. 3** Bio-TEM images of liver tissues of zebrafishes after exposure to AgNPs and AuNPs. Arrows indicate the presence of nanoparticles. *PM* plasma membrane, *CY* cytoplasm, *NU* nucleus

et al. 2016). One of the main routes of AgNPs uptake and toxicity was seen through gills. Griffitt et al. (2009) have observed altered gill filament morphology and global gene expression in adult zebrafish after AgNPs treatment. Effect of AgNPs on  $\text{Na}^+/\text{K}^+$ -ATPase activity in gills was attributed

to the dissolution of  $\text{Ag}^+$  ions from the particles and/or to the presence of  $\text{Ag}^+$  ions at the surface of AgNPs (Griffitt et al. 2009).

Much similar to the gill cells, the control group of liver cells possessed normal cell structures with intact nucleus,

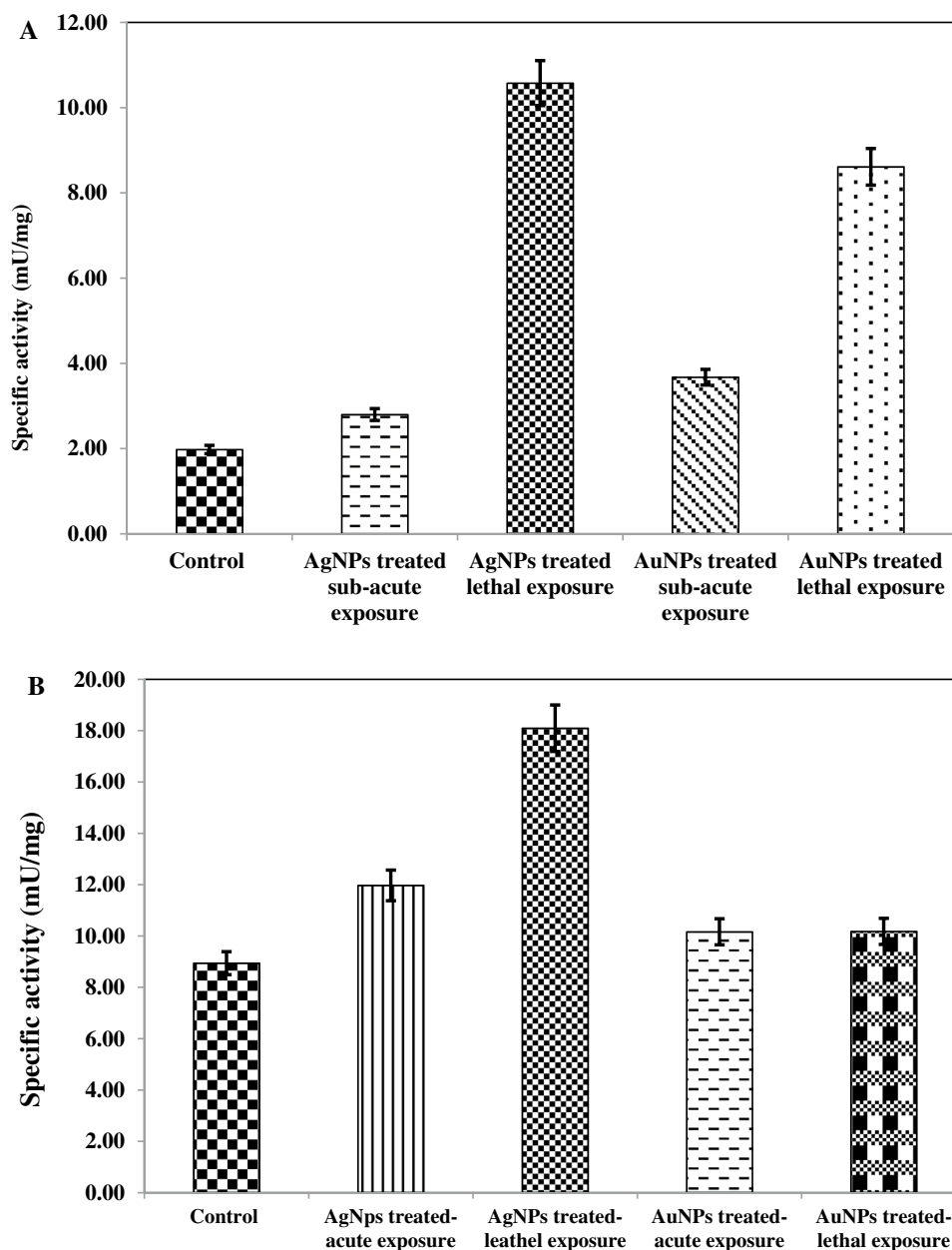
cell organelles, cytoplasm and cell membranes (Fig. 3). However, AgNPs and AuNPs exposed liver cells exhibited clear morphological changes such as cell membrane damage, irregular cell outlines, pyknotic nuclei and complete disruption of liver cells (Fig. 3). In addition to cytological changes, AgNPs and AuNPs were also observed in various parts of the cytoplasm and mitochondria especially in between the nucleus and plasma membrane (Fig. 3). These results were found to be consistent with our recent study in which *M. crista* synthesized AgNPs caused oxidative stress to adult zebrafish (Krishnaraj et al. 2016). Oxidative stress was often considered as an important factor in NP-induced toxicity (Nel et al. 2006). Recently, Choi et al. (2010) have well-demonstrated that oxidative damage, DNA damage and

apoptosis have been associated with AgNP-induced hepatotoxicity in adult zebrafish. In accordance with our results, Kim et al. (2009), Massarsky et al. (2013) and Girilal et al. (2015) have also reported that oxidative stress played a crucial role in the toxicity of AgNPs and Ag<sup>+</sup>.

### Liver marker enzymes

To check the liver toxicity caused by NPs, AST and ALT assays were performed in the liver tissues of control, AgNPs and AuNPs treated adult zebrafishes. The levels of both the marker enzymes were found to be higher in NPs treated groups than the control (Fig. 4a, b). Even so, increased enzyme levels were observed in AgNP-treated group than

**Fig. 4** Levels of **a** aspartate aminotransferase and **b** alanine aminotransferase in the liver tissues of zebrafishes after exposure to AgNPs and AuNPs. Values are presented as mean  $\pm$  standard deviation of three independent experiments



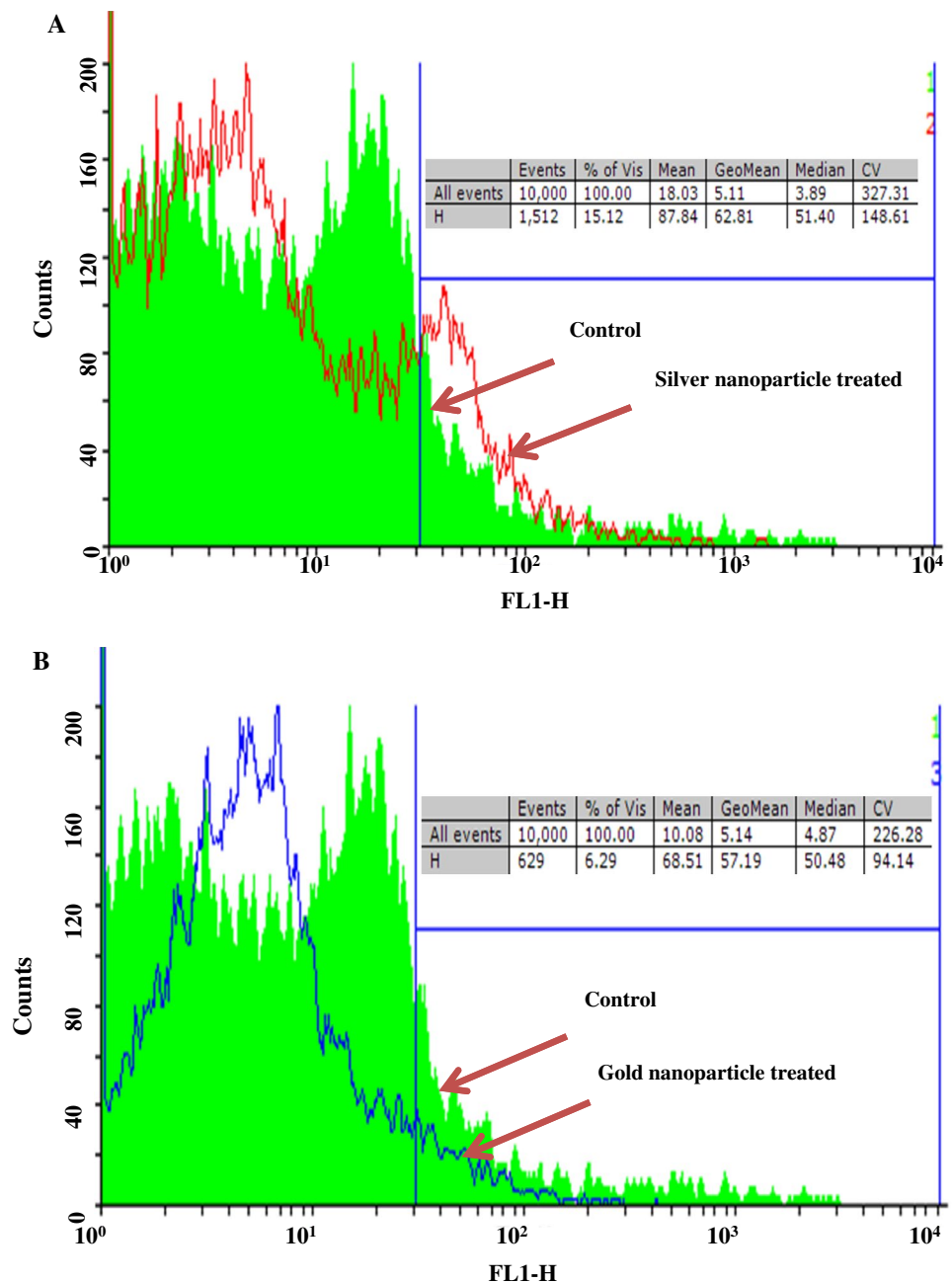
the AuNPs, indicating the toxicity of AgNPs (Fig. 4a, b). Lethal exposure of AgNPs showed increased levels of liver marker enzymes compared to the sub-acute exposure. Similarly, *M. crista* synthesized AgNPs showed elevated levels of AST and ALT in the liver tissues of adult zebrafish owing to the toxicity of AgNPs (Krishnaraj et al. 2016).

### Measurement of reactive oxygen species generation

ROS assay was carried out to understand the toxic mechanism mediated by the NPs. In the present study, the level of intracellular ROS generated by the AgNP-treated liver

cells was higher than the control (Fig. 5a). On the other hand, AuNPs treated liver cells showed lower levels of ROS generation than the control (Fig. 5b). The increased generation of ROS, as observed in AgNP-treated liver cells, was likely due to the cellular damage caused by the NPs. Oxidative stress or stress on an organism was probably due to the increase in ROS such as hydroxyl and peroxy radicals (Saqib et al. 2012). Therefore, oxidative stress was considered as an important toxic mechanism for AgNP-induced toxicity (Nel et al. 2006; Kim et al. 2009; Choi et al. 2010; Saqib et al. 2012; Massarsky et al. 2013; Girilal et al. 2015; Krishnaraj et al. 2016).

**Fig. 5** Flow cytometric analysis of intracellular ROS generation in the liver tissues of zebrafishes after exposure to **a** AgNPs and **b** AuNPs





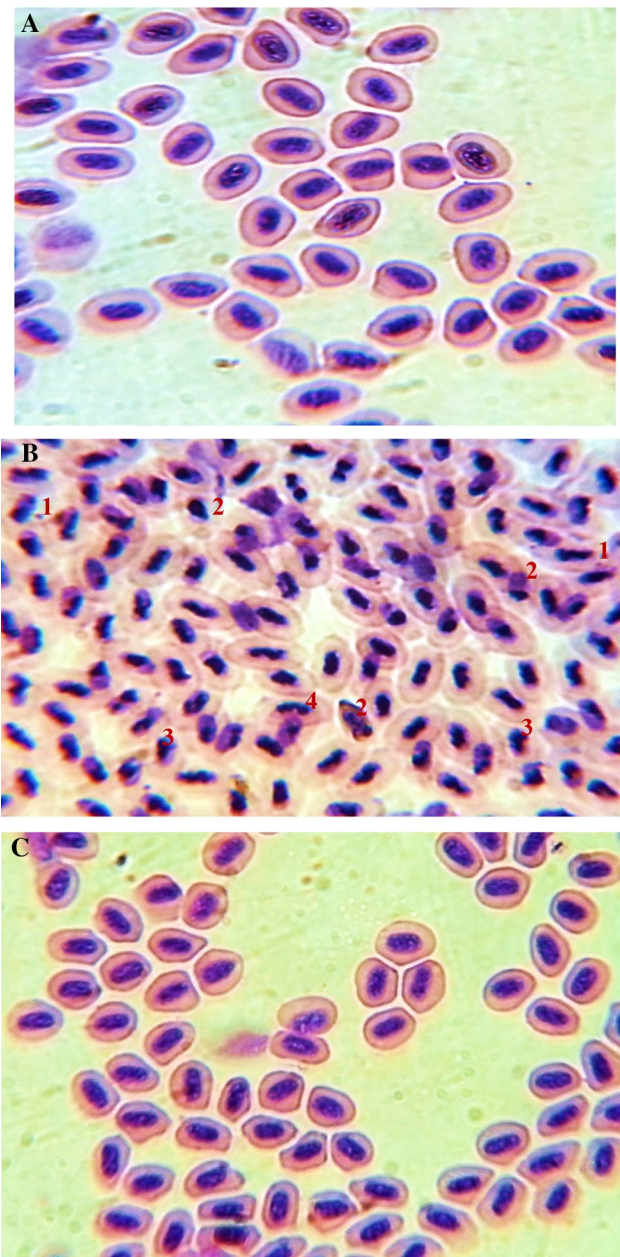
### Micronuclei formation and nuclear abnormalities

As evidenced from Fig. 6b, AgNPs treatment resulted in the formation of micronuclei; nuclear abnormalities such as blebbed nuclei, lobed nuclei and notched nuclei were also marked in the blood cells. The observed changes were linked to the genotoxicity of AgNPs (Ayllon and Garcia-Vazquez 2000). In agreement with our present results, *M. crista* mediated AgNPs also showed micronuclei formation and nuclear abnormalities (Krishnaraj et al. 2016). However, AuNPs tested in the present study did not show such signs of genotoxicity (Fig. 6c). Therefore, it is evident from these observations that AgNPs were more toxic to adult zebrafish than their corresponding AuNPs.

### Expression of oxidative stress and innate immune response related genes

mRNA expression levels of oxidative stress related genes, metal transcription factor 1 (MTF1) and heat-shock protein 70 (HSP70) and innate immune response related genes such as CCAAT-enhancer binding protein (C/EBP), transferrin (TRSF), lysozyme (LYZ), Toll-like receptor 4 (TLR4), Toll-like receptor 22 (TLR22), Nuclear factor- $\kappa$ B (NF- $\kappa$ B), myeloid-specific peroxidase (MPO) and interleukin 1 beta (IL1 $\beta$ ) were analyzed in the liver tissues of control, AgNPs and AuNPs treated zebrafishes. The stress gene, MTF1 was significantly down regulated in AgNP-treated lethal group than the sub-acute group, whereas, in case of AuNPs treatment, this gene was more down regulated in sub-acute exposure than the lethal exposure (Fig. 7a, b). MTF1 has been shown to regulate the transcription of genes with regard to different kinds of stimuli such as hypoxia and oxidative stress. MTF1 may lead to increase the labile cellular zinc, nuclear translocation, DNA binding and transcriptional activation of metallothionein genes (Gunes et al. 1998; Smirnova et al. 2000; Aerie et al. 2013). Another stress gene, HSP70 was down regulated in AgNP-treated lethal group and up regulated in sub-acute group; however, under AuNPs treatment, this gene was more down regulated in lethal exposure than the sub-acute exposure (Fig. 7a, b). Measurement of heat-shock protein induction, especially HSP70, has been recommended as a positive technique in toxicological screening and environmental monitoring to analyze a wide variety of stressors such as AgNPs, heavy metals, teratogens and anoxia (Williams et al. 1996; Scown et al. 2010; Girilal et al. 2015).

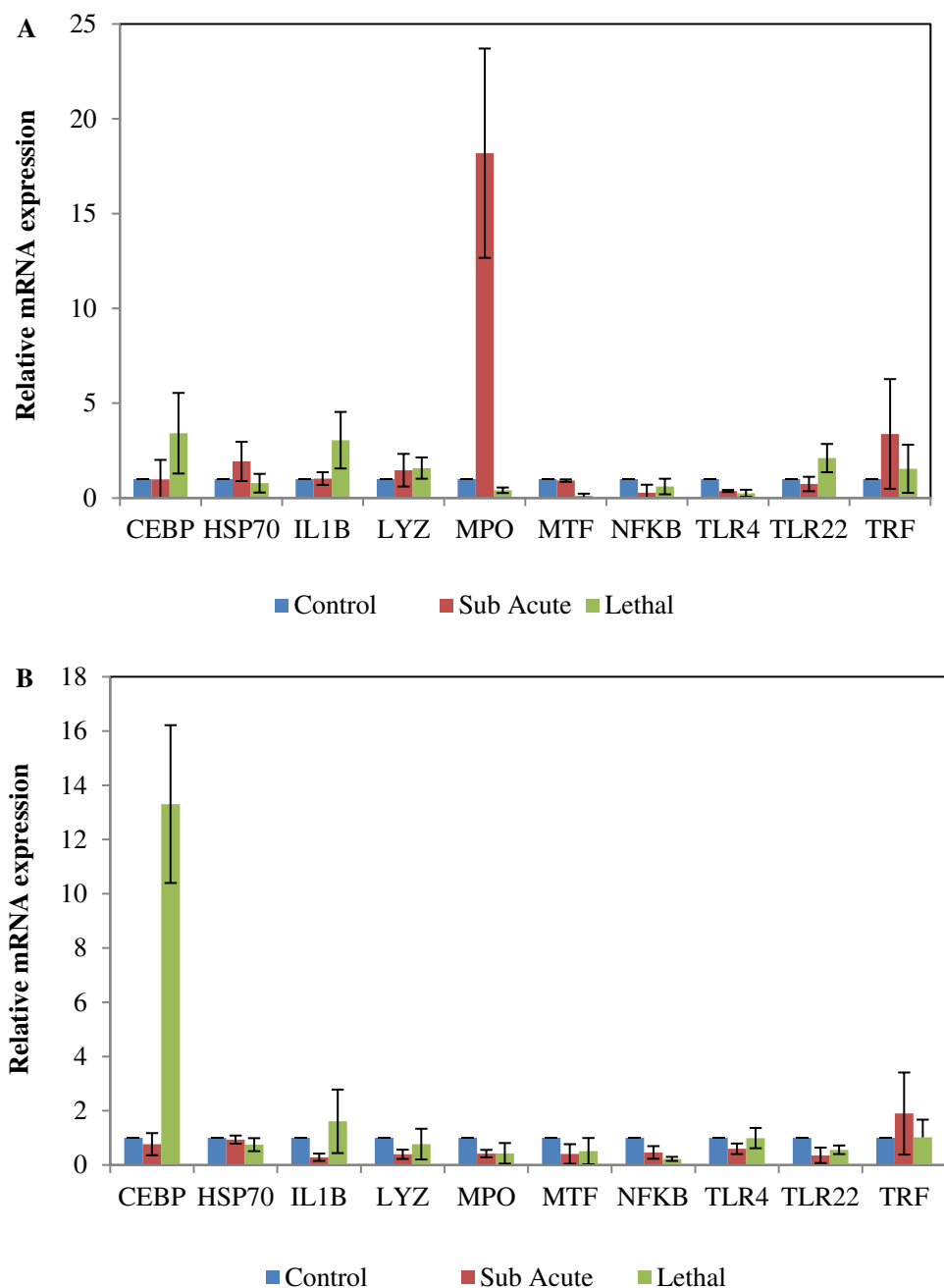
Interestingly, in this study, C/EBP was predominantly up regulated in both AgNPs and AuNPs treated lethal groups and down regulated during sub-acute exposure (Fig. 7a, b). C/EBP has been involved in cell growth, proliferation, cell differentiation, cell cycle arrest and apoptosis (Tsukada et al. 2011). IL1 $\beta$  and LYZ were more up regulated in AgNP-treated lethal group than the sub-acute exposure (Fig. 7a).



**Fig. 6** Photomicrographs of peripheral blood erythrocytes of zebrafishes ( $\times 100$ ). **a** Control blood cells, **b** AgNPs exposed blood cells, **c** AuNPs exposed blood cells. (1) Micronuclei, (2) blebbed nuclei, (3) lobed nuclei, (4) notched nuclei

MPO was significantly up regulated in AgNP-treated sub-acute group and significantly down regulated in lethal group, whereas TLR22 was up regulated in AgNP-treated lethal group and down regulated during sub-acute exposure (Fig. 7a). NF- $\kappa$ B was significantly down regulated in AgNP-treated sub-acute group than the lethal, while predominant down regulation of TLR4 was seen in AgNP-treated lethal group than the sub-acute exposure (Fig. 7a). In this study, surprisingly, opposite results were obtained in AuNPs

**Fig. 7** mRNA expression levels of oxidative stress and innate immune response related genes in the liver tissues of zebrafishes after exposure to **a** AgNPs and **b** AuNPs. Values are presented as mean  $\pm$  standard deviation of three independent experiments



treated liver cells. LYZ, TLR4 and TLR22 were predominantly down regulated in AuNPs treated sub-acute group than the lethal, whereas NF- $\kappa$ B was significantly down regulated in lethal exposure than the sub-acute exposure (Fig. 7b). NF- $\kappa$ B has been linked to cellular responses to stimuli including stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and bacterial or viral antigens (Brasier 2006; Gilmore and Herscovitch 2006). IL1 $\beta$  was up regulated in AuNPs treated lethal group and down regulated during sub-acute exposure, while MPO was equally down regulated in sub-acute and lethal treatments (Fig. 7b).

Interestingly, in this study, both AgNPs and AuNPs treatment predominantly up regulated the expression of TRSF gene in sub-acute treatment than the lethal exposure (Fig. 7a, b). TRSF is one of the most important factors in cellular processes and plays a complex physiological role associated with cell function, differentiation and proliferation (Zakin 1992).

In this study, the toxicity of plant extract derived AgNPs and AuNPs was compared for the first time using zebrafish as a model system. 96 h LC<sub>50</sub> for AgNPs was 24.5  $\mu$ g/L, while for AuNPs, it was 41 mg/L (Fig. 1a, b). Although

AuNPs caused 100% mortality to adult zebrafish at higher concentrations (48.5 and 58.2 mg/L), no extravasations of blood were observed. Even though AuNPs were present in gill and liver tissues, however, the cell membrane, cell organelles, nucleus and cytoplasm were found near to normal (Figs. 2, 3). Interestingly, the levels of liver marker enzymes such as AST and ALT in AuNPs treated zebrafish were found to be low compared to AgNP-treated ones (Fig. 4a, b). Furthermore, in AuNPs treatment, intracellular ROS generation was found to be lower than the control (Fig. 5b). No signs of genotoxic effects such as micronuclei formation and nuclear abnormalities were observed in AuNPs treated blood cells (Fig. 6c). On the other hand, only the AgNP-induced toxicity was specifically associated with all the observations of oxidative stress and immunotoxicity. In accordance with our present findings, some studies have demonstrated that AgNPs were more toxic to zebrafish embryos than their corresponding AuNPs (Bar-Ilan et al. 2009; Asharani et al. 2011; Rajan et al. 2017). This salient feature between AgNPs and AuNPs was probably due to their difference in physico-chemical properties, surface functionalization, biokinetics and, of course, the particle chemistry (Oberdörster et al. 2005; Nel et al. 2006; Buzea et al. 2007; Bar-Ilan et al. 2009).

## Conclusions

In this study, biologically synthesized AgNPs were found to be more toxic to adult zebrafishes than the AuNPs. However, recent studies have clearly demonstrated that biologically synthesized AgNPs were least toxic than the chemically synthesized AgNPs and Ag<sup>+</sup> ions (Sarkar et al. 2014; Girilal et al. 2015). This is likely due to the presence of biomolecules on the surface of NPs. So far, the biological entities have not been reported to induce physiological stress reactions and reduced the toxic effects of biogenic AgNPs (Girilal et al. 2015). In line with this observation, the present findings clearly reveal the significance of relatively safe and less toxic bionanomaterials for possible biomedical applications.

**Acknowledgements** RR sincerely acknowledges University Grants Commission (UGC), Government of India for awarding UGC-BSR Meritorious Fellowship in Sciences. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2007953) and also funds from Chonbuk National University, Republic of Korea.

## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interest.

## References

- Aerle RV, Johnston BD, Lange A, Bastos ED, Moorhouse A, Booth T, Paszkiewicz K, Tyler CR, Ball K, Santos EM (2013) Molecular mechanisms of toxicity of silver nanoparticles in zebrafish embryos. *Environ Sci Technol* 47:8005–8014
- Asharani PV, Wu YL, Gong Z, Valiyaveetil S (2008) Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology* 19:255102
- Asharani PV, Wu YL, Gong Z, Valiyaveetil S (2011) Comparison of the toxicity of silver, gold and platinum nanoparticles in developing zebrafish embryos. *Nanotoxicology* 5:43–54
- Ayllon FE, Garcia-Vazquez (2000) Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. *Mutation Res* 467:177–186
- Balakumaran MD, Ramachandran R, Balashanmugam P, Mukeshkumar DJ, Kalaichelvan PT (2016) Mycosynthesis of silver and gold nanoparticles: optimization, characterization and antimicrobial activity against human pathogens. *Microbiol Res* 182:8–20
- Bar-Ilan O, Albrecht RM, Fako VE, Furgeson DY (2009) Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. *Small* 5:1897–1910
- Bilberg K, Hovgaard MB, Besenbacher F, Baatrup E (2012) In vivo toxicity of silver nanoparticles and silver ions in zebrafish (*Danio rerio*). *J Toxicol* 2012:293784
- Brasier AR (2006) The NF- $\kappa$ B regulatory network. *Cardiovasc Toxicol* 6:111–130
- Buzea C, Pacheco II, Robbie K (2007) Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases* 2:MR17–MR71
- Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu DY (2010) Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquat Toxicol* 100:151–159
- Gilmore TD, Herscovitch M (2006) Inhibitors of NF- $\kappa$ B signaling: 785 and counting. *Oncogene* 25:6887–6899
- Girilal M, Krishnakumar V, Poornima P, Fayaz AM, Kalaichelvan PT (2015) A comparative study on biologically and chemically synthesized silver nanoparticles induced heat shock proteins on fresh water fish *Oreochromis niloticus*. *Chemosphere* 139:461–468
- Griffitt RJ, Hyndman K, Denslow ND, Barber DS (2009) Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicol Sci* 107:404–415
- Gunes C, Heuchel R, Georgiev O, Muller KH, Lichtlen P, Bluthmann H, Marino S, Aguzzi A, Schaffner W (1998) Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. *Embo J* 17:2846–2854
- Haynes CL (2010) The emerging field of nanotoxicology. *Anal Bioanal Chem* 398:587–588
- Hotchkiss RD (1948) A microchemical reaction resulting in the staining of polysaccharide structure in fixed tissue preparations. *Arch Biochem* 16:131–141
- Hu YL, Qi W, Han F, Shao J, Gao J (2011) Toxicity evaluation of biodegradable chitosan nanoparticles using a zebrafish embryo model. *Int J Nanomedicine* 6:3351–3359
- Impellitteri CA, Tolaymat TM, Scheckel KG (2009) The speciation of silver nanoparticles in antimicrobial fabric before and after exposure to a hypochlorite/detergent solution. *J Environ Qual* 38:1528–1530
- Kiernan JA (1999) *Histological and histochemical methods: theory and practice*, 3rd edn. Butterworth-Heinemann, Oxford
- Kim S, Choi JE, Choi J, Chung K-H, Park K, Yi J, Ryu D-Y (2009) Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. *Toxicol In Vitro* 23:1076–1084



- Krishnaraj C, Ramachandran R, Mohan K, Kalaichelvan PT (2012) Optimization for rapid synthesis of silver nanoparticles and its effect on phytopathogenic fungi. *Spectrochim Acta A Mol Biomol Spectrosc* 93:95–99
- Krishnaraj C, Muthukumar P, Ramachandran R, Balakumaran MD, Kalaichelvan PT (2014) *Acalypha indica* Linn: biogenic synthesis of silver and gold nanoparticles and their cytotoxic effects against MDA-MB-231, human breast cancer cells. *Biotechnol Rep* 4:42–49
- Krishnaraj C, Harper SL, Yun S-I (2016) In vivo toxicological assessment of biologically synthesized silver nanoparticles in adult zebrafish (*Danio rerio*). *J Hazard Mater* 301:480–491
- Marcolin E, Miguel BS, Vallejo D, Tieppo J, Marroni N, Gallego JG, Tunon MJ (2012) Quercetin treatment ameliorates inflammation and fibrosis in mice with nonalcoholic Steatohepatitis 1–3. *J Nutr* 142:1821–1828
- Massarsky A, Dupuis L, Taylor J, Eisa-Beygi S, Streck L, Trudeau VL, Moon TW (2013) Assessment of nanosilver toxicity during zebrafish (*Danio rerio*) development. *Chemosphere* 92:59–66
- Nel A, Xia T, Mädler L, Li N (2006) Toxic potential of materials at the nanolevel. *Science* 311:622–627
- Nowack B, Bucheli TD (2007) Occurrence, behavior and effects of nanoparticles in the environment. *Environ Pollut* 150:5–22
- Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113:823–839
- OECD (1992) Test No. 203: fish, acute toxicity test. OECD Publishing, Paris
- Olasagasti M, Gatti AM, Capitani F, Barranco A, Pardo MA, Escuredo K, Rainieri S (2014) Toxic effects of colloidal nanosilver in zebrafish embryos. *J Appl Toxicol* 34:562–575
- Project on Emerging Nanotechnologies (2018) Consumer products inventory. <http://www.nanotechproject.org/cpi/products/>. Accessed 7 June 2018
- Rajan R, Chandran K, Harper SL, Yun S-I, Kalaichelvan PT (2015) Plant extract synthesized silver nanoparticles: an ongoing source of novel biocompatible materials. *Ind Crops Prod* 70:356–373
- Rajan R, Chandran K, Sivakumar AS, Prasannakumar P, Abhay Kumar VK, Shim KS, Song C-G, Yun S-I (2017) Anticancer activity of biologically synthesized silver and gold nanoparticles on mouse myoblast cancer cells and their toxicity against embryonic zebrafish. *Mater Sci Eng C Mater Biol Appl* 73:674–683
- Saqib Q, Al-Khedhairi AA, Siddiqui MA, Abou-Tarboush FM, Azam A, Musarrat J (2012) Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. *Toxicol In Vitro* 26:351–361
- Sarkar B, Netam SP, Mahanty A, Saha A, Bosu R, Krishnani KK (2014) Toxicity evaluation of chemically and plant derived silver nanoparticles on zebrafish (*Danio rerio*). *Proc Natl Acad Sci India Sect B Biol Sci* 84:885–892
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative  $C_T$  method. *Nat Protoc* 3:1101–1108
- Scown TM, Santos EM, Johnston BD, Gaiser B, Tyler CR (2010) Effects of aqueous exposure to silver nanoparticles of different sizes in Rainbow Trout. *Toxicol Sci* 115:521–534
- Shah D, Savaliya R, Patel P, Kansara K, Pandya A, Dhawan A, Singh S (2018) Curcumin Ag nanoconjugates for improved therapeutic effects in cancer. *Int J Nanomed* 13:75–77
- Shankar SS, Rai A, Ahmad A, Sastry M (2004a) Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using *Neem (Azadirachta indica)* leaf broth. *J Colloid Interface Sci* 275:496–502
- Shankar SS, Rai A, Ankamwar B, Singh A, Ahmad A, Sastry M (2004b) Biological synthesis of triangular gold nanoprisms. *Nat Mater* 3:482–488
- Singh S, D’Britto V, Prabhune AA, Ramana CV, Dhawan A, Prasad BLV (2010) Cytotoxic and genotoxic assessment of glycolipid-reduced and -capped gold and silver nanoparticles. *New J Chem* 34:294–301
- Smirnova IV, Bittel DC, Ravindra R, Jiang H, Andrews GK (2000) Zinc and cadmium can promote the rapid nuclear translocation of MTF-1. *J Biol Chem* 275:9377–9384
- Srivastava M, Singh S, Self WT (2012) Exposure to silver nanoparticles inhibits selenoprotein synthesis and the activity of thioredoxin reductase. *Environ Health Perspect* 120:56–61
- Tsukada J, Yoshida Y, Kominato Y, Auron PE (2011) The CCAAT/enhancer binding (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine* 54:6–19
- Williams JH, Farag AM, Stansbury MA, Young PA, Bergman HL, Petersen NS (1996) Accumulation of hsp70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. *Environ Toxicol Chem* 15:1324–1328
- Zakin MM (1992) Regulation of transferrin gene expression. *FASEB J* 6:3253–3258x