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In vitro assay for the toxicity of silver nanoparticles using heart and gill cell lines of *Catla catla* and gill cell line of *Labeo rohita*

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ABSTRACT

Silver nanoparticles (Ag-NPs) are used in commercial products for their antimicrobial properties. The Ag-NPs in some of these products are likely to reach the aquatic environment, thereby posing a health concern for humans and aquatic species. The silver nanoparticles were synthesized and characterized using, UV–vis spectra, Dynamic light scattering (DLS) and Transmission electron microscopy (TEM) analysis. Acute toxicity tests on fish were conducted by exposing *Catla catla* and *Labeo rohita* for 96 h to $AgNO_3$ and Ag-NPs under static conditions. The cytotoxic effect of $AgNO_3$ and Ag-NPs in Sahul India *C. catla* heart cell line (SICH), Indian *C. catla* gill cell line (ICG) and *L. rohita* gill cell line (LRG) was assessed using MTT and neutral red (NR) assay. Linear correlations between each in vitro EC_{50} and the in vivo LC_{50} data were highly significant. DNA damage and nuclear fragmentation (condensation) were assessed by comet assay and Hoechst staining, respectively in SICH, ICG and LRG cells exposed to Ag-NPs. The results of antioxidant parameter obtained show significantly increased lipid peroxidation (LPO) level and decreased level of GSH, SOD and CAT in SICH, ICG and LRG cell lines after exposure to increasing Ag-NPs in a concentration-dependent manner. This work proves that fish cell lines could be used as an alternative to whole animals using cytotoxicity tests, genotoxicity tests and oxidative stress assessment after exposure to nanoparticles.

1. Introduction

Nanotechnology is considered as one of the key technologies of the 21st century and promises a revolution in our world. Nanoparticles are used in bioapplications such as targeted drug delivery systems (Hallaj-Nezhadi et al., 2010; Falanga et al., 2011), rapid diagnostics (Daaboul et al., 2010; Cao et al., 2011), and biomolecular sensing (Miranda et al., 2010), antimicrobial agents (Yoon et al., 2007), transfection vectors (Tan et al., 2007), stain-resistant clothing (Hood, 2004) and fluorescent labels (Su et al., 2008) as well as nanoparticle based cancer therapeutics (Kennedy et al., 2011). The fisheries and aquaculture industry can be revolutionized by using nanotechnology with new tools like rapid disease diagnosis, enhancing the ability of fish to absorb drugs like hormones, vaccines and nutrients etc. rapidly (Rather et al., 2011). Silver nanoparticles have received considerable attention as antimicrobial agents and have been shown to be effective mainly as an antibacterial against both Gram-positive and Gram-negative bacteria aquatic pathogenic bacteria. They are used extensively in clothing, water purification, baby products (e.g. nipples and bottles) personal care products (e.g. shampoos, toothpastes, deodorants, etc.), and bedding and appliances (e.g. washing machines, humidifiers and refrigerators) (Woodrow Wilson International Center for Scholars, 2008). The increased use of nano-sized metallic materials is likely to result in the release of these particles to the environment (Chae et al., 2009). Therefore, in the investigation of the potential aquatic toxicity of nanomaterials adequate information is lacking and hence it has become an important issue.

The toxicity of silver nanoparticles to living systems has been investigated extensively using both in vitro and in vivo systems. Griffitt et al. (2008) reported toxic effects of nanosilver in zebra fish juveniles and adults, daphnids and two algal species with a 50% lethal concentration (LC_{50}) in the low mg/L range. Silver in its ionic form (Ag^+) is well known to be toxic to aquatic organisms at μg/L concentrations and its toxic mode of action being inhibition of Na^+/K^+ ATPase and the following disturbance of ion balance (Wood et al., 1996). This effect has been found to be similar for different aquatic animals like fish, *Daphnia* sp. and crayfish (Bianchini and Wood, 2002; Grosell et al., 2002). Chae et al. (2009) studied the impact of silver nanoparticles on the environment and health of Japanese medaka by studying the changes in the expression of stress-related genes using real time RT-PCR analysis and comparing these results with those of medaka exposed to soluble silver ions. Scown et al. (2010) also reported the effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout.

Numerous results from in vitro studies report that silver nanomaterials induced cytotoxicity and reactive oxygen species (ROS) generation (Carlson et al., 2008; AshaRani et al., 2009; Park et al., 2011; Piao et al., 2011). Carlson et al. (2008) showed a significant dose-dependent and size-dependent decrease in mitochondrial function (MTT assay), mitochondrial membrane integrity (LDH assay), ROS

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generation, release of inflammatory cytokines (TNFα, MIP2, and IL1β) and glutathione depletion in rat alveolar macrophages. AshaRani et al. (2009) reported increased ROS generation and excess oxidative stress, resulting from the intercellular production of hydrogen peroxide and superoxide in Ag-NPs exposed IMR-90 (human lung fibroblast cells) and U251 (human glioblastoma) cells. AshaRani et al. (2009) also reported the decreased metabolic activity associated with a reduction in ATP production in Ag-NP exposed cells, suggesting mitochondrial dysfunction. The in vitro (human lung fibroblast cells, human glioblastoma and human liver cell lines) findings demonstrated that exposure to Ag-NPs can result in genotoxicity from increased oxidative stress (AshaRani et al., 2009; Park et al., 2011; Piao et al., 2011). A recent study reveals that silver nanospheres have cytotoxic and genotoxic effects on the OLHNI2 cell line derived from fin tissue of medaka (*Oryzias latipes*) (Wise et al., 2010). The hepatocyte cell line derived from rainbow trout (*Oncorhynchus mykiss*) was used as in vitro model for toxicity assessment of silver and gold nanoparticles (Farkas et al., 2010).

Silver nanoparticles (Ag-NPs) used in the present study were prepared by a proprietary process as a part of an on-going program in our laboratory to develop a topical antimicrobial agent for the treatment of aquatic pathogens. Since the mechanisms of toxicity of silver nanoparticles are unclear, the goal of this study, was to synthesize and characterize chitosan based silver nanoparticles (Ag-NPs), and to assess its cytotoxic effect on Sahul India *Catla catla* heart cell line (SICH) developed by Ishaq Ahmed et al. (2009), Indian *C. catla* gill cell line (ICG) developed by Taju et al. (2013) and *Labeo rohita* gill cell line (LRG) developed by Abdul Majeed et al. (2013, 2014), as measured by the loss of cellular metabolic inhibition (MTT assay) and lysosomal damage (NR uptake assay). DNA damage and nuclear fragmentation were assessed by comet assay and Hoechst staining for genotoxic effect of silver nanoparticles. The cellular antioxidant activity was also determined to assess the potentiality of the silver nanoparticles to induce cellular oxidative stress. The results of in vitro cytotoxicity studies were correlated with the results of in vivo lethality test using whole fish (*C. catla* and *L. rohita*).

2. Materials and methods

2.1. Synthesis and characterization of chitosan based silver nanoparticles (Ag-NPs)

Silver nanoparticles were synthesized using chitosan redox solution chemical techniques according to published methods (Dongwei et al., 2009), with a slight modification. 5 mL of 52.0 mM $AgNO_3$ and 10 mL of a solution of chitosan (6.92 mg mL^{-1}) were mixed and stirred until homogenous. This, mixture was then transferred to a 12.5 × 2-cm cuvette and allowed to stand for 12 h at 95 °C. The color of the solution progressed from colorless to light yellow and finally to yellowish brown within hours after the reaction was initiated.

2.1.1. Characterization of silver nanoparticles (Ag-NPs)

UV–vis absorption spectroscopy (Shimadzu, UV-2450, Japan) was used primarily to confirm the presence of an absorbance peak centered around 423 nm, consistent with that expected for the plasmon resonance of silver nanoparticles. TEM studies were carried out using electron microscope (Model Philips Morgagni 268) operating at 80 kV at Veterinary College, Chennai, India. The dynamic light scattering (DLS) experiments were performed on a Photocor FC (VIT, Vellore, India).

2.2. Experimental tank water

The physico-chemical properties of test water, namely temperature, pH, dissolved oxygen (DO), total conductivity, and total hardness were analyzed by standard methods (APHA, 1998).

2.3. In vivo fish acute toxicity test

Young specimens of *C. catla* (Hamilton, 1822) and *L. rohita* (Hamilton, 1822) were collected from a local pond located at Walajapet, Vellore, Tamil Nadu. The experimental fishes were about 2–3 g in body mass. Fish were transported live in oxygen bags or buckets to the laboratory, acclimatized and maintained for 10 days. They were fed with commercial pellet feed twice a day and fasted for 24 h before and during the experiments. Fish acute toxicity tests were conducted by exposing *C. catla* and *L. rohita* (N = 10 per aquarium) for 96 h to $AgNO_3$ and Ag-NPs under static conditions (OECD 203, 1992). Six concentrations of $AgNO_3$ and Ag-NPs (2, 4, 8, 16, 32 and 64 μg/mL) diluted with freshwater and control with freshwater alone were tested to determine the LC_{50} (concentration at which 50% of the fish population dies). The aquaria had a working volume of 30 L based on the body mass of fish (1 g/L). All the experiments were conducted in triplicates (3 tanks/treatment). Mortalities were recorded following the guideline for fish acute toxicity (OECD 203, 1992).

2.4. In vitro cytotoxicity assay using fish-derived cell lines

The SICH, ICG and LRG cell lines were maintained at 28 °C in Leibovitz's L-15 medium (pH 7.0–7.4) with 2 mM L-glutamine, 10% fetal bovine serum (FBS), penicillin 100 IU/mL and streptomycin 100 μg/mL. The cells were sub-cultured every 2–3 days using standard procedure. Cells at exponential growth phase were harvested and diluted to a concentration of 10^5 cells/mL in Leibovitz's L-15 medium with 10% FBS. After agitation, the cells were added to each well of 96-well tissue culture plates at the concentration of 2×10^4 cells/well and incubated overnight at 28 °C. After incubation, the medium was removed and the cells were re-fed with fresh medium containing 0 (control), 2, 4, 8, 16, 32 and 64 μg/mL of $AgNO_3$ and Ag-NPs for 24 h EC_{50} analysis.

2.4.1. Cell viability by MTT reduction assay

MTT assay described by Borenfreund et al. (1988) is based on inhibition by chemical injury of the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT-formazan product by mitochondrial succinate dehydrogenase. After a 24-h exposure period, the test medium was replaced by 20 μL of 5 mg/mL MTT in PBS. After the incubation for 4 h at 20 °C, the solution was removed carefully, and the cells were rinsed twice with PBS rapidly. Then dimethyl sulfoxide was added at the rate of 150 μL/well to solubilize the purple formazan crystals produced. Absorbance of each well was measured at 490 nm and the EC_{50} value was calculated.

2.4.2. Neutral red uptake assay

NR uptake assay was carried out based on the procedure described by Borenfreund and Puerner (1985). This assay measures the inhibition of cell growth, which is based on the absorbance of the vital dye NR by live, but not by dead cells. After a 24-h exposure, the test medium in each well was replaced by 200 μL L-15 medium containing 50 μg/mL of NR and incubated in situ for 3 h at 28 °C. The cells were then rinsed with warm phosphate-buffered saline (PBS) to remove the NR dye and then de-stained with 200 μL solution containing glacial acetic acid, ethanol and water in the ratio of 1:50:49, respectively. After rapid agitation for 10 min at room temperature, the absorbance of the solution in each well was measured at 550 nm with a microplate reader (Multiscan EX Thermo Electron Corporation) and the EC_{50} value (concentration of test agent which causes 50% inhibition in NR uptake) was determined. Cell viability was expressed as a fraction of the negative control (cells with media alone). % of cytotoxicity = OD of test sample / OD of control × 100.

2.4.3. Cell morphology analysis

Cells were plated into a 35 mm tissue culture plate at a density of 2×10^5 cells. After overnight growth, supernatants from the culture plates were aspirated and fresh aliquots of growth medium containing different concentrations of Ag-NPs (0, 2, 4, 8, 16, 32 and 64 μg/mL) were added. Upon incubation, cells were washed with phosphate-buffered saline (PBS, pH 7.4) and the morphological changes were observed under an inverted phase-contrast microscope (Carl Zeiss, Germany) at 100× magnification.

2.4.4. Comet assay

The comet assay was performed on SICH, ICG and LRG cell lines according to the method of Singh et al. (1988). 5×10^4 cells on 500 μL of complete culture medium were seeded per well in a 24-well-plate. After a 24-h incubation, cells were exposed to Ag-NPs using the following concentrations: 0 (control), 2, 4, 8, 16, 32 and 64 μg/mL. At the end of the exposure period, cells were collected through trypsinization, followed by centrifugation at 580 *g* for 2 min to obtain the pellet and avoid cell loss. After the centrifugation, the supernatant was discarded and the pellet resuspended in 100 μL of 0.9% agarose in milliQ water (low-melting point agarose, Sigma Aldrich chemicals, USA). The suspensions of cells in agarose were then applied dropwise to microscope slides containing an agarose layer (agarose electrophoresis grade, prepared with a 1% concentration in milliQ water), and kept in a freezer for 10 min. The cells were lysed in freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% DMSO, 1% Triton X-100, pH 10), for 1 h at 4 °C. After rinsing with redistilled water, the slides were placed on the horizontal gel box, covered with the cold alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) and left for 20 min. Electrophoresis was run in the same buffer at 25 V (0.83 V/cm) at 300 mA for 20 min at 4 °C. After electrophoresis the slides were neutralized in a cold neutralization buffer (0.4 M Tris–HCl, pH 7.5), for 2 to 5 min, fixed in methanol:acetic acid (3:1) for 5 min and stored in the dark at room temperature. Prior to examination, the slides were rehydrated and stained with 10 μg/mL ethidium bromide and examined using a Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Germany). A positive control (5 μM H_2O_2) was also included in every batch of samples. This strategy was chosen to compare the variation in the distance of migration. The positive control was not included in evaluation.

Slides were examined at 400× magnifications using a fluorescence microscope equipped with two filters one for ethidium bromide (excitation 510–560 nm, emission 590 nm) and another for fluorescein diacetate. For each experimental condition 100 randomly chosen cells from two duplicate slides were examined (50 from each slide). In all 100 comets were scored visually according to the relative intensity of the fluorescence in the tail and given a value of 0, 2, 4, 8, 16, 32 and 64 μg/mL [0 — undamaged (Plate. 1 A, B and C, in SICH, ICG and LRG cells, respectively), and maximally damaged, 64 μg/mL (Plate. 1 D, E and F in SICH, ICG and LRG cells, respectively)]. The extent of DNA migration was determined as a percentage of DNA in the tail (% tDNA) using an image analysis system comet 5, Kinetic Imaging Ltd.

2.4.5. Nuclear fragmentation by Hoechst 33258

Nuclear fragmentation of SICH, ICG and LRG cells was analyzed with Hoechst 33258. The cells were seeded in 12-well microplates and incubated overnight. Then the cells were treated with different concentrations of Ag-NPs (0, 2, 4, 8, 16, 32 and 64 μg/mL). Cells were fixed in 4% paraformaldehyde in PBS for 30 min, washed with PBS, and stained with 1 μg/mL Hoechst 33258 in PBS for 30 min. Stained cells were washed twice with PBS. The changes in nuclei were observed with a fluorescent microscope through a UV filter.

2.5. Preparation of cell extract and biochemical assays

The SICH, ICG and LRG cell lines were exposed to different concentrations of Ag-NPs (0, 2, 4, 8, 16, 32 and 64 μg/mL) on 25 cm^2 flasks for 24 h. After 24 h they were trypsinized and pelleted by centrifugation at 500 *g* for 5 min. The cell pellet was washed with PBS (0.1 M, pH7.4), resuspended in 500 μL chilled homogenizing buffer (250 mM sucrose, 12 mM Tris–HCl, 0.1 mM DTT, pH 7.4) and lysed using a Dounce homogenizer. The lysate was centrifuged (8000 *g*, 10 min, 4 °C) and the supernatant (cell extract) was used for various biochemical assays. Protein concentration in the cell extract was estimated by the method of Lowry et al. (1951).

2.5.1. Assay of superoxide dismutase (EC 1.15.1.1)

SOD activity was determined according to the method of Kono (1978) using Nitro blue tetrazolium (NBT) in the presence of riboflavin.

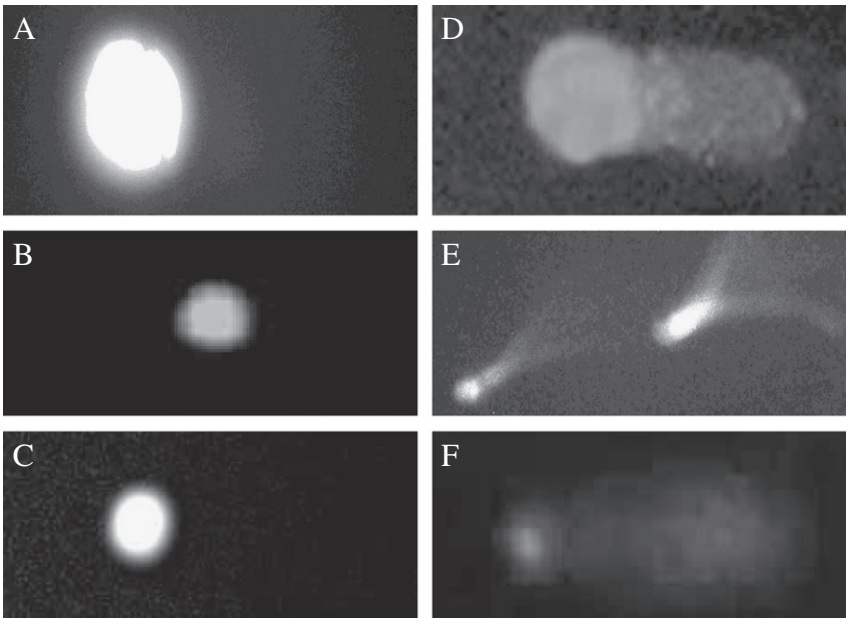

Plate 1. Fluorescence photomicrograph of SICH, ICG and LRG fish cell lines (× 400). DNA damage levels as determined on SICH, ICG and LRG fish cell lines exposed to Ag-NPs (64 μg/mL) for 24 h. In control cell of SICH (A), ICG (B) and LRG (C) and Ag-NPs (64 μg/mL) exposed to SICH (D), ICG (E) and LRG (F) cells.

Briefly, the reaction mixture (2.1 mL) contained 1924 μL sodium carbonate buffer (50 mM), 30 μL nitrobluetetrazolium (1.6 mM), 6 μL Triton X-100 (10%) and 20 μL hydroxylamine–HCl (100 mM). Subsequently 100 μL cell extract was added and absorbance (560 nm) was read for 5 min against blank (reaction mixture sans cell extract). In this experiment, a specific control containing reaction mixture with cell extract (unexposed cells) was also run. The results were expressed as relative enzyme activity μmol/mg protein.

2.5.2. Assay of total reduced glutathione (EC 1.8.1.7)

GSH was estimated by the method of Saldak and Lindsay (1968). The reaction mixture containing 1.2 mL EDTA (0.02 M), 1 mL distilled water, 250 μL 50% trichloroacetic acid and 50 μL Tris buffer (0.4 M, pH 8.9) was centrifuged at 300 *g* for 15 min. Clear supernatant (500 μL) was mixed with 1 mL of 0.4 M Tris buffer (containing 0.02 M EDTA, pH 8.9), 100 μL of 0.01 M DTNB [5,5-dithio-bis-(2-nitrobenzoic acid)] and 100 μL cell extract. The mixture was incubated at 37 °C for 25 min and the yellow color developing was read at 412 nm against blank. The amount of glutathione is expressed as μmol/mg protein.

2.5.3. Assay of catalase (EC 1.11.1.6)

Catalase activity was measured using the method described by Aebi (1984). In this, absorbance (240 nm) of 1 mL reaction mixture containing 0.8 mL H_2O_2 phosphate buffer (H_2O_2 diluted 500-folds with 0.1 M phosphate buffer of pH 7), 100 μL cell extract and 100 μL distilled water was recorded for 4 min against blank (H_2O_2–phosphate buffer). The enzyme activity was expressed as μmol/mg protein.

2.5.4. Estimation of lipid peroxidation

The lipid peroxidation was measured in fish cells exposed to toxicants using the method described by Beuge and Aust (1978). A mixture of 100 μL Tris buffer (150 mM, pH 7.1), 10 μL ferrous sulfate (100 mM), 10 μL ascorbic acid (150 mM), 780 μL distilled water and 100 μL cell extract was incubated at 37 °C for 15 min. Thiobarbituric acid (0.375%, 2 mL) was then added to the mixture and allowed to react at 100 °C (in water bath) for 60 min. The reaction mixture was then centrifuged (800 g for 10 min) and supernatant was read at 532 nm against blank. The results were recorded as μmol of TBA reactive substances/mg protein.

2.6. Data analysis

Experiments were performed in triplicate (in vivo) with eight replicates (in vitro) for each exposure concentration. Absolute values of each assay were transformed to control percentages. The results of LC_{50} and EC_{50} values were expressed as dilution percentage of the sample calculated using computerized software Spearman–Karber method (version 1.5). The individual data points of the concentration response

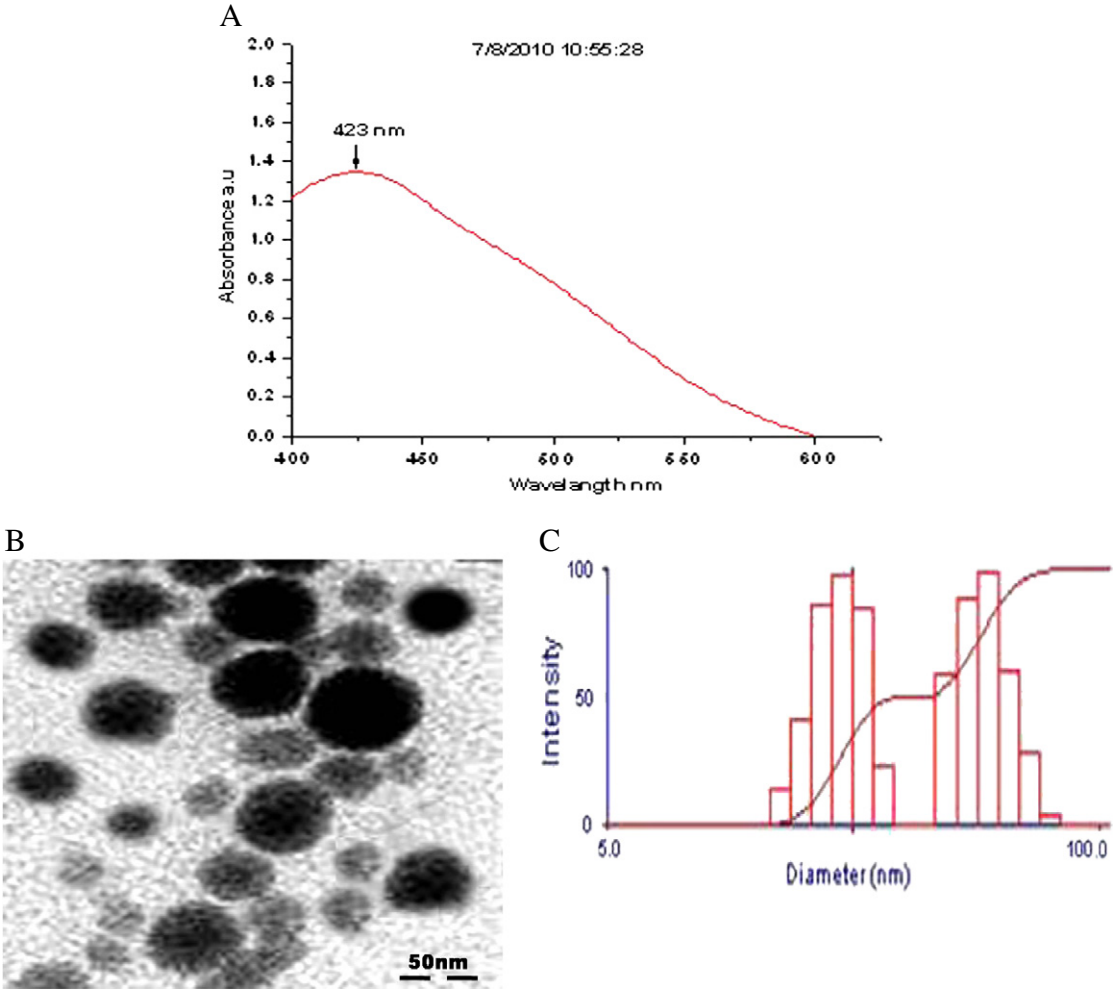

Fig. 1. Synthesized silver nanoparticles were characterized by UV–vis spectrum (A), Dynamic light scattering (B) and transmission electron microscope (C), images of spherical shaped silver nanoparticles.

cytotoxicity graph were presented as the arithmetic mean percent inhibition relative to the control standard error (SE). Significant differences between the mean values were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests (SPSS version 16, SPSS Inc., Chicago, IL, USA). A significance level of $p < 0.05$ was used for statistical testing.

3. Results

The formation of Ag-NPs was revealed by the UV–vis spectra. Silver nitrates were reduced with chitosan at 95 °C, and the typical UV–vis absorption spectra of the resulting solutions are shown in Fig. 1A. It is clear that spectra display the characteristic surface plasmon resonance (SPR) band of silver nanoparticles centered at about 423 nm, indicating the formation of silver nanoparticles. In addition, size and morphology of the nanoparticles was noted from the DLS and TEM analyses. The TEM images also indicate that the Ag-NPs had a narrow size distribution, with an average size of 50 nm (ranging within 40–60 nm) (Fig. 1B). The particles appeared to be spherical in shape. The DLS measurements show, in agreement with the TEM analysis, that there was a restricted size distribution of the Ag-NPs, ranging from 40.3 to 55.2 nm (Fig. 1C).

The physico-chemical features of the test water ($AgNO_3$ and Ag-NPs) were determined. The ranges of temperature, pH, DO, conductivity and total hardness were: 26.7 to 27.8 °C, 7.85–7.93, 4.5 to 5.8 mg/L, 240 to 298 μM/cm and 168–196 mg/L, respectively in $AgNO_3$ exposed tanks, whereas in Ag-NP exposed tanks, the ranges of temperature, pH, DO, conductivity and total hardness were: 26.5 to 27.3 °C, 7.2–7.5, 6.5–7.5 mg/L, 236–289 μM/cm and 155–170 mg/L, respectively.

The cumulative percentage survival in *C. catla* and *L. rohita* fishes exposed to different concentrations of $AgNO_3$ and Ag-NPs was determined and the results are presented in Fig. 2A and B. The toxic effect of $AgNO_3$ and Ag-NPs on the survival of fish was found to be concentration and time dependent. The LC_{50} values of $AgNO_3$ and Ag-NPs for *C. catla* and *L. rohita* were calculated at a 96-h exposure and LC_{50} was 3.60 μg/mL and 12.70 μg/mL for $AgNO_3$ and Ag-NPs respectively in *C. catla* and LC_{50} 4.01 μg/mL and 12.28 μg/mL $AgNO_3$ and Ag-NPs respectively in *L. rohita*.

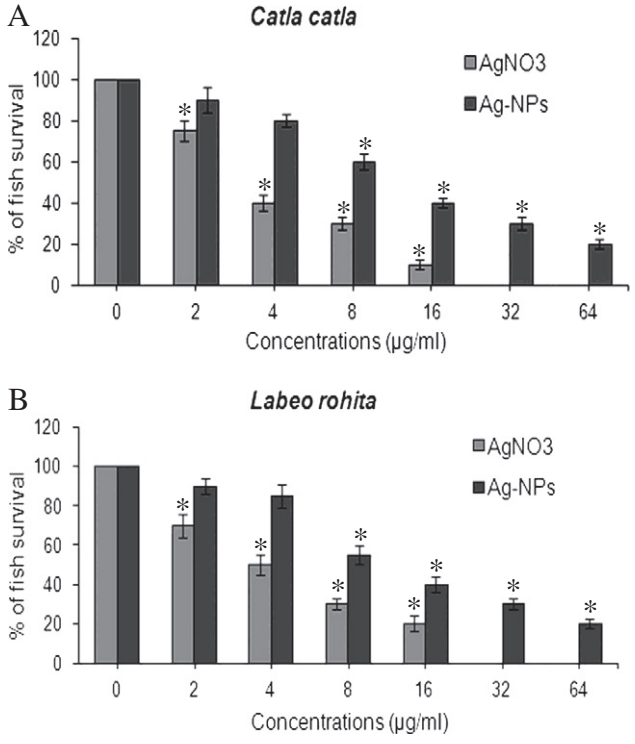

Fig. 2. Survival of fishes exposed to different concentrations of Ag-NPs after a 96-h exposure, A) *Catla calta* and B) *Labeo rohita*. The individual data points are expressed as the arithmetic mean percentage of control (mean ± SE) (n = 3 triplicate). *significantly different from control; $p < 0.05$.

SICH, ICG and LRG cell lines were exposed to $AgNO_3$ and Ag-NPs at different concentrations for 24 h and cytotoxicity was determined using MTT reduced and neutral red uptake (NR) assays. A total of six concentrations ranging from 2 to 64 μg/mL of $AgNO_3$ and Ag-NPs were used to carry out in vitro cytotoxicity assay in fish cell lines using two basal cytotoxicity end points and the results are shown in Fig. 3A–F. The lowest concentration of $AgNO_3$ (2 μg) was found to be toxic in all the cell lines with the effect being more pronounced in the ICG cell line, whereas the lowest concentration of Ag-NPs (2 μg) was not markedly cytotoxic to SICH, ICG and LRG cells, when compared to the control cells. The progressive increase in the concentration of $AgNO_3$ and Ag-NPs led to an increase in toxicity when compared to control. EC_{50} (95% confidence interval) values of $AgNO_3$ and Ag-NPs were calculated for SICH, ICG and LRG cells using MTT and NR assays and the results are given in Table 1. Three cell lines were tested for their sensitivity to $AgNO_3$ and Ag-NPs and the results revealed that ICG cell line was found to be slightly sensitive to $AgNO_3$ and Ag-NPs, followed by SICH and LRG cell lines in the two endpoints employed in the present study. Correlations among the endpoints employed in the three cell lines to study cytotoxicity of $AgNO_3$ and Ag-NPs have been determined. A general tendency in the sensitivity between these two endpoints could be observed and statistical analysis revealed good correlation with $R^2 = 0.91$ for all combinations between endpoints.

Linear correlations between each in vitro EC_{50} value and the in vivo LC_{50} data, of Ag-NPs were significant $p < 0.00$ l with $R^2 = 0.914$ and 0.928 for MTT_{50} and NR_{50}, respectively between *C. catla* vs. SICH cells (Fig. 4A and B); $p < 0.00$ l with $R^2 = 0.851$ and 0.945 for MTT_{50} and NR_{50}, respectively between *C. catla* vs. ICG cells (Fig. 4C and D); and $p < 0.001$ with $R^2 = 0.837$ and 0.951 for MTT_{50} and NR_{50}, respectively between *L. rohita* vs. LRG cells (Fig. 4E and F).

The SICH, ICG and LRG cells were incubated with 0, 2, 4, 8, 16, 32 and 64 μg/mL of Ag-NPs for 24 h, and then observed with optical microscope under 100× magnifications. SICH (Fig. 5A), ICG (Fig. 5C) and LRG (Fig. 5E) cell lines in the control (untreated) had a regular shape. The cells which were exposed to low concentration of Ag-NPs (2 μg/mL) appear similar to the control cells, indicating that lower concentration of Ag-NPs did not harm the SICH, ICG and LRG cells. With increasing concentrations of Ag-NPs, the cells started to detach, became less polyhedric, more fusiform, showed shrinkage, became rounded and finally irregular in shape. When the concentration reached 64 μg/mL, apoptosis, necrosis and decrease in number of cells were observed in SICH cells (Fig. 5B), ICG cells (Fig. 5D) and LRG cells (Fig. 5F).

The percentage of DNA damage, the score of damage and the cumulative tail length from 100 cells per sample (in triplicate) were measured in SICH, ICG and LRG cells exposed to different concentrations (0, 2, 4, 8, 16, 32 and 64 μg/mL) of Ag-NPs and the results are shown in Fig. 6. The percentage of DNA migration or tail DNA in SICH, ICG and LRG cells exposed to 2 μg/mL of Ag-NPs was estimated to be about 1.7%, 1.5% and 1.9%, respectively at a 24-h exposure, and Ag-NPs at the concentration of 64 μg/mL caused 9.8%, 8.4% and 10.7% of tail DNA migration in SICH, ICG and LRG cells, respectively (Fig. 6). Comet results of Ag-NPs-exposed SICH, ICG and LRG cells showed a concentration-dependent increase in tail DNA (%) compared to the control cells, which gave the extent of DNA damage.

The SICH, ICG and LRG cells were exposed to Ag-NPs for 24 h at different concentrations (0, 2, 4, 8, 16, 32 and 64 μg/mL). Nuclear staining was performed using 1 μg/mL Hoechst and the cells were observed under a fluorescence microscope. The results are shown in Fig. 7A–F. Apoptotic cells were identified by Hoechst staining of condensation and fragmentation of the nuclei as shown in SICH cells (Fig. 7D), ICG cells (Fig. 7E) and LRG cells (Fig. 7F) at higher concentration

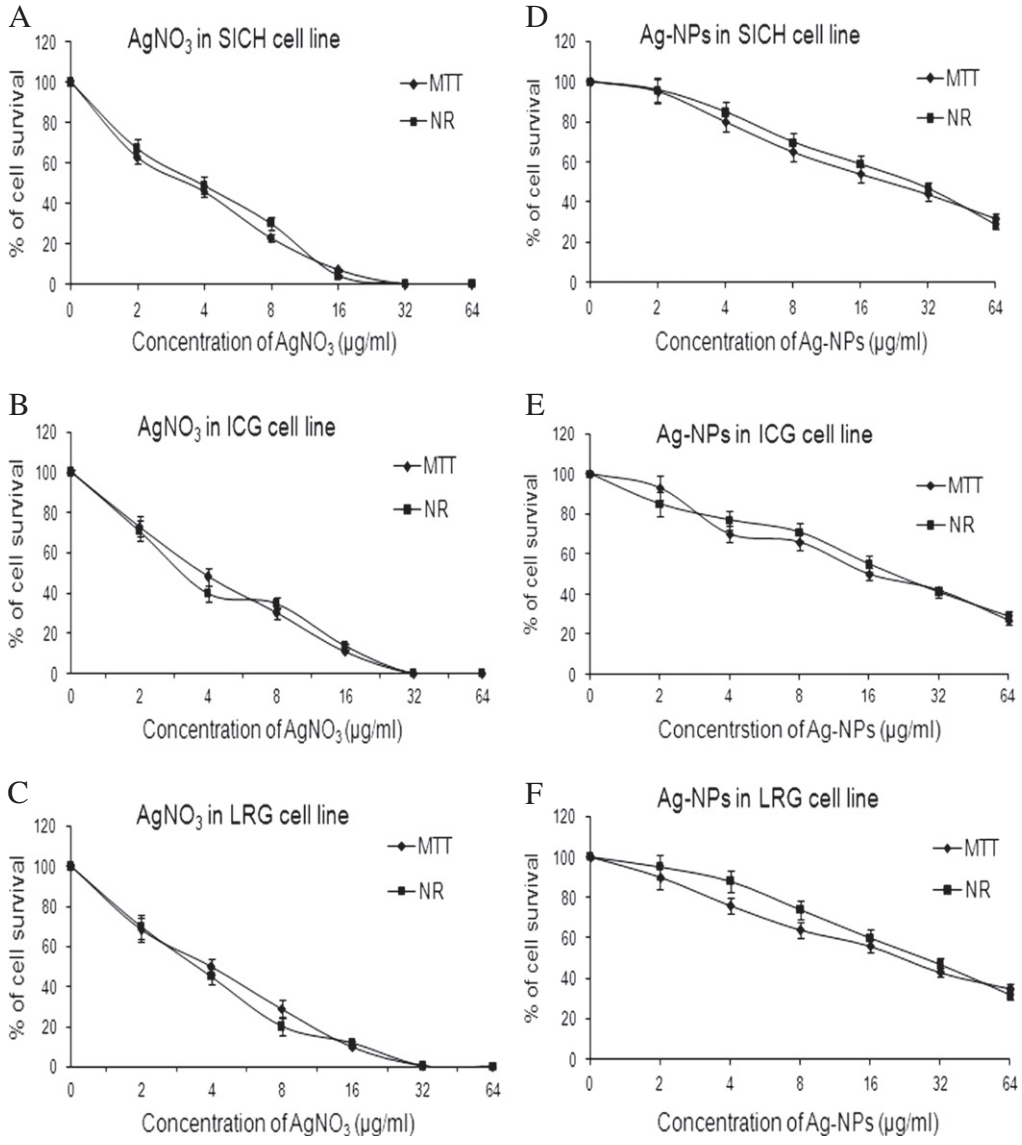

Fig. 3. In vitro cytotoxicity of $AgNO_3$ exposed to SICH (A), ICG (B) and LRG (C) cell lines, and Ag-NPs exposed to SICH (D), ICG (E) and LRG (F) after a 24-h exposure by MTT and NR assays. The individual data points are expressed as the arithmetic mean percentage of control (±SE) (n = 8).

i.e. 64 µg/mL of Ag-NPs exposed for 24 h, while no nuclear changes were observed in control cells are shown in SICH cells (Fig. 7A), ICG cells (Fig. 7B) and LRG cells (Fig. 7C).

The level of antioxidant parameters such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH) and lipid peroxidase (LPO) was measured in SICH, ICG and LRG cell lines exposed to different concentrations Ag-NPs and the results were shown in Fig. 8A–D. Regarding oxidative stress biomarkers, no appreciable change was observed in SOD, CAT, GSH and LPO levels in the SICH, ICG and LRG cells exposed to lower concentrations i.e. 2 µg/mL of Ag-NPs when compared to the control cells (Fig. 8A–D). However, when these cell lines were exposed to 64 µg/mL (higher concentration) of Ag-NPs, the activity of SOD (~2.5 folds, ~3.1 folds and ~3.7 folds decrease in SICH, ICG and LRG cells respectively), CAT (~2.1 folds, ~1.1 folds and ~2.6 folds decrease in SICH, ICG and LRG cells respectively) and level of GSH (~1.9 folds, ~2.2 folds and ~3.3 folds decrease in SICH, ICG and LRG cells respectively) was decreased. The decreased was found to be significant ($^*p < 005$) when compared to the control cell lines. The level of LPO was increased (~2.0 folds, ~3.4 folds and ~3.1 folds increase in SICH, ICG and LRG cells respectively). The level however increased significantly ($^*p < 0.05$) in Ag-NP exposed cells when compared to the control cell lines. Ag-NPs resulted in the dose-dependent reduction of SOD, CAT, GSH levels and LPO generation, which reflected the oxidative stress in the SICH, ICG and LRG cells.

Table 1
EC_{50} (±SE) values with 95% lower and upper confidence limits of three fish cell lines to $AgNO_3$ and Ag-NPs (µg/mL) measured by MTT reduction and NR uptake assay at a 24-h exposure.

Cell lines	Assays	$AgNO_3$	Ag-NPs
SICH	MTT	3.98 (3.30–4.80)	20.77 (15.14–29.37)
	NR	3.80 (3.17–4.86)	22.36 (17.24–32.04)
ICG	MTT	3.71 (2.98–4.62)	18.64 (14.25–24.38)
	NR	3.34 (2.60–4.30)	20.90 (18.70–30.55)
LRG	MTT	4.01 (3.20–5.01)	21.54 (15.53–32.72)
	NR	3.74 (3.06–4.56)	23.81 (21.14–36.59)

4. Discussion

In the present study, silver (Ag-NPs) nanoparticles were synthesized and characterized. Their effect on in vivo acute toxicity using fish (*C. catla* and *L. rohita*) was studied. In vitro cytotoxicity, genotoxicity

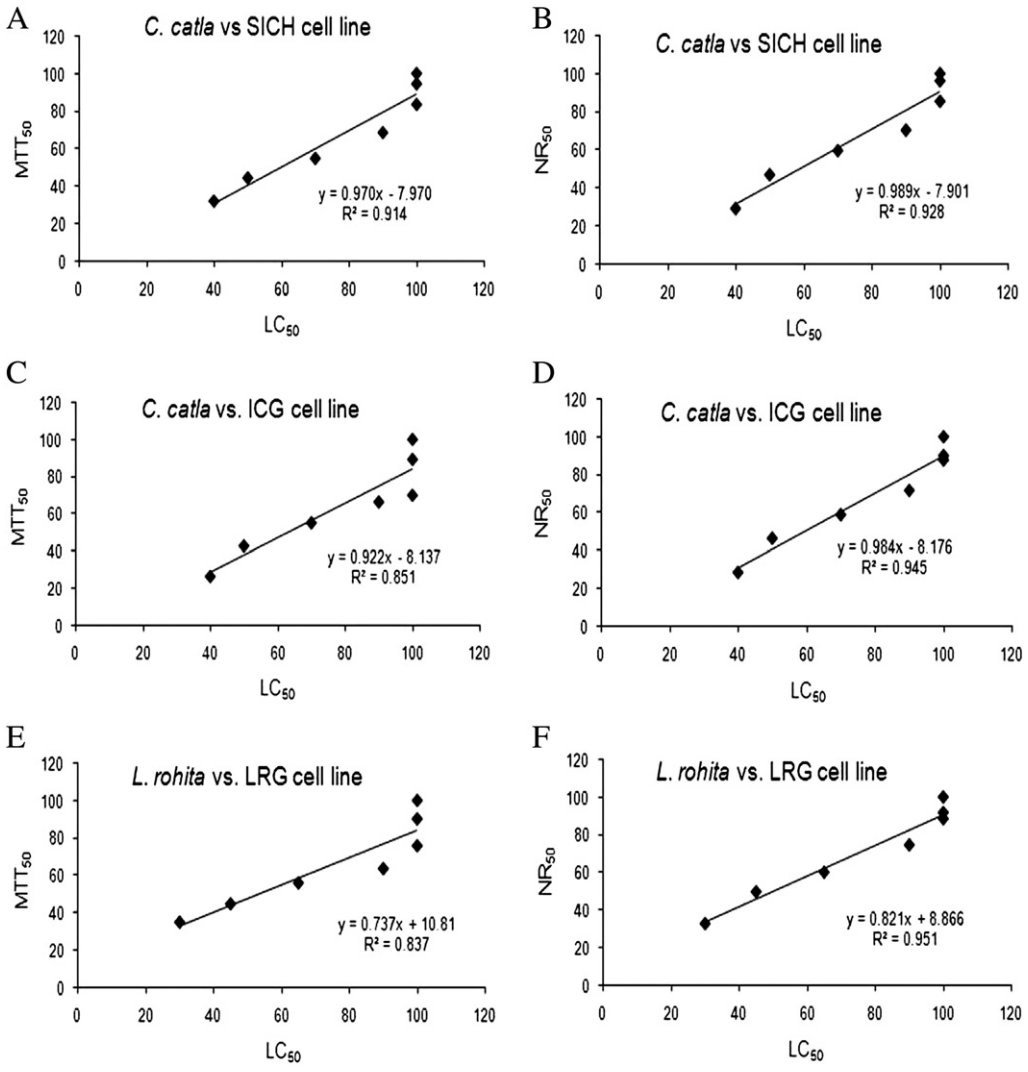

Fig. 4. Linear regression between MTT_{50} and NR_{50} (A), MTT_{50} and AB_{50} (B), NR_{50} and AB_{50} (C), MTT_{50} and CB_{50} (D), NR_{50} and CB_{50} (E), and AB_{50} and CB_{50} (F) for SICH, ICG and LRG cells exposed to Ag-NPs.

and oxidative stress were investigated using established fish cell lines (SICH, ICG and LRG) as a possible alternative tool for toxicological studies. Such studies on toxic effects of nanomaterials are very few and no clear guidelines are available to quantify these effects. Previous reports show that silver nanoparticles can be toxic to fish. Griffitt et al. (2008) reported that silver nanopowder (20–30 nm) induced a 48 h LC_{50} of 7.07–7.20 mg/L in juvenile zebrafish (*Danio rerio*) depending on whether the exposure was to an adult or juvenile fish. In a study on zebrafish, Choi et al. (2010) reported the 24-h LC_{50} to be 250 mg/L for silver nanoparticles (5–20 nm). Recently, Chae et al. (2009) reported that in Japanese medaka (*O. latipes*), the 96-hour LC_{50} was 34.6 μg/L for silver nanoparticles (50 nm). Accordingly, it is clear that silver nanoparticles of different sizes and with or without different stabilization agents show different degrees of toxicity in different organisms, under different exposure times and conditions. The LC_{50} values of Ag-NPs in the present study were determined as 12.70 μg/mL (or 12.70 mg/L) and 12.28 μg/mL (or 12.28 mg/L) for *C. catla* and *L. rohita*, respectively at a 96-h exposure. The silver ions were around 3.52 (*C. catla*) and 3.06 (*L. rohita*) times more toxic than the silver nanoparticles by mass of silver added to the experimental tanks. Bilberg et al. (2012) reported that silver ions exposed to zebrafish were approximately 3.4 times more toxic than the silver nanoparticles by mass of silver added to the tanks.

Cytotoxicity of $AgNO_3$ and Ag-NPs was evaluated by studying cellular morphology, metabolic inhibition (MTT assay) and lysosomal damage (NR uptake assay) under control and exposed conditions. A previous study, reported that silver and gold nanoparticles were highly cytotoxic to rainbow trout hepatocytes and gill cells as shown by a clear reduction of metabolic activity and membrane integrity (Farkas et al., 2010, 2011). In another study, Cerium oxide nanoparticles were tested for its cytotoxicity in H4IIE rat hepatoma cell line and the RTG-2 rainbow trout gonadal cell line by means of four standard (MTT, NR, LDH and total cellular protein assay) cytotoxicity assays (Rosenkranz et al., 2012). Ahamed et al. (2011) reported that MTT, NR and LDH assays could be used to study the cytotoxicity of Nickel ferrite nanoparticles in A549 cells. The neutral red uptake (NRU) and MTT assays were used to measure the cytotoxicity of TiO_2 nanoparticles in human amnion epithelial (WISH) cells (Saquib et al., 2012). In the present study, decreased mitochondrial function (MTT assay) and lysomal damage (NR assay) were observed in cells exposed to $AgNO_3$ and Ag-NPs (2–64 μg/mL) in a dose-dependent manner. Arora et al. (2008) reported similar results in decreased mitochondrial function in a dose-dependent manner in HT-1080 and A431 cells exposed to SNP (concentration range 3.12–50 μg/mL) by XTT assay. Our in vitro cytotoxicity results demonstrate that the cytotoxic effect of $AgNO_3$ and Ag-NPs in both the NR and MTT assays are basically the same and therefore, both cytotoxicity assays gave equal results. This is consistent with

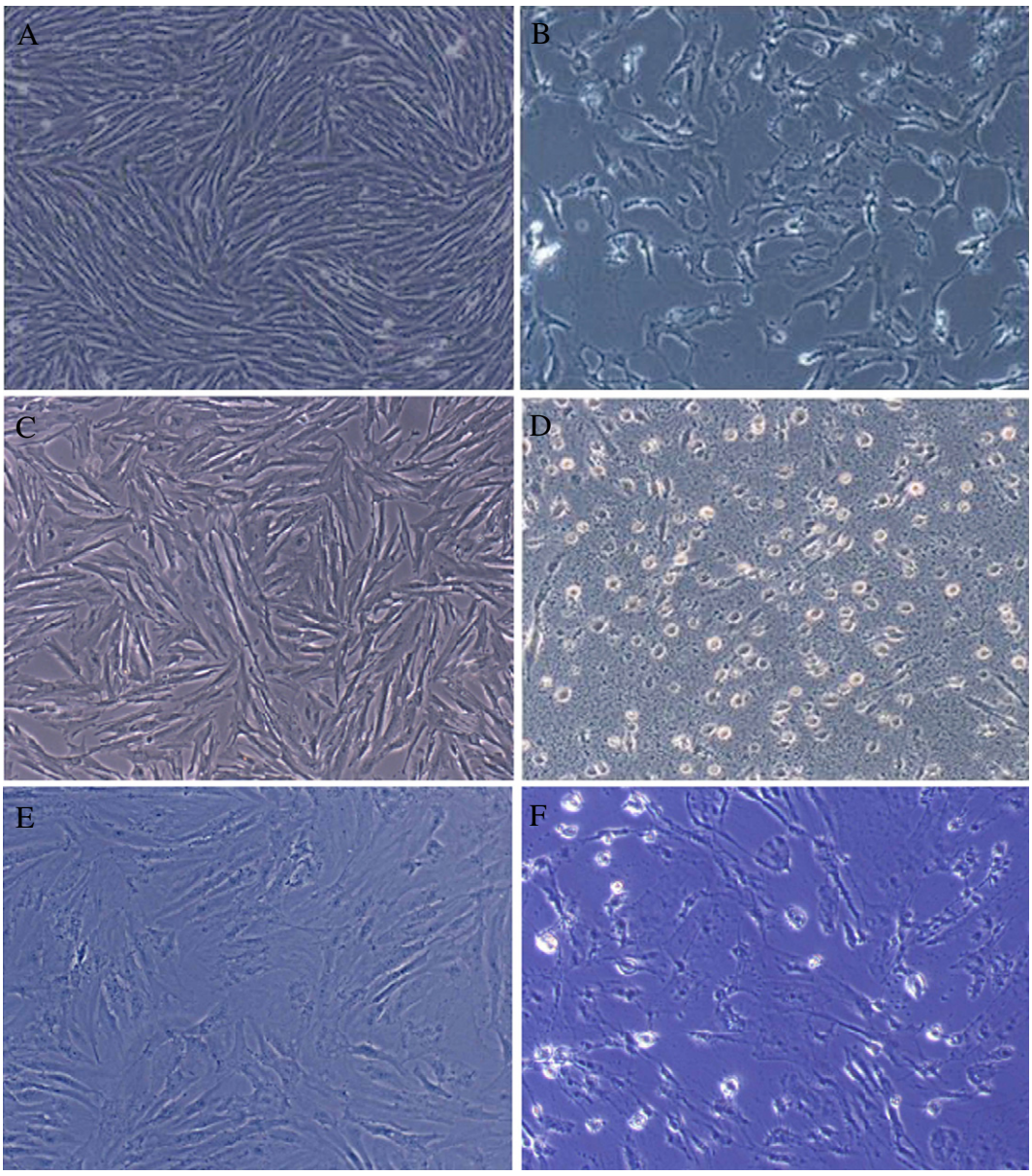

Fig. 5. Morphological alterations (cell detachment, fusiform, shrunken and round cells) in SICH (B), ICG (D) and LRG (F) cells exposed to high concentration (64 μg/mL) of Ag-NPs, while no morphological changes were observed in control SICH (A), ICG (C) and LRG (E) cells.

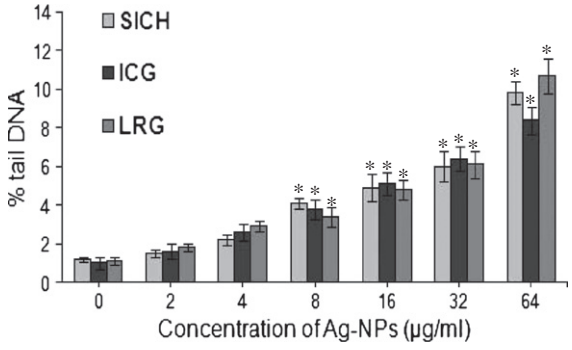

Fig. 6. Percentage of DNA damage in SICH, ICG and LRG fish cell lines cultured for 24 h and then exposed to various concentrations of Ag-NPs. Three slides were prepared per assay and 100 nuclei were counted per slide. Bars are means of SE. Results of the comet assay (*significantly different from control; $p < 0.05$).

previous studies, where the EC_{50} values in the NR and MTT assay were in the same order of magnitude for a series of toxicants (Caminada et al., 2006). A significant correlation between these two assays was ascertained by a regression equation and this closely represented the ideal straight line case of $y = x$, with y corresponding to the EC_{50} (MTT assay) and x corresponding to the EC_{50} (NR assay). The results of in vitro cytotoxicity assays revealed that the cytotoxicity in all the three cell lines were similar, although the ICG cell line was slightly sensitive than the SICH and LRG cell lines. Our results are consistent with the observation made by Caminada et al. (2006) on PLHC-1 and RTG-2 fish cell lines among which PLHC-1 was more sensitive than RTG-2. All the data in the current study taken together indicate that silver nanoparticles can be cytotoxic to fish cells as measured by MTT and NR an assay which indicates that these two assays are suitable for cytotoxic assessment.

In a number of cell lines and different fish species, good correlations between cytotoxicity in vitro and lethality in vivo have been reported (Fent, 2001; Na et al., 2009; Taju et al., 2012, 2013; Abdul Majeed

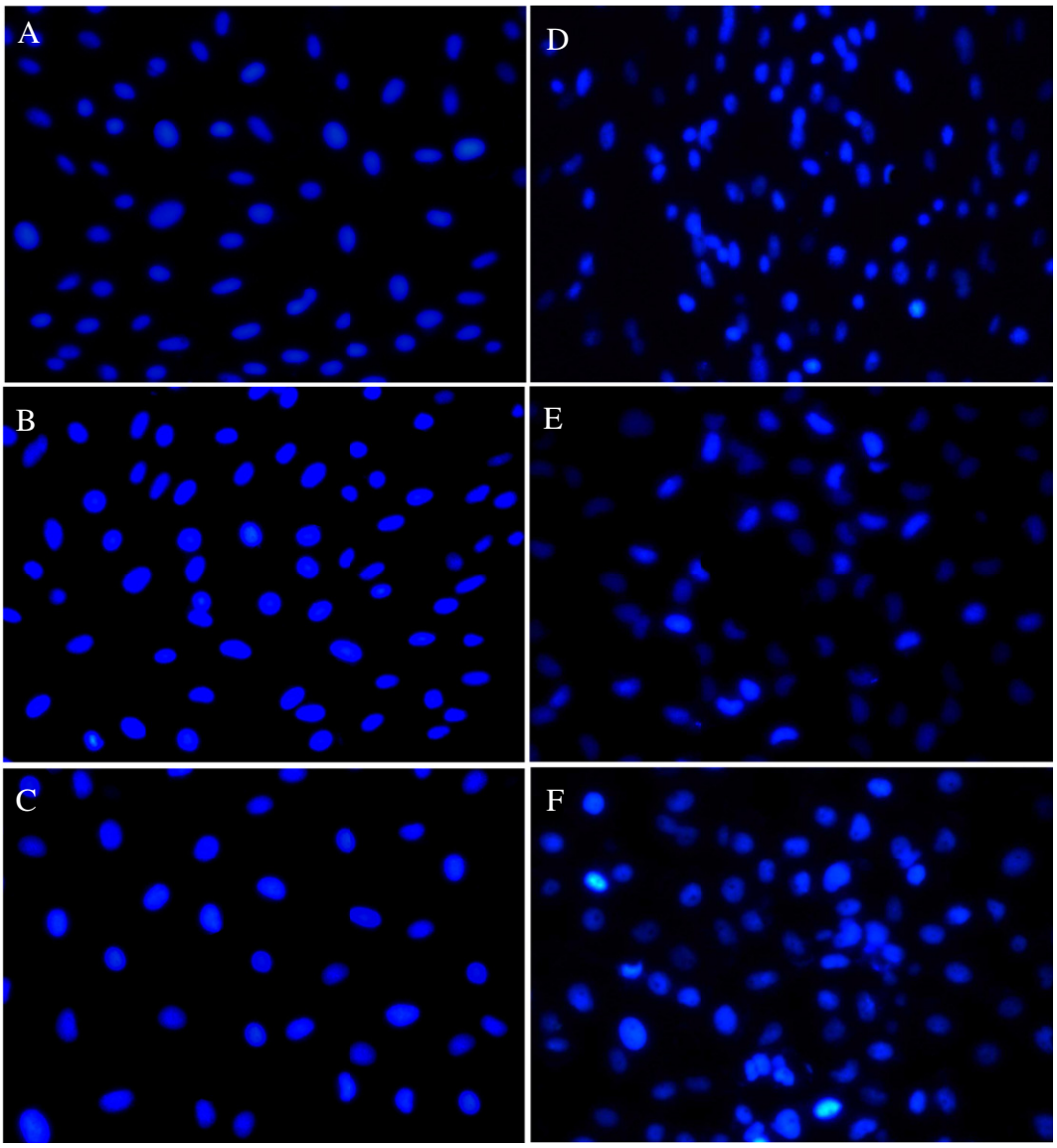

Fig. 7. SICH, ICG and LRG cells were exposed to Ag-NPs (0 and 64 μg/mL) for 24 h. Nuclear morphology staining was performed using 1 μg/mL Hoechst and cells were analyzed with a fluorescence microscope. Condensation and fragmented nucleus were observed in SICH (D), ICG (E) and LRG (F) cells those exposed to higher concentration of Ag-NPs whereas in no nucleus in control cells SICH (A), ICG (B) and LRG (C) cells.

et al., 2013, 2014). The results of the present study revealed that the two EC_{50} (Ag-NPs) values were closely correlated with whole fish LC_{50} (Ag-NPs) values and that the linear correlation between each in vitro parameter and the LC_{50} data were found to be highly significant. The results of in vitro assays using an SICH and ICG cells of *C. catla* and LRG cells of *L. rohita* were correlated with those obtained from in vivo assay using the same species of fishes (*C. catla* and *L. rohita*).

The comet assay is a widely used in vitro assay in fundamental research for assessing DNA damage and repair, in genotoxicity testing of novel chemicals and pharmaceuticals, environmental biomonitoring and human population monitoring (Speit and Rothfuss, 2012). In the present study, the comet assay showed how Ag-NPs can induce genotoxic damage in SICH, ICG and LRG cells. Our comet assay results compare favorably with recent studies on mammalian cells which have been used to investigate the genotoxic potential of Ag-NP nanoparticles at similar concentrations (Singh et al., 2010). Kim et al. (2013) conducted the comet and MN assays to confirm the genotoxicity of Ag-NPs in CHO-K1 cells, and reported a dose-dependent DNA breakage in the comet assay. In the current work, DNA damage, as measured by single cell gel electrophoresis (SCGE), was also dose-dependent and more prominent in the SICH, ICG and LRG cells.

Oxidative stress as a common mechanism for cell damage when exposed to nano and ultrafine particles is well documented (Nel et al., 2006; Yang et al., 2009). A possible role of oxidative stress in the induction and mediation of DNA damage and apoptosis has been documented (Simonian and Coyle, 1996). Nanoparticles may induce oxidative stress, leading to generation of free radicals and alteration in antioxidants, oxygen free radicals, the scavenging enzyme system, and lipid peroxidation. Several studies have demonstrated the role of oxidative stress in Ag-NPs exposed in vivo fish toxicity (Chae et al., 2009; Choi et al., 2010; Scown et al., 2010). Numerous reports have asserted the role played by oxidative stress in Ag-NP cytotoxicity (Hussain et al., 2005; Arora et al., 2008; Choi and Hu, 2008; AshaRani et al., 2009). Some forms of oxidative stress may impair the biomembrane as well as affect cell permeability and fluidity, leaving cells more susceptible to osmotic stress or decreasing nutrient uptake (Wiesner et al., 2006). To prevent impairment due to oxidative stress, primary antioxidant protection is provided by enzymes such as SOD, catalase (CAT), and GSH (non-enzyme). GSH is also an

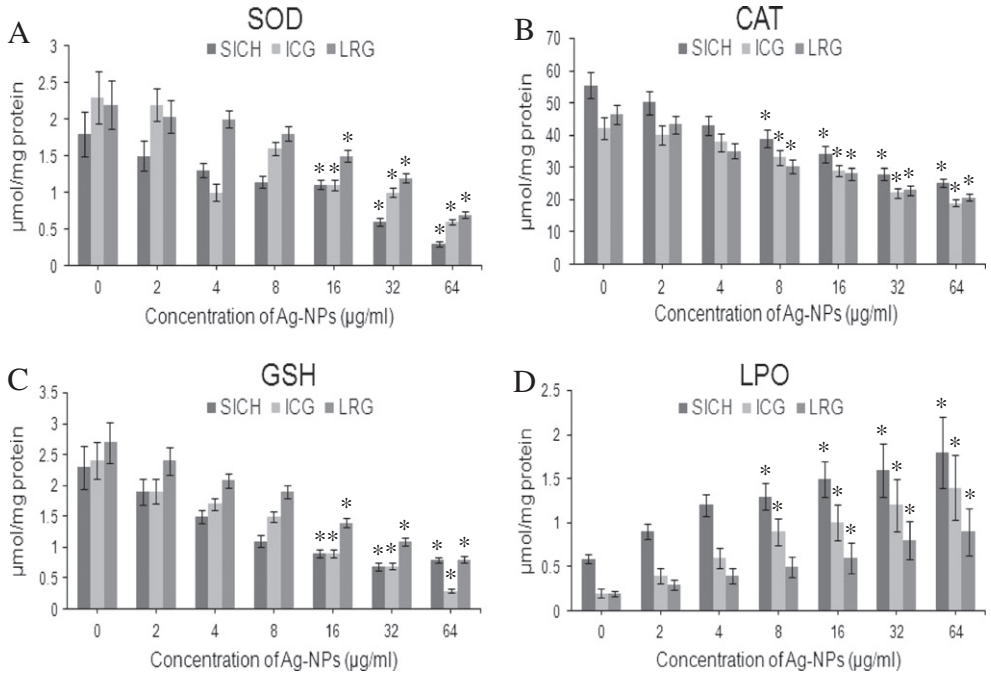

Fig. 8. Levels of SOD, catalase, GSH and lipid peroxidation in unexposed (control) and Ag-NPs exposed (2, 4, 8, 16, 32 and 64 µg/mL for 24 h) SICH, ICG and LRG cells. The data are expressed as mean ± standard error (S.E.) of three independent experiments. *significant change ($p < 0.05$).

important intracellular molecule that protects cells against endogenous and exogenous oxidative stresses (Freeman and Crapo, 1982).

Studies on rat liver derived cell line (BRL 3A) by Hussain et al. (2005) showed that there was a significant increase in ROS and decrease in GSH levels at 25 and 50 µg/mL of Ag (15, 100 nm). Arora et al. (2008) reported that silver nanoparticles induced oxidative stress as a mechanism(s) of toxicity which was assessed by depletion of SOD, CAT and GSH in HT-1080 and A431 cells and as well as increased lipid peroxidation (LPO). The depletion of GSH by oxidants may alter the redox status of the cell and present a stressful and toxic situation since GSH plays a critical role in maintaining cellular redox homeostasis. Alterations in GSH homeostasis can be monitored as an indication of functional-damage to cells. Oxidative stress is also known to increase the LPO level. In the current study, a significant GSH depletion in the three fish cell lines caused by Ag-NPs was observed early after exposure compared to the control cells. Such an effect has been demonstrated for Ag-NPs by in vitro tests (Hussain et al., 2005; Arora et al., 2008). The potentially decreased biological action of GSH-dependent enzymes is probably related to lipid peroxide control (Ferrari et al., 2008).

The superoxide dismutase and catalase system provides the first defense against oxygen toxicity. SOD is the enzyme which deals with oxyradicals and is responsible for catalyzing the dismutation of highly superoxide radical $O2^{-}$ to O_2 and H_2O_2 (Kappus, 1985), and CAT facilities to conversion of H_2O_2 to water and oxygen (Stanic et al., 2006). Wu and Zhou (2012) reported that the GSH levels in the tissues of liver, gills, and brain of the Ag-NPs treated medaka (*O. latipes*) significantly differed from the control values after 14 days of exposure and a dose-dependent GSH depletion was also observed in all tissues, whereas increased lipid peroxidase (LPO) concentrations in the liver and gill were observed, signifying dose-related responses. In the present study, the SOD and CAT activities in the SICH, ICG and LRG cells exposed to 2 µg/mL of Ag-NPs, showed no significant difference when compared with control cells, whereas increased concentration showed a significantly decreased SOD and CAT activities in a concentration dependent manner. This might be due to the synthesis of new enzymes or the enhancement of pre-existing enzyme levels under a lower concentration. A dose-dependent GSH depletion, SOD activities reduction and lipid peroxidation (MDA) generation, which reflected the oxidative stress in the PMEF cells exposed to four different types of nanoparticles were reported (Yang et al., 2009). The results of antioxidant parameters obtained from this study show significantly increased lipid peroxidation (LPO) and decreased GSH, SOD and CAT in SICH, ICG and LRG cells after exposure to increasing Ag-NPs in a concentration-dependent manner. Hence the significant depletion of GSH and marked elevation in LPO in the SICH, ICG and LRG cells exposed to Ag-NPs clearly suggest oxidative stress. On the whole, data obtained by us clearly suggest that oxidative stress is the cause of ensuing cytotoxicity and DNA damage in the case of Ag-NP exposed SICH, ICG and LRG cells. The present work is the first report that silver nanoparticles induce cytotoxic, genotoxic and oxidative stresses in SICH and ICG cells of *C. catla* and LRG cells of *L. rohita*.

5. Conclusions

In conclusion, chitosan based silver nanoparticles were synthesized and characterized by using UV–vis spectra, DLS and TEM analyses to assess the toxicity of Ag-NPs using SICH, ICG and LRG cells. Acute toxicity tests on fish were conducted by exposing *C. catla* and *L. rohita* for 96 h to $AgNO_3$ and Ag-NPs under static conditions. The results showed that mitochondrial function and lysosomal damage in SICH, ICG and LRG cells exposed to $AgNO_3$ and Ag-NPs were in a concentration-dependent manner. Linear correlations between each in vitro EC_{50} and the in vivo LC_{50} data were highly significant. Our results indicate that synthesized silver nanoparticles are less toxic when compared to $AgNO_3$ exposed fishes (*C. catla* and *L. rohita*) and fish cell lines. The microscopic observation demonstrated that cells exposed to Ag-NPs at higher concentration became rounded, more fusiform, showed shrinkage and acquired an irregular shape. Comet and Hoechst assays were found to be useful for the analysis of genotoxicity in Ag-NP exposed SICH, ICG and LRG cells. With increasing dose, DNA damage is more serious, and the migration distance is longer under the same electrophoresis conditions. The results of antioxidant parameters obtained show significantly increased lipid peroxidation (LPO) level and decreased levels of GSH, SOD and CAT in all the three cell lines after exposure to increasing Ag-NPs in a concentration-

dependent manner. In addition, the inverse correlation between cell viabilities decline vs. oxidative stress level elevation further proved that oxidative stress was probably the key route by which nanoparticles induced cytotoxicity. Our data are consistent with reports showing that Ag-NPs are not only cytotoxic and genotoxic but also induce oxidative stress to the three fish cells tested in a concentration-dependent manner. This indicates that the fish cell lines could be used as an alternative to whole animals not only for cytotoxicity tests and genotoxicity tests but also for studying oxidative stress when exposed to environmental contaminants such as nanomaterials and nanoparticles. The use of these cell lines as potential bio-indicator systems to monitor and detect other hazardous pollutants in the aquatic environment could become a reality in the near future.

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