Advances in Biotechnology

Chapter 5

A comparative in-vitro cytotoxicity study of biogenic and chemically synthesized metal (Ag and Au) nanoparticles

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Abstract

In the current scenario, the most frequently asked query regarding the metal nanoparticles is related to its toxic effects despite of their vast potential application in the field of health, pharmaceutical and medicine. Even though there are lots of studies carried out focusing on the toxicity of metal nanoparticles, a proper comparative study of biologically and chemically synthesized metal nanoparticles is lacking. The main aim of the study is a comparative morphological, biological and genotoxic studies of biogenic as well as chemically synthesized silver and gold nanoparticles on the cell line- African monkey kidney cells (Vero) and human spermatozoa. Chemically synthesized nanoparticles had significant cytotoxic activity leading to the cell death and the comet assay in the spermatozoa make us clear that it creates significant DNA damage too. The release of lactate dehydrogenase, nitric oxide and reactive oxygen species (ROS) after its exposure to the metal nanoparticles finally resulting damage to most biomolecules, including DNA and protein points to the effect of biogenic and chemically synthesized nanoparticles on different biological system.

Keywords: Toxicity; Metal nanoparticles; biologically synthesized; chemically synthesized

1. Introduction

Potential applications of metal nanoparticles due to their unique optical, thermal and antimicrobial properties made them the centre of attention during the recent years in the field of health, pharmacy, food and medicine industries. That being said, potential undesirable noxious effects of the same metal nanoparticles have been reported through many studies. Among the different metal nanoparticles, silver and gold were reported to be imperative because of their unique optical, thermal and antimicrobial properties with increased surface area and surface to volume ratio [1,2]

A different choice of biological systems like bacteria, fungi and plants were exploited and reported for the biosynthesis of silver and gold nanoparticles which theoretically claims that they are green synthesis and toxic free due to the biomolecules involved in the synthesis process. The toxicity of nanoparticles is mainly due to the hazardous chemicals involved in their synthesis process as reducing agents, which limits their usage. But the use of biological systems for the synthesis of nanoparticles which are comparatively green, offers several advantages such as ease in production, procedure, economical and environment friendliness. A proper study involving the comparison of chemically synthesized metal nanoparticles, biologically synthesized nanoparticles and their corresponding metal ion form is noble to counter such important queries relating the toxicity [3].

A comparative morphological, biological activity and genotoxicity study was carried out using myco-based as well as chemically synthesized silver and gold nanoparticles was carried out and analyzed. This is compared with corresponding metal in its ionic form which is considered to be more toxic. The IC50 value was calculated with respect to their cellular metabolic toxic activity using the African monkey kidney cells (Vero) and comet assay was performed to check the genotoxic activity of the silver and gold nanoparticles in human spermatozoa.

2. Materials and methods

2.1 Chemicals

Silver nitrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate heptahydrate, ammonium sulfate and Muller-Hinton agar were procured from SRL (Mumbai, India).

2.2 Source of microorganisms

The fungus Trichoderma atroviride was obtained from the Culture Collection of CAS in Botany, University of Madras, India and maintained on potato dextrose agar (HiMedia, Mumbai, India) slants at 27˚C.

2.3 Production of biomass

To prepare the biomass for biosynthesis of silver nanopartices, the fungus was grown aerobically in liquid broth containing dihydrogen potassium phosphate (7g/L), dipotassium hydrogen phosphate(2g/L),magnesium sulfate heptahydrate(0.1g/L), ammonium sulfate (1g/L), yeast extract (0.6g/L), and glucose (10g/L). This was incubated on an orbital shaker at 27°C at 150 rpm, and the biomass were harvested after 72h of growth by sieving through a plastic sieve, followed by extensive washing with sterile double distilled water [3].

2.4 Biosynthesis of metal nanoparticles

The fungal biomass (20g wet weight) was mixed with 100 mL of sterile double distilled water and agitated on an orbital shaker at 150 rpm for 48h at 27°C. After incubation, the cell filtrate was filtered through Whatman filter paper no. 1. To 100mL of cell filtrate in Erlenmeyer flask, $AgNO_3$ and $AuCl_4$ was added and kept undisturbed for 24 hours in dark conditions to get an overall ionic concentration (Ag+ and Au+) of 10^{-3}M [3].

2.5 Chemical synthesis of metal nanoparticles

Silver and gold nanoparticles were chemically synthesized using chemical reduction method according to Asta et al., (2006) [4] and McFarland et al., (2004) [5] respectively.

2.6 Characterization of metal nanoparticles

Surface plasmon resonance of silver and gold nanoparticles was characterized using a UV–Vis spectrophotometer (Cary 300 Conc spectrophotometer) at a resolution of 1nm from 250 to 800nm. For transmission electron microscopy (TEM), the sample was prepared by placing a drop of colloidal solution on a carbon-coated copper grid and setting a completely dried drop by vacuum desiccators. The image of the sample was obtained using a transmission electron microscope (JEOL 2000 FX MARK II) equipped with an EDX attachment. X-ray diffraction pattern of dry nanoparticles was obtained using Philips MDL PW 1050 diffractometer with CuKα radiation (λ=1.5406 Å with Ni filter). The FTIR spectrum of the sample was recorded by Perkin-Elmer Fourier transform infrared spectroscopy; the spectrum ranged from 2000 to 1000cm-1 at a resolution of 4 cm-1 by making a KBr pellet with metal nanoparticles.

2.7 Toxicity study of metal nanoparticles in cell lines

Effect of biological and chemically synthesized silver and gold nanoparticles on African monkey kidney cells (Vero) were assayed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) assay [6]. The half maximal inhibitory concentration (IC50) of nanoparticles and the positive controls were found out by the experiment and were compared.

2.7.1 Lactate dehydrogenase (LDH) assay

LDH activity was determined by the linear region of a pyruvate standard graph using regression analysis and expressed as percentage (%) leakage as described previously by Ulmer et al., (1956) [7]. The amount of LDH released was expressed in percentage.

2.7.2 Nitric oxide (NO) assay

The amount of nitrite was determined by the method of Stueher and Marletta (1989) [8]. The pink colour developed was measured at 540 nm in a microquant plate reader (Biotek Instruments).

2.8 Toxicity study of metal nanoparticles on spermatozoa

Semen sample was collected from a healthy donor in 3 days interval and was used for the study.

2.8.1 Assessment of semen vitality and morphology

The vitality and morphology of the semen sample was analyzed by staining using eosin-nigrosin method [9] and examined under optical microscope.

2.8.2 Staining method (Eosin & Nigrosin method)

Twenty five micro litres of semen sample was mixed with 2 drops of 0.5% eosin solution in microcentrifuge tube and waited for 10 seconds. To the above mixture added 2 drops of 10% nigrosine stain were added and mixed gently. From the above mixture thin smears were made using another clean edged glass slide. The slides were air dried and were used for examining the morphology of sperms under oil immersion (100x magnification). Dead sperms took eosin stain and live sperms remained white in colour. The sperm vitality was expressed by counting the percentage of live and dead sperms. A large number of live but immotile sperm cells may indicate an abnormality in the axoneme.

Biologically and chemically synthesized silver and gold nanoparticles, silver nitrate and tetra choloroauric acid was added to the semen sample and was observed under normal optical microscope in 30 and 60 minutes interval at 40x and 100x oil immersion to study the sperm vitality as well as the morphology of the sample.

2.8.3 Comet Assay

The comet assay or single cell gel electrophoresis (SCGE) is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells. DNA damage in the sperm suspension was analysed by following the methodology of Cabrita et al. (2005)

[10]. The lengths of migrated DNA (Comet tail) were measured using the CASP software and about 10-50 comets/point were scored.

2.8.4 Application of CASP software

The comets were analyzed using the CASP software. The images were used to estimate the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of total DNA in the tail.

2.9 Statistical analysis

All the experimental data obtained in the present study were subjected to statistical analysis. Statistical Package for the Social Studies (SPSS) software, Version 11.5 for Windows was used to perform all the statistical analysis. Limits of significance for all critical ranges were set at $P<0.05$. For post-hoc comparisons the Least Significant Difference test (LSD) was employed.

3. Results and discussion

3.1 Silver and sold nanoparticles

Silver and gold nanoparticles were synthesized biologically as well as chemically and were used in the study. The characterization of biogenic silver and gold nanoparticles is shown in figure 1 and 2.

3.2 Toxicity study of metal nanoparticles in cell lines

3.2.1 MTT assay

Cell proliferation analysis results by MTT assay using different concentrations of metal nanoparticles is shown in figure 3 and 4. Results revealed that the complexes could reduce the cell viability of kidney cells (Vero) in a dose dependent manner. For tetra chloroauric acid alone, IC50 value was found to be 5µg/mL, for chemically synthesized gold nanoparticles it was 40 µg/mL and for biologically synthesized gold nanoparticles 80 µg/mL was the obtained IC50 value.

Along with the experiments of silver nanoparticles, Silver nitrate (AgNO3) in the same concentration was used as positive control and IC50 values for silver nitrate was found to be 10µg/mL, for chemically synthesized silver nanoparticles it was 20 µg/mL; and for biologically synthesized silver nanoparticles IC50 value was calculated to be 30 µg/mL [3].

3.2.2 Release of lactate dehydrogenase (LDH)

LDH leakage is routinely used as an indicator of damage to plasma membrane integrity

and in assessing cytotoxic nature of a compound as dead cells release LDH into the culture medium. LDH is a cytosolic enzyme released into cell culture supernatant due to compromised membrane integrity, which is associated with cell death [11]. Figure 5 shows the percentage of LDH released from the vero cells after 48 hours of treatment with gold and silver nanoparticles respectively. There was a significant increase of LDH level in the culture supernatant of the cells that were treated with chemically synthesized nanoparticles compared to that of biologically synthesized. Nanoparticle induced cytotoxicity can be demonstrated by the increase in LDH leakage and associated loss of cell viability which may be due to the destabilization of membrane phospholipids.

3.2.3 Nitric oxide (NO) assay

The nitrite is the stable product of the nitric oxide released in response to oxidative stress. The amount of nitrite in the culture medium corresponds to the level of nitric oxide. Hence the level of nitrite was estimated to measure the nitric oxide produced after complex treatment [12]. Treatment of Vero cells with the nanoparticles resulted in enhanced Nitric oxide release than the untreated cells as shown in figure 6 for gold and silver nanoparticles respectively. Nitric oxide is a gaseous signalling molecule and a well-known, short-lived free radical which is produced non-enzymatically by iNOS and causes damage to most biomolecules, including DNA and protein.

Two major NOS-isoenzymes are present in cells: endothelial, membrane-bound, constitutively active NOS (eNOS), and cytoplasmic inducible NOS (iNOS) [13]. Previous studies showed that increased ROS generation lead to the activation of iNOS which in turn releases NO. RNS are by-products of Nitric oxide production in living cells. Up-regulated RNS production can cause cell damage or death, through nitration of biological target molecules such as DNA, lipids, and proteins [14]. Hence from the present study it is obvious that the nanoparticles could induce the Nitric oxide production in vero cells in which positive control ($AuCl_4$ and $AgNO_3$) and chemically synthesized gold and silver nanoparticles are being more potent in inducing Nitric oxide production. This confirmed that the biologically synthesized forms are less cytotoxic than other synthesized forms.

3.3 Toxicity study of metal nanoparticles in spermatozoa

3.3.1 Assessment of semen vitality and morphology

The vitality of the sperms was examined using eosin stain through optical microscope. Eosin could penetrate dead sperms and it did not occur in live sperms. This was taken as an indication to differentiate between live and dead sperm cells. Spermatozoa were found to be more damaged in positive controls, where $AgNO_3$ and $AuCl_4$ were used, than in chemically synthesized and least in biologically synthesized metal nanoparticles (figure 7a & b). The per-

centage cell death rate of the spermatozoa after 30 and 60 minutes of exposure is illustrated in figure 8.

In case of normal spermatozoa, they are oval in shape with a smooth contour and an acrosome occupying 40-70% of the head having straight midpiece and tail. The abnormalities or differences are noticed in the head, midpiece, tail and acrosome cap. In the case of control and biologically synthesized silver and gold nanoparticles treated cells, the morphology of the cells was found to be normal. In the case of $AgNO_3$ and $AuCl_4$ treatments, most of the cells had taken eosin dye inside the cells, which confirmed the damage of the cell membrane. Many of the cells were devoid of the midpiece and tail due to effect of the chemically synthesized nanoparticles and metal ions. In many of the cells treated with chemically synthesized nanoparticles and metal ions, the damage of the cells was obvious with the intracellular matter coming outside which confirmed their cytotoxic effect.

3.3.2 Comet assay

Cells were analysed with the comet assay to measure DNA damage caused by the silver and gold nanoparticles and a comparative study under florescent microscope was carried out as shown in figure 9. The control DNA (untreated) was observed as round in shape, confirms an unaffected DNA, where as other DNA images shows significant damage and tailing of DNA. The detailed data obtained from the comet assay with the help of CASP software has been presented in table 1. The head and tail DNA damage was found to be higher in the case of silver and gold ions treated than in silver nanoparticles synthesized by chemical method and the least damage in biologically synthesized silver and gold nanoparticles.

As per the previous studies and findings, many engineered nanoparticles including gold and silver have been found to have genotoxic effects, such as DNA-strand breaks, point mutations and oxidative DNA adducts [15,16,17]. In our previous studies with different concentrations of biologically and chemically synthesized particles against fresh water fish, it was demonstrated the effect of metal nanoparticles in different tissues like muscle, liver and kidney in comparison with the intensity stress proteins (HSP 70) released after the exposure to nanoparticles [3]. Nanoparticles easily cross the nuclear membrane and they can therefore interact with DNA directly or indirectly even though the exact mechanism for this interaction is not well known. Some of these alterations or damage to DNA, when occurring in spermatozoa may cause spermatogenic defects that could eventually result in trans-generational defects and the genomic stability of sperm cells [18].

When the biological system is challenged with metal ions or nanoparticles, the chance of forming Reactive Oxygen Species (ROS) is more. Even though several metabolic processes may use ROS in a good way, many participate in an oxidative burst and act not only as direct

protecting agent against stress, but also as signals activating further reactions. Generally a biological system try to keep the concentration of ROS at the possible low level because they are more reactive than molecular oxygen (O_2) [19] and they react with almost every organic constituent of the living cell. The high reactivity of ROS is based on the specificity of their electronic configuration. ROSs is known to damage cellular membranes by inducing lipid peroxidation [20]. They also can damage DNA, proteins and lipids [21] leading to cell death, damage and viability. It may be concluded that a similar process had happen when the cells were exposed to nanoparticles leading to cell death.

4. Conclusion

A detailed comparative morphological, biological, and genotoxicity study of biogenic metal nanoparticles with chemically synthesized metal nanoparticles was carried out since a cost effective existing competitive alternatives for biologically synthesized metal nanoparticles are chemically synthesized ones. Regarding the half maximal inhibitory concentration, chemically synthesized nanoparticles have IC50 value less than that of their biogenic nanoparticles In the comparative genotoxicity study on the spermatozoa, the chemically synthesized metal nanoparticles caused more cell damage and DNA damage which was confirmed by Comet assay. The overall findings from LDH assay, nitric oxide assay, the photomicrographs and comet assay points to the effect of nanoparticles on different cells and it has been observed that the chemically synthesized nanoparticles could vigorously induce cell death, damage and viability more significantly than their biological counterparts.

5. Figures

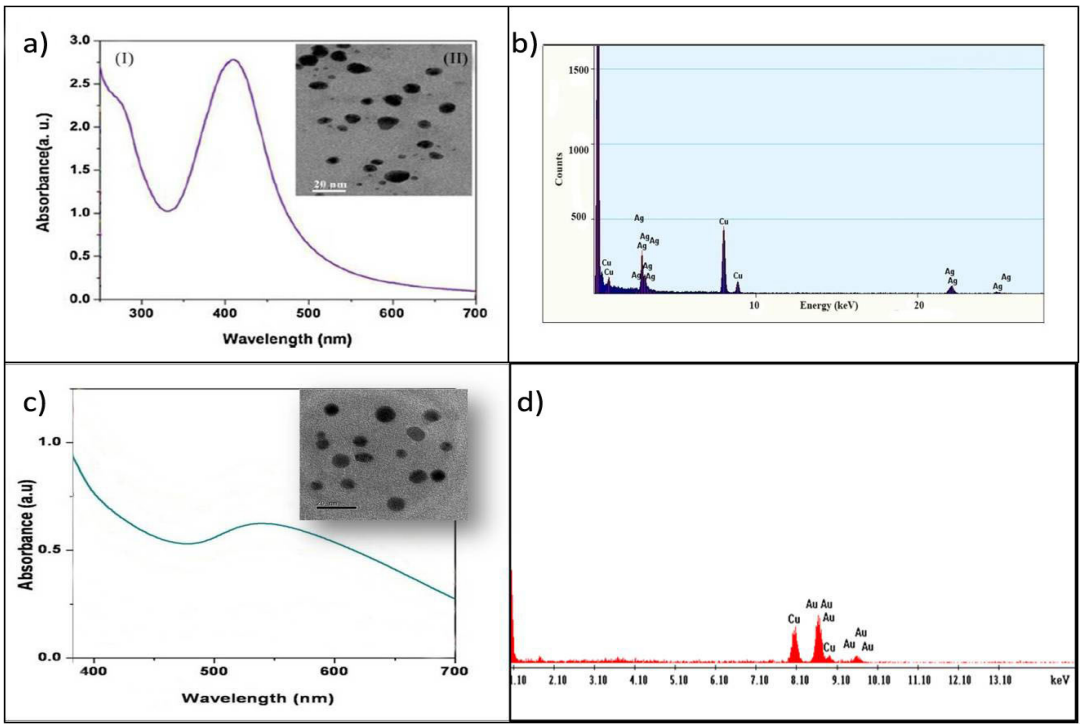

Figure 1: a. i) UV-Vis spectra recorded after the reaction of 1 mM AgNO3 solution with culture filtrate of fungal biomass. ii) TEM micrograph of biogenic silver nanoparticles

b. EDAX spectrum of silver nanoparticles

c. i) UV-Vis spectra recorded after the reaction of 1 mM AuCl4 solution with culture filtrate of fungal biomass. ii) TEM micrograph of biogenic gold nanoparticles

d. EDAX spectrum of gold nanoparticles

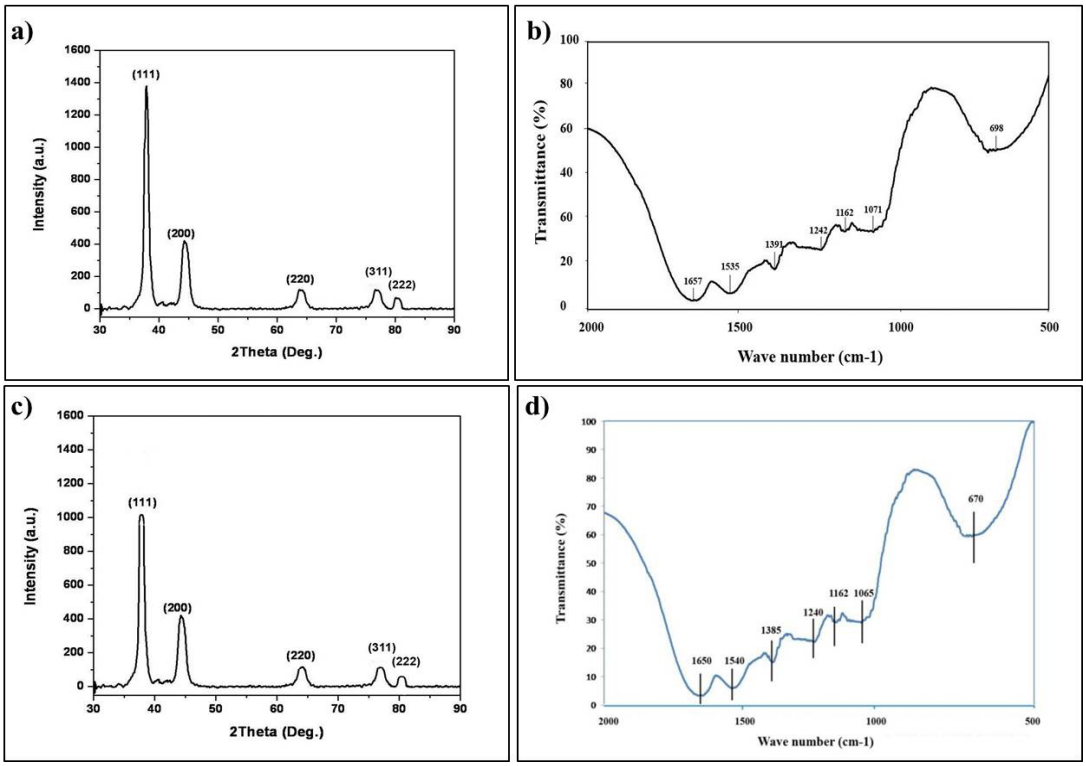

Figure 2: a) XRD pattern and b) FTIR spectrum of biogenic silver nanoparticles. c) XRD pattern and d) FTIR spectrum of biogenic gold nanoparticles

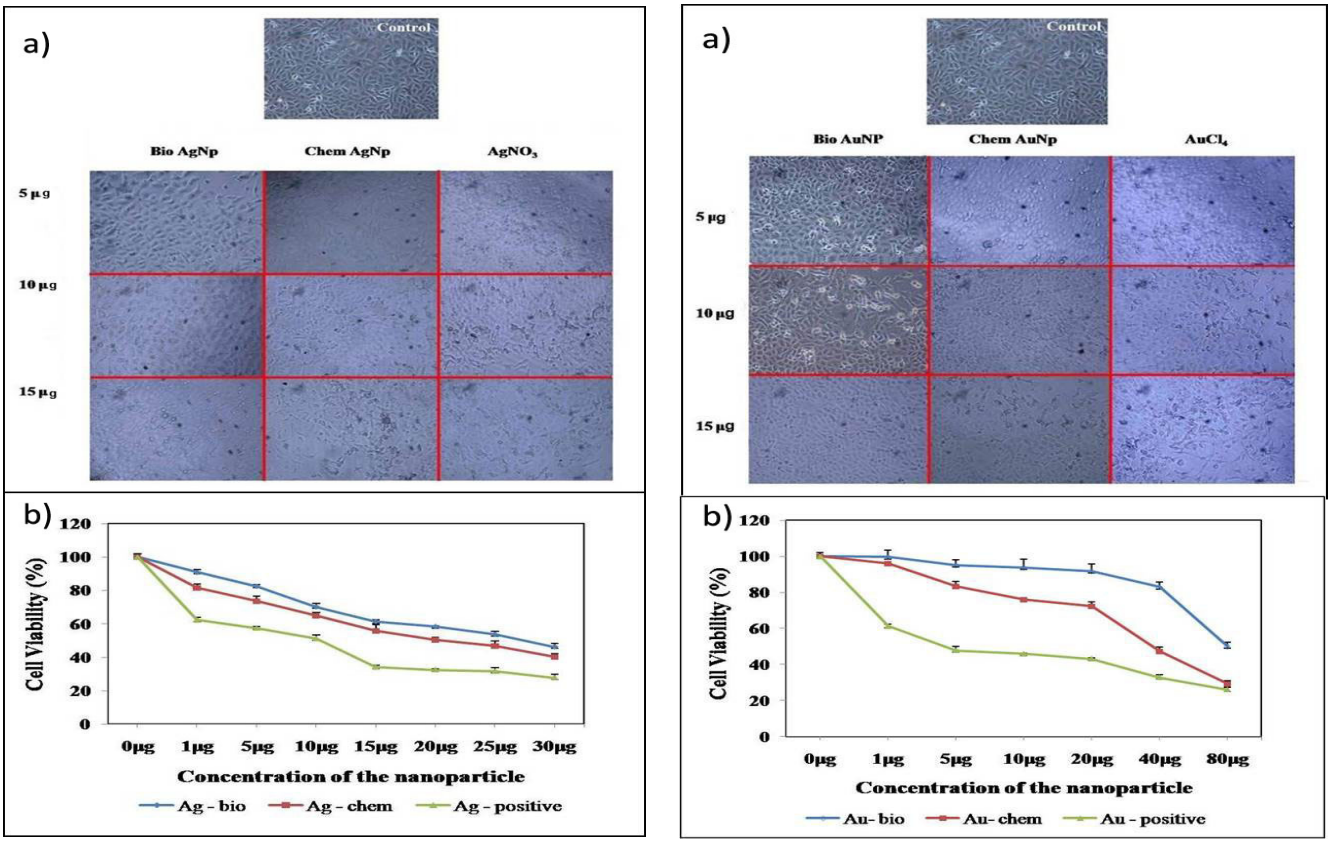

Figure 3: a) Optical microscope view and b) MTT assay performed on African monkey kidney cells (Vero) and exposed to different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

Figure 4: a) Optical microscope view and b) MTT assay performed on African monkey kidney cells (Vero) and exposed to different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid

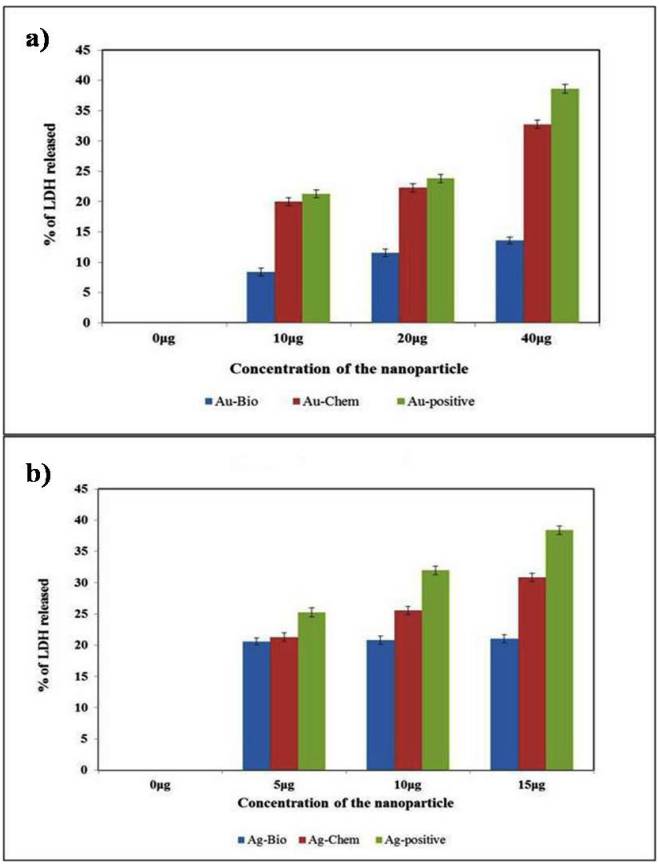

Figure 5: Lactate dehydrogenase assay performed on cell lines (a) with different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid (b) with different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

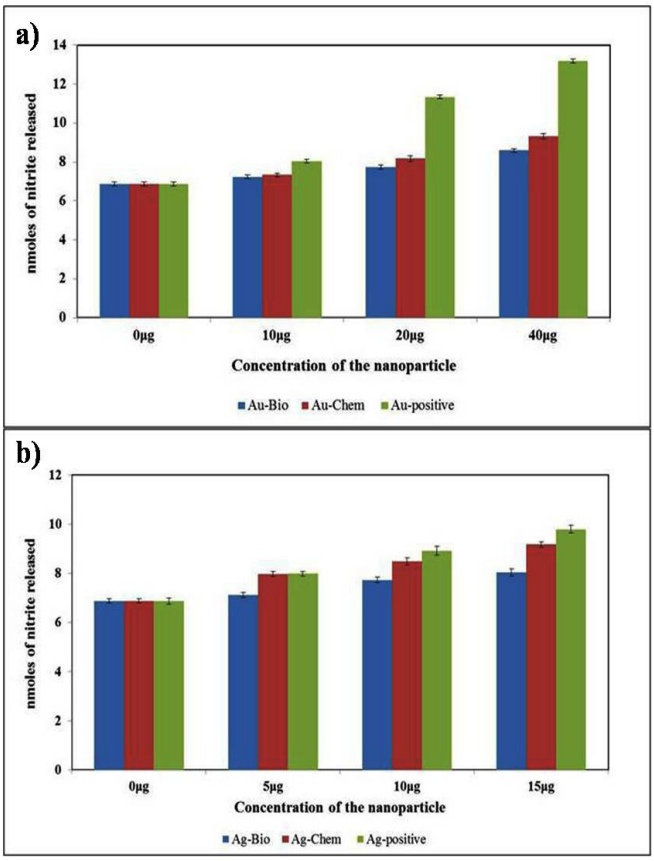

Figure 6: Nitric oxide assay performed on cell lines (a) with different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid (b) with different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

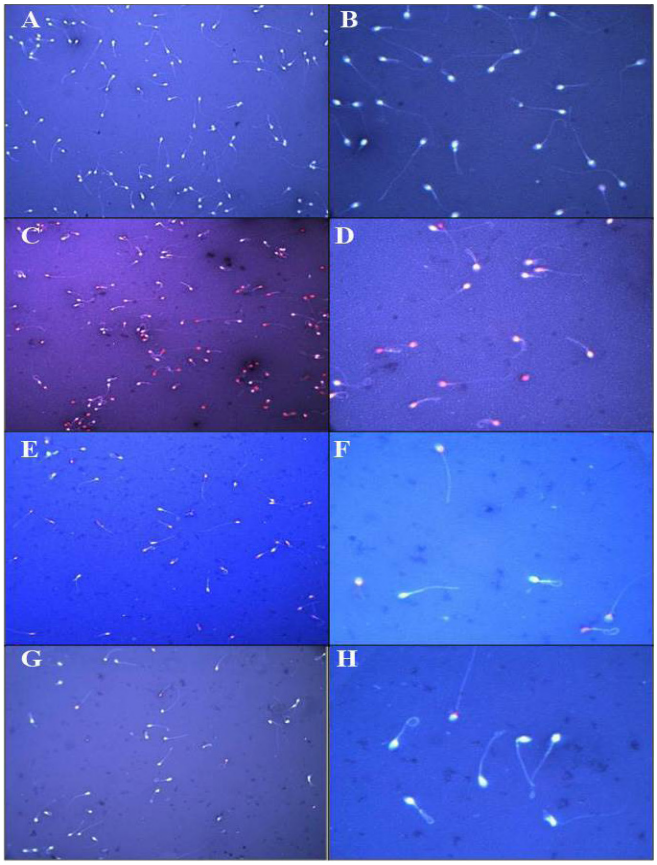

Figure 7a: Effect of silver nanoparticles on human spermatozoa after 30 min of incubation. Control cells after 30 min at (A) 40x and (B) 100x magnification, Cells treated with silver nitrate at (C) 40xand (D) 100x magnification, Cells treated with chemically synthesized silver nanoparticles at (E) 40x and (F) 100x magnification and Cells treated with biologically synthesized silver nanoparticles at (G) 40x and (H) 100x magnification

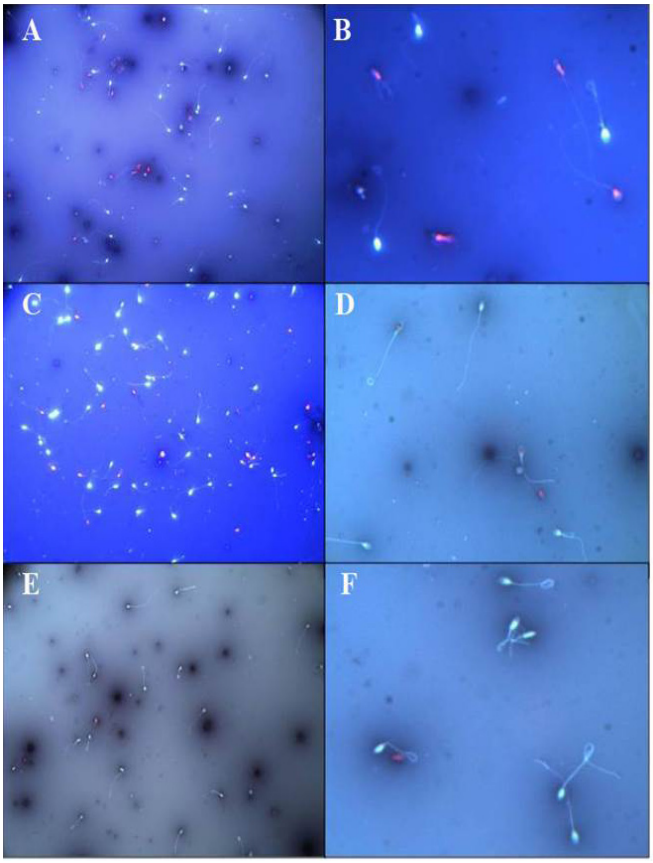

Figure 7b: Effect of gold nanoparticles on human spermatozoa after 30 min of incubation. Cells treated with gold chloride at (A) 40x and (B) 100x magnification, Cells treated with chemically synthesized gold nanoparticles at (C) 40x and (D) 100x magnification and Cells treated with biologically synthesized silver nanoparticles at (E) 40x and (F) 100x magnification

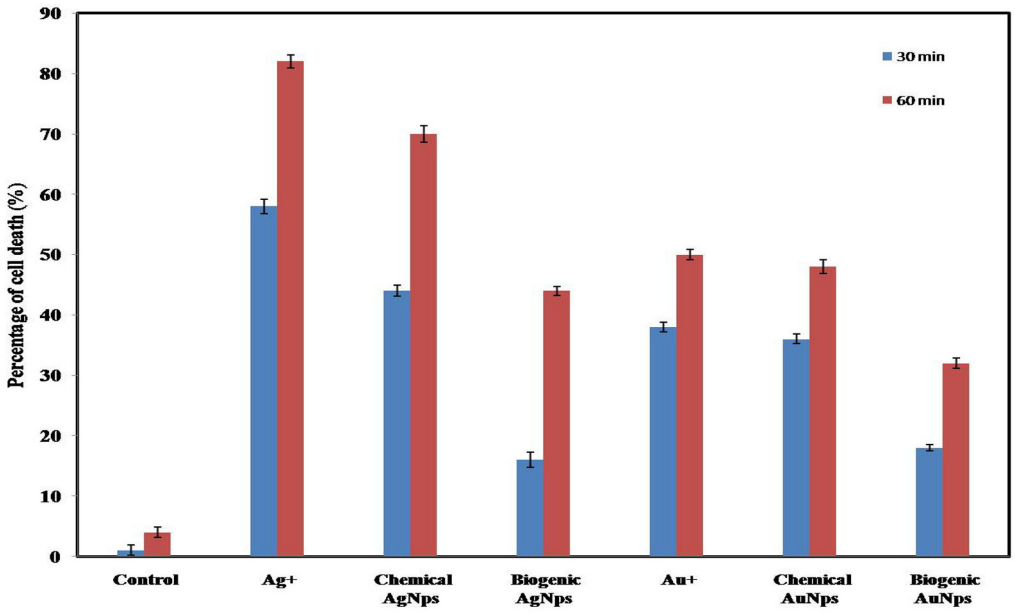

Figure 8: Percentage cell death rate of spermatozoa after 30 minutes and 60 minutes of exposure to different treatment

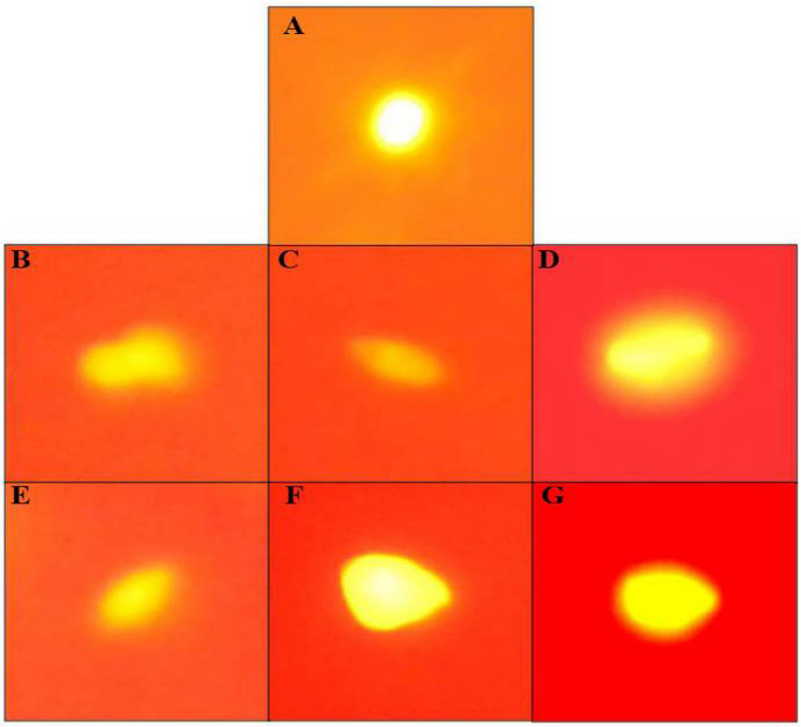

Figure 9: Fluorescent microscope view (100x) after Comet assay on human spermatozoa after different treatment with silver and gold nanoparticles

(A) Control, (B) Silver nitrate treated, (C) Chemically synthesized silver nanoparticles treated, (D) Biologically synthesized silver nanoparticles treated, (E) Tetra chloroauric acid treated, (F) Chemically synthesized gold nanoparticles treated and (G) Biologically synthesized gold nanoparticles treated

6. Table

Table 1: Comet assay analysis of human spermatozoa using CASP software

Treatment	Head DNA	Tail DNA	Tail Movement
CONTROL	99.956	0.044	0.001
$AgNO_3$	85.53	14.47	3.471
AgNPs (Biological)	90.83	9.17	1.375
AgNPs (Chemical)	87.55	12.45	1.493
$AUCl_4$	90.789	9.202	1.288
AuNPs (Biological)	96.848	3.152	0.347
AuNPs (Chemical)	96.199	3.801	0.342

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