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Research Article

GENOTOXICITY ASSESSMENT OF LOW CONCENTRATION AGNPS TO HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

Silver nanoparticles (AgNPs) are extensively used in most of the consumer products. To study the genotoxic effects of AgNP to human peripheral blood lymphocytes at lower concentrations is the objecqtive of the present study. We studied the genotoxic effect by lymphocyte proliferation assay and hemolysis assay. Metal ion analysis was also done to check the uptake of nanoparticles in the cell pellet of human peripheral blood cells to ensure the generation of ROS, a possible mechanism by which the DNA damage could happen. The results indicate that the aberrant cell damage for the exposure period of 24hrs was found to be 0, 8 and 24% for 5, 15 and 25µg/mL of AgNPs respectively. Interestingly, cytotoxicity studies on erythrocytes also reveal the toxic effect of silver nanoparticles with membrane damage of the lipo-polysaccharide layer through haemolysis assay. Here we report that AgNPs exhibit geno-toxicity in human peripheral blood lymphocytes through the fragmentation of DNA and other chromosomal aberrations. Ag^+ ion and AgNP concentration were analysed at 15µg/mL of AgNP by Atomic absorption spectroscopy (AAS) and was estimated to be 0.725±0.02 µg/mL and 1.859±0.03 µg/mL in culture medium and cell pellet respectively. Taken together, silver nanoparticles are found to be cytotoxic and genotoxic even at a lower concentration such as 1.8µg/mL.

Keywords: Silver nanoparticles, Silver ions, Genotoxicity, Human peripheral blood lymphocytes, Chromosomal aberrations, DNA damage, Haemolysis

INTRODUCTION

Nano-geno-toxicology is an emerging field in which there are only few studies so far conducted [1, 2]. There is a rapid development in the field of nanotechnology and it has resulted in a vast array of nanoparticles with varying size, shape, surface charge chemistry, coating and solubility behaviour. Nanoparticles are defined as particles less than 100 nm in one dimension at atomic, molecular and macromolecular scales [3, 4]. The nanoparticle differs from its own bulk form in its physical properties [5, 6] and more toxic than its bulk form [7, 8]. Silver was the second most referenced (25 products) in various medical and general products due to its antibacterial properties followed by silica (14), titanium dioxide (8), zinc oxide (8), and cerium oxide (1). [9]. Silver nanoparticles have been well studied of its antimicrobial properties and used increasingly in many consumer products such as deodorants, clothing materials, bandages, cleaning solutions, sprays, biosensors, antimicrobial agents, cosmetics, therapeutic agents, biomaterials, house-hold products [10,11,12,13]. Over usage of silver nanoparticles on the other hand, also had created negative impact to human and non-human biota [14]. In addition to the general toxic properties of nanomaterials, the knowledge of the possible interactions with DNA becomes essential, given the importance of the effects of genetic damage in human health. Thus, genotoxic effects are intimately related to the incidence of cancer and other health effects, such as infertility, aging, atherosclerosis and the occurrence of genetic disorders in subsequent generations, when germ cells have been affected. For all these reasons, extensive studies on the genotoxicity of nanomaterials are absolutely necessary [2]. Nanotechnology has its own exceptional therapeutic and pharmaceutical properties [15, 16]. The published research on environmental risk assessments and toxicological profile of nanoparticles as such is therefore very limited [17].

Silver nanoparticles (AgNPs) are also highly toxic to mammalian cells even in its bulk form and extremely toxic to bacteria, fungi, algae, fish, and certain plants etc. The toxicity of a metal nanoparticle is influenced by several factors like solubility, binding specificity to a biological site, etc. Many reports have been published on the fact that silver nanoparticles are toxic to human cells which has clarified the properties required for polymers that resist bacterial colonisation for use in medical devices. The increase in antibiotic-resistant microorganisms has prompted interest in the use of silver as an antimicrobial agent [18, 19]. Jeong and co-workers have identified potential harmful effects of silver nanoparticles (Ag NPs) on human health and a comprehensive toxicity assay was conducted on human Jurkat T cells, using oxidative stress-related endpoints [20]. Sathya and co-workers have reported on structural and numerical chromosomal alterations induced by metal and metal oxide nanoparticles performed on *in vitro* chromosomal and bacterial assays [21].

As a preliminary evaluation of toxicity of nanoparticles, in the present study we investigated the genotoxicity of silver nanoparticles at a very lower concentration to human peripheral blood lymphocyte cultures and the chromosome analysis was done at the metaphase stage to check for the DNA damage and aberration. The effects of metal ion (Ag^+) leaching on DNA damage and chromosomal aberrations were further identified to ensure the generation of ROS which could be the underlying mechanism of action of DNA damage.

MATERIALS AND METHODS

Chemicals

99.5% trace metals basis silver nanoparticles (CAS Number: 7440-22-4) diameter of approximately <100 nm (primary size) was obtained from Sigma Aldrich, USA. The physical characterization of AgNPs: surface area 5.0 m^2g^{-1}, density 10.49 g/cm^3. RPMI-1640 medium, Fetal bovine serum (FBS), Penicillin and Streptomycin were purchased from GIBCO. Phyto-heamagglutinin (PHA), colchicine, Hypotonic solution (KCl), Fixative (Methanol: Acetic acid: 3:1) were of analytical grade. All other chemicals used were of the highest purity available from commercial sources.

Nanoparticle characterization

AgNPs were dispersed in deionised water (Milli-Q) by means of using ultrasonic vibrations at 40% amplitude (Sonics, Vibracell 130W, 20 kHz, USA) for 5-10 minutes to get three different concentrations of 5, 15, 25µg/ml. These concentrations were determined from the literature survey as 25µg/ml was reported as toxic by Asharani et al, 2009 [23]. AgNP solutions were characterized (200 – 700 nm) using UV-Vis spectrophotometer for its plasmon resonance peak (UV-Vis Doublebeam spectrophotometer, Systronics 2201, India). Dynamic Light Scattering measurements were done for the particle size

distribution (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA).

Toxicity studies

Lymphocyte culture

Chromosome spreads were made from PHA stimulated human peripheral blood lymphocytes.

Culture medium

RPMI-1640 supplemented with Fetal Bovine Serum (10% v/v), 1mM glutamine and 2mM $NaHCO_3$ was used for the culture of whole blood cells. PHA served as the mitogen for stimulating the lymphocytes to enter into mitosis.

Source of blood

Venous blood was drawn from six (n=6) healthy volunteers with informed consent (age 21-25) in vacutainers. It was stored at 4°C until brought to lab.

Culture setup

The lymphocytes cultures were set up by adding 0.5 mL of heparinized whole blood to 4.5mL of RPMI-1640 with glutamine supplemented with 10% FBS, 100 UI/mL Penicillin G and 100 µg/mL streptomycin, 1% (v/v) of L-glutamine and 1% (v/v) heparin. Lymphocytes were stimulated with 1.5% (v/v) phytohaemagglutinin (PHA). Experiments were performed in triplicates were for each individual concentration and exposure time, and incubated at 37°C in a chamber containing 5% CO_2. The lymphocyte cultures were exposed to AgNPs (5, 15, 25µg/mL) and incubated for 24h. At 67th hr of incubation period, the dividing cells were arrested at a stable metaphase stage by adding 0.025µg/mL colchicine solution to each culture vials. The cultures were incubated further for 5 hours at 37°C. Lymphocyte cultures were then harvested at 72 h. The cells were collected by centrifugation at 1000 rpm for 10mins and washed twice with RPMI 1640 with l-glutamine, supplemented with 2% (v/v) FCS. The supernatant was aspirated, after gently tapping the cells containing pellet. 5ml of pre-warmed (37°C) hypotonic solution (0.075M KCl) was added to the tubes and the contents were mixed gently using a Pasteur pipette and incubate for 5 minutes at 37°C [21, 22].

The cells were smeared and fixed onto slides with freshly prepared Carnoy's fixative (3:1: Methanol: Glacial acetic acid) at 20°C for one hour. A test slide was prepared by gently placing a drop of the cell suspension on a cleaned glass slide and dried immediately by using hot plate at 40°C. Staining was achieved with 10% (v/v) Giemsa in phosphate buffer pH 6.8 for 8 to 5 min. The test slides were examined under the microscope for cell density and metaphase spreads.

Preparation of Giemsa stain:

4% working solution was prepared by mixing 2ml of Giemsa stock solution and 2ml of 10% disodium hydrogen phosphate which was made up to 50ml with double distilled water. The test slides were stained in Giemsa solution for 5 minutes and washed in distilled water for 2 minutes and air dried.

Scoring and microphotography

A minimum 50 good metaphase spreads were analysed in each sample for each concentration. Scoring of chromosomal aberrations including tri-radial and chromosomal beaks and gaps were carried out in well spread and stained cells was observed under oil immersion objective lens (100X) of the light microscope (Olympus, CX 31, USA). The selected metaphase spreads were photographed using Cytovision software.

Metal ion analysis

When AgNPs are dispersed in cell culture medium, the chances of metal ions getting released into medium are high. Briefly, centrifugation was done at 12,000 rpm for 10 minutes, the clear supernatant was filtered through 0.22µm (Anapore) membrane disc. To it, 2 m1 of 1% nitric acid was added and the released ions were measured using AAS (Varian, AA-240).

Haemolysis assay

Erythrocyte suspension were washed thrice by iso-osmotic PBS (pH 7.4) and then diluted in washing solution at the concentration of 500µl and stored in 4°C for not more than 24 hours. 500µl of erythrocyte suspension was interacted with AgNPs at the final doses (5, 15 & 25µg/ml) and incubated for 1 h at 4°C. The erythrocyte treated with PBS (pH 7.4) was taken as the control. The degree of haemolysis was determined by measuring the absorbance of the supernatant at 540 nm, after centrifuged. The absorbance of the control group was used as the blank as previously reported. [28].

RESULTS

UV-Vis Spectrophotometry

AgNPs exhibit optical properties due of their surface plasmon resonance (SPR) which depends upon shape, size and size distribution of the nanoparticles. The maximum wavelength was at 422.1 nm. (Fig. 1)

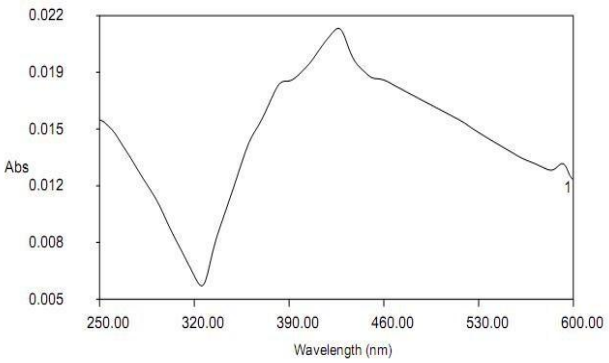

Fig. 1: It shows UV Vis spectrum of silver nanoparticles (5µg/mL)

Dynamic Light Scattering

The effective diameter was found to be 46 nm by dynamic light scattering method in deionised water. (Fig. 2)

Toxicity studies

Chromosomal aberrations

The metaphase spreads shown in Fig.3A was the control sample, in which no AgNP was added which resulted in a clean metaphase spread. 5µg/ml of AgNP treated cell showed no appearance of aberrations on 24 hr and 48 hr exposure. The AgNP uptake is less and the metaphase chromosomes were normal. The aberrant cell percentage was zero. 15µg/ml of AgNP treated cell showed tri-radial chromosomes, a morphological alteration on 24 hr exposure (Fig 3B).

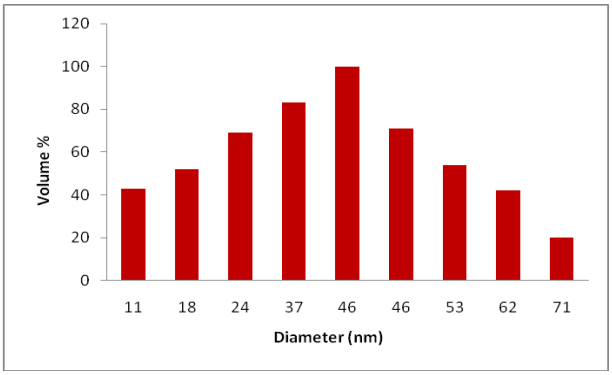

Fig. 2: It shows Particle size distribution of silver nanoparticles in deionized water

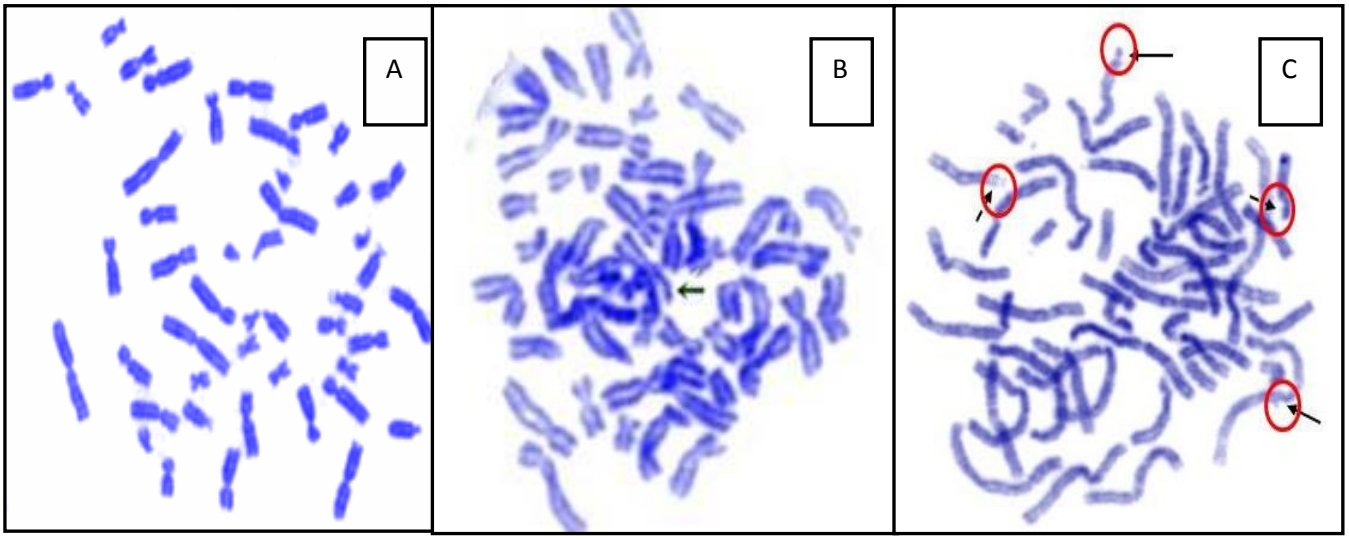

Fig. 3: It shows Metaphase chromosomal aberrations at different concentrations of silver nanoparticles. A – Healthy individual with no aberration (control), B- Metaphase spread with arrow mark indicates tri radial chromosome in 15 µg/mL of AgNPs, C- Metaphase spread showing breaks, gaps and fragments (25µg/mL)

Table 1: It shows Effect of AgNPs on human chromosomes

Ag NP (mg/L)	Metaphases analysed	Chromosome number Mean ± SE	Fragments	Breaks	Gaps	Tri-radials	Aberrant Cell Damage (%)
5	50	46±0	0	0	0	0	0
15	50	46±0	0	0	0	4	8
25	50	46±0	0	5	7	0	24

The AgNP uptake has occurred and survival of the cells from nanoparticle mediated damage depends on their ability to expel the nanoparticles from the inside of the cell environment. The aberrant cell percentage was found to be 8%. 25µg/ml of AgNP treated cell showed gaps and breaks on 24 hr exposure resulting in aberrant cell damage of 24% (Table 1).

Six healthy individual samples were analyzed for 50 metaphases per concentration. Percentage of aberrant cells was expressed as number of cells which showed aberrations/number of metaphases analysed × 100.

Exclusion of AgNPs followed a slow and time dependant pattern. Though the cells were able to remove nanoparticles efficiently, the nanoparticle concentrations in cells were well within detectable limits of Ag concentration even after 48 hours of recovery, chances of a continuous and prolonged AgNPs mediated stress. A minimum of 50 metaphases per sample was scored for the chromosome analysis.

Metal ion analysis

The concentration of silver (Ag ion) in cell culture medium and cell pellet was analysed (Table 2). Despite lesser uptake of silver ions by the blood cells, it proves to be toxic by producing chromosomal aberrations, by the generation of ROS through mitochondrial dysfunction, a possible mechanism for DNA damage as proposed by AshaRani et al. [23]

Table 2: It shows Ag ion release in RPMI and cell pellet

Concentration of AgNP added (µg/ml)	Total ion concentration (µg/ml)Mean±S.E.	
	In medium (RPMI-1640)	In cell pellet
25	1.166±0.02	3.169±0.16
15	0.725±0.02	1.859±0.03
5	0.211±0.01	0.605±0.01

Haemolysis assay

Erythrocytes were incubated along with silver nanoparticles and the resulted ghost cells were seen through microscope (data not shown). Erythrocytes are incapable to produce SOD and catalase, hence are vulnerable to the extraneous toxicants since the cell membrane may be easily damaged during lipid peroxidation. Ghost cells resulted from the breakage of erythrocyte membrane exposed to AgNPs. Another result of the breakage was haemolysis, which happened when the content escaped from the inner to the outer of erythrocyte [24, 25].

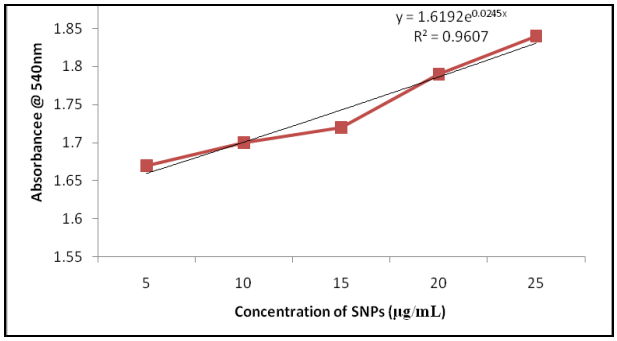

Fig. 4: It shows Haemolysis of erythrocyte under AgNPs. The absorbance was measured at 540 nm

Table 3: It shows the dose dependent haemolysis was better fitted by exponential curve fitting for AgNPs.

Trendline/ Regression	Function	R^2
Linear	y = 0.043x+1.615	0.957
Exponential	$y=1.619e^{0.024x}$	0.960
Logarithmic	y=0.099ln(x)+1.648	0.834
Power	$y=1.65x^{0.057}$	0.842

As shown in Fig. 4, haemolysis increased, sharply with the dose rising and the contact with plasma membrane occurs in haemolysis. The dose dependent haemolysis was better fitted by exponential curve fitting for AgNPs (Table 3).

DISCUSSION

The present study reveals the fact that even at lower concentration of AgNPs chances are more for its toxic effect to human cells. The dose response shows an increase in DNA damaging effect with higher the treatment nanoparticle concentration. The uptake of nanoparticles inside human cell is still an unidentified area but there are probable mechanisms being suggested which sheds light to the mechanisms of toxicity as well as potential therapeutic application of nanoparticles. Attempts to identify the uptake routes of AgNPs led to the suggestion that, AgNPs were taken up primarily through endo-cytosis and diffusion. Further experiments disclosed a concentration-dependent genotoxicity by chromosomal aberrations. AgNPs treated cells exhibit chromosome instability and mitotic arrest in human cells. Cell organelles show intensive toxic effects on the mitochondrial function leading to generation of ROS, thereby leading to oxidative stress. By the generation of ROS through mitochondrial dysfunction which is a possible mechanism for DNA damage has been proposed by Asharani et al. [23] Nanoparticle treated cells appeared to be clustered with a few cellular extensions which could be due to disturbances in cytoskeletal functions as a consequence of nanoparticle treatment. In eukaryotic cells, DNA damage caused the arrests of cell cycle progression at the G_2/M boundary, allowing cells extra time to repair damage prior to segregation of chromosomes [26].

In vitro exposure of human peripheral blood cells to silver nanoparticles resulted in inhibition of PHA induced proliferation at a concentration ≥15 µg/ml. [27]. Effects on cytokine production were already seen at non-cyto-toxic concentration of as low as 3µg/ml. In 25µg/ml, the AgNP treatment affects the cell cycle and mitotic index. Our present work brings out the exposure of AgNPs resulted in chromosomal abnormalities, inhibition of proliferation that AgNP has potential deleterious effect on erythrocyte in a dose-dependent way *in vitro*. The AgNPs were adsorbed to the erythrocyte membrane due to their high surface-volume ratio. Once the surface was covered by AgNP erythrocyte showed a tendency to be agglutinated because the membrane-bound AgNP deformed erythrocyte and hence decreased the repulsion among erythrocytes. The erythrocyte membrane is composed of a lipid bi-layer primarily with protein embedded in, which keeps the membrane in dynamic equilibrium between fluidity and solidity. Erythrocyte is extremely vulnerable to oxidative damage because of the high poly-unsaturated fatty acid content in the membranes. AgNPs ultimately leads to the decrease of erythrocyte survival haemolysis and generation of ghost cells [28].

Metallothioneins are regarded as essential biomarkers in metal-induced toxicity which facilitates metal detoxification and protection from free radicals. Ghost cells resulted from the breakage of erythrocyte membrane when exposed to AgNPs. Due to the damage of cell wall membrane was haemolysis, which happened when the content escaped from the inner to the outer of erythrocyte [25, 28].

Taken together, in the present study, we also examined the toxic effects of silver nanoparticles on human peripheral blood lymphocytes. It is shown that the AgNPs could produce fragments and gaps as a result of ROS generation and increased ROS levels leading to DNA damage. It is yet to be elucidated whether the toxic effects of AgNPs are specific to any chromosome, or any type of nanomaterials would have similar effects on chromosomes. Further, karyotyping studies are to be carried out for locating the aberration thereby using it as a "potential marker" fragment which can be restricted and used in oncotherapy. In depth studies are needed to assess the risks of AgNPs and to comprehend the underlying mechanisms.

CONCLUSION

This research validates that even at 15 µg/mL concentration of AgNPs (which releases 1.8 µg/mL of Ag ions) has the potential to cause toxicity as analyzed by a range of cyto and genotoxicity parameters. The chromosomal aberrations and cell cycle arrest issues the safety related to AgNPs. Chromosomal aberrations are believed to be the key factors resulting in cell cycle arrest. AgNPs are proved to be cytotoxic and genotoxic. Prospective application of AgNPs as an anti-proliferative agent could be narrowed by the fact that it is similarly toxic to normal cells. The mitotic arrest of cells at G_2/M boundary can be further investigated on onco-therapy. Besides embracing the antimicrobial potential, the biological applications employing AgNPs should be given special attention for its toxicity.

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REFERENCES

1. Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch, F. Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artefacts and limitations—Many questions, some answers. Mutat Res Rev Mutat Res 2009;681: 241-258.
2. Singh N, Manshian B, Jenkins GJS, Griffiths MS, Williams MP, Maffeis GGT, Wright JC, Doak H.S. NanoGenotoxicology: The DNA damaging potential of engineered nanomaterials. Biomaterials 2009; 30: 3891–3914.
3. Osterberg R, Persson D, Bjursell G. The condensation of DNA by chromium (III) ions. Biomol Struct Dyn 1984; 2: 285-290.
4. The Royal Society and Royal Academy of Engineering, UK. Nanoscience and Nanotechnology, Opportunities and Uncertainties. UK: The Royal Society: 2004
5. Munzuroglu O, Geckil H. Effects of metals on seed germination, root elongation, and coleoptile and hypocotyl growth in *Triticum aestivum* and *Cucumis sativus*, Arch Environ Contam Toxicol 2002; 43: 203-213.
6. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles Environ Health Perspect 2005; 113: 823-839.
7. Anastasio C, Martin ST. Atmospheric nanoparticles. Rev Miner Geochem 2001; 44: 293-349.
8. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the Nanolevel, Science 2006; 311: 622-627.
9. Kahru A, Dubourguier HC. From ecotoxicology to nanoecotoxicology, Toxicology 2010; 269: 105-119.
10. Chen X, Schluesener HJ. Nanosilver: a nanoproduct in medical application. Toxicol Lett 2008; 176: 1-12.
11. Tripathy A, Chandrasekran N, Raichur AM, Mukherjee A. Antibacterial applications of silver nanoparticles synthesized by aqueous extract of *Azadirachta indica* (Neem) leaves. J Biomed Nanotech 2008; 4: 1–6.
12. John DB, Steena RS. Biosynthesis of silver nano particles and its antibacterial activity against human pathogens. Int J Pharm Pharm Sci 2013; 5(1): 257-259
13. Joyce NM, Shiny PJ, Vinita E, Sindhu PD, Samundeeswari A, Mukherjee A, Chandrasekaran N. A review on safer means of nanoparticle synthesis by exploring the prolific marine ecosystem as a new thrust area in nanopharmaceutics. Int J Pharm Pharm Sci 2013; 5(1): 23-29
14. Toxicological profile for Silver. Contract No: 205-88-0608. Prepared by Clement international corporation, U.S. Public Health Service, ATSDR/TP-90-24. ATSDR (Agency for toxic substances and Disease Registry) 1990.

15. Park EJ, Yi J, Kim Y, Choi K, Park K. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism, Toxicol in Vitro 2010; 24: 872-878.
16. Miura N, Shinohara Y. Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells. Biochem Biophys Res Commun 2009; 390: 733-737.
17. Helland A. Nanoparticles: A closer look at the risks to human health and the environment, *IIIEE*, 2004 ISSN 1650-1675.
18. Monteiroa DR. Gorupb LF, Takamiyaa AS, Ruvollo AC, Camargob ER, Barbosaa DB. The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. Int J Antimicro Ag 2009; 34: 103–110.
19. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials, Biotech Adv 2009; 34: 76-83.
20. Eom HJ, Choi J. p38 MAPK Activation, DNA Damage, Cell Cycle Arrest and Apoptosis As Mechanisms of Toxicity of Silver Nanoparticles in Jurkat T Cells. *Environ* Sci Technol 2010; 44: 8337–8342.
21. Sathya TN, Vardhini NV, Balakrishnamurthy P. Revolution of Nano in *in-vitro* genetic toxicology, Cell and Tissue Res 2010; 3: 2389-2396.
22. Ramos DL, Gaspar JF, Pingarilho M, Gil OM, Fernandes AS, Rueff J, Oliveira NG. Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione *S*-transferase genotypes, Mutat Res Genetic Toxicol Environ Mutagenes 2011; 724: 28-34.
23. AshaRani PV, Hande MP, Valiyaveettil S. Anti-proliferative activity of silver nanoparticles. BMC Cell Biology 2009; 10: 65.
24. Hebbel RP, Leung A, Mohandas N. Oxidation-induced changes in micro-rheologic properties of the red blood cell membrane. Blood 1990; 76: 1015-1020.
25. Duchnowicz P, Koter M, Duda W. Damage of erythrocyte by phenoxyacetic herbicides and their metabolites. Pestic Biochem Physiol 2002; 74: 1–7.
26. AshaRani PV, Grace Low KM, Hande MP, Valiyaveettil S. Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells, *ACS Nano* 2009; 3: 279–290.
27. Shin SH, Ye MK, Kim HS, Kang HS. The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells. Int Immunopharmacol 2007; 13: 1813-1818.
28. Qiang LS, Rong Z, Hong Z, Meng X, Yu SX, De YS, Long WS. Nanotoxicity of TiO_2 nanoparticles to erythrocyte in vitro *Food Chem Toxicol* 2008; 46: 3626 -3631.