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CYTOTOXIC, ANTI-PROLIFERATIVE AND APOPTOTIC EFFECTS OF SILVER NANOPARTICLES (AGNPS) AGAINST HUMAN NEUROBLASTOMA (SK-N-SH) CANCER CELL LINES

Dr. B.Gowramma*   D. Muralidhara Rao**
**Department of Botany, Vijayanagara Sri Krishnadevaraya University, Ballari , Karnata, India.*
***Department of Biotechnology, Sri Krishnadevaraya University, Anantapuram , Andhra Pradesh, India.*

Abstract
Cytotoxicity, anti-proliferative and apoptotic effect of silver nanoparticles produced from Corynebacterium was estimated in Human neuroblastoma (SK-N-SH) cancercell lines. Cytotoxicity of silver nanoparticles in the cell lines was demonstrated by MTT assay using dose - dependent manner. Half maximum inhibitory concentrations (IC50) were evaluated. Silver nanoparticles were exhibited good cytotoxic effects in SK-N-SH cell lines. Reactive oxygen species (ROS) production in all the cell lines was examined by dihydroethedium (DHE) staining method. Analysis of apoptosis and necrosis was carried out by annexin-V/PI staining method using flowcytometry. Therefore, this study was designed to recognize the anticancer property of silver nanoparticles against SK-N-SH cell lines and to the best of our knowledge this is the first report on cytotoxic activity of silver nanoparticles against human neuroblastoma cancer.

Key words: Silver nanoparticles, Cytotoxicity, Apoptosis, IC50, MTT, DHE, ROS, SK-N-SH.

INTRODUCTION

In nanotechnology, a nano particle is defined as a small object or particle that behaves as a whole unit in terms of its transport and properties. Nanotechnology takes advantage of the fact that when a solid material becomes very small, its specific surface area increases, which leads to an increase in the surface reactivity and quantum-related effects (Buzea et al., 2007). Even today, the majority of AgNP applications in the medical field are due to the antimicrobial nature of silver (Lansdown, 2010). AgNPs are associated with various types of toxicities including systemic toxicity, cytotoxicity and genotoxicity (Asharani et al., 2009). Hence, a lot of interest was raised and many current studies are dealing with the biological and environmental effects of AgNPs. S. M. Hussain et al. used rat liver cells as an *in vitro* model and found that AgNPs caused cytotoxicity due to oxidative stress through glutathione depletion and ROS production (Hussain, Hess, Gearhart, Geiss, & Schlager, 2005). Recently, P Asharani *et al.* reported that AgNPs led to the arrest of G2/M phase of human cell cycle along with metabolic arrest associated with oxidative stress (Asharani et al., 2009).

Singh and Ramarao (2012) demonstrated that Ag-NPs induced stress pathways from ROS and cytokine production in Neuronal (Neuro 2A) cell lines. Reactive oxygen species (ROS) are continually generated and eliminated in biological systems. They play an important role in a variety of normal biochemical functions, and abnormality in their function results in pathological processes. Excessive production of ROS in the cell is known to induce apoptosis (Martindale et al., 2002; Sastre et al., 2000]. Rahman et al. (2009) reported that intraperitoneal administration of silver nanoparticles, at a very high concentration, may lead to alterations of gene expression, suggesting the neurotoxic effects of these nanoparticles. Furthermore, a more recent study has indicated that the expression of neural-lineage-specific transcription factors *Ascl1*, *Brn2* (also called *Pou3f2*) and *Myt1l* can directly convert fibroblasts into functional neurons *in vitro* (Vierbuchen et al., 2010) . More studies of the toxicity of nanoparticles, in particular genotoxicity, are imperative. Interesting studies that discuss the methodologies currently available for genotoxic studies were recently published. These studies presented a survey of the in vitro and in vivo genotoxicological studies of nanomaterials conducted in recent years, including silver nanoparticles (Ng et al., 2010; Johnston et al., 2010; Rico et al., 2011).

Hence the study is focused on to regulate the growth of Human neuroblastoma (SK-N-SH) cancer cell lines.

MATERIALS AND METHOD

Silver nanoparticles, Penicillin-Streptomycin, Amphotericin-B solution,L-Glutamine,Trypsin-EDTAsolution,Dihydroethedium (DHE), Fetal bovine serum (FBS), Dimethly sulfoxide (DMSO), V-FITC (Fluorescein isothiocyanate) / PI (Propidium Iodide, Ethanol, H_2O_2 (25% w/w, AR), PBS, Tryphan Blue 0.4%, RNase, Paraformaldehyde (PFA)and MTT.

Cell Line used: SK-N-SH and the cells were cultured in SK-N-SH in Modified Eagle's Medium DMEM/F-12 (1:1) supplemented with 10% heat-inactivated FBS, 200mM L-Glutamine, 2% Penicillin-Streptomycin and 2.5 µg/mL Amphotericin-B solution then incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for 24-48 hrs, the adherent cells were separated using Trypsin-EDTA solution 1X/0.25 %. The ell density was carried out using cell counter based on trypan blue dye exclusion method.

Cell Viability Assay (MTT Assay)

The cell viability of Ag-np treated SK-N-SH cells was measured using 96-well microplates. SK-N-SH cell line was seeded in 96-well microplates at a density of $2.5x10^4$ cells/well and incubated for 24hrs, after which the cells were exposed to an increasing concentration of AgNPs (25, 50, 100, 125 and 150µg/mL) cells were seeded in duplicates and incubated in a CO_2 incubator for 24 hrs. Silver nanoparticle treated cells were thereafter incubated with MTT for 3 hrs. To the each well 100µL dimethyl sulfoxide (DMSO) was added. The AgNPs-untreated cells were used as controls. Cell viability was determined by measuring the absorbance on a microplate reader at 570nm. Viability was calculated as a percentage of viable cells at different test concentrations relative to the control (untreated) cells (% cell viability = (A_{570} of treated cells / A_{570} of control cells) ×100%). The AgNPs concentration that resulted in 50% inhibition of cell growth was calculated as the half maximal inhibitory concentration (IC_{50}) by constructing a dose-response curve. IC_{50} value was estimated by using the linear equation y= mx+c

REACTIVE OXYGEN SPECIES (ROS) MEASUREMENT

Generation of ROS (superoxide) was measured using a cell permeable fluorescent marker DHE (dihydroethidium), which upon oxidation by ROS yields the red-fluorescent product 2-hydroxyethidium Murugavel et al., 2007]. cell suspension at a density of $1\text{-}2X10^4$ cells per well was seeded in a 6-well plate with cover slips and allowed to grow for 24h at 37°C, 5% CO_2. Silver nanoparticles was added at the concentration of 65µg/ml (1ml solution) and incubated for 24h in CO_2 incubator. After incubation, the cells were washed 1-2 times with 1mL PBS and stained the cells with 20µM DHE (1mL per well) and incubated for 30-45 mins at 37°C, in dark. The cells were washed 1-2 times with 1mL PBS, followed by addition of 1mL PBS. 25% H_2O_2 treated cells were used as positive controls. Images were captured using fluorescent microscope with appropriate channel settings.

FLOW CYTOMETRIC AND ANNEXIN V-FITC/PI STAINING ANALYSIS

Apoptosis analysis was performed using Annexin V-FITC (Fluorescein isothiocyanate)/PI (Propidium Iodide) assay with flow cytometer. Cells were cultured in 6-well plates for 24h at 37°C in 5% CO_2 humidified atmosphere and were later exposed to 65µg/ml AgNPs for 24h. Cells treated with 25% H_2O_2 were used as positive controls. After incubation, both attached and detached cells were harvested and washed once with 1X cold buffer and once with 1X Annexin V binding buffer. Cells were then suspended in 100µL binding buffer at $1X10^4$ c/ml concentration and stained with with Annexin V–FITC (Ann V) and propidium iodide (PI) at 24 h following drug treatment, followed by flow cytometry analysis. Simultaneous staining with Ann V and PI enables a distinction between viable cells (Ann V_/PI_), early apoptosis (Ann V+/PI_), late apoptosis (Ann V+/PI+), or necrotic death (Ann V_/PI+)(Vermes et al.,1995).

RESULTS AND DISCUSSION

Cell viability

The cell-viability assay is one of the important parameters for toxicology analysis that explains the cellular responses to toxic materials, and it can provide information on cell death, survival, and metabolic activities (Asharani et al., 2009). To find out the effect of AgNPs on mitochondrial activity, cells were treated with various concentrations of AgNPs between 25 and 150µg/ml, and toxicity was measured. The data from this experiment suggest that with higher concentrations less the cell viability in SK-N-SH cells was founded. The cell-viability assay suggested that the IC50 was 60.0 µg/mL, which is significantly better than earlier findings.

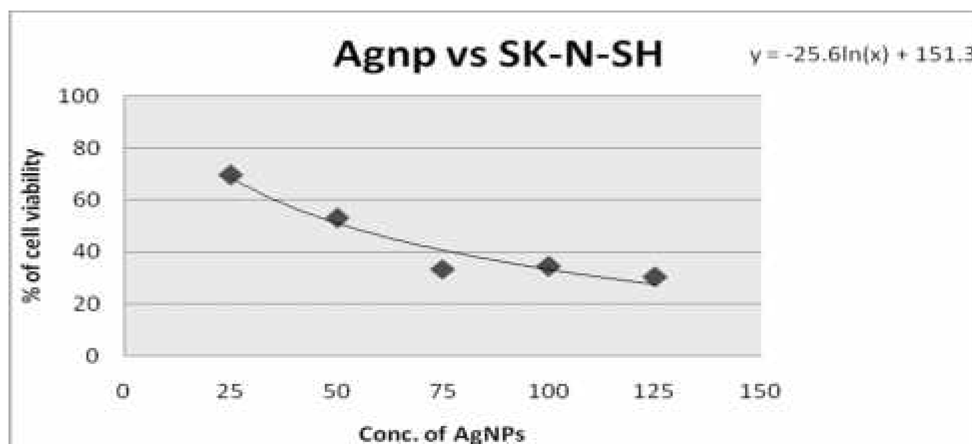
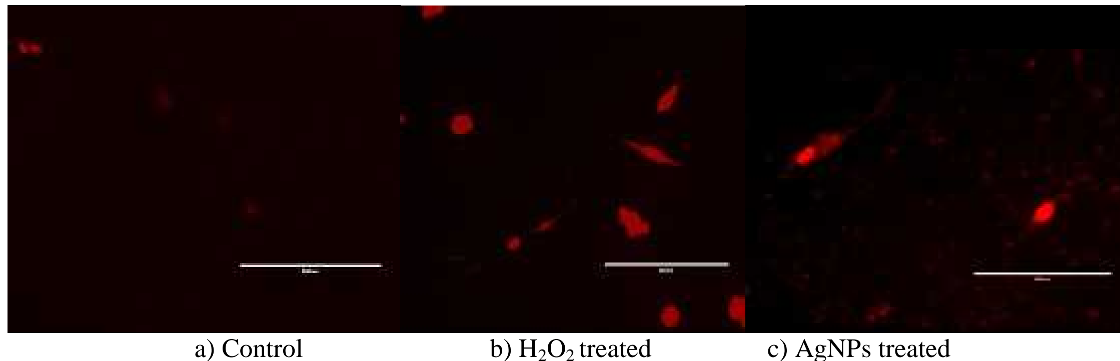


Fig.1 Graph showing the decreased SK-N-SH cell viability with increasing the silver nanoparticle concentration

AgNPs induced Cellular Reactive Oxygen Species in SK-S-SH cell lines

Oxidative stress in turn plays an important role in many types of cellular injury, which can result in DNA damage and apoptotic cell death. ROS are chemical species that are produced as by-products of cellular oxygen metabolism, which occurs via mitochondrial respiration in eukaryotic cells (Carlson C, 2009; Franco JL, 2009; Braydich-Stolle, 2010). Present studies provide the evidence for a molecular mechanism of AgNPs inducing generation of ROS, and it could be one of the factors for apoptosis. Earlier studies show that AgNPs could induce generation of ROS in macrophages (Parket et al., 2011). To know the effect of AgNPs in oxidative stress, we measured ROS generation using the DHE assay. Cells were also treated with a characteristic ROS generating agent, H_2O_2 (1 mM), as a positive control. DHE fluorescence was detected in cells treated with AgNPs for 24h. As shown in Fig.2, the ROS levels generated in response to AgNPs were significantly higher in AgNPs treated cells than control. Hence from the results it can be analyzed that, the synthesized silver nanoparticles was successfully inducing reactive oxygen stress in the cancerous cell lines and stopping the cell proliferation.



a) Control b) H_2O_2 treated c) AgNPs treated

Fig. 2 The expression of oxygen free radicals was analyzed by labeling with 20µM dihydroethidium (DHE). a) Control: SKNSH cell were not treated with AgNPs b) SKNSH cell were treated with 200mM H_2O_2 c) SKNSH cell were treated 60µg/ml with AgNPs

FLUORESCEIN ISOTHIOCYANATE (FITC)–ANNEXIN V ASSAY

The annexin-V-Fluorescein assay was used to determine the mode of cell death. Treatment with AgNPs significantly increased phosphatidylserine (PS) translocation in SKNSH cells compared to the control. Statistical data were extracted from the dot plots (not included), from the results, SK-N-SH cell was showing almost 100% of apoptosis was not found late apoptosis and any residual damage when treated with AgNPs. Untreated/controlled cells were showing 0.3% of apoptosis, 1% of late apoptosis and no residual damage. Fig: 3 show the flow cytometric analysis based on the percentages of viable (LL1), early apoptotic (LR1), apoptotic (UR1) and residual damaged (UL1) cells. Nanoparticles were Inducing apoptosis by arresting the cell proliferation. Recent reports have identified apoptosis as a major mechanism of cell death in exposure to nanomaterials (Pan et al., 2007). The important regulatory mechanisms of apoptosis include death receptors, activation of caspases, mitochondrial responses and Bax, and the regulation of Bcl-2 gene expression (Cell et al., 2006).

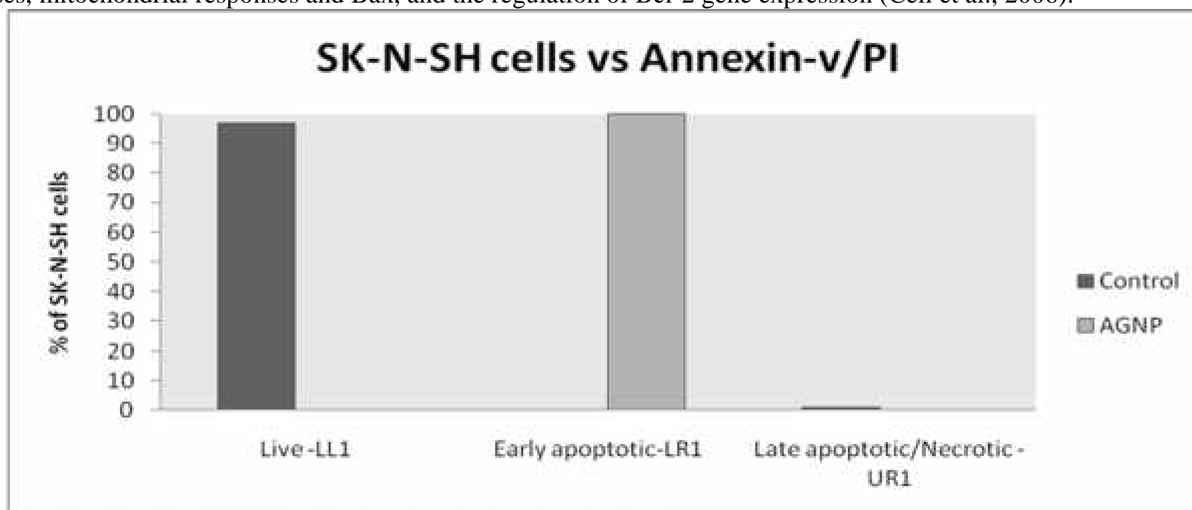


Fig. 3 Graphical showing of live, apoptosis and necrosis in AgNPs treated SK-N-SH cell lines after 24hours, untreated cells was used as control.

CONCLUSION

In conclusion, our data indicated that the biosynthesized Ag-NPs induced apoptosis on SK-N-SH cell line. The overall results indicated that the biologically synthesized AgNPs have antiproliferative activity through induction of apoptosis in cancerous cell lines, suggesting that biologically synthesized AgNPs might be a potential alternative agent for cancer therapy. This study demonstrates the possibility of using AgNPs to inhibit the growth of the tumor cells and their cytotoxicity for potential therapeutic treatments and offers a new method to develop molecule for cancer therapy. Biosynthesized Ag-NPs from *Corynebacterium glutamicum* could be considered a potential chemotherapeutic agent in the treatment of human neuroblastoma cancer.

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