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Original Article

Diverse Cytotoxic Capability of Silver Nanoparticles against the Normal and Cancerous Lymphocytes

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ABSTRACT

Despite the toxic effects on numerous organs, silver nanoparticles can be used to cease cell cycle and promotes apoptosis in cancerous cells. Therefore, by choosing the right dosage nanoparticles can be effective in cancer treatment, whilst do not harm normal tissues. In the current study, it has been tried to measure silver nanoparticles toxicity against HPB-ALL leukemia cell line and normal human lymphocytes. The cytotoxicity of 20-nanometer silver nanoparticles was investigated by MTT test. Also, nanoparticles effect on apoptosis was assessed by flow cytometry. DNA fragmentation analysis was done to investigate the genotoxicity. MTT colorimetric assay revealed that the maximal half inhibitory concentration (IC50) of silver nanoparticles were at 8.43 µg/mL and 15.74 µg/mL in cancer cell-line and normal cells, respectively, at 24-hour exposure. The IC50 doses of silver nanoparticles were used to assess the induction of apoptosis by flow cytometry. The apoptosis occurred in 12.34% of normal cells and 36.88% of HPB-ALL cells. The difference between these two groups were statistically significant ($P<0.0001$). Besides, the size and the complexity of cancer cells treated by nanoparticles decreased more than normal cells. The obtained IC50 doses were used for genotoxicity assay, too. The DNA fragmentation results were in accordance with cytotoxicity results and showed more fragmentation in cancer cells. It could be concluded that the nanoparticles of silver showed lower toxicity to normal lymphocytes in comparison to leukemia HPB-ALL cell-line. Hence, it may be useful to concern it as a potential chemotherapeutic agent having lowered side effects in cancer therapy.

Key words: Leukemia, Silver nanoparticles, Cytotoxicity

Introduction

Acute leukemia is diagnosed by reducing hematopoietic elements and accumulation of immature cells (blasts). According to the lineage origin, two major leukemia can be distinguished: acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) (Bodet-Milin *et al*., 2016). ALL is a malignancy caused by the irrepressible proliferation of lymphoid progenitor cells from bone marrow, blood and extra-medullary sites

(Terwilliger and Abdul-Hay, 2017; Pieters and Carroll, 2008). Common symptoms of ALL are fatigue, weakness, pale skin, fever, frequent infections, gum bleeding and bone pain (Clarke *et al*., 2016). Chemotherapy, radiotherapy, bone marrow transplantation and stem cell transplantation are used to treat ALL according to patient's age, stage of disease and patient's reaction to the treatment (Pui and Evans, 2006). Despite the progress toward treatment methods in children suffering from ALL, the 5 year old survival rate for adult patients remains very low (Pui *et al.,* 2015). Chemotherapy is the primary therapeutic strategy against ALL. It induces apoptosis pathways in cancer cells and regularly dividing normal cells as well (Topp *et al.,* 2011). This phenomenon evokes the side-effects that are usually observed in patients under chemotherapy (Ohnishi and Takeda, 2015). Hence, scientists are continuously searching for agents being more effective on cancerous cells rather than normal cells, to reduce cancer therapy side-effects.

To achieve this goal, nanotechnology seems to offer potential and promising approaches. One of the most studied nanoparticles is silver. They have 1-100 nm-length in one dimension (Cameron *et al.,* 2018). Silver nanoparticles (AgNPs) have been extensively used in biological, industrial and medical fields (Ávalos Fúnez *et al.,* 2013). Because of their wide usage, numerous studies have been done to explore their properties and to evaluate their toxicity and hazard to both normal and cancer cells. It has been proven that AgNPs are of antimicrobial properties (Dasgupta and Ramalingam, 2016; Gunawan *et al.,* 2017; Hsu *et al.,* 2010). Several studies have shown that nanosilver has anticancer effects (Chauhan, 2015; Li *et al.,* 2017; Reshi *et al.,* 2016; Xue *et al.,* 2018). Other studies on the toxicity of nanosilver have shed light on its toxicity on normal human cells which is because of its ability to cause DNA damage and apoptosis even at non-toxic doses (Ahamed *et al.,* 2010; Guo *et al.,* 2019; Yu and Xu, 2016). It has been revealed that silver nanoparticles interact with membrane proteins and activate signaling pathways inhibiting cell growth. AgNPs can also enter cells through diffusion or endocytosis and cause malfunction of mitochondria, damage cellular macromolecules and ultimately inhibit cell growth (McShan *et al.,* 2014). Mitochondria seem to be the potential target of silver nanoparticle-induced apoptosis (Maurer and Meyer, 2016). Apoptosis is the mechanism by which a cell undergoes programmed death. It has a pivotal role in normal development and tumor suppressing. Activation of apoptotic pathway can be regulated by internal and external signals. In cancers, apoptosis is dysregulated and inhibited. Hence, using substances which have ability to induce apoptosis in cancerous cells, are addressed in chemotherapy research (Pfeffer and Singh, 2018). Accordingly, silver nanoparticles, having aforementioned properties, are assumed to possess a potential efficiency as promising anticancer; however, their toxicity effects on normal cells have been documented. Most of the investigations have focused on introducing a well modified nanoparticle for the chemotropic propose against cancer cells (Zhang *et al.,* 2018; Zangeneh, 2019); meanwhile, lack of a comparison between silver nanoparticles effect on normal and cancerous cells exists.

Therefore, it appears to be crucial to compare the effect of nanosilver on cancer cells and normal cells. The current study was designed to assess whether nanosilver affects normal and cancerous cells equally and whether it is possible to introduce a suitable dose to avoid cytotoxic effects of nanosilver on normal cells. Hence, we investigated the

cytotoxicity and genotoxicity of silver nanoparticles *in vitro* on HPB-ALL cell line as a cancerous cell line and normal human lymphocytes.

Experimental

Materials and methods

Preparation of silver nanoparticles

Silver nanoparticles were purchased from US Research Nanomaterials, Inc. with following characteristics: Silver (Ag) Nano Powder (Ag 99.99, 20 nm, metal basis).

Cell Culture

Human leukemia cell-line HPB-ALL (C213) was purchased from the Cell Bank of Pasteur Institute of Iran. Human normal white blood cells were obtained from peripheral blood of a healthy person. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2.

Cytotoxicity assays

Cell viability analysis

To assess cell viability, microculture tetrazolium test (MTT) was conducted (Mohammadi Kian *et al.,* 2018). Before utilizing silver nanoparticles, 1x 10^4 cells were plated in 96-well plate in RPMI for 24 hours. The cultured cells were treated by nanosilver with different concentrations (1, 5, 10, 15, 25, 50 and 100 µg/mL). After 24 hours, the effect of silver nanoparticles was examined using colorimetric 3-(4,5-dimethylthiazol2-yl)-2,5, iphenyl-tetrazolium bromide (Atocel, Budapest). Absorbance was analyzed at 570nm using ELISA Reader (AWARENESS TECHNOLOGY, Inc.). Obtained results were used to calculate the half-maximal inhibitory concentration (IC50).

Apoptosis assay

Treated white blood cells and HPB-ALL cells were seeded at a density 3x10^5 cells/well and incubated for 24 hours in the presence of nanosilver at a dosage lower than IC50. To determine the percentage of apoptosis induction, samples were stained by Annexin V-FITC/propidium iodide (PI) (Sigma) (Mohammadi Kian *et al.,* 2018). Samples were analyzed by BD flow cytometer instrument and FLOWJO (Tree Star Inc., version 9.6.3, USA) software.

Genotoxicity assay

To analyze the genotoxicity of silver nanoparticles, DNA fragmentation assay was done. Briefly, the cells were plated at a density of 2 x 10^5 cells/well and treated by IC50 dosage of nanoparticles for normal lymphocytes and HPB-ALL cells. DNA fragmentation was assessed according to the protocol previously presented by Horie *et al.,* (Horie *et al.,* 2016). The DNA was electrophoresed on a 0.8% agarose gel in Tris-borate-EDTA buffer. Electrophoresis was done at 25 °C and 80 V for 45 minutes.

Data analysis

Experiments were performed in three replications. Results were described as mean±standard deviation (SD). The obtained results were analyzed by student *T*-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The differences at $P<0.05$ were considered as statistically significant. The SPSS version.24 was used to perform analysis.

Results

Cell viability

MTT assay was done to achieve IC50 of silver nanoparticles on normal human lymphocytes and human leukemia HPB-ALL cell-line. The results of the MTT assay are presented in Figure 1. It should be mentioned that in the current study, to control cell death occurring and to avoid limiting factor of nanoparticle concentrations, the concentration lower than IC50 was used. The calculated IC50 for HPB-ALL cells was 8.43 µg/mL over a 24 hour period. Besides, deduced IC50 for normal lymphocytes was 15.74 µg/mL. The observed difference was statistically significant ($P<0.05$). The obtained data indicated that normal cells showed more resistant to silver nanoparticles than cancerous counter-part. Hence, to induce apoptosis in normal lymphocytes, a higher concentration of nanosilver is needed.

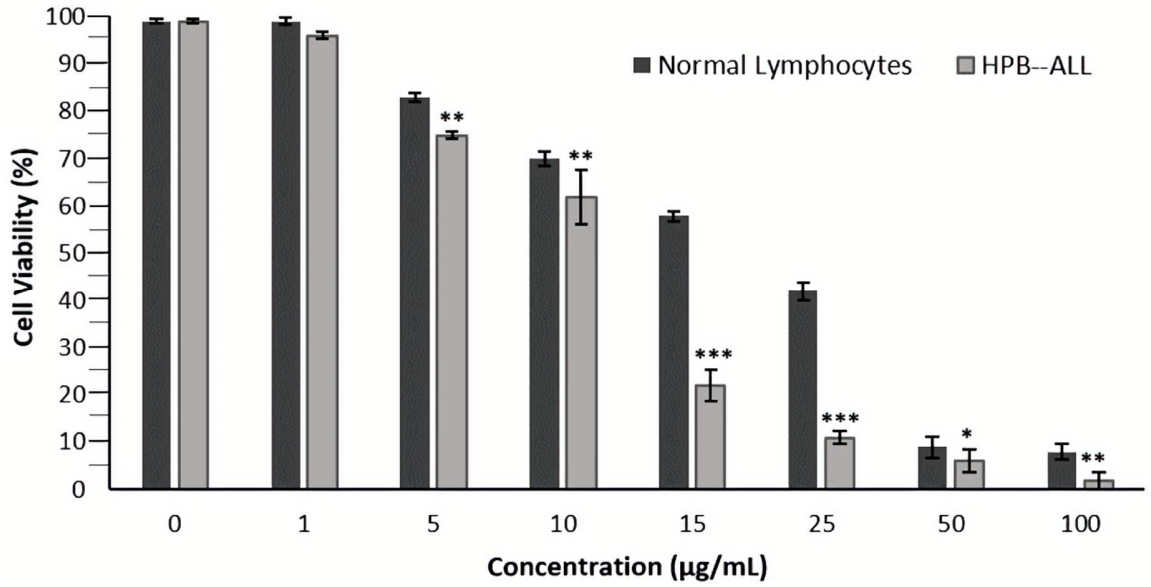

Figure 1. Direct effect of silver nanoparticles with different concentrations on human normal lymphocytes and cancerous HPB-ALL cell-line in 24-hour exposure. The data are expressed as the mean ± SD (n=3, *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$)

Cell apoptosis detection by flow cytometry

To measure the ability of nanosilver to induce apoptosis, the normal lymphocytes and HPB-ALL cells were treated by the concentration of nanoparticles' IC50 (8.43 µg/mL and 15.74

µg/mL for HPB-ALL and normal lymphocytes, respectively). The cells were stained by Annexin V/PI and analyzed by flow cytometry. The viable cells are Annexin V- and PI-, early apoptotic cells are Annexin V+ and PI- and the necrotic cells are represented by Annexin V+ and PI+. As presented in Figure 2 and 3, apoptosis occurred in 12.34% of normal cells. This number for HPB-ALL cells was 36.88% ($P<0.0001$). In addition, necrosis occurrence in cancer cells in comparison to normal cells was significantly higher ($P<0.0001$); however, the necrosis was insignificant and did not exceed 12.06%. By considering results released from flow cytometry, it can be concluded that the size and the complexity of cancer cells treated by nanoparticles decreased more than normal cells. The results indicated that HPB-ALL cells were cytotoxically more susceptible to nanosilver than normal cells.

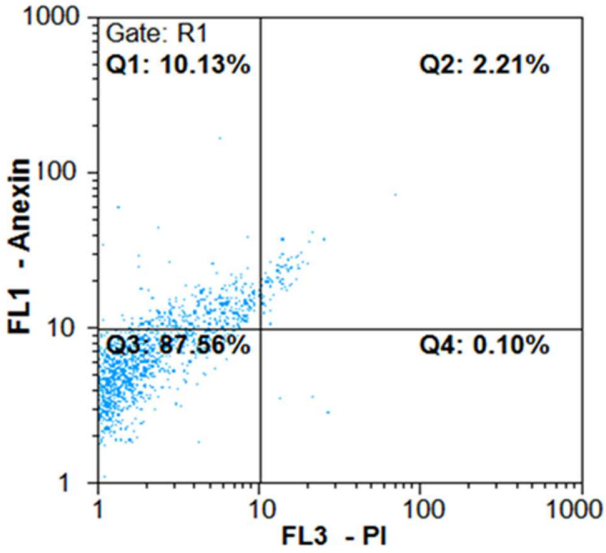

Figure 2. Flow cytometry of normal lymphocytes treated by silver nanoparticles (7.5 µg/mL). Q1: early apoptotic cells, Q2: late apoptotic cells, Q3: viable cells and Q4: necrotic cells

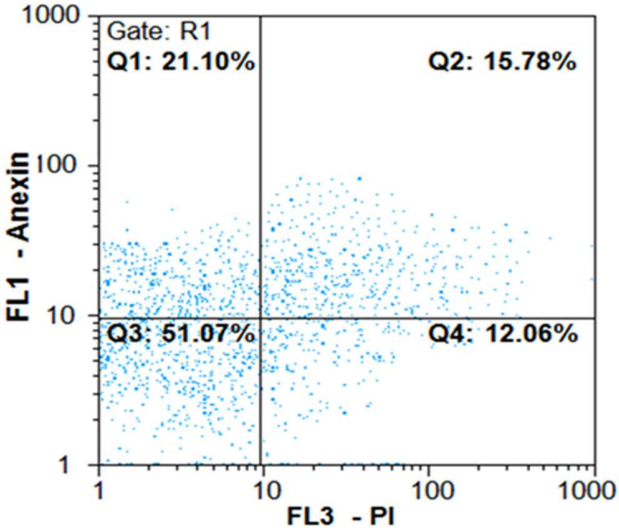

Figure 3. Flow cytometry of HPB-ALL cells treated by silver nanoparticles (7.5 µg/mL). Q1: early apoptotic cells, Q2: late apoptotic cells, Q3: viable cells and Q4: necrotic cells

DNA fragmentation

To further clarify the apoptotic damage caused by silver nanoparticles involving genomic DNA, the previously mentioned DNA of normal lymphocytes and HPB-ALL cells were treated with 15.74 and 8.43 µg/mL silver nanoparticles, respectively. Then, genotoxicity was analyzed using gel electrophoresis. As seen in Figure 4, the exposure to nanosilver produced smear of DNA, but the formed smear was more in HPB-ALL cells than normal cells. The DNA of cancer cells has been fragmented more in comparison to normal cells. Accordingly, the cytotoxicity of silver nanoparticles against cancer cells was higher than normal cells. Furthermore, DNA damage to normal cells was less than cancerous. In other words, DNA of the normal cells showed more resistant to silver nanoparticles.

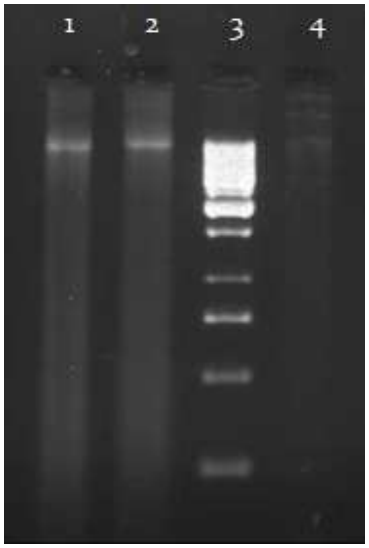

Figure 4. Induction of genotoxicity by silver nanoparticles. Normal cells were exposed to 15.74 µg/mL and HPB-ALL cells were exposed to 8.43 µg/mL of nanosilver. Lane 1 and 2: HPB-ALL cells, lane 3: marker, lane 4: normal lymphocytes

Discussion

Treatment failure of ALL in adult patients (Coustan-Smith *et al.,* 2011), increasing the occurrence of leukemia globally (Arber *et al.*, 2016) and lack of a long-lasting and safe treatment (Park *et al.,* 2018) have led researchers to do studies rigorously to find novel efficient therapy for ALL and also to decrease the side effects of chemotherapy. Currently, various studies have aimed to reduce the systemic toxicity of anticancer agents (*e.g.,* Rashmezad *et al.,* 2015). Nanosilver seems to be an outstanding alternative in this regard. Silver nanoparticles by 100 nm or less size are now used widely as delivery, targeting and diagnostic agents in pharmaceutical projects of cancer medicine (Rashmezad *et al.,* 2015).

The current study was conducted to analyze the toxicity of nanosilver with the size of 20 nm against acute lymphoblast leukemia cells in comparison to normal lymphocytes. The IC50 for HPB-ALL cells was 8.43 and for normal cells was 15.74 µg/mL. Other studies reported the toxicity of silver nanoparticle on cancer cell-line. For instance, a survey conducted in 2013, the effect of green-synthesized silver nanoparticles were tested against MCF-7 breast cancer cells in different concentrations for 24 h. Nanoparticles caused apoptosis and DNA fragmentation in the

MCF-7 cells (Gurunathan *et al.,* 2013). In another experiment, it was shown that the nanoparticles in size range of 6.5 to 43.8nm were used as a colloidal aqueous suspension in concentrations of 1.0–5μg/mL (Shahabzzadeh *et al.,* 2011). Their results were similar to those of ours. In 2011, Shahoon (2013) studied cytotoxic effects of silver and hydroxyapatite nanoparticles biocompatibility on L929 fibroblast cells. Nanoparticles that were used by this group was rod-like and less than 100 nm size. The results showed concentrations of over 20 ppm, within 24, 48 hours are toxic to fibroblast cells (Shahoon, 2013). However, the results of this study showed that spherical silver nanoparticles at concentrations of less than 20 ppm was also toxic to cells and caused apoptosis of the cell. In fact, cells are more sensitive to spherical silver nanoparticles than the rod-shaped, because the shape of the particles is effective on the contact surface of the particles and the number of silver ions released (Kim *et al.,* 2007). Moreover, this may be due to more sensitive peripheral blood mononuclear cells than fibroblasts cells to silver nanoparticles (Hsu *et al.,* 2010). The different toxicity observed was likely to be the result of the nanoparticles' size. Particles with smaller size could cause more toxicity than the bigger particle; because they have a larger surface area (Zhang *et al.,* 2016). Parnsamut and his colleague tested the effects of silver and gold nanoparticle on Jurkat cells and U937 cells. Their results showed IC_{50} values of AgNPs were 9.8 and 12.6 ppm, and IC_{50} values of AuNPs were 43.3 and 45.6 ppm, respectively (Parnsamut AND Brimson, 2015). Our study showed that AgNPs inhibited the proliferation of Hpb-ALL leukemia cells at lower concentrations with an IC_{50} value of 2.68 μg/ml. The reason for this difference could be that silver nanoparticles compared to gold nanoparticles were more easily oxidized and caused oxidative stress in the cell membrane (Reidy *et al.,* 2013). Overall, the activity of silver nanoparticles depends on their size-, shape-, concentration-, cell type- and time-dependent.

Results of our investigation showed different cellular reactions to the same nanoparticles in normal and cancerous cells ($P<0.05$). In their study, Paknejadi and colleagues demonstrated that the toxicology of silver nanoparticles on human normal fibroblast cell-line was both concentration and time dependent (Paknejadi *et al.,* 2018). There is a lack of information about the different influence of silver nanoparticles on cancerous cells. A study of Choi and colleagues represented differential cytotoxicity of nanosilver in human ovarian cancer cells and ovarian cancer stem cells (Choi *et al.,* 2016).

Our results revealed that silver nanoparticles were approximately 2 times more cytotoxic on HPB-ALL than normal cells. So far, none of the previous studies has addressed this comparison and only the effects of nanoparticles on cancer cells, and the apoptosis was investigated *in vitro*. This study promoted a perspective toward the ability of nanosilver to act differently against cancerous cells.

Conclusions

The current study speculated that the cytotoxicity of silver nanoparticles on human acute lymphoblastic leukemia cells was approximately twice more than that of normal lymphocytes, and they had significant effects on apoptosis induction in cancerous cells. These results indicated a potential anti-leukemia role for silver nanoparticles. Therefore, our results can be confirmed after the final studies on animal models because nanoparticles can be applied *in vivo* tests, and the selective toxicity is significant in healthy and cancer cells. Also, an investigation of molecular mechanisms by which nanosilver acts differently against cells is of importance.

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