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Comparative Study of the Cytotoxic Effect of Silver Nanoparticles on Human Lymphocytes and HPB-ALL Cell Line: As an In Vitro Study

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Abstract

Background: Cancer is currently the second leading cause of death worldwide that is originated from cell growth and proliferation without control. Acute lymphoblastic leukemia (ALL) is one of the types of leukemia that affects lymphocyte maturation and it is common among children. Silver nanoparticles are considered one of the targeted chemotherapy methods by creating cytotoxicity.
Objectives: In this research, a comparative study of cytotoxic effect of silver nanoparticles was evaluated on human lymphocytes and HPB-ALL cell line as an in vitro study.
Methods: In this experimental study, lymphocytes and HPB-ALL cell line were exposed to silver nanoparticles at RPMI 1640 medium culture in order to assess toxicity for 24 hours. To this aim, MTT assay was used to evaluate the toxicity of the silver nanoparticles. DNA fragmentation and apoptosis were evaluated by Gel Electrophoresis and Flow Cytometry, respectively. Moreover, quantitative PCR was performed on bax, bcl-2, and caspase-9 genes.
Results: The results of MTT assay showed IC_{50} values of silver nanoparticles were 5.87 and 2.68 μg/mL for lymphocytes and HPB-ALL cell line, respectively. The results showed that silver nanoparticles could split DNA of the HPB-ALL cell line more than DNA of the lymphocytes during DNA fragmentation. Flow Cytometry results indicated that the early apoptosis was 6.04% and 22.75% in lymphocytes and HPB-ALL cell line, respectively. Moreover, Q-PCR results showed a significant up-regulation of caspase-9 and bax genes and downregulation of bcl-2 gene in comparison to the control group.
Conclusions: The silver nanoparticles had cytotoxic effects on the lymphocytes and HPB-ALL cell line. The results showed that the silver nanoparticle had a significant cytotoxic effect on HPB-ALL cell line.

Keywords: Anti-Cancer Properties, Leukemia Cells, Lymphocytes, Silver Nanoparticles

1. Background

Cancer is one of the most important mortality causes in the world. The global distribution and diverse pattern of cancer over the past years have resulted in developing a variety of therapies, depending on the type, the extent of the disease and the patient's condition using a combination of different methods for preventing and treating cancer (1). Despite many efforts in preventing and treating cancer, the disease has been growing and is still a deadly global cause. Acute lymphocytic leukemia is a blood cancer and more common in children under the age of 10 years and causes many deaths annually (2). Nowadays, various treatments for cancer are used, including surgery, chemotherapy, and radiation therapy that eliminate healthy cells in most cases; however, they can cause toxic and side effects in patients. Therefore, finding new ways to treat cancer is necessary to reduce side effects (3, 4). Recently, one of the important methods in cancer research projects is nanomaterials as carriers and developing anticancer medicines for drug delivery to the cancerous tissue and minimizing side effects (5). Nowadays, metal nanoparticles serve as an attractive candidate for the delivery of many small molecules or large bio-molecules (6, 7). Silver nanoparticles have been used over the past few years for chemical stability, catalytic and antimicrobial activity (8-13). The anticancer properties of silver nanoparticles have been identified in many studies. Also, silver nanoparticles contribute to DNA damage, increase the expression of the caspase 3 protein and trigger apoptosis, and thus lead to the inhibition of proliferation, through transducing cell death signals, which is very important in cancer treatment (14). Silver nanoparticles can interact with membrane proteins and activate cellular signaling pathways that lead to cell division inhibition (15-17). Silver nanoparticles can enter the cell through diffusion or endocytosis to disrupt the mito-

chondria, produce reactive oxygen species (18) and damage to proteins and nucleic acids in the cells and ultimately leads to the inhibition of cell division. When oxidation stress occurs, ROS production is beyond the capacity of the antioxidant defense system (19-22).

2. Objectives

In this research, a comparative study of cytotoxic effect of silver nanoparticles was evaluated on human lymphocytes and HPB-ALL cell line as an in vitro study.

3. Methods

3.1. Cell Culture

HPB-ALL leukemia cell line code C213 was purchased from Cell Bank of Pasteur Institute of Iran. Lymphocyte cells were isolated from peripheral blood healthy human. HPB-ALL cell line and isolated lymphocytes were cultured in RPMI1640 containing 10% FBS (fetal bovine serum), 3.7 g/L sodium bicarbonate, 100 U/mL penicillin-streptomycin in the moist atmosphere with 5% CO_2 in 37°C.

3.2. Nanoparticles

The used nanoparticles with specific sizes were prepared from US Research Nanomaterial, Inc.

3.3. MTT Assay

The effect of silver nanoparticles on the growth and proliferation of cancerous and normal cells was evaluated by colorimetric method using MTT 3-(4, 5-) dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (23, 24). This method was based on the breakdown of Tetrazolium salt by mitochondrial succinate dehydrogenase enzyme in living cells. Lymphocytes and HPB-ALL cell line were plated separately in 96 well plates (1×10^4 cells/well). Then, silver nanoparticles were added to each well with concentrations of 5, 10, 15, 25, 50, and 100 μg/mL. After 24 hours, 20 μL MTT (Atocel) was added to each well plate and incubated for 4 hours. After this time, the culture medium containing MTT was carefully extracted and 100 μL DMSO was added to each well. The optical absorption of each well was measured using ELISA reader at 570 nm after a 15-minute incubation. The trials were repeated three times.

3.4. DNA Fragmentation Assay

Lymphocytes and HPB-ALL cell line were incubated in RPMI 1640 medium for 24 h with different concentration of silver nanoparticles. Cells were harvested and re-suspend with 0.5 mL PBS and 55 μL of lysis buffer (200 μL of 0.5 M EDTA 5 mL of 1 M Tris-Cl buffer pH 8.0 0.5 μL of 100% Triton X-100 50 mL of H_2O) incubated for 20 min on ice. The cell suspension was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was elicited with 1:1 mixture of phenol: chloroform and precipitated in two volumes of cold ethanol and one-tenth volume of sodium acetate (25). The DNA was resolved in 0.8% Agarose Gel.

3.5. Flow Cytometry Assay

To indicate the levels of apoptosis, Annexin incorporated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) were used. The method was executed using the Annexin V-FITC Apoptosis Detection Kit and pursuant to the manufacturer's instructions. Cells were grown in well plates and treated with silver nanoparticles. Then cells were washed in cold PBS and re-suspended in annexin V binding buffer. Afterward, annexin V was added. After being incubated for 10 minutes at room temperature, cells were washed with binding buffer. Then, PI was added and analyzed using a FACSCalibur Flow Cytometer (BD Biosciences). Fluorescence levels of FITC and PI were measured.

3.6. Quantitative Real-Time PCR Analysis

Total RNA was extracted from the treated and untreated cells by using TRIzol reagent. RT was performed using a Prime-Script RT reagent kit (Takara, Japan). Forward and reverse primers used for qPCR are presented in Table 1. Subsequently, qPCR reactions were performed using RealQ Plus Master Mix Green (Amplicon, Denmark) under the following cycling conditions: Initial step of 95°C for 30 secs; followed by 94°C for 30 secs and 60°C for 30 secs for 40 cycles. The relative expression level of bax, bcl-2, and caspase-9 genes was normalized to that of β-actin in the same sample (26, 27). Relative expression of target genes was normalized to β-actin, analyzed by $2^{-\Delta\Delta Cq}$ method, and presented as a ratio compared with the control group.

4. Results

4.1. MTT Assay

The results were considered IC_{50} in the form of a concentration that inhibits cell growth IC_{50} obtained from the effect of the silver nanoparticles on HPB-ALL cell line was 2.68 μg/mL and on lymphocytes were 5.87 μg/mL.

Table 1. Forward and Reverse Primers Used for qPCR

Gene	Forward	Reverse
β-actin	5'-TCCTCCTGAGCGCAAGTAC-3'	5'-CCTGCTTGCTGATCCACATCT-3'
BCL-2	5'-ATTGGGAAGTTTCAAATCAGC-3	5'-TCCTCTGTCAAGTTTCCTT-3'
BAX	5'-GAGCTGCAGAGGATGATTGC-3'	5'-AAGTTGCCGTCAGAAAACATG- 3'
Caspase 9	5'-CGAACTAACAGGCAAGCAGC-3'	5'-ACCTCACCAAATCCTCCAGAAC-3'

4.2. DNA Fragmentation Assay

According to the results of Gel Electrophoresis, silver nanoparticles were shown to break down genomic DNA that is observed as band and smear. Figure 1A shows the effect of silver nanoparticles on lymphocytes. Lane A1 with a very low DNA concentration indicates a lower DNA fragmentation by silver nanoparticles. Figure 1B shows the effect of silver nanoparticles on the HPB-ALL cell line. The formed smears in the Lane B1 had a higher concentration of DNA and showed the effect of silver nanoparticles on the HPB-ALL cell line. It could be concluded that the effect of silver nanoparticles on the HPB-ALL cell line was greater than that of lymphocytes.

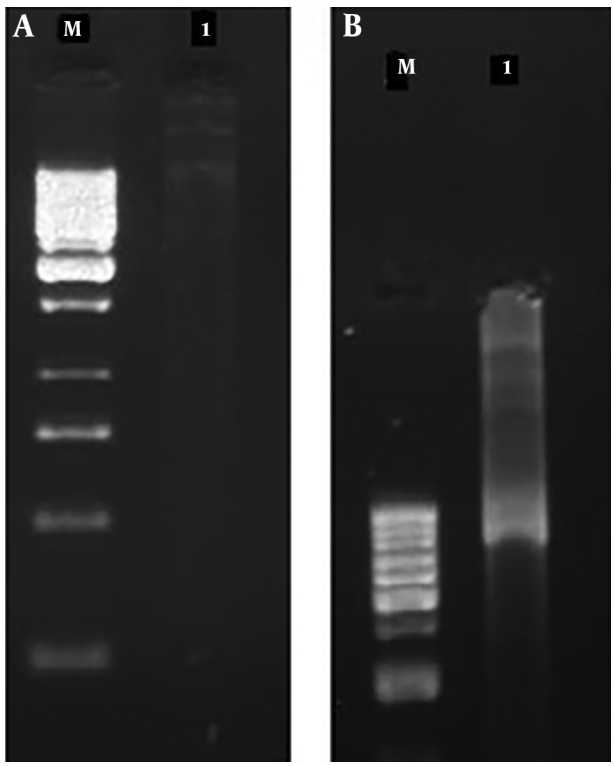

Figure 1. DNA fragmentation assay. A, lane1 lymphocytes, B, lane1 HPB-ALL cell line

4.3. Flow Cytometry Assay

In this study, the distinction between early and late apoptosis was carried out. Annexin V-FITC was used to specify cells at early apoptosis because Annexin V, in the presence of Ca^{2+} ions, is highly susceptible to binding to phosphatidylserine on the surface of apoptotic cells. In addition, cell staining with PI was used to determine cells at late apoptosis (Figure 2).

4.4. Real-Time PCR

The results of this study showed that the expression of bax and caspase 9 genes that induce apoptosis was increased and the expression of bcl-2 gene that suppressed apoptosis was decreased. There was a significant difference between cancer cells and live cells (Figures 3 and 4).

5. Discussion

Acute lymphoblastic leukemia (ALL) is a type of cancer in which the bone marrow makes too many immature lymphocytes. It occurs mostly in children but it is also fatal in adults. Today common cancer treatments include surgery, radiation therapy and chemotherapy, which often destroy healthy cells as well. Recently, many nanoparticles have been targeted in drug delivery to tumor cells by reducing the systemic toxicity of anticancer drugs. Silver nanoparticles disrupt the cellular communication network by disrupting cellular signal transduction. They are also involved in apoptosis by damaging DNA (1-3, 5).

In this research, the cytotoxic effect of silver nanoparticles was investigated using MTT assay on lymphocytes and HPB-ALL after 24 h. In addition, IC_{50} results for HPB-ALL and lymphocytes were 2.68 μg /mL and 5.87 μg /mL, respectively. The calculated IC_{50} for silver nanoparticles in the HPB-ALL cell line was less than the IC_{50} calculated in the lymphocytes. In two similar studies, IC_{50} of silver nanoparticles was 6.33 μg/mL and 3.42 μg/mL on mesenchymal stem cells and the G292 osteoblastic cell lines, respectively (28, 29). Mozaffari Dehshiri et al. (30) investigated the cytotoxic effects of silver nanoparticles examined on primary rat hepatocytes and human cancer cell lines and the size of silver nanoparticles was 64.08 nm. The IC_{50} values of AgNps revealed for A549, HPG2, MCF-7, and MDBK were 5.94, 1.41, 3.68, and, 1.9 ppm, respectively. Kaba and Egorova

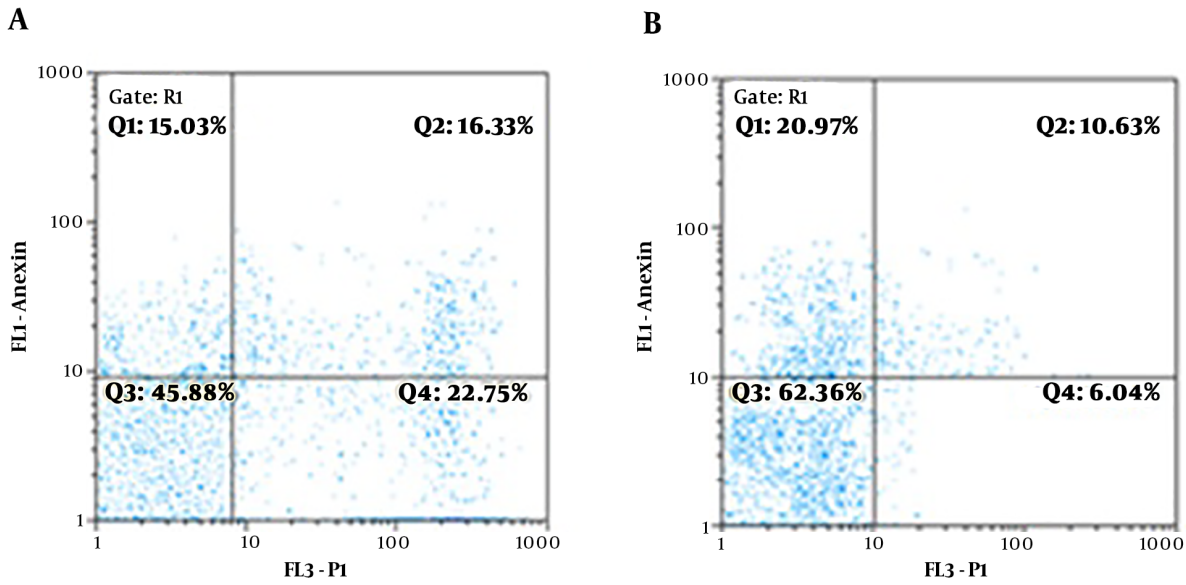

Figure 2. A, flow cytometric results of the effect of silver nanoparticles on the HPB-ALL cell line. Apoptotic cells in the Q4 and Q2 domains represent early and late apoptosis in cells, respectively. B, Flow Cytometry results of the effect of nanoparticles on lymphocytes. Apoptotic cells in areas Q4 and Q2 show early and late apoptosis, respectively. With regard to the above results, it appears that silver nanoparticles have induced more apoptosis in the HPB-ALL leukemia cell line.

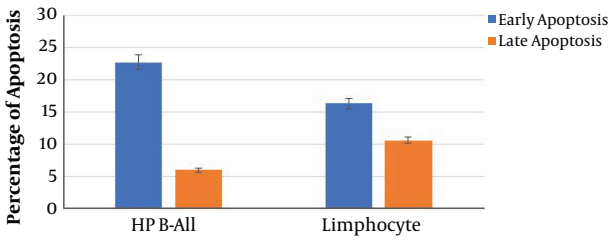

Figure 3. Comparison chart of the percentage of early and late stages of apoptosis in lymphocytes and HPB-ALL cell line.

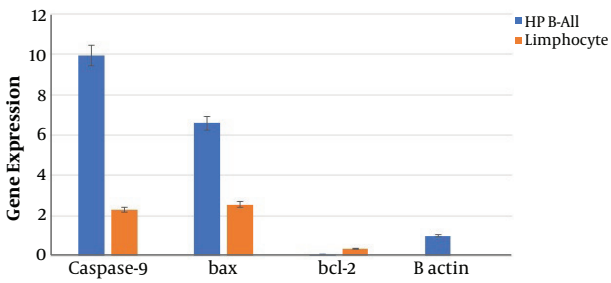

Figure 4. Comparison chart of the expression of Caspase 9, bax, and bcl-2 genes in lymphocytes and HPB-ALL cell line.

(31) demonstrated the antitumor effect of silver nanoparticles on two types of cells, namely HeLa and U937 cells. Silver nanoparticles content of 0.5 - 8.0 μg/mL at 4 and 24 h incubation had notable cytotoxic effect on cancer cells. There are some related diseases to the skin (32, 33) and researchers investigated them. Paknejadi et al. (34) investigated the concentration and time-dependent cytotoxicity of silver nanoparticles in human normal skin fibroblast cell line. The average size of silver nanoparticles was 6.03 nm. The results of MTT assay showed concentration- and time-dependent procedure in the decrease of cell viability and IC_{50} values for 24 and 48 incubation were 30.64 and 14.98 g/mL, respectively. In the present study, the cytotoxic effect of silver nanoparticles was determined on each cell type. Comparison of the results of the analysis of MTT of the HPB-ALL cell line and lymphocytes showed that silver nanoparticles had a more toxic effect on the leukemia cell line. In the study of Perde-Schrepler et al. (23), the cytotoxicity and genotoxicity effects of silver nanoparticles were investigated by the size-dependent method in cochlear cells. They concluded that all sizes of nanoparticles had toxicity to cochlear cells (18). Shahbazzadeh et al. (29) evaluated the cytotoxicity of silver nanoparticles on fibroblasts and mesenchymal stem cells and they showed that these nanoparticles had less toxic effects on healthy cells. In this study, a comparison of the effect of induction of apoptosis was evaluated by silver nanoparticles in HPB-ALL cell line and lymphocytes revealed that these nanoparticles could split the DNA of normal and cancerous cells. These results were similar to the effects of silver nanoparticles on the cancer cells of the HL60 myeloblastic leukemia, Hella's

cancer cells and healthy peripheral blood cells. With the influences of silver nanoparticles on cells and peroxidation of fats, free radicals of ROS are formed that damage DNA and induce apoptosis (35). In addition, in the present study, apoptosis was evaluated in HPB-ALL cell line and lymphocytes by silver nanoparticles and Flow Cytometry that showed the values of 22.75% and 6.04%, respectively. The amount of apoptosis in the HPB-ALL cell line was approximately three times in white blood cells. Also, the expression of bax and caspase 9 genes were effective in apoptosis which was investigated by qRT-PCR. Anti-apoptotic bcl-2 gene expression was down-regulated. Govindaraju (35) investigated the apoptosis induced by silver nanoparticles on HL60 cells who showed similar results with Flow Cytometry. Kulandaivelu et al. (36) checked out cytotoxic effect on cancerous cell lines by biologically synthesized silver nanoparticles. The concentration and time of silver nanoparticles were measured with MTT assays. Significant cytotoxic effects were displayed at 100 μg/mL concentration. The apoptotic activity was determined by caspase-3 activation and DNA fragmentation assays. Western blot showed the release of cytochrome c from mitochondria upon starting bcl-2 inhibition and bax activation. The results showed that synthetic bio-silver nanoparticles could be used to treat breast cancer (36). In a similar study, Yuan et al. (37) investigated the cytotoxic effect and apoptosis of the mixture camptothecin and silver nanoparticles in human cervical cancer cells. They evaluated pro- and anti-apoptotic genes that were measured using qRT-PCR. Accordingly, the mixture significantly inhibited cell viability and growth of HeLa cells (37). In the current study, gene expression of bcl-2 was decreased as anti-apoptotic and gene expression of bax was increased as pro-apoptotic. Baharara et al. (38) used biosynthetic silver nanoparticles extracted from Achillea biebersteinii Flower on MCF-7 cells as well as the apoptosis pathway of several genes was investigated. They showed that there was a relationship between apoptosis and bcl-2 and bax genes expression and caspase activity. Therefore, bcl-2 gene expression showed an inverse relationship with apoptosis and bcl-2 gene expression, while inversely correlated with bax gene expression (38). Kanipandian et al., (23) evaluated the apoptosis signaling pathway in A549 lung cancer cells using nanoparticles extracted from cotton leaf. These nanoparticles were found to increase the expression of bax genes that were effective in apoptosis, while decreasing the expression of bcl-2 genes that inhibited apoptosis (39).

These observations are in agreement with previous studies that reported the ability of silver nanoparticles to produce apoptosis. In the present study, silver nanoparticles had less toxic effects on healthy cells. Considering that nanoparticles are used in in vivo tests, it is important to evaluate their selective toxicity to healthy and cancerous cells. In general, the results of MTT assay, fragmentation of DNA and Flow Cytometry showed that silver nanoparticles had effective cytotoxicity in the HPB-ALL cell line and preferably apoptosis that was induced in these cells and caused the death of cancer cells with less toxicity on living cells. Therefore, the percentage of induced apoptosis by these nanoparticles in the HPB-ALL cell line was approximately three times of apoptosis in white blood cells. Also, the IC_{50} value obtained in the HPB-ALL cell line was less than of the white blood cells which indicates the greater effect of these nanoparticles on cancer cells.

Footnotes

Authors' Contribution: Zahra Farahani was involved in the study concept, design, analysis, interpretation of data, and drafting of the manuscript. Kazem Parivar, Nasim Hayati Roodbari, and Mona Farhadi supervised the conduction of the study.

Conflict of Interests: The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical Approval: The study protocol was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran.

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