Introduction

Breast cancer is one of the most malignant type of cancer among women in United State of America and North West Europe [1-2]. Therefore, we found useful to work on MCF7 human breast carcinoma cells. MCF7 human breast cell line was obtained from metastatic breast cancer patient at 1970 [1].

Metal compounds have been used in medicine for years and shown to be effective on human and animal tumors in vitro [3]. Although there are numerous metal agents with apoptotic/antiproliferative properties used in the treatment of breast cancer, they are known to have side effects and are not efficient. Therefore, this increased interest in the investigation of new metal chemotherapeutic agents against cancer [4]. Lately, silver and silver complexes are investigated on cancer cells [5-6]. Although silver ions are efficient against pathogens, they have low toxicity on healthy cells [7-9]. The effects of silver and a great number of silver compounds have been proved on many pathogens including bacteria, viruses and eukaryotic microorganisms [10-14]. Especially in recent years, silver nitrate from silver compounds has been used in antimicrobial treatments [10-11]. On the other hand, the anticancer effects of silver compounds have recently become the subject of research in the medical science [6]. These complexes are known to interact with DNA which in turn trigger apoptosis. The induction of apoptosis is a common mechanism known to kill cancer cells [15]. Apoptosis is one of the cell death in eukaryotic organisms [16]. Apoptosis is established homestatic balance by eliminating abnormal cells in normal tissues [17-19]. The defects in apoptotic pathways, and mutations lead to many human diseases [20]. Depending on these defects, while increased amount of apoptosis cause AIDS, diabetes, neurodegenerative diseases such as Parkinson’s,
as a result of reduction amount of apoptosis, the cancer development is occurred [21]. Mitochondria plays a key role in the apoptotic process. Mitochondrial control of apoptosis has been described at several levels. One of these levels is mitochondrial membrane potential ($\Delta \Psi_{mt}$) [22].

The aim of current work was to determine the triggered pathways underlying the anticancer effects of silver nitrate against MCF7 breast carcinoma cells. We investigated the apoptotic signal pathways mediated by cytotoxic effects of silver nitrate using Annexin-V FITC/PI, mitochondrial membrane potential assay (JC-1 staining) and TUNEL assay. Morphologically generated apoptotic structures were scanned via confocal microscopy.

**Materials and Methods**

**Materials**

The MCF7 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Silver nitrate (AgNO3) was purchased from Himedia, India. Roswell Park Memorial Institute medium (RPMI 1640) (Sigma Aldrich, USA), the Fetal Bovine Serum (FBS) (Gibco, South America), the penicillin-streptomycin (Gibco, South America), the Dulbecco’s Phosphate Buffered Saline Concentrate (PBS) (10X) (Biological Industries, Israel), Tryptsin/EDTA Solution (Biochrom, Germany), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Alfa aesar, Germany), and the dimethylsulfoxide (DMSO) were maintained from Sigma-Aldrich, USA. Annexin-V FITC/Propidium iodide (PI) apoptosis detection kits were purchased from BD Biosciences, San Diego, USA. The JC1 kit was obtained by BD Biosciences, San Diego, USA. The Annexin-V FITC and acridine orange (Santa Cruz Biotechnology, USA), TUNEL kits were purchased from Santa Cruz Biotechnology, USA. This work was made between January 2014 and March 2014 in Medical Plants, Drugs and Scientific Research Center for cell culture studies at Anadolu University.

**Methods**

**Cell culture model**

MCF7 human breast carcinoma cells were maintained in 75 cm$^2$ sterile plastic tissue culture flasks in RPMI medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at 100 units/mL as adherent monolayers. These cells were grown at 37 °C humidified atmosphere containing 5% CO$_2$ in air.

**Cell survival assay**

To investigate the cytotoxic effects of silver nitrate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay was used. MCF7 human breast cancer cells were seeded into each well of 96 plate at a density of 10,000 cells. Silver nitrate (AgNO3) was administered in increasing doses (1.9 - 3.9 - 7.8 - 15.6 - 31.25 - 62.5 – 125 – 250 – 500 - 1000 µM) on MCF7 cells and incubated for 24, 48 ve 72 hours. The main stock solution of silver nitrate was prepared in DMSO and medium. The silver nitrate was dissolved in DMSO and diluted with medium. Following incubation, the MTT solution prepared in PBS was added to each well as 20µl and kept for 2-4 hrs. Then, the medium was removed and 100 µl DMSO was added to each well, dissolving the sediment for 10 minutes. The cells were measured at 540 nm using microtitre plate reader (Bio. Tec. ELx808IU, USA). The cell viability was calculated as a percent ratio and compared with control cells. Each concentration was repeated in three wells and IC$_{50}$ values were defined as the drug concentrations that reduced absorbance to 50 % of control values. Three independent experiments were conducted in order to obtain the proper IC$_{50}$ value.

**Annexin V-FITC/ PI staining**

The number of apoptotic/necrotic cells was measured using Annexin V/PI (propidium iodide) assay. MCF7 cells were seeded 6 well plates at a concentration of $1\times10^5$/cells/well with RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. The inhibition concentration of the silver nitrate (IC$_{50}$, 10 µM) was added on MCF7 cells. The plates were incubated at 37 °C in an atmosphere of 5% CO$_2$ for 72 hr. After the incubation, the cells were trypsinized and centrifuged, and the cell pellets were twice resuspended in phosphate-buffered saline (PBS). Then, the 100 µl Binding Buffer solution (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl$_2$) (BD Pharmingen, San Diego, USA) was added to each sample, and the cells were marked with Annexin V-FITC (5 µg/mL) and PI (propidium iodide) (5 µg/mL) for 15 minutes in the dark at room temperature (20-25 °C). The data were collected for 10,000 cells in each sample. The samples were analyzed by the BD FACS Aria (I) Cell Sorter, and Diva Software flow cytometry.

**JC-1 labelling**

The mitochondrial membrane potential was measured using JC-1 (5’,$\text{-}6,6’$-tetrachloro-1,1’$,3,3’$-tetraethylbenzimidazolylcarbocyanine iodide) dye. MCF7 cells were seeded in six-well plates at a density of $10^5$/cells/mL and the IC$_{50}$ dose of silver nitrate (10 µM) was added to cells. The cells were incubated 5% CO$_2$ air-conditioned atmosphere at 37 °C temperature. Following 72 hours incubation, the cells were trypsinized, washed with PBS, and centrifuged 400 × g for 5 minutes. JC1 dye solution (1X assay buffer + JC1 stock solution) was added on the cells. The JC1 stock solution was prepared by dissolving the DMSO. Thereafter, the samples were incubated at a temperature of 37 °C for 10-15 min. After incubation, the cells were washed twice with an assay buffer, and read by the BD FACS Aria (I) Cell Sorter Diva Software flow cytometry.

**TUNEL paraffin-embedded assay**

TUNEL paraffin-embedded assay was preferred to determine the DNA strand breakages. The MCF7 human breast cells were maintained in 75 cm$^2$ sterile plastic tissue culture flasks in RPMI medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin.
at 100 units/mL as adherent monolayers. After that, the IC_{50} concentration of silver nitrate (10 µM) was added on cells for 72 hr. Following that, the incubation, the cells in flask were collected in eppendorf tubes. The cells were fixed 10% formaldehyde (neutral-buffered) (900 ml distilled water (pH 7), 4g sodium phosphate monobasic, 6.5 g sodium phosphate dibasic, 100 ml of pure formaldehyde). Thereafter, the cells were embedded in paraffin and sectioned in 4 microns thickness. The sections were incubated in 60%, 70%, 80%, 90% and 96% alcohol series for 1 hr. The sections were reduced with distilled water and exposed to Proteinase K enzyme (in 37 °C) for 15 min. Then, they were washed with PBS and incubated in hydrogen peroxide (3% aqueous). The sections were also incubated in PBS and Equilibration Buffer for 3 min. Tdt enzyme working solution was kept for 1 hr. Digoxigenin peroxidase secondary antibody was incubated on sections for 30 min. DAB Chromogen kept for 3 min. After incubation, acetone and xylene were used for half an hour on sections. The sections were incubated three times in paraﬃn for half an hour.

**Confocal microscopy assay**

The apoptotic structures were observed by confocal microscopy. MCF7 cells were plated onto sterilized coverslips in 6 well-plates, and exposed to the IC_{50} dose of silver nitrate (10 µM) for 72 hours at a temperature of 37°C. After exposure, the control and treated cells were washed with PBS and stained by acridine orange fluorescent dye. Whereupon, the structure of the cell membrane and nucleus morphologically were monitored and the structural changes were detected. The morphology was scanned by the Leica TCS-SP5 II confocal microscopy and the Leica Confocal Software version 2.00 were used.

**Results**

**Silver nitrate induced cytotoxicity**

The cytotoxic effects on cell viability of silver nitrate were defined in MCF7 cells using MTT assay (Figure 1). Silver nitrate induced cytotoxicity in MCF7 cells. The cytotoxicity increased depending on time and dose (Figure 1). The IC_{50} concentration of silver nitrate on MCF7 cells was assigned as 10 µM at 72 hr. Figure 1 shows that the number of living cells decreased almost 40% in the lowest dose 1.9 µM at 72 hr. The highest dose 1000 µM caused to 92% reduced on cells for 72 hr. Based on these results, we determined silver nitrate IC_{50} dose for other apoptosis analyzes. These values were calculated using SPSS software and Microsoft Excel 2010. This experiment was repeated 3 times.

<table>
<thead>
<tr>
<th>Annexin V-FITC/PI staining</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
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</thead>
<tbody>
<tr>
<td>Control (%)</td>
<td>5.6</td>
<td>3.6</td>
<td>90.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Silver nitrate (%)</td>
<td>0.2</td>
<td>4.0</td>
<td>72.7</td>
<td>23.1</td>
</tr>
</tbody>
</table>

Q1, Necrosis; Q2, Late apoptosis; Q3, Viability; Q4, Early apoptosis

Table 1. The Detection of Early/late Apoptotic and Necrotic Numbers by Silver Nitrate (IC_{50}: 10 µM) Administered on MCF7 Cells for 72 hr.

**Figure 1. The Cytotoxic Effects of Silver Nitrate on Cell Growth**

In order to detect the ratio of early/late apoptotic and necrotic cell death induced by silver nitrate (AgNO3), the cells were labelled by Annexin V and PI (Figure 2). Apoptotic cells are stained by Annexin V and PI determines necrotic cells as a result of damaged cell membrane compare to control. Early apoptotic cells increased as 23%. There was not a significant increase in necrotic and late apoptotic cells (Table 1).

**The cells positive of apoptosis and necrosis were triggered by silver nitrate**

The changes in mitochondrial membrane potential (ΔΨ_{mt}) that occur during apoptosis were determined using JC-1 fluorochrome dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide). A reduced ΔΨ_{mt} was observed in response to IC_{50} dose of silver nitrate (10 µM) (Figure 3). Compared to control cells, the mitochondrial membrane potential (ΔΨ_{mt}) decreased approximately 3-fold after administration of silver nitrate for 72 hr (Table 2).

**The mitochondrial membrane was depolarized by silver nitrate**

We performed TUNEL (terminal deoxynucleotidyl transferase [TdT]- mediated deoxyuridinediphosphate [dUTP] nick endlabeling) paraffin embedded to analyse breaks of DNA strand. Silver nitrate caused DNA breaks on MCF7 cells (Figure 4c-d). These breaks of DNA strand
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Figure 3. The Reduction in Mitochondrial Membrane Potential was Assessed by JC-1 Staining. A) The untreated MCF7 cells. B) The treated MCF7 cells with silver nitrate. The mitochondrial membrane potential decreased approximately 3-fold compared to control cells for 72 h. P2, The cells with polarized mitochondrial membrane potential. P3, The cells with depolarized mitochondrial membrane potential. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

Figure 2 Annexin-V FITC and PI Staining of MCF7 Cells to Assay Early/late Apoptosis and Necrosis Induced by the Concentration of Silver Nitrate (IC_{50}, 10 µM) for 72 hr; (A) Control (B) MCF7 Cells were Treated with the Concentration of Silver Nitrate (IC_{50}, 10 µM) for 72 hr. The early apoptotic cells increased significantly 23% compared to control cells (Q1=Necrosis, Q2=Late apoptosis, Q3=Viability, Q4=Early apoptosis). At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

Table 2. The Efficacy of Silver Nitrate (IC_{50}, 10 µM) on Mitochondrial Membrane Potential of MCF7 Cells for 72 hr

<table>
<thead>
<tr>
<th>JC-1 labelling</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (%)</td>
<td>96.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Silver nitrate (%)</td>
<td>90.6</td>
<td>9.5</td>
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</tbody>
</table>


The morphological assay of MCF7 cells treated with silver nitrate

The morphological assay of MCF7 cells treated with silver nitrate (IC_{50} value: 10 µM) was conducted through confocal microscopic evaluation. We investigated the apoptotic effect of silver nitrate (IC_{50}: 10 µM) on MCF7 cells. We detected by the appearance of apoptotic bodies. The apoptotic structures of silver nitrate-treated MCF7 cells were determined at 72 hr by confocal microscopy. The morphological changes were recorded as nucleus condensation, ghost cells and fragmented DNA (Figure 5d-e-f).

Discussion

Our studies suggest that silver nitrate increased cytotoxicity and apoptotic effects on MCF7 cells. Consequently, the administered silver nitrate on MCF7 cells significantly reduced cell growth and was cytotoxic effective at low doses, indicating that apoptotic pathways. In addition, silver nitrate increased depolarized mitochondrial membrane potential. The depolarized mitochondrial membrane potential is an important factor at signal pathways leading to apoptosis. Finally, silver nitrate induced the early/late apoptosis and necrosis in MCF7 cells and the main cause of cell death was determined as early apoptosis. The early apoptosis was
Figure 4. The TUNEL Paraffin-embedded Assay. The light microscope was used for imaging. The apoptotic effect (DNA fragmentation) of silver nitrate on MCF7 cells. (a-b) No staining: in the absence of pretreatment there is no TUNEL access to DNA breaks. (20× and 40×). (c-d) The IC$_{50}$ concentration (10 µM) of silver nitrate for 72 hour was used on MCF7 cells (20× and 40×). The apoptotic nuclei were stain dark brown. The brown nuclei are indicated by the black arrow.

Figure 5. Confocal Microscopy Assay. The cells were stained by acridine orange. a-b-c) the nucleus of untreated cells. d-e-f) the nucleus of treated cells with silver nitrate (IC$_{50}$: 10µM). The apoptotic bodies were showed by arrows.
19% more activity than late apoptosis and necrosis.

For the past 20 years, silver compounds, particularly silver nitrate, has been used intensely in antimicrobial treatment [10-11]. The dilute solutions of silver nitrate have been used to prevent bacteria in newborn babies’ eyes. It has been administered in the treatment of extensive burns and has been used in the treatment of many diseases such as ulcers and wart [23]. The reports indicate that silver compounds have in vitro anticancer activity in last years. In these days, researchers have focused on silver nitrate from silver compound and have shown to trigger apoptosis [8]. Silver nitrate has been tested in immortalized murine fibroblast cell line L929 and the cytotoxic effects have been observed [24]. Park et al have demonstrated that silver nitrate increased cytotoxic activity and IC_{50} value of silver nitrate has found as 7.1 µg/ml in L929 cells for 24 hr [25]. In present study, IC_{50} value of silver nitrate was found as 130 µM in MCF7 cells. Kaba and Egorova (2015) have suggested that silver nitrate has been less cytotoxic toward U937 (human leukaemic monocyte lymphoma cell line) at various concentrations [26]. In another study, the different concentrations of silver nitrate (1-10-50-100 µg) have been used to study the viability of MDA-MB-231 cells and the toxicity has been measured. The silver nitrate has treated cancer cells and it has been observed more than 60% viable cells at 100 µg/ml concentration [27]. In our study, it was determined 8% viable cells at highest concentration of silver nitrate (1000 µM) (Figure 1). Silver nitrate (AgNO₃) has administrated on OVCAR-3 (ovarian), MB157 (breast) ve Hela (cervical) human cancer cell lines and the inhibition effects of silver nitrate (IC_{50} value) have been obtained respectively in 35 µM, 5 µM and 50 µM for 72 hours. Consequently, the silver nitrate has been observed most effective in MB157 cell line compared to OVCAR-3 and Hela cell lines due to ultra low dose (5 µM) as IC_{50} value [5]. Our results showed that IC_{50} value of silver nitrate was obtained as 10 µM in MCF7 cell lines. This means that silver nitrate is more effective on MCF7 cells compare to Hela and OVCAR-3 cells. But it is not as effective as in MB157 cells. These values are similar to the our results. After silver nitrate administrated at low doses (<1 µM), chromatin changes have been observed on K562 cells. The nuclear changes such as condensed chromatin have occured by silver nitrate. The concentrations of silver nitrate (10-15 µM) have caused nuclear shrinking with an infrequent formation of apoptotic bodies [35]. In another work, fluorescent microscopic images have showed that the silver nitrate has caused apoptotic bodies in MDA-MB-231 human breast cancer cells [27]. The our work reported that the inhibition concentration of silver nitrate (IC_{50}:10 µM) created condensed nuclear, fragmented DNA and various apoptotic bodies (Figure 5d-e-f).

In another work, it has been analyzed cytotoxicity of silver nitrate on normal cell line (GES-1) and BEL-7404 (human hepatoma cells), A549 (human non-small cell lung cancer), HepG2 (human hepatoma cells), NCI-H1650 (human non-small cell lung cancer cells) cell line and, it has exhibited excellent anticancer activity after a 48 h treatment. The IC_{50} values of AgNO₃ are respectively 38.67 µg/ml, 22.42 µg/ml, 33.824 µg/ml, 5.363 µg/ml and 29.949 µg/ml [36]. Silver nitrate has been tested for cytotoxic activities against A2780 ovarian cancer cells and, IC_{50} of silver nitrate has been found as 16.1 µM [37]. In our study, it has been found as 10 µM on MCF7 cells. This indicates that it has higher anticancer effect on MCF7 cells. Silver nitrate has treated with increasing concentrations of the compounds (0.01–10 µM) for 72 h on B16F10 (metastatic murine melanoma) cells.
and on Melan-a (non-tumorigenic murine melanocyte) cells and cell viability has been determined by the MTT colorimetric assay. Silver nitrate has been less active than cisplatin on non-malignant Melan-a cells, indicating that it might promote less damage on normal cells, which is an important characteristic of new anticancer drug candidates [38]. It has been found that Ag ions alone at 20 μM decreased cell viability of A549 cells by ~80%, but no cytotoxicity has been seen at doses less than 10 μM [39]. Silver nitrate has been assessed cytotoxicity by MTT, neutral red uptake (NRU), total protein content (TPC) and leakage lactate dehydrogenase (LDH) assays on Balb/c 3T3 and HepG2 human liver cancer cells. Inhibitory concentrations (IC_{50}, μM) of silver nitrate have been determined as 2.13 ± 0.88 in Balb/c 3T3 and 6.48 ± 1.60 in HepG2 cells by MTT assay [40]. In the lights of all these findings, it can be said that silver nitrate has different cytotoxic and apoptotic effects on several cancer cells.

In conclusion, we preferred to silver nitrate (AgNO_{3}) as silver compound, which is for testing cytotoxicity and apoptotic effects. The previous studies have suggested that silver nitrate has anticancer and apoptotic potential in various cell lines. The our work has revealed that silver nitrate has a high cytotoxic potential at low concentrations on MCF7 human breast carcinoma cells and the apoptosis proportion of cells was increased by treatment of silver nitrate in MCF7 human breast carcinoma cells depolarizing mitochondrial membrane potential. These findings demonstrate that silver nitrate could be used as a pharmaceutical agent against breast cancers.

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References


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