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Treatment of Murine Mammary Adenocarcinoma Cell Line (AMN3) With Polyvinylpyrrolidone Coated Silver Nanoparticles

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Abstract. In this research, we have synthesized silver nanoparticles with a pulsed laser using a metallic silver (purity; 99.99%), the targeted metallic silver piece was submerged in a liquid containing Polyvinylpyrrolidone (PVP). The produced nanoparticles were observed with different methods included UV-Vis Spectrometer, Atomic Absorption (AAS) and Electron Microscope (TEM). All peak absorption spectra for the two types of nanoparticles were ranged between 401 and 411 nm whereas nanoparticles size were in the range between 1 and 70 nm. The malignant anti-proliferation activity of nanoparticles was tested on murine mammary gland cancer cell line (AMN3). This activity was with potent inhibitory effect on the cellular proliferation rate of the cells under investigation. The inhibitory action was elevated when both the concentration of silver nanoparticles (AgNPs) and time of exposure were increased in comparison with the control untreated cells. Staining the cells with crystal violet stain revealed an increase in the level of cellular destruction. The results demonstrated that exposing the malignant cells to the nanomaterial of silver increases the proportion of the cells that undergo mitochondrial membrane disruption and DNA fragmentation and that will result in the induction of apoptosis. These results confirm that PVP-coated AgNPs induces apoptosis and cancer cell death.

INTRODUCTION

Nanoparticles (NPs) have been known since a long time, the “nano” term derived from Greek word meaning dwarf, these particles must be, at least in one dimension, at range between 1 and 100 nm. Nanotechnology encloses the design, production, characterization, and application of nanometer-scaled objects (nanoparticles, nanotubes, nanorods, nanosheet) in different fields [1]. Nanoparticles have many potential biomedical applications, as therapeutic agents, novel diagnostic agents, and as drug delivery systems [2]. Biological interactions of NPs differ from their bulk materials, its physical and chemical aspect attributable to their ability to pass through the cellular wall and membranes [3]. Several studies confirmed that nanoparticles have a superior cytotoxic profile compared with their bulk counterparts [4]. Environmental concerns were raised against these nanoparticles; these concerns resulted from the increment of their reactivity with biological Eco-systems [5]. Silver nanoparticles (AgNPs) are fine particles of metallic silver known as colloidal silver [6]. Colloidal silver was experimented to treat many infectious diseases [7]. Silver nanoparticles are the first and most widely commercialized nanomaterial to the medical and healthcare sectors. It was tested in a range of different biomedical applications, owing to its significant antibacterial [8], antifungal [9] and antiviral properties[10]. Nano-silver toxicity is attributed to the ability to release free silver ions in the biological system [11]. Cancer considered being one of the hardest diseases to treat; majorly most causes

failed treatment and die. The initial response of some cancers to chemotherapy may significantly decrease afterward resulting from its development of chemo-resistance [12]. It was shown that cancer cells of the breast were more sensitive than normal breast cells to oxidative stresses induced with such agents [13]. In consistent with this stream we hypothesized using nano-silver to treat breast cancer cells *in vitro*. These nanoparticles are known to produce reactive oxygen species (ROS)[14]. Therefore, we thought to benefit of this trait to study the proliferation inhibition and apoptosis induction in a murine mammary adenocarcinoma cell line (AMN3).

MATERIALS and METHODS

Culture Media and other Preparation

Tissue culture medium RPMI-1640 (US Biological -USA) supplemented with 10% Fetal Calf Serum (FCS) (Gibco -USA), L-glutamine, HEPES, sodium bicarbonate, penicillin (100 U/ml), and streptomycin (100 µg/ml) was prepared according to the manufacturer procedure. Murine mammary gland carcinoma cells (AMN3) was provided by the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). This media was used to maintain and growing of the AMN3 cells.

Synthesis of Silver Nanoparticles

This was carried out as mentioned in[15] with the modification that metallic silver plate (99.99% purity) was submerged in two types of solutions, Polyvinylpyrrolidone (PVP) (1.5mg/ml DDW) and deionized distilled water (DDW) separately at laser energy 800mJ/pulse for 15 min.

Characterization of Silver Nanoparticles

The ultraviolet-visible spectral determination was carried out using a spectrophotometer (UV-1800 series SHIMADZU, Japan) directly after synthesis and the concentrations were measured by atomic absorption (AA350, Germany). The size and morphology along with particle distributions was determined by TEM (CM10 pw6020, Philips-Germany)[16].

Cellular Viability

The viability of the cells exposed to nano-silver was evaluated by MTT assay method as described by [17]. Cellular morphology changes were observed using inverted microscope and images were collected using a CDD camera (DM510 Leica, Germany).

Mitochondrial Transmembrane Potential (MTP) Assay

Carried out as described by [18] using MitoCapture BioAssay Kit (USBiological, USA).

Nuclear Morphology Assay

Carried out using acridine orange and propidium iodide stain mixture as described in [19]. Cells were observed and Micro-photographed using inverted fluorescent microscope supplied with a digital camera using the blue and green filters (DMI6000 Leica, Germany).

DNA Fragmentation Assay

Carried out as described in [20], the DNA was visualized by Scie-Plus gel documentation system (Scie-Plus, England).

RESULTS

Characterizations of Synthesized AgNPs by Pulse Laser Ablution

Formation of AgNPs was noticed after the change of solution color from colorless to gray-yellowish. The absorption spectra for AgNPs (PVP and DDW) at laser energy (800mJ/pulse) showed peaks at 401 and 411nm respectively (Fig.1). The highest concentration obtained (AgNPs PVP and DDW) at laser energy 800mJ/pulse were 50 µg/ml. nano-silver particle sizes type AgNPs-DDW at laser energy 800mJ/pulse ranged between 7.5 to 63 nm, while particle sizes type AgNPs-PVP ranged between 5.1 to 51.9 at laser energy 800mJ/pulse, both were within the nano-scale range. The nanoparticle shapes observed by TEM were almost of spherical shape (Fig. 2).

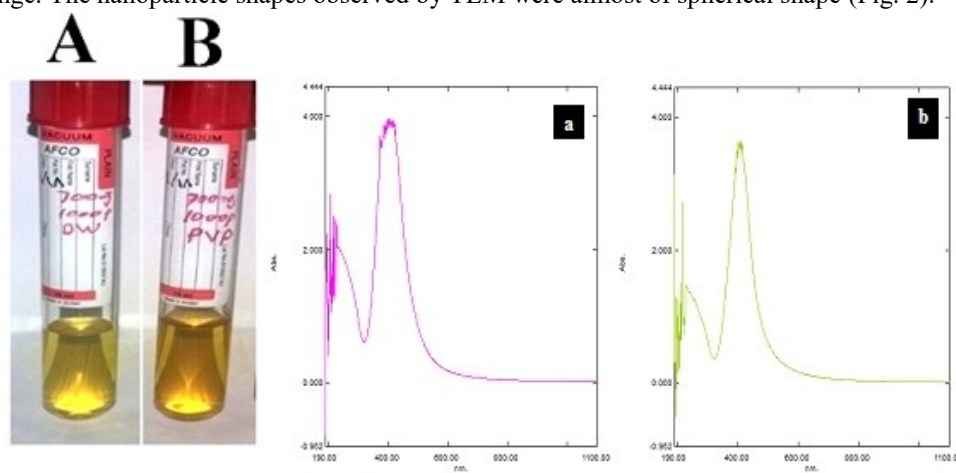


FIGURE 1. nano-silver synthesized by pulse laser ablation: A, AgNPs-DDW (800 mJ/pulse); B, AgNPs-PVP (800 mJ/pulse) and a, AgNPs-PVP with peak absorbance 401nm; b, AgNPs-DDW with peak absorbance 411nm.

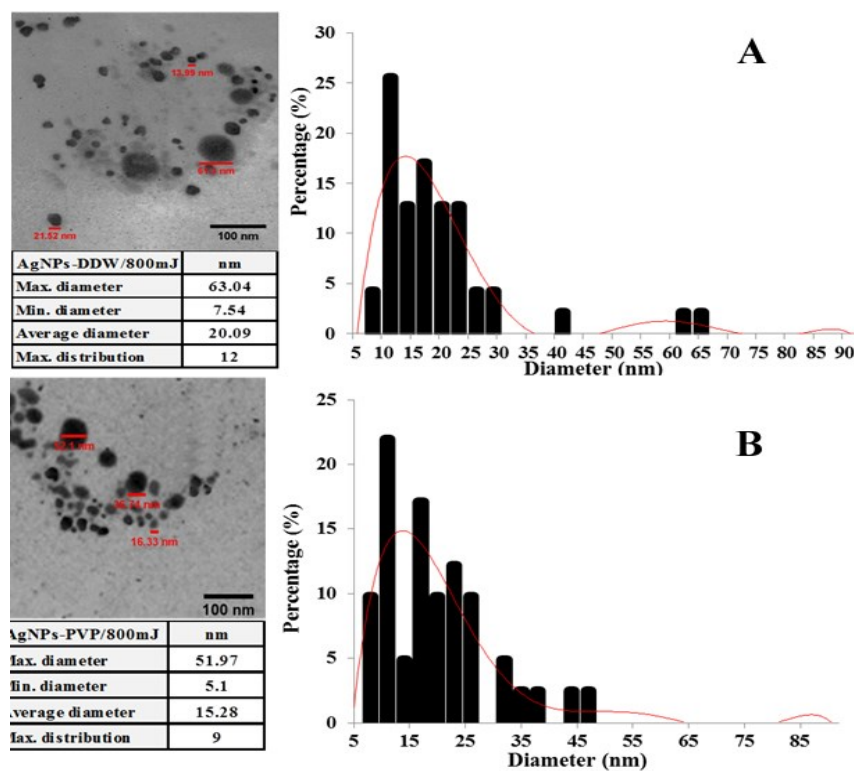


FIGURE 2. Nano-silver analysed by TEM. (A) AgNPs-DDW at laser energy 800mJ/pulse, (B) AgNPs-PVP at laser energy 800mJ/pulse.

Measurement of Cellular Viability

The anti-proliferative effect after 24 hrs of exposure of AMN3 cell line to both types of nano-silver was demonstrated by the results. AgNPs with (PVP or in DDW) were able to abridge the cellular viability of AMN3 cancer cell line with a concentration-ascending manner ($P < 0.05$). Obviously, even the lowest concentration used of AgNPs was effective significantly. The inhibitory concentration 50 (IC_{50}) for 24hrs values of both types of nano-silver were obtained. The synthesized AgNPs coated with PVP and non-coated showed no significant differences between them in the level of anti-proliferative (Fig.3).

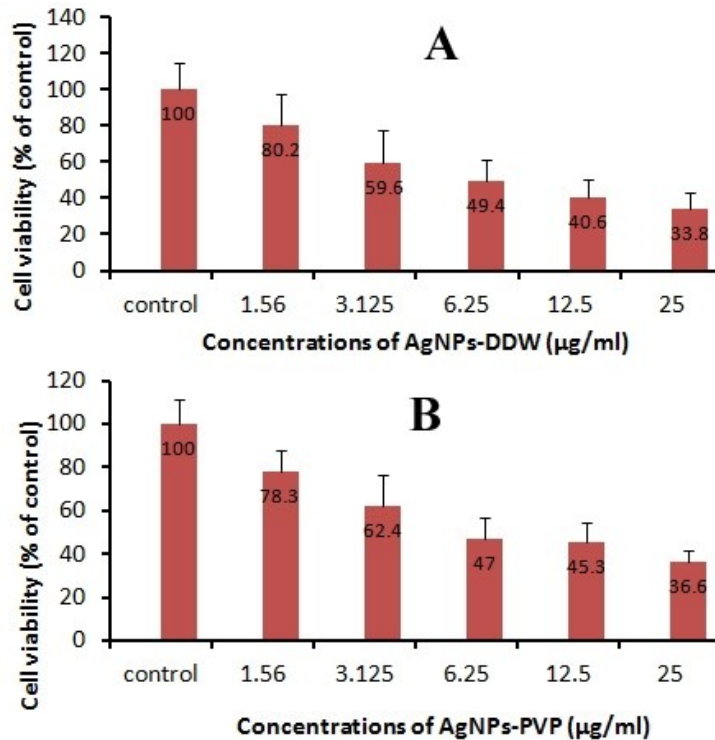


FIGURE 3. Nano-silver and AMN3 cells viability. Cells were exposed to AgNPs at versatile concentrations for 24 hrs, and cellular viability was tested by MTT method. The results presented as means of three separate experiments. **(A)** Cells treated with AgNPs-DDW at laser energy (800mJ/pulse), **(B)** Cells Treated with AgNPs-PVP at laser energy (800mJ/pulse). Significant differences statistically at level ($P < 0.05$) was observed among treated and untreated control groups.

Changes in Cellular Morphology

The results strongly informed that exposure to nano-silver produces massive cellular morphological changes in the cell line under investigation. They diverted between cell membrane disintegration, de-attachment to the surface, cytoplasmic shrinkage, and even signals of apoptosis were clearly noticed such as cell membrane blebbing and increment of apoptotic bodies in the media (Fig. 4).

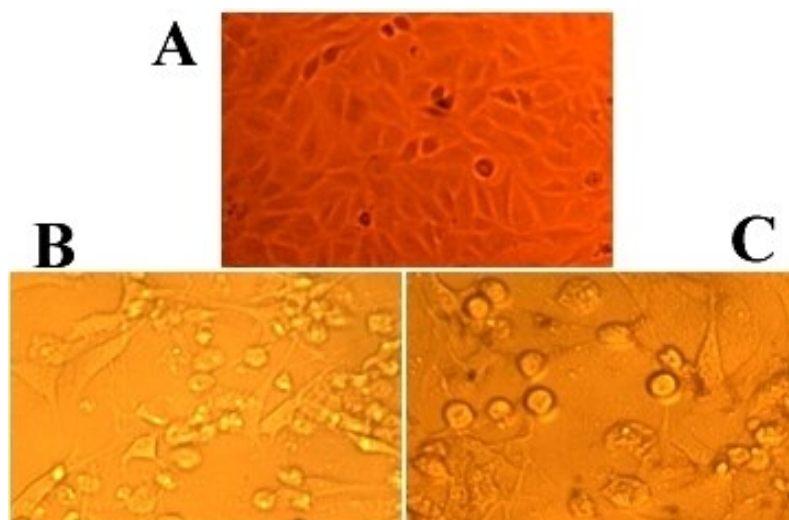


FIGURE 4. Optical micrographs of AMN3 cells after 24 hrs exposure to the IC₅₀ of nano-silver. (A) control untreated cells, (B) AgNPs-PVP (800mJ/pulse) treated cells in culture media, (C) AgNPs-DDW (800mJ/pulse) treated cells in culture media.

Changes in MTP

The early cellular apoptosis event was assessed using MitoPT apoptosis Kit. In untreated cells (control) the stain fluorescence appeared in red color (red cells) when accumulated in the middle space of the mitochondrial membrane because it became an aggregate compound. In cells suffering from apoptosis, the stain does not accumulate in the mitochondria due to the altered mitochondrial transmembrane potential (MTP), so the stain remains in the cytoplasm in its soluble form, emitting green wavelength (green cells). After 24 hrs of treating the cells with AgNPs IC₅₀, the proportion of green cells was very high compared to control untreated cells (Fig. 5).

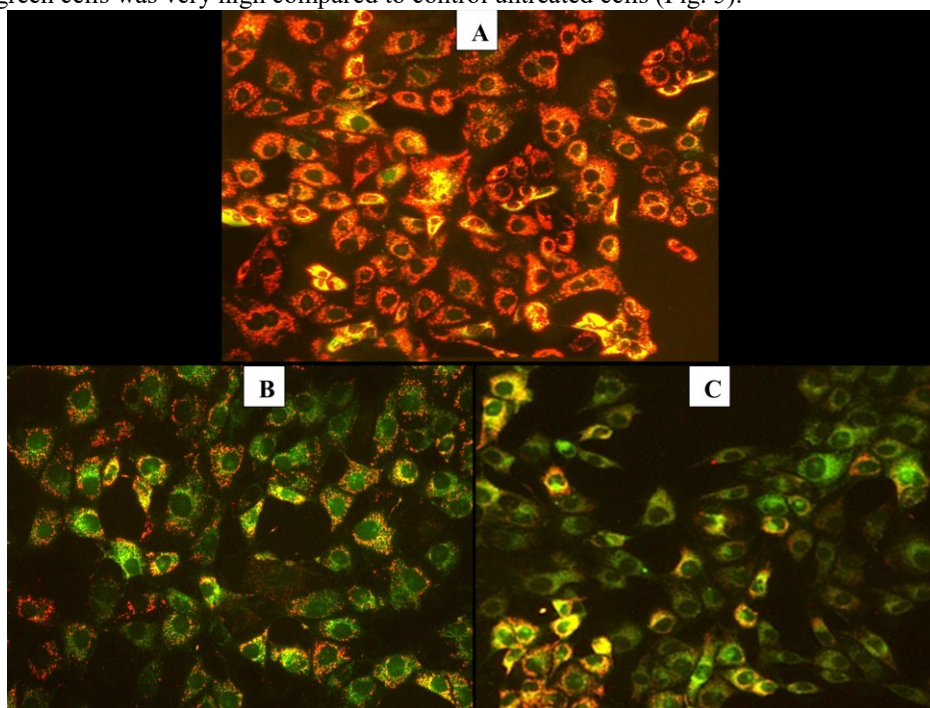


FIGURE 5. Fluorescent microscopy images demonstrate mitochondrial membrane distraction brought on by AgNPs. Cells treated with IC₅₀ for 24h and stained with MitoPT for 30 min. (A) untreated cells (control); (B) AgNPs-PVP (800mJ/pulse) treated cells; (C) AgNPs-DDW (800mJ/pulse) treated cells.

Nuclear Morphological Changes

Nano-silver induced different aspects of nuclear morphological changes in the treated cells; it's evident from the way that cells stained with. The green nucleus indicates viable cells, while cells suffering apoptosis appeared in green-yellowish or intensive green that resulted from the condensation of chromatin. The orange and red-colored nucleus with fragmentation indicates nonviable end-stage apoptotic cells (Fig. 6).

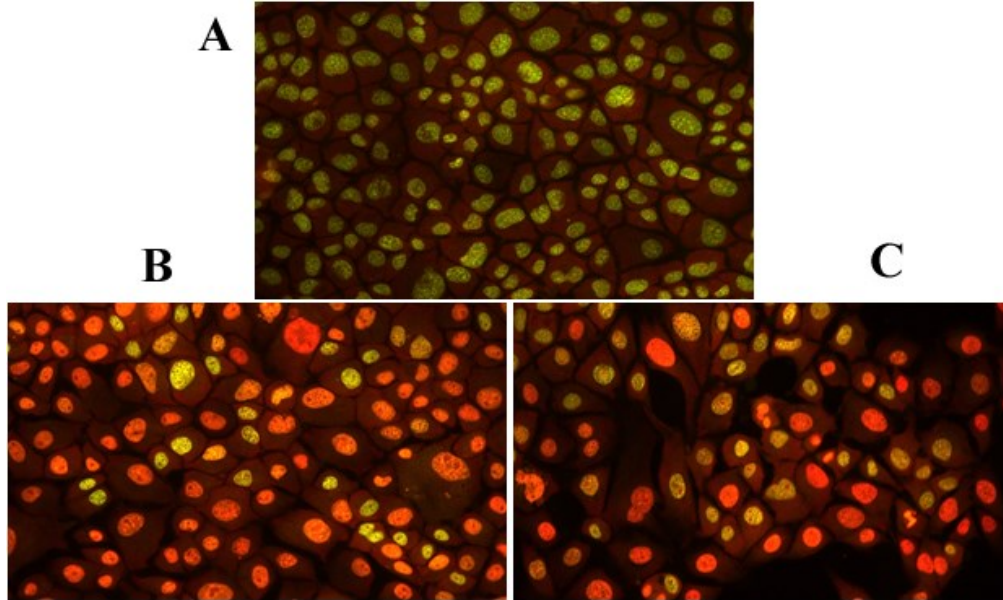


FIGURE 6. Fluorescent microscopy images of AO/PI-double-stained AMN3 cells that exposed to IC_{50} of AgNPs for 24 h. (A) control untreated AMN3 cells exhibit normal structures; (B) AgNPs-PVP (800mJ/pulse) treated cells; (C) AgNPs-DDW (800mJ/pulse) treated cells.

DNA Fragmentation

To demonstrate the late event of apoptosis accrued in AMN3 cells treated with nano-silver, the DNA fragmentation pattern was followed after agarose electrophoresis. Characteristics of DNA smears were readily noticed (Fig.7).

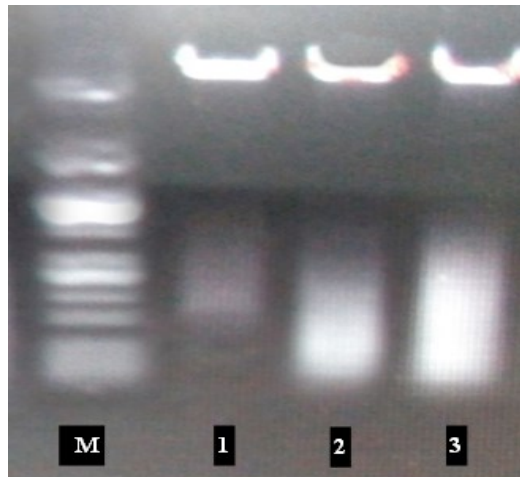


FIGURE 7. Late event of apoptosis upon treatment of AMN3 cells with the IC_{50} of nano-silver for 24 hrs. The fragmented DNA was resolved by agarose electrophoresis. Lane M: 1 kb ladder; lane 1: control untreated cells; lane 2: AgNPs-PVP (800mJ/pulse) treated cells; lane 3: AgNPs-DDW (800mJ/pulse) treated cells.

DISCUSSION

In this research, pulse laser ablation in liquid (PLAL) technique was invested to synthesized particles of nano-silver in two types of liquids, de-ionized double distilled water (DDW-AgNP) and PVP solution de-ionized double distilled water (PVP-AgNP). The results extracted from the experiments referred obviously to a successful formation of nanoparticles in both types of liquids. Afterword the formed nano-silver was tested against mammary carcinoma cell line to look for if there will be any difference in the anti-proliferative effectiveness between the two methods. We did not investigate the presence of Ag ions in the culture media that exposed to both types of nano-silver, because it's now widely accepted that the successful formation of these nanoparticles they will eventually produce Ag ions and as a results ROS in cancer cell line culture media [21]. In a previous study our team approved that nano-silver prepared by the same technique were able to induce cytotoxicity and proliferation inhibition of human breast cancer cell line AMJ13, while the prepared nano-silver possess minimal growth inhibition effect toward normal lymphocytes according to survival determination by MTT assay[22]. We used two types of nano-silver the first contained PVP the other prepared by the same technique; results indicated both types (AgNP-PVP and AgNP-PVP) possess similar IC50. The presence of PVP in the solution did not affect the anti-proliferative activity of the nano-silver as indicated with MTT assay. Both types of nano-silver demonstrated the early and late events of apoptosis. The looking for innovative therapies of cancer is highly requested demand, this is stems' from the nature of this disease. The traditional chemo-radiotherapies that was invented during the last five decades and still used nowadays encounter elevated resistant in many cases [23]. Most of these therapies either attack cancer cells DNA replication machinery, or interact with spindle microtubules to prevent proliferation of these cells throw force them to enter the apoptosis [24]. In this regard nano-silver may serve more efficiently the treatment of cancer because this martial capable of attack cancer cells from so many different directions and interfere with many essential mechanisms of cellular functions that cancer cells cannot resist and lead them to die[25].

AUTHORS CONTRIBUTION

Nawfal N. and Asmiet R. prepared AgNPs by pulse laser ablation method and characterization of AgNPs. Evaluation of AgNPs cytotoxicity was done by Nawfal N. and Amer T. Tawfeeq All the authors contributed ideas and contributed to the writing of this paper.

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