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Effect of Silver Nanoparticles on Improving the Efficacy of 5-Aminolevulinic Acid-Induced Photodynamic Therapy

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ABSTRACT

Introduction: The most important limitation of 5-aminolevulinic acid (5-ALA)-induced photodynamic therapy (PDT) is the efficacy of the cells in converting 5-ALA to protoporphyrin IX. The present study aimed to investigate the effectiveness of silver nanoparticles (AgNPs) with the photosensitivity at the surface plasmon resonance wavelength on 5-ALA-mediated PDT.

Material and Methods: First of all, the toxicity of 5-ALA, AgNPs, and combined 5-ALA and AgNPs was evaluated on DFW cell line derived from melanoma. After choosing the optimal concentration, both pulsed and continuous light irradiations were conducted at different doses using a light-emitting diode source in the groups receiving 5-ALA, AgNPs, as well as 5-ALA and AgNPs combination, and the controls. The cell survival was evaluated 24 h after irradiation using MTT assay.

Results: According to the results, light exposure did not significantly change cell survival in the absence or presence of AgNPs. However, light exposure in the presence of 5-ALA and AgNPs/5-ALA combination caused a significant reduction in the cell survival. The necessary light exposure to induce 50% cell death (ED50) in the presence of 5-ALA was 1280 mJ/cm2; however, this value was 280 mJ /cm2 in the presence of combined AgNPs and 5-ALA.

Conclusion: As the findings indicated, PDT had a higher efficacy in the presence of combined 5-ALA and AgNPs than in the sole 5-ALA presence. Nonetheless, further studies are required to evaluate the definitive mechanism of these findings.

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Introduction

Cancer refers to the uncontrollable growth of cells that can attack, metastasize, and spread to the adjacent cells [1]. Melanoma is a common form of skin cancer caused by a type of skin cells called melanocytes with malignant tenderness. Melanocyte is the reason behind the growth of melanin, dark colored skin, hair, eyes, and spots on the body. Melanoma tumors are mostly brown or black. If diagnosed early, melanoma is 100% curable, but in invasive cases when it spreads to other tissues of the body, there is a low chance of treatment.

Local surgeries, chemotherapy, and radiation therapy are the most commonly used therapeutic procedures for relapses and metastatic melanoma. Nonetheless, there has been little progress in metastatic melanoma treatment due to the ineffectiveness of these systemic treatments, which is mainly caused by the resistance of the melanoma cells to apoptosis-based therapeutic agents [2]. Given the side effects of these therapeutic approaches and the resistance of melanoma cells to treatment, it is essential to find new and non-invasive methods.

Recurrent photodynamic therapy (PDT) for skin metastases has been shown to be an effective method. The PDT as a photochemical and minimally invasive approach has been known for the treatment of various diseases, such as cancer. This therapeutic approach is underlined by three basic elements, namely light, photosensitizer, and oxygen .None of these elements are toxic; nevertheless, their combination triggers a photochemical reaction and produces single oxygen species or reactive oxygen species (ROS) with cellular toxicity effect through apoptosis, necrosis, and autophagy [3, 4].

5-Aminolevulinic acid (5-ALA) is a chemical that has been successfully used for the diagnosis and treatment of cancerous tissues. The 5-ALA is a naturally occurring substance found in the body, which is not sensitive to light and serves as a pathway for the synthesis of heme as a biological precursor [5].

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According to the literature, 5-ALA-mediated PDT induces severe cell apoptosis caused by mitochondrial pathway activation, which can effectively inhibit the growth of the cancer cells and increase patients' survival. This kind of phototherapy has been suggested to result in a significant induction of apoptosis in apoptosis-resistant human glioma cells [6].

The 5-ALA has been widely used in PDT due to low dark toxicity to cells, rapid clearance from the body (24-48 h), and rapid conversion into protoporphyrin IX (PpIX) (i.e., 1-8 hrs). However, its hydrophilic property and bipolarity impede its effect on the site of the lesion and cell membrane [7]. Some of the limitations of PDT mediated by 5-ALA include the dependence of PpIX concentration on 5-ALA dose and the specific uptake of cells from ALA-5, low efficiency of some cell lines in converting 5-ALA into PpIX, and inhomogeneity and inadequate production of PpIX in the tumor.

Several approaches have been proposed to overcome these limitations, including: 1) enhancement of the activity of the enzymes involved in the pathway for PpIX synthesis, 2) inhibition of the biological process of PpIX conversion into heme in two ways, namely the removal of the substrate ferrous ions (Fe^{2+}) required for the reaction and inhibition of enzyme ferrochelatase that catalyzes the reaction, and 3) inhibition of PpIX efflux pump, especially constraining ABCG2 transporter using inhibitors [8].

The application of nanotechnology in PDT and the transfer of photosensitizer represent an important achievement since nanoparticles boost the effectiveness of PDT. Recent advances in the application of nanotechnology in PDT include the production of biodegradable and non-biodegradable nanoparticles as photosensitizer or passive carriers and the synthesis of targeted photosensitizer compounds for active transmissions [9]. To compensate for these limitations in 5-ALA-induced PDT, a combination of nano-structures with a 630-nm plasmonic absorption peak may help improve the efficacy of this treatment.

Among these nanostructures, special attention has been paid to silver nanoparticles (AgNPs) due to their distinctive features, optical and radiation sensitivity, photodynamic ability, as well as anti-bacterial, antifungal, antiviral, and anti-venous properties [10-12]. Due to their size and geometric shape, AgNPs show plasmonic resonance surface [13], as well as anti-cancer properties. The anti-cancer effects of AgNPs have been investigated under various in vitro conditions for different tumor types, such as lung, blood, breast, and other tumors. The AgNPs has been unanimously reported to be able to damage DNA, impede the growth of cancer cells, stop cell cycle, and subsequently cause cell apoptosis [14, 15].

According to the recent studies, the cytotoxicity of AgNPs is the function of the size, concentration, and incubation time of the target cell [16]. The nanoparticles of smaller size and higher concentrations are more toxic [17]. With this background in mind, the present study was conducted with the aim of investigating the effect of AgNPs on the efficacy of 5-ALA-mediated PDT.

Materials and Methods

Chemicals

The separation of the cells from the floor of the flasks was performed using $AgNO_3$, trypan blue, 5-ALA (95% purity), and trypsin-ethylenediaminetetraacetic acid. MTT powder was obtained from the Sigma Company (St. Louis, USA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) medium-1640 were purchased from the Gibco Corporation (Lot No: 371748, USA).

Cell line and cell culture conditions

The DFW cell line is a dipigmented subunit derived from human dermal fibroblast cells obtained from melanoma cancer. The cells were grown in 75 cm^2 plastic tissue culture flasks as a monolayer in RPMI-1640 medium and supplemented with 10% FBS and 1% antibiotics (50 units/mL penicillin and 50 mg/mL streptomycin). The cells were incubated at 37℃ in a humidified atmosphere in a CO_2 incubator containing 5% CO_2.

When the cells were proliferated and the flask was filled, they were detached from the flask bed using trypsin-ethylenediaminetetraacetic acid. The cell culture medium was changed every 2-3 days. The trypan blue method was used for cell counting and ensuring a cell survival of more than 98% before undertaking the designed treatments.

Silver nonaparticles synthesis

The AgNPs synthesis was carried out as described by Hengzima et al. [18]. To this end, 24.75 mL aqueous solution of AgNO3 (0.05 M), 0.5 mL tryptose sulphite cycloserine (75 mM), 200 µL (17.5 mM), and 60 µL H2O2 (30 wt %) were added to a flask and rapidly stirred at room temperature. During this process, 250 µL NaBH4 (100 mM) was rapidly injected into the obtained solution, which resulted in the immediate production of a light yellow solution. After 35 min, a deep yellow colloidal solution was obtained, which was an outcome of the formation of large AgNPs. Subsequently, the color changed from deep yellow to red, green, and finally blue in several seconds.

The size distribution curve of synthesized particles and their zeta potential were recorded by a particle size analyzer (Molvern Instruments, Southborough, Massachusetts). In addition, an spectrophotometer (Shimadzu Model UV-1700, Japan) was utilized to determine the ultraviolet visible spectrum of AgNPs.

Cytotoxicity of the treatment agents

In this step, the inhibitory concentration of 10% (IC_{10}) and IC_{50} of AgNPs and 5-ALA were determined and their optimum concentrations with the least toxic effects of 5-ALA and AgNPs were evaluated for the DFW cells. To obtain these data, after seeding 1.5×10^4

of cells/well in the 96-well plates, they were incubated for 24 h in the culture medium containing RPMI-1640 with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2.

Subsequently, the cell incubation continued in the presence of six concentrations of 5-ALA (i.e., 0.25, 0.05, 1, 2, 4, and 8 mM) and seven concentrations of AgNPs (i.e., 0.011, 0.11, 1.1, 5. 5, 11, 16.5, and 19.8 µg/mL) for 4 h. A control group with no additional agent was also considered at all experimental series. To determine the cytotoxicity induced by the synchronized presence of 5-ALA and AgNPs, the cells were incubated in the presence of 1 mM 5-ALA with different concentrations of AgNPs for 4 h. The MTT test was used to assay the cell survival and cytotoxicity of the agents.

Treatment groups

After determining the optimum concentration for the therapeutic agents, the PDT effect on cell survival was investigated by subjecting DFW cells to different experiments as shown in Table 1.

Table 1. Treatment conditions of the studied groups

Groups No.	Therapeutic agent concentration (µg/mL)		Optical doses (mJ/cm^2)		
	5-ALA	AgNPs			
1	0	0	0	0	0
2	0	0			
3	0	11	435	1305	3915
4	167.6	0			
5	167.6	11			

5-ALA: 5-aminolevulinic acid, AgNPs: silver nano-particles

After 24 h incubation of cells with 1.5×10^4 cells per cc and evacuation of the cellular environment, therapeutic agents (i.e., AgNPs, 5-ALA, combined 5-ALA and AgNPs) were incubated on cells for 4 h at specific concentrations. In the next step, during the evacuation of the cellular environment, 100 µl of the medium with 3% FBS was added to each well of the plate. To determine the phototoxic effect, the cells were irradiated into the optical doses of 435, 1305, and 3915 mJ/cm^2 by a light-emitting diode system (633 nm, 1 W, 7.25 mW/cm^2). After treatment, 100 µl of the culture medium accompanied with 10% FBS was added to each well, and incubation was continued for 24 h. In the final step, the cell survival was determined.

Cell viability assessment

The viability of the treated cells was determined by MTT assay. In order to perform this test, at first, the culture medium was removed from the cell wells. Then, 100 µL culture medium without FBS and 10 µL MTT (0.5 mg/mL) were added, and the plates were transferred to the culture incubator. The plates were covered by the aluminum foils in order to avoid MTT reduction by the background light.

After 4 h, 200 µL dimethyl sulfoxide was added into each well. After 5 min of stirring, the optical density of the wells were determined to be 570 nm against 630 nm by an enzyme-linked immunosorbent assay reader system (Awareness, model STATFAX, USA). The experiments were performed for at least three times.

Judgment parameters

Effective dose (ED_{50})

In addition to the use of cell survival percentage following different designed treatments, ED_{50} (the required dosage for inducing 50% cell death) was utilized to estimate and compare the efficacy of the PDT in the presence of 5-ALA, AgNPs, as well as 5-ALA and AgNPs combination.

Synergistic ratio

The synergistic ratio was calculated as the ratio of total cell death caused by light exposure and therapeutic agent to cell death caused by exposure in the presence of each agent. Values smaller than 1 indicated the effect of light-induced synergy in the presence of each agent.

Data analysis

The data obtained from the experiments were analyzed by SPSS software, version 24. First, the Kolmogorov-Smirnov test was used to assess the normality of the data. When the normality was confirmed, one-way ANOVA and Tukey's test were employed. To ensure the reliability of the obtained results, each test was repeated for at least three times.

Results

Silver nanoparticle characterization

The AgNPs were characterized using zeta potential, ultraviolet-visible spectroscopy, and transmission electron microscope (TEM). Figure 1 depicts the size distribution curve. According to the curve, the maximum distribution of AgNPs was observed at a diameter of about 19.01 nm. Figure 2 illustrates the zeta potential of AgNPs, which is negative according to the shape.

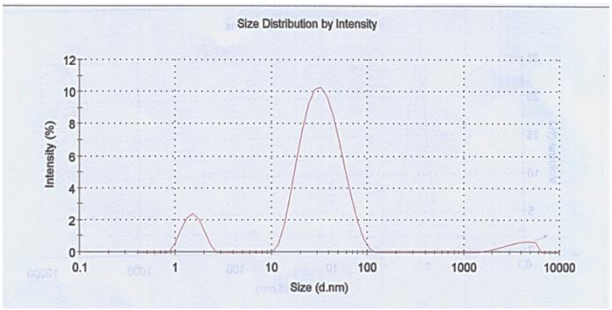

Figure 1. Size distribution curve of silver nanoparticles

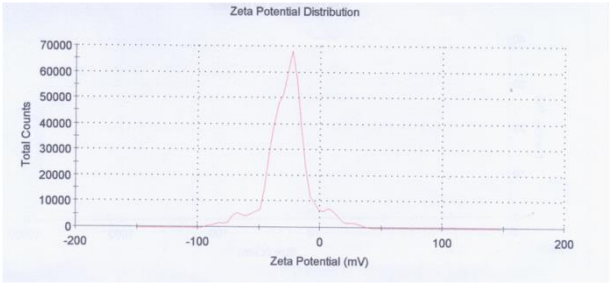

Figure 2. Zeta potential distribution curve of silver nanoparticles

Ultraviolet-visible spectra were recorded to determine the absorption peak of AgNPs. As displayed in Figure 3, the absorption peak was reached at 635 nm. Furthermore, TEM was utilized to determine the shape of AgNPs. Figure 4 shows that AgNPs are not spheroid or regular.

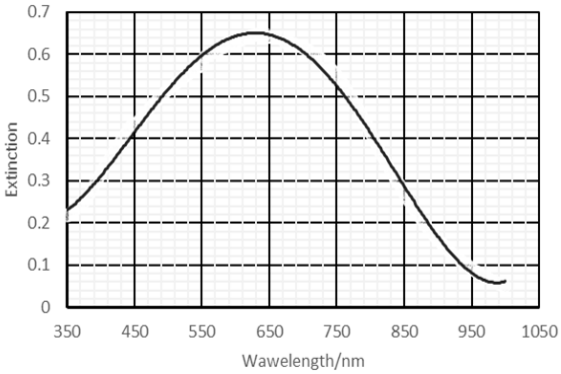

Figure 3. Absorption spectrum of silver nanoparticles

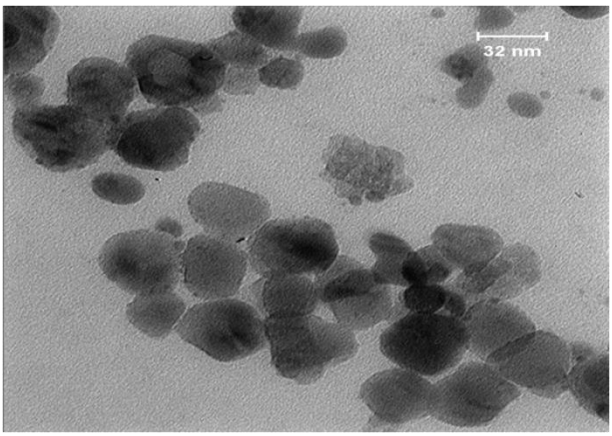

Figure 4. An image of silver nanoparticles taken by a transmission electron microscope

Cytotoxicity data

In the presence of 5-ALA, an increase in the concentration of 5-ALA raised the percentage of the DFW cell line survival (Figure 5). The cytotoxicity experiments of the AgNPs suggested that cell viability dropped by up to 60% at the concentration of 19.8 µg/mL (Figure 6).

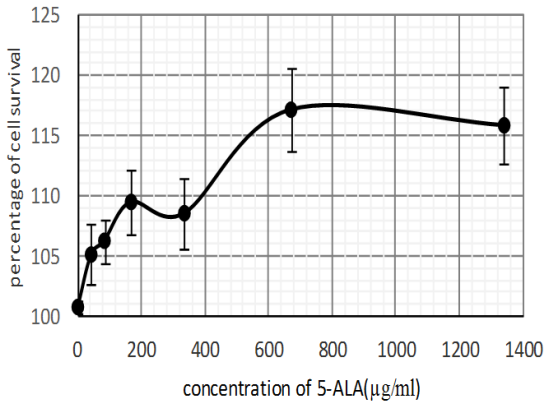

Figure 5. Mean cell survival obtained by MTT test after four hours of cell incubation with 5-aminolevulinic acid (mean±standard error; the experiments were performed for at least three times)

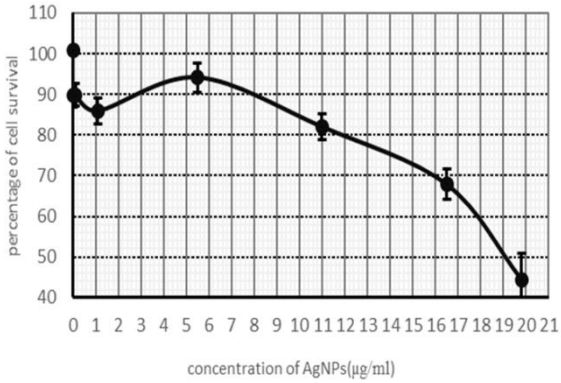

Figure 6. Mean cell survival obtained by using MTT test after four hours of cell incubation with silver nanoparticles (mean±standard error; the experiments were performed for at least three times)

A significant difference was observed in the control group regarding the given concentrations, except for 58.5 µg/mL. In the analysis of 5-ALA toxicity with AgNPs, cell survival rate at a concentration of 19.8 µg/mL, AgNPs was significantly different from the control group and other concentrations (Figure 7).

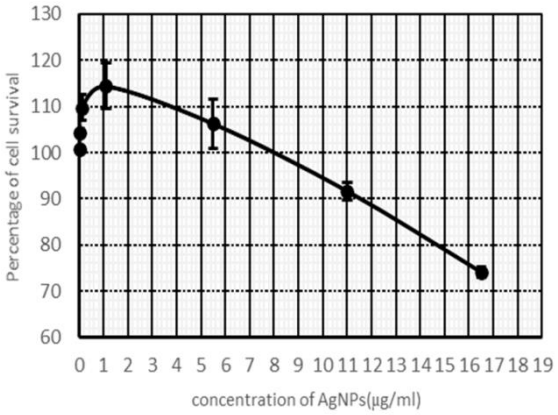

Figure 7. Mean cell survival obtained by MTT test after four hours of cell incubation in the presence of different concentrations of silver nanoparticles with 5-aminolevulinic acid (167.6 µg/mL) (mean±standard error; the experiments were performed for at least three times)

Experimental results

The PDT with the optical doses of 435, 1305, 3915 mJ/cm^2 was independently applied to the four groups. In this section, the continuous and pulsed presentations of data obtained from 1-watt light irradiation with a wavelength of 633 nm and intensity of 7.25 mW/cm^2 were presented in four independent groups. As shown in figures 8 and 9, continuous and pulsed light exposure did not have any significant effect on cell survival, and the control group was not significantly different from any of the groups. In the presence of 5-ALA with continuous light, the highest cell death at an optical dose of 3915 mJ/cm^2 was about 77%, while this value was about 60% for pulsed exposure as 10:5.

In the presence of AgNPs, continuous exposure was not significantly different from pulsed exposures in terms of cell survival. In the presence of 5-ALA and AgNPs combination, the highest cell death was 77% at an optical dose of 1305 mJ/cm^2 in the continuous exposure and 76% as 10:10 in the pulsed exposure. Furthermore, all experimental groups were significantly different from the control group.

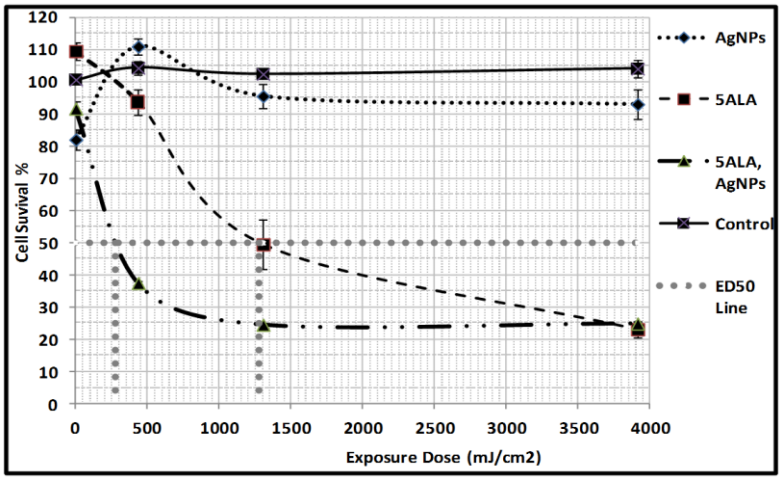

Figure 8. Cell survival percentage for different continuous exposure doses in the presence of 5-aminolevulinic acid, silver nanoparticles, and combination of 5-aminolevulinic acid silver nanoparticles, as well as the absence of any agent 24 hours after treatment (mean±standard error for the mean obtained from three experiments)

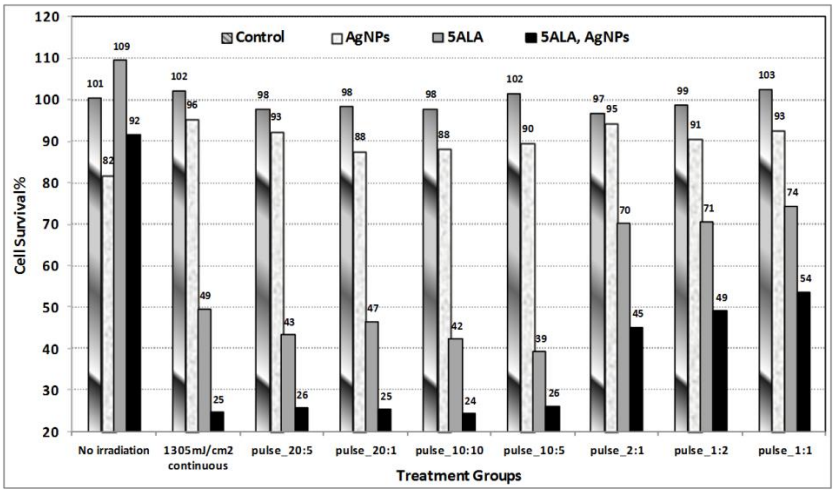

Figure 9. Cell survival percentage for 1305 mJ/cm^2 continuous and pulsed exposure in the presence of 5-aminolevulinic acid, silver nanoparticles, and combination of 5-aminolevulinic acid silver nanoparticles, as well as the absence of any agent 24 hours after the treatment (mean±standard error for the average obtained from three experiments)

The ED_{50} estimated for the group receiving 5-ALA was significantly higher than that in the group receiving 5-ALA and AgNPs combination. The values of synergistic ratio and ED_{50} calculated with continuous and synergistic ratios determined for the pulsed exposures are presented in Table 2.

Table 2. Values of ED_{50} and synergistic ratio in groups with continuous photodynamic therapy and synergistic ratio in the groups with pulsed exposures in the presence of the therapeutic agents

		Therapeutic agents		
		AgNPs with 5-ALA	5-ALA	AgNPs
Continuous exposure	Synergistic ratio	0.07±0.01	-0.85±1.12	2.25±1.13
	ED_{50} (mJ/cm^2)	280	1280	>3909
Pulsed exposure	Synergistic ratio	0.15±0.04	-0.21±0.13	2.26±0.9

5-ALA: 5-aminolevulinic acid, AgNPs: silver nano-particles

Discussion

The present study was conducted to use triangular AgNPS with a plasmonic absorption peak at 635 nm with 5-ALA for applying PDT to DFW cells. The limitations of 5-ALA PDT include hydrophilic and bipolar effects, which impede its permeability in the lesion site and cell membrane [7], dependence of protoporphyrin concentration on 5-ALA dose, and consequently the specific withdrawal of cells from ALA-5, low efficacy of some cell lines in converting 5-ALA into PpIX, and inhomogeneity and inadequate production of PpIX in the tumor [8].

The DFW cell line was a resistant cancer cell line. The toxicity of AgNPs was enhanced with increased concentrations, leading to a decline in the cell survival rate. The toxicity dependence on the nanoparticle concentration is consistent with the results reported in most of the studies investigating the cytotoxicity of AgNPs. The nanoparticle density that inhibits the cell growth and proliferation by 50% (IC_{50}) is about 19 µg/mL. A non-toxic concentration of AgNPs was selected for the PDT experiments, which guaranteed 82% survival rate for the cells.

The incubation time of cells with AgNPs was estimated at 4 h, following the study of Yadegari et al. [19] and considering the required time for converting 5-ALA to PpIX [7]. The identical incubation time of AgNPs with 5-ALA offers the advantage of using AgNPs and 5-ALA nanoparticles in the nanostructures to improve PDT in the future. The toxicity of 5-ALA on DFW cells was evaluated at six concentrations of 5-ALA. The review of literature, including a study performed by Kim et al. in 2011, suggested that 5-ALA concentration, PpIX accumulation, ROS production, and cellular mass are directly related to ALA-mediated PDT.

In addition, at 1 mM concentration of ALA, the accumulation of PpIX in the cell is maximized, with PDT producing the highest ROS and highest rates of apoptosis and cell necrosis. Therefore, in the present study, the concentration of 1 mM (167.6 µg/mL) was used for treatments [20]. We investigated the cytotoxicity of 167.6 µg/mL 5-ALA in combination with different concentrations of AgNPs for the DFW cells.

The toxicity derived from the combination of 5-ALA and AgNPs may indicate the absorption of AgNPs by cancer cells. Given the lack of 5-ALA toxicity for this cell line, this toxicity could be attributed to AgNPs. However, this toxicity was lower, compared to the toxicity created by AgNPs, which could be due to the presence of 5-ALA and its associated cellular survival. In the concentration of 11 µg/mL AgNPs, direct exposure to light nanoparticles had no effect on cell death.

Accordingly, it can be concluded that silver does not have sufficient photodynamic effect. This is inconsistent with the results obtained by Tinga et al., demonstrating the photodynamic capacity of AgNPs on breast and lung cancer cells [10]. This discrepancy could be due to different concentration, size and shape, incubation time, synthesis method, and cell line type. The comparison of the exposure results in the presence of 5-ALA and combined 5-ALA and AgNPs suggested that in PDT with the latter exposure type, the same treatment efficiency could be obtained at a higher dose with less optical efficacy.

This improved 5-ALA PDT efficacy due to the presence of AgNPs could be explained by at least one of the following mechanisms:

- The concomitant presence of AgNPs and 5-ALA leads to a greater cellular uptake from 5-ALA, compared to the presence of 5-ALA alone. This may result in the formation of more PpIX, and consequently increased effect of PDT in the 5-ALA and AgNPs received cells.
- The presence of AgNPs improves PDT efficacy by the enhancement of the activity of the enzymes involved in the pathway of PpIX synthesis or inhibition of the activity of PpIX extraction pumps in the cell.
- Finally, if the presence of AgNPs does not contribute to the formation of PpIX, failing to produce direct toxicity through the formation of oxygen and silver ions, then it is anticipated that the surface properties of nanoparticles increase the light flux between AgNPs and PpIX. In this way, the reception of more photons by PpIX leads to an increase in the production of reactive oxygen species, and ultimately enhances the therapeutic efficacy.

The comparison of the results of pulsed and continuous exposure indicated no significant difference between these two exposure modes in terms of the improved efficacy of the PDT. PulsedHowever, pulsed exposure is expected to be more useful in clinical treatments, which is crucial to the oxygenation of the cell during PDT.

Conclusion

As the findings indicated, the cytotoxicity of AgNPs did not increase with light exposure. The concomitance of AgNPs with 5-ALA led to a significant decline in cell survival after PDT. The improved efficacy of PDT could

be explained by a greater uptake of 5-ALA cells, the surface properties of AgNPs, and amplification of the activity of the enzymes involved in the pathway for PpIX synthesis. Further studies are required to answer these questions.

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