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Original Article

Cytotoxic and Oxidative Stress Effects of Zinc Phthalocyanine–Loaded Nanoemulsion–Based Photodynamic Therapy in a Skin Fibroblast Cell Line

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Abstract

Introduction: Photodynamic therapy (PDT) is a treatment modality in which a light-sensitive substance is used to destroy cancer cells and treat skin diseases. Recently, zinc phthalocyanine (ZnPc) has gained special attention as an efficient photosensitizer. In this study, we evaluated cytotoxicity, assessed the potential effects on cell migration, and measured oxidative stress induced by PDT using a ZnPc-loaded nanoemulsion (NE) in a skin fibroblast cell line.

Methods: Cytotoxicity was assessed by the MTT assay, the ability of cells to repair tissue by the wound-healing assay, and the level of oxidative stress by measuring malondialdehyde (MDA) and total antioxidant capacity (TAC). Data were analyzed using SPSS software version 22.

Results: Results showed that the lowest cytotoxicity on fibroblast cells was observed at a drug concentration of 20 µg/mL, a laser dose of 12J/cm^2, and an incubation time of 1 hour in the dark. The scratch test confirmed that although cell migration was higher in the control group, the difference at the optimized dose was not statistically significant. At two concentrations of 40 and 60 µg/mL, MDA and TAC levels showed a significant increase ($P \leq 0.05$) and decrease ($P \leq 0.05$), respectively, compared to the control group.

Conclusion: The time-dependent efficacy of NE/ZnPc as a photosensitizer in PDT was revealed in this study. The highest cell viability alongside minimal non-significant changes in oxidative stress indicators (i.e., MDA and TAC) indicates that NE/ZnPc-PDT may have great potential for use in skin therapeutic applications where the preservation of the health of non-target cells, such as fibroblasts, is essential.

Keywords: Photodynamic therapy, Cancer, Zinc phthalocyanine, Scratch test, Cell migration

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Introduction

As the largest organ of the body and primary defense barrier against environmental factors, the skin plays a vital role in maintaining homeostasis and protecting the organism. Dermal fibroblasts, the main cells of the dermis, synthesize and organize components of the extracellular matrix, such as collagen and elastin, and play a major role in wound healing and skin regeneration. During the proliferative phase of wound healing, fibroblasts migrate to the injury site, proliferate, and differentiate into myofibroblasts, contributing to wound contraction and closure (1). Therefore, evaluating and enhancing fibroblast viability and migratory capacity are crucial for developing novel wound-healing therapies. Photodynamic therapy is a non-invasive and light-activated therapeutic technique that has gained considerable attention in recent decades. This method relies on a photochemical reaction between 3 components: a photosensitizer, a light source with an appropriate wavelength, and molecular oxygen. Following light activation, the photosensitizer transfers energy to oxygen, producing reactive oxygen species (ROS), including singlet oxygen and free radicals, that damage cellular macromolecules and ultimately induce cell death via apoptosis or necrosis (2). PDT offers several advantages, including high selectivity toward target cells, minimal damage to adjacent healthy tissue, and a low possibility of antibiotic or chemotherapy resistance. Consequently, it has been successfully applied in treating various skin cancers, precancerous lesions, microbial infections, and inflammatory dermatoses (3). The efficacy of PDT largely depends on the physicochemical characteristics of the photosensitizer. Phthalocyanines, a family of macrocyclic compounds, have been recognized as potent second- and third-generation photosensitizers due to their strong absorption in the red-near-infrared region, high chemical stability, and efficient singlet-oxygen production. Among them, zinc phthalocyanine has shown great promise for PDT in both in vitro and in vivo studies because of its low dark toxicity and high photoactivation efficiency (4). However, limitations such as poor aqueous solubility and

molecular aggregation can hinder its clinical translation. To overcome these drawbacks, nanoemulsions have recently been introduced as effective delivery systems for hydrophobic photosensitizers. They enhance solubility, stability, and cellular uptake while enabling controlled release and reduced systemic toxicity. These features significantly improve the photodynamic performance of the encapsulated drug (5). Given the increasing significance of PDT and the need for safe and efficient photosensitizers, the present study aimed to evaluate the cytotoxicity, cell migration, and oxidative stress response of fibroblasts treated with a nanoemulsion-based zinc phthalocyanine under PDT conditions. This approach assesses the biocompatibility of NE/ZnPc and its potential as a photosensitizer formulation for skin photodynamic therapy, with selective phototoxicity and minimal damage to healthy fibroblasts.

Materials and Methods

Cell Preparation and Culture

The healthy mouse skin fibroblast cell line (L929) was purchased from the Cell Bank of Isfahan University of Medical Sciences. The cells were cultured in 25 cm^2 tissue culture flasks containing DMEM medium supplemented with 1% L-glutamine (Sigma-Aldrich, USA), 10% fetal bovine serum (FBS) solution (Kiasist, Iran), and 1% penicillin/streptomycin (U 100). After 48 hours of incubation (reaching a cell density of 80%) under conditions of 95% humidity, 5% CO2, and pH 7.2 at 37°C, the cells were washed with PBS solution and then trypsinized using 0.05% trypsin and 0.02% EDTA. Briefly, 2×10^4 fibroblasts were seeded in 96-well plates in triplicate. Wells containing untreated cells and culture medium alone served as positive and negative controls, respectively (6). Next, the cytotoxic effects of NE/ZnlPc on the intact mouse skin fibroblast cell line were evaluated using the MTT assay. Finally, cell migration was examined using the scratch assay.

Evaluation of Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Following the designated treatment periods, the cell culture medium was carefully aspirated and replaced with 50 µL of freshly prepared sterile MTT solution in each well. The plates were then incubated for 3 hours at 37°C under a 5% CO_2 atmosphere. Subsequently, the MTT solution was discarded, and 150 µL of Dimethyl Sulfoxide (DMSO; Merck, Germany) was added to each well to dissolve the resulting formazan crystals. The plates were incubated for an additional 5-10 minutes in a shaker incubator to ensure complete dissolution. Finally, light absorption was quantified using an ELISA reader set to 570 nm (6).

Optimization of Photodynamic Therapy Conditions

To optimize the photodynamic therapy conditions, 3 main parameters, including drug concentration, incubation time, and laser irradiation power, were investigated separately and step by step. In each step, changes in one of the factors were made at different intervals. In contrast, the other two factors were held constant to evaluate the effect of each factor independently and determine the optimal conditions. It should be noted that due to preliminary patenting efforts, information regarding the synthesis of the nanoparticles used in this study was not included in the article, and the focus of this study was on evaluating their drug delivery and health performance rather than providing details on their synthesis process.

Irradiation Device and Procedure

PS was irradiated using a laser diode (Aftabrayaneh, Iran) with red light at a wavelength of 670 nm and a stable output power of 100 mW/cm^2. The laser doses evaluated in this study were 0, 12, 24, and 36 J/cm^2. In experimental conditions requiring lysis, the wells were simultaneously covered with a dark mask with a rectangular hole matching the well size. The wells surrounding the irradiated area were left empty to prevent potential radiation exposure. Control cells were placed in wells of the same plate and treated exactly as the irradiated cells, but without drug or light. Each experiment was repeated three times (7). Cell viability was assessed 24 hours after the PDT using the MTT assay.

Evaluation of Optimal Drug Concentration

To achieve optimal drug concentration, a laser dose of 12 J/cm^2 and an incubation time of 2 hours were selected, and wells containing cells were treated with drug concentrations of 0, 20, 40, 60, and 80 µg/mL.

Evaluation of Optimal Laser Dose

To achieve the optimal laser dose, a drug concentration of 20 µg/mL and an incubation time of 1 hour were used, and the wells were irradiated with laser doses of 0, 12, 24, and 36 J/cm^2.

Evaluation of Optimal Incubation Time in the Dark

To achieve optimal incubation, a laser dose of 12 J/cm^2 and a concentration of 20 µg/mL were selected, and wells containing cells were incubated in the dark for 30 minutes and 1, 2, and 4 hours.

Scratch Test

In this test, 5×10^5 cells from the normal mouse skin fibroblast cell line were seeded in a 6-well plate and incubated at 37°C for 24 hours. After the cells adhered to the bottom of the plate and filled the bottom, a scratch was made at the bottom of the plate with a sterile pipette tip. To remove suspended cells and cell debris resulting from scratching, the wells were washed 3 times with phosphate-buffered saline. Subsequently, DMEM culture medium supplemented with 10% FBS was added, and the wells were treated with NE/ZN PC at 20 µg/mL for 1 hour, followed by laser irradiation at 12 J/cm^2. Fibroblast migration was examined at 0, 6, and 12 hours using an inverted imaging microscope and analyzed using ImageJ

software (8).

Assessment of Oxidative Balance in Fibroblasts (MDA and TAC)

To perform the experiment, cells were counted using a hemocytometer and distributed at a density of 1.5×10^4 cells per well in 96-well cell culture plates. After 24 hours of incubation to ensure cell adhesion, the plates were ready for treatment. Treatment was performed at drug concentrations of 20, 40, and 60 µg/mL, with a laser dose of 12 J/cm² and 1 hour of incubation in the dark. After the treatment period, the cells were harvested to assess lipid peroxidation and antioxidant levels. TAC and MDA assays were performed according to the standard TBARS (Thiobarbituric Acid Reactive Substances) assay using commercial kits (MDA Assay Kit and TAC from Kia Zist). The contents of each well (including cells and culture medium) were collected, and the cells were lysed with lysis buffer (RIPA buffer with 1% SDS) and BHT 100X at 4°C. In the next step, the homogenate was centrifuged, and the supernatant was collected to assess the concentration of MDA and TAC. The concentrations of TAC and MDA in each sample were calculated from a standard curve prepared with MDA and TAC solutions at defined concentrations (according to the kit instructions). The final results were expressed as nm/mL of protein (9).

Statistical Analysis

All data were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test in SPSS software version 22. $P \leq 0.05$ was considered statistically significant. Graphs were generated using Microsoft Excel version 2010.

Results

Optimization Results

The results of fibroblast cell viability (Figure 1) showed that the least lethal effect on fibroblast cells was observed at a drug concentration of 20, a laser dose of 12, and an incubation time of 1 hour in the dark.

Investigation of Skin Fibroblast Migration

To evaluate the migration of healthy mouse skin fibroblasts, a scratch test was performed on the cells treated with NE/ZnPc at a concentration of 20 µg/mL and laser at a dose of 12 J/cm². Then, they were compared to the fibroblasts of the control group at times 0, 6, and 12 hours (Figure 2). The results of the scratch test indicated a significant effect of NE/ZnPc treatment with laser irradiation on the migration rate of mouse skin fibroblast cells. At the initial time after scratch, no significant migration was observed in any of the experimental groups, indicating a natural delay phase in the response of cells to injury. However, as time progressed and reached the time points of 12 and 6 hours, the migration of fibroblasts into the scratch area steadily increased. This increase in migration was more pronounced in the control group than in the group treated

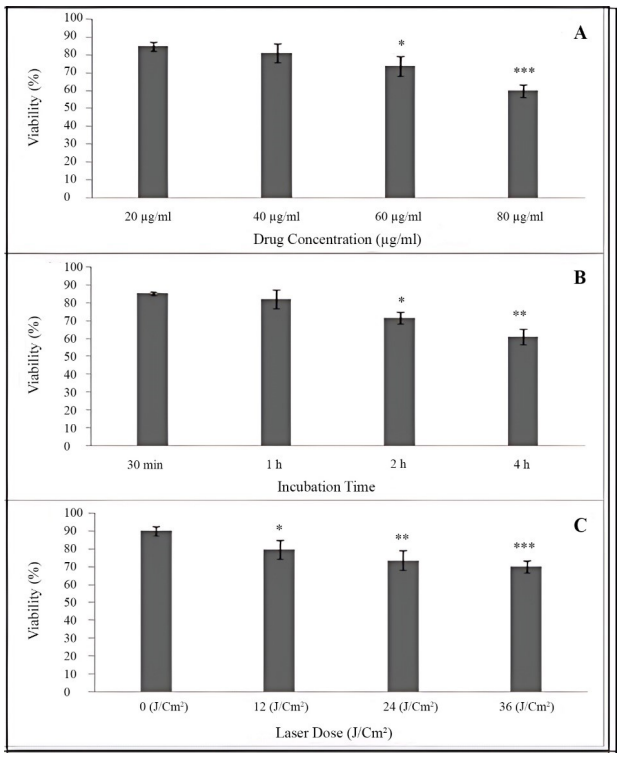

Figure 1. Results of Evaluation of Optimization Conditions. A: Laser dose and incubation time were 12 J/cm² and 1 hour, respectively, and drug concentration was variable. B: Laser dose and drug concentration were 12 J/cm² and 20 µg/mL, respectively, and incubation time was variable. C: Incubation time and drug concentration were 1 hour and 20 µg/mL, respectively. Data are expressed as mean ± standard deviation. The significance levels are presented as follows: *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$

with NE/ZnPc and laser. These findings indicate that although migration in the control group was higher than in the treatment group, cell migration also increased with increasing time in the treatment group, indicating low cytotoxicity of the drug on this cell line. Photodynamic treatment with NE/ZnPc and laser irradiation not only does not exert a negative toxic effect on the viability and migratory ability of mouse skin fibroblasts but also significantly accelerates cellular processes involved in wound healing, especially cell migration.

Evaluation of TAC and MDA

According to Figure 3A, with increasing drug concentration, the level of MDA in fibroblasts increased. This increase was not significant at a concentration of 20 µg/mL compared to the control group. Following comparison of the level of this factor between the 3 concentrations, a significant increase in the level of MDA was observed at the concentration of 60 µg/mL compared to the concentration of 20 µg/mL. In comparison, no significant difference was observed between the two concentrations of 20 µg/mL and 40 µg/mL. Unlike MDA, the TAC (Figure 3B) decreased with increasing drug concentration compared to the control group. This decrease was not significant at a concentration of 20 µg/mL. Following comparison of the level of this factor among the 3 concentrations, a significant decrease in TAC level

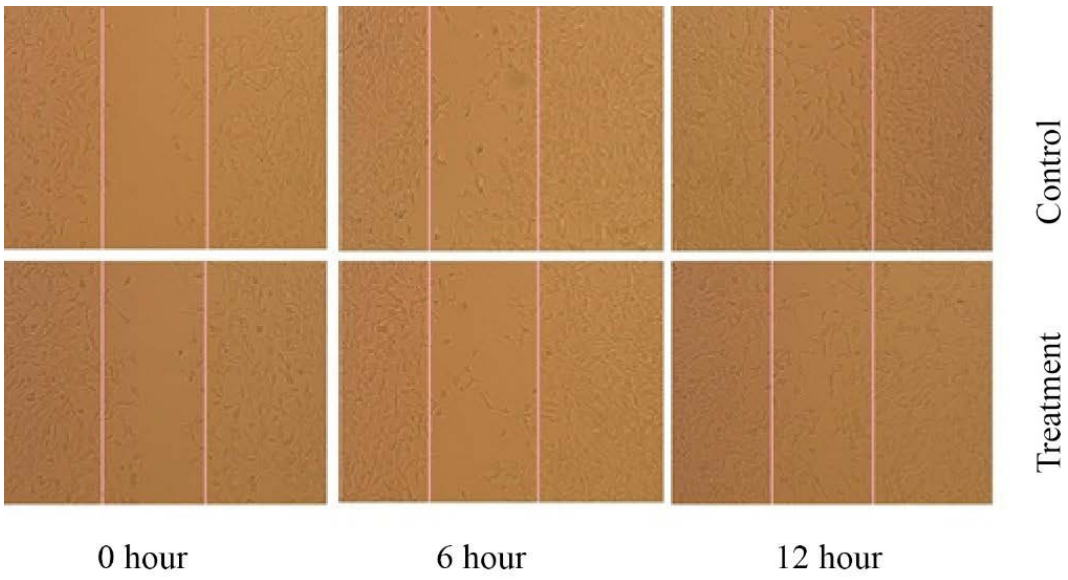

Figure 2. The Investigation of the Wound Healing of the Fibroblast Cell Line after Photodynamic Therapy Using the Scratch Test Dotted lines indicate the area of the scratch at 0, 6, and 12 hours after the treatment

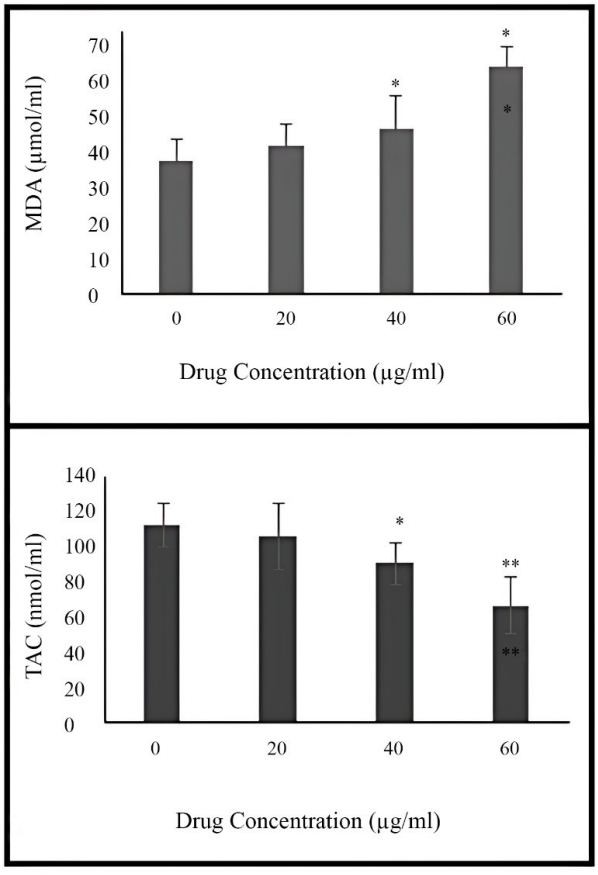

Figure 3. The Investigation of the Effect of Different Concentrations of NE/ZnPc on MDA and TAC Levels in Fibroblast Cells under Laser Irradiation Data are expressed as mean ± standard deviation. The significance level is indicated by $*P \leq 0.05$, $**P \leq 0.01$

was observed at the concentration of 60 µg/mL compared to the concentration of 20 µg/mL. In comparison, no significant difference was observed between the two concentrations, 20 µg/mL and 40 µg/mL.

Discussion

This study aimed to optimize photodynamic therapy conditions using a NE/ZnPc formulation to promote wound healing. Given the crucial role of fibroblasts in the proliferative and migratory phases of wound repair (1), it was important to ensure that PDT conditions exerted minimal toxic effects on the viability and function of these cells before assessing reparative outcomes. The MTT assay served as the foundational tool for our optimization strategy (10). Our findings demonstrated that a NE/ZnPc concentration of 20 µg/mL, combined with a laser dose of 12 J/cm^2 and an incubation time of 1 hour, represented the optimal parameters, exhibiting the least toxic effect on fibroblast cells. This targeted approach prioritized the preservation of cellular health, thereby establishing a suitable foundation for subsequent functional evaluations (11, 12). Further assessment of cellular function using the scratch assay corroborated the MTT assay results. Immediately after scratching, no significant cell migration was observed across groups, reflecting the inherent delay phase in cellular responses to injury and the time required for migratory mechanisms to activate (13,14). However, with increasing incubation time (at 6 and 12 hours), fibroblast migration into the scratch area progressively intensified. Notably, migration was most pronounced in the control group compared to the NE/ZnPc and laser-treated groups. Despite this difference, the sustained increase in cell migration in the treatment groups over time is a critical observation. It suggests that while NE/ZnPc-PDT may modulate the migration rate compared to untreated cells, it does not induce significant cytotoxicity under these specific scratch test conditions. This preservation of migratory capacity, a vital aspect of wound healing and tissue repair (15), indicates that NE/ZnPc-PDT is compatible with fibroblast function at the optimized dose. This contrasts sharply with the severe reduction or cessation of migration often observed with

cytotoxic treatments, thereby highlighting the potential of ZnPc-PDT to selectively target diseased cells (e.g., cancer) while minimizing damage to healthy surrounding tissues and maintaining their reparative capabilities (16). Such biostimulatory effects of low-dose PDT in facilitating repair processes have also been reported by Topaloglu et al (17). To comprehensively evaluate the biological impact of varying drug concentrations and PDT techniques, the oxidative balance within fibroblasts was meticulously measured after the treatment. We assessed MDA levels, a marker of lipid peroxidation, and total antioxidant capacity, an indicator of cellular antioxidant defense, across 3 different drug concentrations (18). The observed fluctuations in these markers, with both increases and decreases at higher concentrations, collectively illustrate the complex cellular response to PDT-induced production of reactive oxygen species. Specifically, elevated MDA levels at higher concentrations signify the onset of oxidative damage due to excessive ROS generation. Concomitantly, changes in TAC reflect the adaptive efforts of cells to counteract this stress through their intrinsic defense mechanisms. Findings on oxidative stress further underscore the success of our dose optimization phase. No statistically significant differences were observed in oxidative stress markers at the optimized 20 µg/mL. This strongly suggests that this specific PDT regimen produces low nontoxic levels of ROS. Crucially, such controlled ROS levels are believed to be sufficient to activate beneficial cellular signaling pathways associated with wound repair, rather than inducing cellular damage. This observation aligns with the results of the study conducted by Souza et al. They demonstrated that low-dose PDT, particularly with agents like AlClPc in a 3D microenvironment, can enhance collagen synthesis, myofibroblast differentiation, and ROS production (19). The synergy between the light source and the NE/ZnPc nanoemulsion in modulating cell signaling is consistent with the understanding that while high ROS levels impede wound repair, optimal ROS levels can increase ATP production and accelerate wound closure (20). This finely tuned ROS generation represents the underlying biological mechanism by which low-dose NE/ZnPc-PDT achieves its wound-healing potential without compromising fibroblast viability or function.

Conclusion

This study established an optimal PDT protocol for wound healing using NE/ZnPc (20 µg/mL), 12 J/cm^2 laser, and 1-hour incubation. This method effectively balances therapeutic benefits with cellular integrity, maintaining fibroblast viability, preserving migratory function, and avoiding significant oxidative stress. This knowledge could pave the way for the development of novel and more targeted PDT treatments.

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Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals. This study was also approved by the Ethics Committee of Islamic Azad University, Falavarjan Branch (IR.IAU.FALA.REC.1399.012).

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