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Optimization of 5-aminolevulinic acid-based photodynamic therapy protocol for breast cancer cells

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ABSTRACT

Background: Photodynamic therapy (PDT) is a therapeutic strategy for the treatment of cancer. 5-aminolevulinic acid (5-ALA) as a precursor of the protoporphyrin IX (PpIX) has a great potential for PDT application. Although 5-ALA-based PDT has been studied in many pre-clinical and clinical studies for breast cancer, there are different PDT application protocols in the literature. Therefore, the aim of this study was to determine the optimal *in vitro* protocol for 5-ALA-based PDT in breast cancer treatment.

Methods: The therapeutic effects of 5-ALA (1 and 2.5 mM) on two different subtypes of breast cancer cell line (MCF-7 and MDA-MB-231) were evaluated by PpIX-fluorescence accumulation and WST-1 analysis. Then, the cells were irradiated with diode laser at different doses (1.5, 3, 6, 9 and 12 J/cm^2). After irradiation, the anticancer effects of 5-ALA were analyzed through cell viability and cell death analysis.

Results: Our results showed that 5-ALA exhibited a higher PpIX fluorescence in both breast cancer cells for 4 h incubation. After irradiation, 1 mM 5-ALA significantly reduced the proliferation of breast cancer cells in a laser dose-dependent manner and induced apoptotic cell death upon 24 h incubation ($p < 0.05$). However, MDA-MB-231 cells were more sensitive to 5-ALA-based PDT than MCF-7 cells in a dose of 9 J/cm^2 and 12 J/cm^2.

Conclusion: Our preliminary findings proposed an optimal *in vitro* protocol of 5-ALA-based PDT by using a laser diode for breast cancer. However, there is a need to investigate the underlying molecular mechanisms of 5-ALA/PDT sensitivity among the subtypes of breast cancer.

1. Introduction

Photodynamic therapy (PDT) is an approach for the treatment of cancer as well as non-oncological diseases. PDT is based on the use of photosensitizer (PS) and then activation of PS upon light irradiation at a specific wavelength. After irradiation, PS generates reactive oxygen species (ROS) in the presence of oxygen. Increased ROS levels can result in apoptotic cell death and necrosis in targeted cancer cells [1–4]. Therefore, the efficacy of PDT in cancer therapy depends on the type, the concentration and the exposure time of PS used, the dose and source of light, the penetration level of the light into tissue and the level of sufficient oxygen [5,6].

5-aminolevulinic acid (5-ALA) is the biological precursor of the protoporphyrin IX (PpIX) and thus 5-ALA is approved for several clinical applications. In the literature, 5-ALA induces the accumulation of PpIX in the mitochondria and enhances the production of intracellular ROS under irradiation [7,8]. Thus, 5-ALA based PDT can potential therapeutic strategy for the treatment of different tumors. In pre-clinical and clinical studies, the therapeutic effects of 5-ALA on breast cancer have been studied in detail [9–14]. For instance, the study of Frei et al. (2018) states that the accumulation of 5-ALA-induced PpIX in breast cancer cells is higher than prostate, ovarian and brain cancer cells. Therefore, 5-ALA treatment is proposed to be effective for breast cancer therapy [13]. Additionally, Morita et al. (2019) evaluate the differences the accumulation of 5-ALA induced PpIX in two different sub-types of breast cancer cells (MCF-7 and MDA-MB-231). After treatment with 5 mM 5-ALA for 2 h, the accumulation of PpIX is different between these cells due to possible association with either phenotypic or genotypic differences [14]. However, there are different *in vitro* experimental protocols for the treatment of 5-ALA (the different

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concentration (1, 2, 5 and 10 mM) and exposure time) in breast cancer cells after irradiation with different source of light (laser and LED) doses (1, 3, 5, 6 and 8 J/cm^2) [15–19]. Thus, there is urgently need to determine the optimal *in vitro* protocol for 5-ALA based PDT in the treatment of each breast cancer cell type due to its heterogeneity.

In the current study, we proposed the *in vitro* protocol for 5-ALA based PDT in the treatment of breast cancer. For this purpose, we selected two different subtypes of human breast cancer cell line [MCF-7 (Luminal A; estrogen, progesterone receptor positive (ER+, PR+), human epithelial receptor 2 negative (HER2-) and MDA-MB-231 (triple negative; ER-, PR-, HER2-). MCF7 and MDA-MB-231 cells are suitable as a breast cancer cell model due to their phenotypic and genotypic differences. MCF-7 cells are hormone-dependent and exhibit the epithelial phenotype whereas MDA-MB-231 cells are insensitive to hormone therapy and express markers of mesenchymal phenotype. Additionally, these cells are different in terms of metabolic activity. MCF-7 cells produce ATP through oxidative phosphorylation while MDA-MB-231 cells prefer glycolysis for ATP production [20,21]. We firstly investigated the cytotoxic effects of 5-ALA on breast cancer cells before irradiation. Then, the different density of laser energy and exposure time inducing 5-ALA was performed to determine the best 5-ALA based PDT protocol. Finally, the therapeutic potential of 5-ALA based PDT on each breast cancer cell line was evaluated through cell viability, cell photosensitization and apoptotic cell death analysis.

2. Material and methods

2.1. Cell culture

In this study, two breast cancer cell lines (MCF-7 and MDA-MB231) were used to evaluate the effects of 5-ALA/PDT on the molecular subtypes of breast cancer. Both cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). Cell lines were cultured in DMEM (Dulbecco's Modified Eagle Media, Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cells were incubated at 5% CO_2 and 37 °C in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. 5-ALA incubation and cell photosensitization

5-aminolevulinic acid hydrochloride (5-ALA, Sigma, A3785) was dissolved in sterile distilled water. Cells were seeded in 96-well and 24-well cell culture plates with DMEM medium and incubated for 24 h. After incubation, cells were treated with freshly prepared 5-ALA at a concentration of 1 and 2.5 mM in DMEM medium without FBS for 2 h and 4 h at 37 °C. After incubation, the medium was removed and replaced with fresh medium with FBS and irradiated with different light doses.

2.3. Photodynamic treatment in vitro

In all experiments, the wavelength of the laser light source was 635 nm with ± 3 FWHM (full width half maximum). The laser light source was capable of working in the various pulse frequencies from DC to 20 MHz. In the experiment, continuous wave (CW) mode was used for laser irradiation at 1.5 J/cm^2, 3 J/cm^2, 6 J/cm^2, 9 J/cm^2 and 12 J/cm^2. The optic power and wavelength spectrum validation were made with power meter and spectrometer (PM100 and C series spectrometer, Thorlabs, Germany). The fluence rate was 30 mW/cm^2 to achieve different light doses as summarized in Table 1. Furthermore, 1 and 2.5 mM 5-ALA concentration was used for each light dose to compare the effects of different light doses on the efficacy of 5-ALA in breast cancer cells. Control cells were incubated in the same medium without irradiation.

Table 1
The radiation parameters of the 5-ALA based PDT experiments.

Radiation Parameters	
Wavelength (nm)	635 ± 3 nm
Mode	CW
Power Output	30 mW
Exposure time (s)	50 s, 100 s, 200 s, 300 s, 400 s
Energy density (J/cm^2)	1.5, 3, 6, 912 (J/cm^2)
Photosensitizer	5-ALA

2.4. Cell viability assay

Cell viability was determined using the WST-1 assay (Biovision, San Francisco, CA, USA). The cells were incubated in the 96-well plates overnight. Then, cells were treated with 1 mM and 2.5 mM of 5-ALA for 4 h and irradiated with different light doses. After irradiation, the cells were incubated for 24 h in the incubator. Following incubation, 10 µL of WST-1 was added to each well and incubated for 45 min at 37 °C in the dark. Cell viability analysis was performed by reading the absorbance values at 450 nm with the microplate reader (Allsheng, China).

2.5. Annexin V assay

The percentage of apoptotic cells was analyzed by the Muse Annexin V & Dead Cell assay kit (Millipore, Germany). The cells were incubated in the 24-well plates overnight. Thereafter, the cells were treated with 1 mM of 5-ALA for 4 h and irradiated with effective light doses (6, 9 and 12 J/cm^2) according to WST-1 results. After 24 h incubation, each experimental group was collected, washed in cold phosphate-buffered saline (PBS) and then stained with the Muse Annexin V & Dead Cell reagent for 30 min at room temperature. Finally, the analysis was performed using a Muse Cell Analyzer (Millipore, Germany). (Annexin at excitation/emission = 485/535 nm; 7-AAD at excitation/emission = 555/655 nm)

2.6. Acridine orange staining

The cells were incubated in the 24-well plates overnight. Then, the cells were treated with 1 mM of 5-ALA for 4 h and exposed to laser irradiation. After 24 h incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were stained with acridine orange (1 µg/mL) for 30 min in the dark and washed with PBS. Images were obtained with the EVOS FL Cell Imaging System (Thermo Fisher Scientific, USA). (Excitation: 502 nm (dsDNA), 460 nm (ssDNA and RNA) and Emission 525 nm (dsDNA), 650 nm (ssDNA and RNA).

2.7. Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with the Post-hoc Tukey test was used for multiple comparisons. Additionally, a two-way ANOVA analysis was performed to compare groups. All experiments were repeated at least 3 times and the obtained data presented as the mean ± standard deviation. $P < 0.05$ was considered significant.

3. Results

3.1. Accumulation of PpIX-fluorescence in 5-ALA-treated breast cancer cells

To observe the levels of PpIX accumulation in MCF-7 and MDA-MB-231 cells, we treated cells with two different concentrations (1 and 2.5 mM) of 5-ALA for 2 and 4 h as shown in Fig. 1. In general, the intracellular level of PpIX increased in a time and concentration-

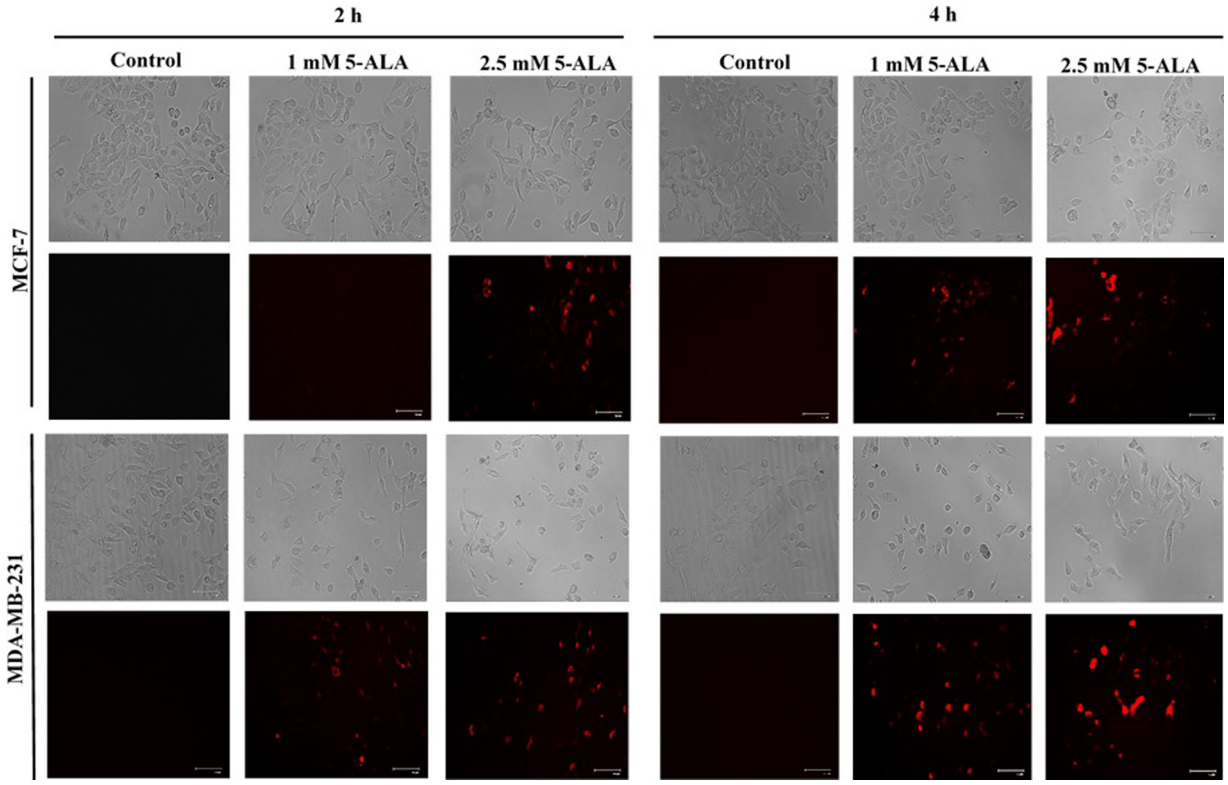

Fig. 1. Imaging of MCF-7 and MDA-MB-231 cells after treatment with different concentrations (1 and 2.5 mM) of 5-ALA for (A) 2 h and (B) 4 h compared with control cells.

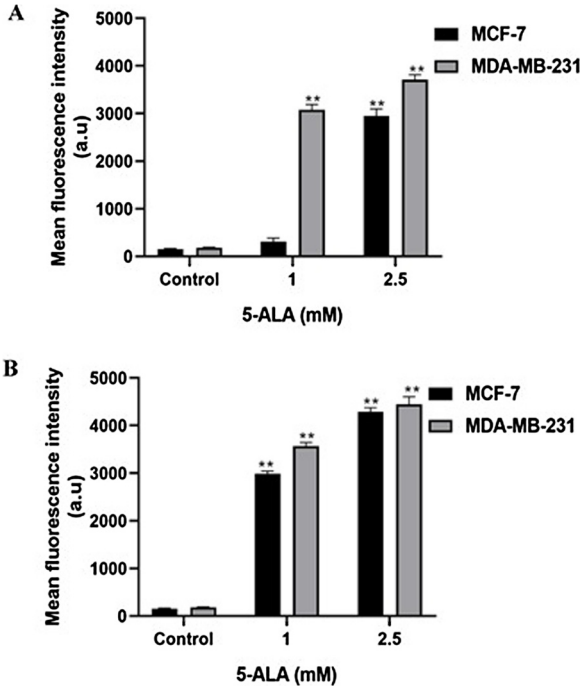

Fig. 2. Mean fluorescence intensity of 5-ALA-induced PpIX (mean ± SD) after a (A) 2 h and (B) 4 h incubation with 1 and 2.5 mM 5-ALA exposure. ($p < 0.05$*, $p < 0.01$**).

dependent manner for both breast cancer cells. However, the red PpIX-fluorescence was higher in MDA-MB-231 cells than MCF-7 cells. Additionally, the fluorescence intensity of images was measured by NIH ImageJ software. The mean PpIX-FI was significantly higher in MDA-MB-231 cells than MCF-7 cells after 2 h and 4 h incubation with 5-ALA in Fig. 2. Therefore, 4 h ALA incubation time was selected for the optimal condition for further experiments.

3.2. Cell viability of breast cancer cells after laser irradiation

To determine the best cytotoxic effect of 5-ALA based PDT on breast cancer cell, the cells treated with two different concentrations (1 and 2.5 mM) of 5-ALA and then irradiated with a dose of 1.5, 3, 6, 9 and 12 J/cm^2 (Fig. 3). In the absence of irradiation, 5-ALA displayed no significant toxic effects on the viability of MCF-7 and MDA-MB-231 cells for 4 h. However, increasing light doses caused a significant decrease in cell viability after treatment with 1 mM 5-ALA for 4 h. After laser irradiation with 1.5, 3, 6, 9 and 12 J/cm^2, the viability of MCF-7 cells was 112.8 ± 5.1 %, 80.6 ± 2.3 %, 80.1 ± 2.4 %, 61.8 ± 0.7 % and 58.9 ± 0.6 %, respectively at 1 mM 5-ALA ($p < 0.05$). However, a significant decrease (89.5 ± 2.0 %, 84.9 ± 0.3 %, 73.1 ± 1.8 %, 55.9 ± 3.2 % and 46.1 ± 3.9 %) was analyzed in a dose of 1.5, 3, 6, 9 and 12 J/cm^2, respectively in MDA-MB-231 cells ($p < 0.01$). Furthermore, 1 mM 5-ALA suppressed the viability of breast cancer cells more efficiently compared with 2.5 mM 5-ALA after irradiation with different light doses. Therefore, our findings demonstrated that 1 mM 5-ALA considerably inhibited the proliferation of both breast cancer cells after irradiation with 9 and 12 J/cm^2. However, 5-ALA was more effective in MDA-MB-231 cells than MCF-7 cells. Furthermore, the percentage of cell viability for each dose of light and breast cancer cell line was analyzed by two-way ANOVA analysis (Table 2). There was a significant interaction between cell type and the dose of light in terms of the cell viability data ($p < 0.05$). According to WST-1 results, 1 mM dose of 5-ALA treatment followed by 6, 9 and 12 J/cm^2 of laser light was determined the optimal condition for effective 5-ALA/PDT treatment for further analysis.

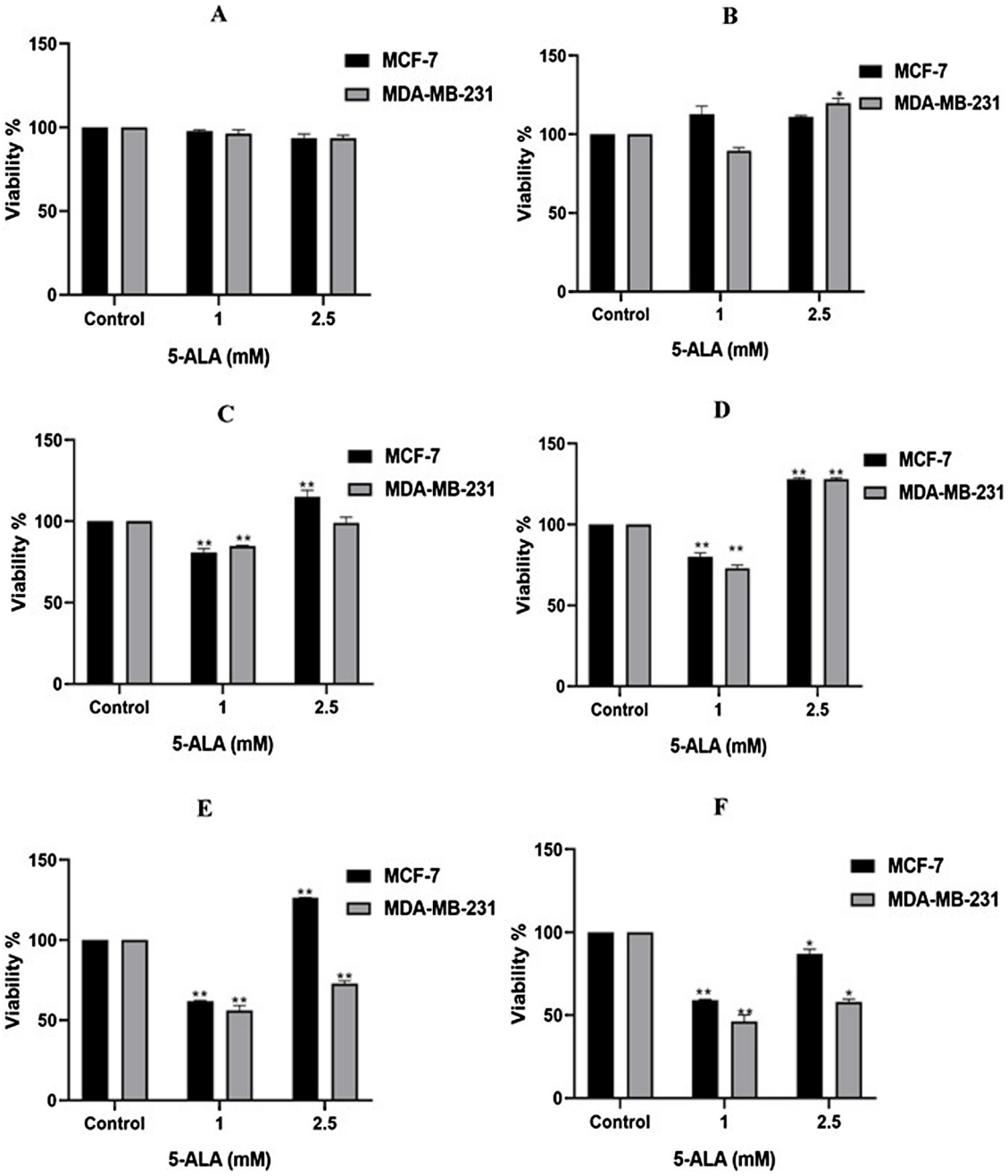

Fig. 3. Comparison of the viability of MCF-7 and MDA-MB-231 cells after 5-ALA treatment. Exposure to 5-ALA (A) before irradiation for 4 h and after irradiation at (B) 1.5 j/cm^2, (C) 3 j/cm^2, (D) 6 j/cm^2, (E) 9 j/cm^2 and (F) 12 j/cm^2 ($p < 0.05$*, $p < 0.01$**).

Table 2
Comparison of cell type with different light doses in breast cancer cell viability.

	Cell Type			
	MCF-7	MDA-MB-231	Total	
Light dose	$\overline{X}$ ± Sd	$\overline{X}$ ± Sd	$\overline{X}$ ± Sd	
6 J/cm^2	80.03 ± 1.76	72.37 ± 1.80	76.20 ± 4.49	F = 248.643; p < .0.001
9 J/cm^2	61.35 ± 0.84	55.82 ± 2.30	58.59 ± 3.40	
12 J/cm^2	58.39 ± 1.11	45.54 ± 3.02	51.96 ± 7.33	
Total	66.59 ± 10.22	57.91 ± 11.91	62.25 ± 11.66	
	F = 89.537; p < 0.001			*J/cm^2 X Cell Type Interaction* F = 5.617; p = 0.019

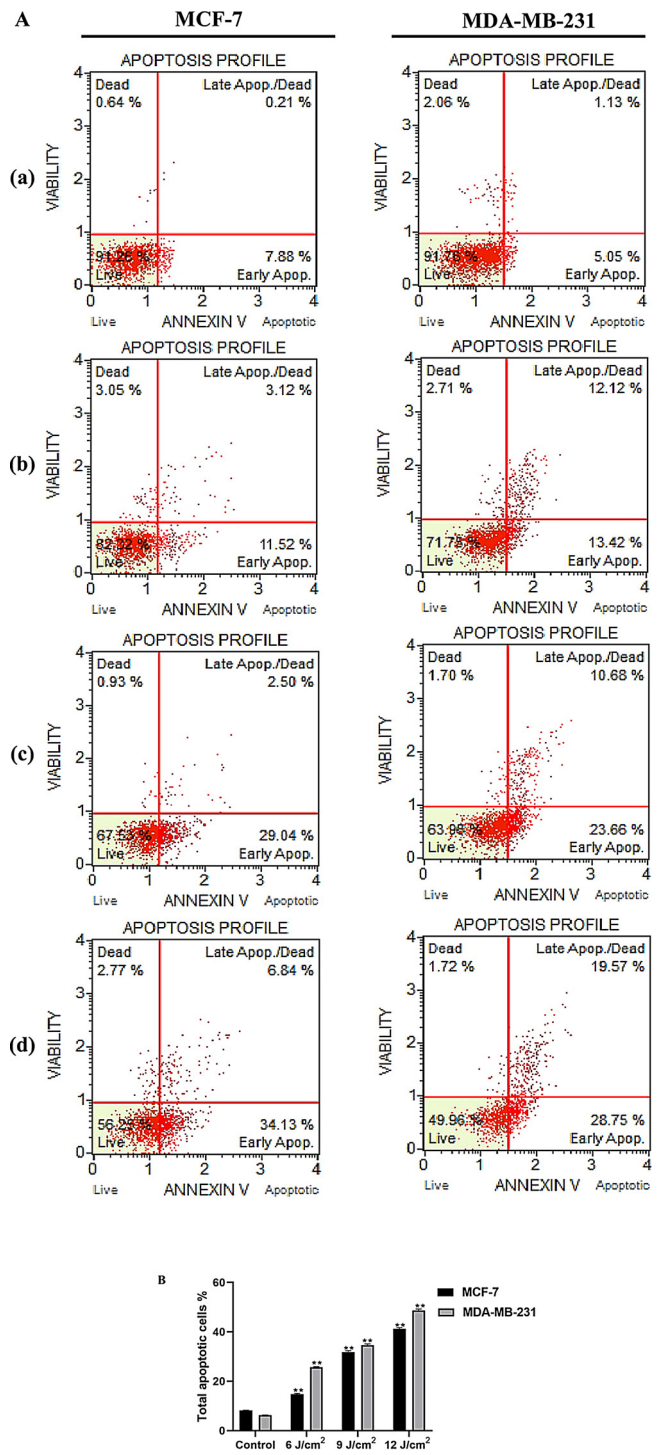

Fig. 4. 5-ALA based PDT induced apoptotic cell death in breast cancer cells. (A) Annexin V analysis of MCF-7 and MDA-MB-231 cells after irradiation with a dose of (b) 6 j/cm^2, (c) 9 j/cm^2, and (d) 12 j/cm^2 compared with (a) control. (B) Statistical comparison of the mean of percentage of total apoptotic cell death in breast cancer cells after irradiation ($p < 0.05$*, $p < 0.01$**).

3.3. Apoptotic effects of 5-ALA after laser irradiation

The impact of 5-ALA based PDT on apoptotic cell death, we performed Annexin V analysis and the obtained results were summarized in Fig. 4. After irradiation with 6, 9 and 12 J/cm^2, the total apoptotic cells significantly increased from 8.2 ± 0.5%–14.8 ± 0.3 %, 31.9 ± 0.6 % and 41.3 ± 0.4 %, respectively in 1 mM 5-ALA treated MCF-7 cells ($p < 0.01$). Furthermore, a significant increase was detected in the percentage of total apoptotic cells (25.7 ± 0.2 %, 34.6 ± 0.5 % and 48.6 ± 0.6 %) at 6, 9 and 12 J/cm^2, respectively in MDA-MB-231 cells ($p < 0.01$). Therefore, 5-ALA/PDT induced particularly early apoptotic cell death in a laser dose-dependently. Our findings were consistent with WST-1 results.

3.4. Cell morphological changes

AO staining was performed to observe morphological changes in breast cancer cells as shown in Fig. 5. After irradiation with different doses of light, cell shrinkage and membrane blebbing, some vacuolization and marked chromatin condensation were observed in a laser dose-dependent manner in MCF-7 and MDA-MB-231 cells compared with control cells. Particularly, 5-ALA induced much more damage in cells at 12 J/cm^2. Furthermore, rounded cells showed higher red fluorescence than flat cells due to the depolymerization of DNA [22]. Consistent with our previous Annexin V results, MDA-MB-231 cells were more sensitive to 5-ALA/PDT than MCF-7 cells.

4. Discussion

Herein, we investigated 5-ALA based PDT protocol for MCF-7 Luminal A (ER+, PR+, HER2-) and MDA-MB-231 triple negative (ER-, PR-, HER2-) breast cancer cell lines. Our findings demonstrated that the intracellular accumulation of PpIX fluorescence varied between cells and 5-ALA based PDT was more effective for MDA-MB-231 cells than for MCF-7 cells. Administration of 5-ALA for 4 h followed by laser irradiation at 6, 9 and 12 J/cm^2 was the best obtained PDT protocol for breast cancer cells.

The utility of 5-ALA through the characterization of PpIX fluorescence has been studied in different breast cancer cell lines to discriminate residual of breast cancer cells from the normal mammary epithelial cells [14,23]. In these studies, breast cancer cells are incubated with 5-ALA for 2 h to detect the accumulation of PpIX. They demonstrate that a significant increase is observed in the fluorescence of PpIX in the breast cancer cell lines compared to normal mammary epithelial cells and the accumulation of PpIX is different in terms of a variety of cell phenotypes [14,23]. However, Millon et al. (2010) state that the fluorescence intensity of PpIX is not affected by ER expression levels in breast cancer cell lines [23]. In the present study, the intracellular accumulation of PpIX varied in two biological subtypes of breast cancer cell lines following administration with 1 and 2.5 mM 5-ALA for 2 and 4 h. Our results showed that PpIX fluorescence increased in a concentration and time-dependent manner and a more concentrated PpIX level was observed in MDA-MB-231 cells (Fig. 1 and 2). However, breast cancer cell viability in 2.5 mM 5-ALA was higher than that in 1 mM concentration of 5-ALA following irradiation with different light doses. Alterations in heme biosynthetic enzymes, mitochondrial functions and porphyrin transporters (porphyrin importer and exporter activity) are associated with the level of PpIX accumulation in cancer cells [24]. For instance, increased ABCG2 activity in bronchoalveolar carcinoma cells leads to a decrease in intracellular PpIX level after 5-ALA stimulation [25]. Therefore, resistance mechanisms can be associated with changes in the accumulation of PpIX and the level of 5-ALA toxicity.

Additionally, a light source is a significant factor for effective PDT treatment. The study of Daniel et al. (2014) states that the different light wavelengths could also produce essential tissue damage upon the absorbance spectrum of PS [26]. In the present study, a laser system developed in an earlier study [27] could potentially produce high intensity monochromatic light by optimizing the LD temperature and laser optical output for PDT experiments. Importantly, the laser system prevented wavelength shifts and captured the peak absorbance point of the 5-ALA (635 nm) through precise parameter control for more effective PDT treatment.

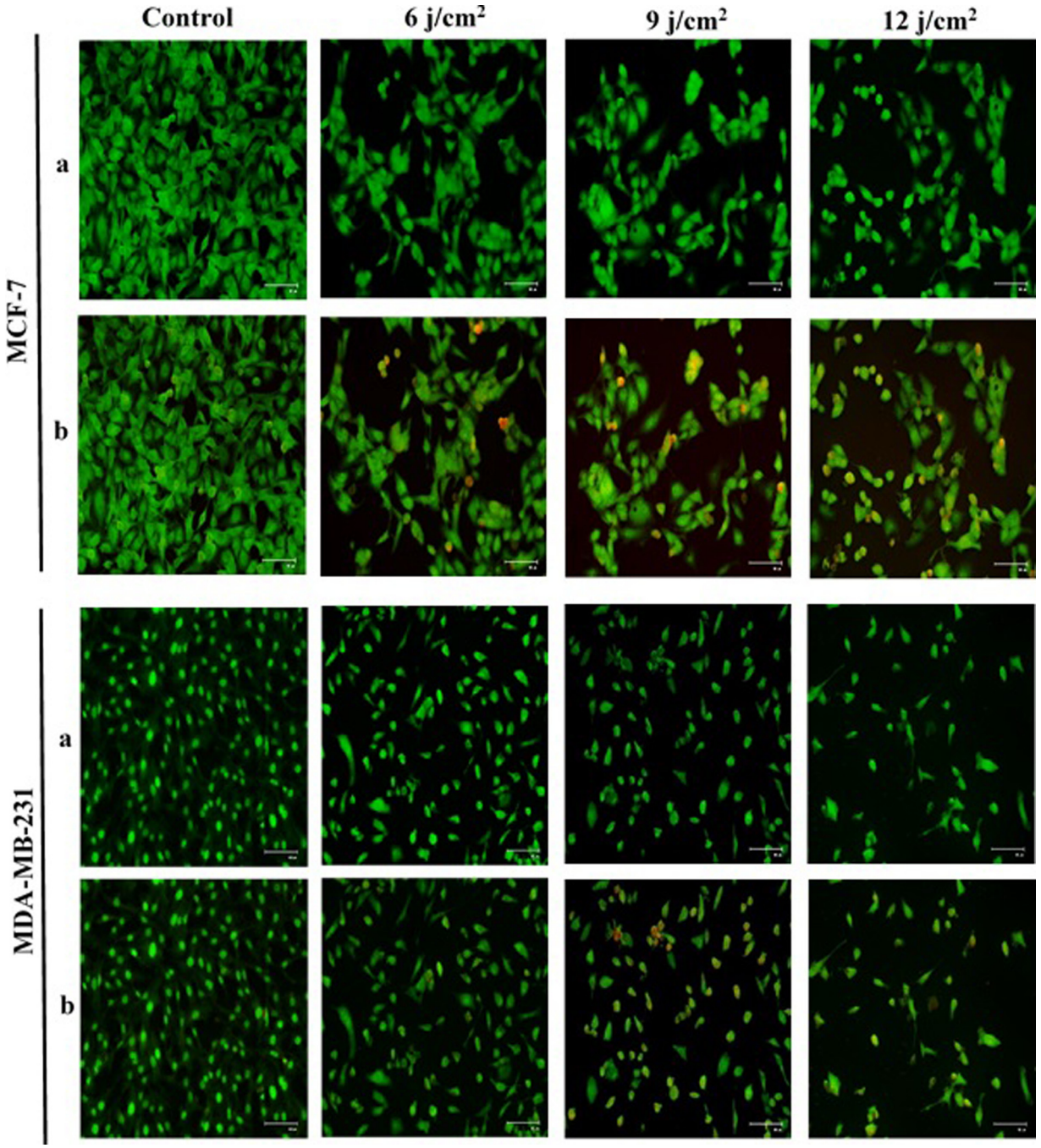

Fig. 5. AO staining indicated that 5-ALA based PDT induced apoptotic cell death in a laser dose-dependent manner. Images were obtained at (a) 510 ± 42 nm green filter and (b) both green and 628 ± 40 nm red filters.

Different PS (porphyrins and chlorins) based PDT is promising technology for diagnosis and treatment of different cancer types including skin, lung, bladder, brain, esophagus, breast, prostate, *etc.* in clinical trials [28]. In *in vitro* studies, 5-ALA based PDT has been extensively studied through the different light sources in oral, lung, colon, bladder and cervical cancer and head and neck squamous cell carcinoma. In these studies, different PDT protocols are performed to irradiate 5-ALA and kill cancer cells. For instance, a dose of 5.86–10.54 J/cm^2 with LED for oral cancer [29], a dose of 15 − 100 J/cm^2 energy with different lights for bladder cancer [30], a dose of 5 J/cm^2 for cervical cancer by using a laser generator [31], a dose of 1, 5 and 10 J/cm^2 with laser light for lung cancer [32], a density of 1 and 2 J/cm^2 with Gallium-Aluminum-Arsenide diode laser for head and neck squamous cell carcinoma [33] and a dose of 3.0 J/cm^2 with 3 types of LED for colon cancer [34] have been used for 5-ALA based PDT. Furthermore, there are different PDT protocols leading to different outcomes for the treatment of breast cancer, *in vitro* [15–19]. In order to determine antitumor effects of 5-ALA, different breast cancer cell lines (transfected with a mutated Her2/Neu oncogene MCF10A human breast epithelial cells, MCF-7, MCF-7/ADR (ADR resistant), 4T1 and EMT6 mouse mammary tumor cells) are irradiated with a diode, argon and He-Ne ion laser and LED lights at a dose of 1, 3, 5, 6 and 8 J/cm^2 in these studies. The study of Mohammadpour and Fekrazad (2016) states that 1 mM 5-ALA based PDT induces cell death at 6 J/cm^2 (5.73 ± 0.77 %) in 4T1 breast cancer cells. Therefore, 4T1 cells are resistant to 5-ALA/PDT [16]. In another study, 1 mM 5-ALA for 3 h treatment leads to significant phototoxicity with LD_{50} around 8 J/cm^2 in MCF-7 cells. Additionally, MCF-7/ADR resistant cells are less sensitive to ALA–PDT

than MCF-7 cells despite the similar amounts of PpIX [18]. The study of Abo-Zeid et al. (2018) show that the viability of MCF-7 cells is significantly decreased (from 75.0%–46.3%) after treatment with 0.5 mM and 2 mM 5-ALA, respectively for 4 min using He-Ne ion laser at 5 J/cm^2 [19]. Therefore, optimization of PDT protocol in the subtype of breast cancer should be required to obtain the best effective results and prevent differences in the effectiveness of PDT therapy. For this purpose, we selected different light doses (1.5, 3, 6, 9 and 12 J/cm^2) to irradiate 5-ALA and determined the effects of 5-ALA/PDT on two different subtypes of breast cancer cell line. Our results indicated that the proliferation of breast cancer cells significantly reduced after irradiation with a laser at a density of 6, 9 and 12 J/cm^2 (Fig. 3). Moreover, 5-ALA treatment induced apoptotic cell death after irradiation. However, the efficacy of 5-ALA based PDT was different in each breast cancer cell line.

This study has some limitations. Firstly, the obtained results should be validated in other breast cancer cell lines. Secondly, the therapeutic efficacy of 5-ALA may be evaluated after irradiation with different laser mode and higher energy densities. Thirdly, the different responses of each sub-type breast cancer cell to 5-ALA based PDT and the underlying molecular mechanisms of apoptotic cell death should be investigated. Finally, our findings should be further supported by *in vivo* studies due to the limitations of an *in vitro* study.

In conclusion, we proposed an *in vitro* 5-ALA based PDT protocol for the treatment of breast cancer cells. 5- ALA/PDT using diode laser reduced cell proliferation in a laser dose-dependent manner and induced apoptotic cell death in two different subtypes of breast cancer cell. However, aggressive breast cancer cells were more susceptible to 5-ALA/PDT than hormone sensitive breast cancer cells. Although our preliminary findings provide optimization of 5-ALA-based PDT for breast cancer cells, further studies should be performed to investigate the differential cytotoxicity between molecular subtypes of breast cancer.

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