Lasers in Medical Science
https://doi.org/10.1007/s10103-021-03398-8

ORIGINAL ARTICLE

Investigation of the therapeutic effect of 5-aminolevulinic acid based photodynamic therapy on hepatocellular carcinoma

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Received: 24 April 2021 / Accepted: 6 August 2021
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Abstract
Hepatocellular carcinoma (HCC) is a heterogeneous type of cancer and current treatment options limit successful therapy outcomes. Photodynamic therapy (PDT) has attracted attention as an alternative approach in the treatment of different types of cancer. However, there is no study in the literature regarding the effect of PDT on HCC, in vitro. Therefore, the aim of this study was to determine the cytotoxic and apoptotic effects of 5-aminolevulinic acid (5-ALA)/PDT on two different HCC cell lines in terms of hepatitis B virus (HBV) infection. The therapeutic effects of 5-ALA-based PDT on HCC cell lines (Huh-7 and SNU-449) were evaluated by PpIX-fluorescence accumulation, WST-1 analysis, Annexin V analysis, and acridine orange/ethidium bromide staining after irradiation with different light doses through diode laser. The results showed that 1 mM 5-ALA displayed higher PpIX fluorescence in the SNU-449 cell line than the Huh-7 cell line after 4 h of incubation. After irradiation with different light doses (3, 6, 9, and 12 J/cm^2), 5-ALA significantly reduced the proliferation of HCC cells and induced apoptotic cell death ($p < 0.01$). Furthermore, SNU-449 cells were more responsive to 5-ALA-based PDT than Huh-7 cells due to possibly its molecular features as well as viral HBV status. Our preliminary data obtained from this study may contribute to the development of 5-ALA/PDT-based treatment strategies in the treatment of HCC. However, this study could be improved by the elucidation of the molecular mechanisms of cell death induced by 5-ALA/PDT in HCC cells, the use of different photosensitizer, light sources, and in vivo experiments.

Keywords Hepatocellular carcinoma · Photodynamic therapy · 5-Aminolevulic acid (5-ALA) · Apoptosis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide that ranks fourth among cancer-related causes of death. Several treatment options, including curative resection, liver transplantation, radiofrequency ablation, chemoembolization, radioembolization, and systemic targeted agents, are available for HCC patients. Despite significant improvements in surgical and regional treatments over the past few years, recurrent disease remains a significant concern. Therefore, the success of HCC treatment depends on tumor stage, patient performance status, liver function reserve, and hepatitis B virus (HBV) infection, which is the main cause of HCC and requires multidisciplinary new approaches [1–5].

Photodynamic treatment (PDT) is a clinically approved, minimally invasive procedure and exerts cytotoxic activity against malignant cells. PDT involves applying a photosensitizer (PS) followed by irradiation at wavelengths within the PS absorption band. In this mechanism, PS absorbs photons from the light source and extracts electrons from the outside. During this process, free radicals, also known as singlet oxygen, damage the cellular structures through oxidation without recycling. 5-Aminolevulic acid (5-ALA) is a commonly used PS for PDT applications. However, 5-ALA is not light sensitive on its own. This sensitivity is achieved through

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protoporphyrin IX (PpIX) within the mitochondria. Some clinical studies show that PDT can improve the survival rate and the quality of life for patients with non-surgical cancer [6–9].

In general, oxygen, light, and photosensitizer alone have no toxic effect on cancer and normal cells. With the PDT therapy, combined photochemical reactions result in the death of the targeted cell by different signaling pathways and cell death types, including apoptosis, necrosis, and autophagy. The type of cell death in PDT is regulated by the type, the physiochemical properties and concentration of PS, the position of the cell, the oxygen concentration, the wavelength and intensity and source of light (laser light sources with monochrome wavelengths and non-polychromatic laser sources with broadband wavelengths), and the cell type. Laser light sources have advantages in light transmission for providing fiber optic equipment, limiting irradiation time due to monochromatic wavelength. Therefore, the effects of PDT on cancer cells are regulated by different parameter. For instance, PDT induces more apoptotic cells at lower light doses, whereas higher light doses can result in more necrotic cells [10, 11]. Therefore, the optimal conditions for each type of cancer should be identified for effective PDT treatment. In this context, the aim of this study was to investigate the therapeutic role of 5-ALA based PDT in HCC in vitro in terms of HBV status.

Material and method

Cell culture

In this study, SNU-449 (ATTC® CRL-2234) infected with HBV cell line was obtained from the American Type Culture Collection (ATCC). The Huh-7 (ATTC ® HB-8065) cell line was gifted by Associate Professor Yasemin Eraç of the Department of Pharmacology at the Faculty of Pharmacy at Ege University.

The medium for the SNU-449 cell line was prepared in RPMI 1640 medium containing sodium bicarbonate (2.0 g/L) and L-glutamine (0.3 g/L) by adding 10% FBS and 1% penicillin/streptomycin. For Huh-7 cell line, the DMEM medium was prepared by adding 10% FBS and 1% penicillin/streptomycin. Huh-7 and SNU-449 cells were cultured in suitable media at 37 °C in an incubator with 5% carbon dioxide (CO_2).

5-ALA application and cell light sensitization

5-Aminolevulinic acid hydrochloride (5-ALA, Sigma, A3785) was dissolved with water. The cells were seeded at a density of 2×10^4 per well in a 96-well plate in appropriate medium and incubated for 24 h. After incubation, the cells were incubated at 37 °C for 4 h with freshly prepared 5-ALA at a concentration of 1 mM in DMEM without FBS. After the incubation, the medium was changed with fresh medium containing FBS and laser applications were performed. Control cells were incubated in fresh medium without 5-ALA administration and irradiation. Additionally, after incubating 1 mM 5-ALA for 4 h, the intracellular PpIX levels before irradiation was observed with EVOS Cell Imaging System (Thermo Fisher Scientific, USA) at 628 ± 40 nm red filter and analyzed by Image J program.

Photodynamic treatment application

In the experiments, the wavelength of the laser light source was used at 635 nm [12]. Laser irradiation was applied using continuous wave (CW) mode at 3 J/cm^2, 6 J/cm^2, 9 J/cm^2, and 12 J/cm^2. The exposure time to laser light was 100, 200, 300, and 400 s, respectively. Optical power verification was performed by power meter and spectrometer (PM100 and C series spectrometer, Thorlabs, Germany) for wavelength spectrum verification. The power output from the laser device was 30 mW/cm^2.

Cell viability assay

The cytotoxic effects of 5-ALA on HCC cells were determined by WST-1 (BioVision, San Francisco, CA, ABD). For the WST-1 experiment, the cells were seeded on 96-well plates at 2×10^4 cells in each well. After treatment with 1 mM 5-ALA for 4 h, the laser irradiation at 3 J/cm^2, 6 J/cm^2, 9 J/cm^2, and 12 J/cm^2 were performed. Following 24-h incubation, the WST-1 solution was added and incubated in the incubator for 45 min. After incubation, the absorbance was measured at 450 nm wavelength. The viability of the control group, which was not treated with 5-ALA, was considered 100% viable, and the viability rates of the irradiated cells were calculated as % compared to the control.

Annexin V analysis

To detect the apoptotic effect of 5-ALA on the cells after irradiation by the most effective irradiation doses (9 and 12 J/cm^2) according to WST-1 data, Annexin V analysis was performed. After the incubation with 1 mM 5-ALA for 4 h, the laser irradiation at 9 and 12 J/cm^2 was performed. After 24 h of incubation, the cells were removed with trypsin and centrifuged at 1500 rpm for 5 min. The supernatant was removed and the cell pellet was suspended in PBS and incubated with 100 µL Muse® Annexin V and Dead Cell Assay Kit for 30 min at room temperature in the dark. Stained cells were analyzed in the Muse® Cell Analyzer (Merck Millipore, Germany).

Acridine Orange/Ethidium Bromide staining

AO/EB staining was performed to evaluate the morphological effect of 5-ALA on HCC cells. After the incubation with 1 mM 5-ALA for 4 h, the laser irradiation at 9 and 12 J/cm^2 was performed. After the application, 1 mL of 4% paraformaldehyde was added to each well for fixation. After fixation, each well was washed three times with PBS. Then, AO/EB solution (100 mg/ml) was added to each well and incubated for 30 min in the dark. After staining, the cells were visualized by the EVOS Cell Imaging System (Thermo Fisher Scientific, USA).

Statistical analysis

The GraphPad Prism 6.0 program was used for statistical analysis s. The differences between groups were assessed by One Way ANOVA (Post-hoc Tukey) analysis. $p < 0.05$ was considered statistically significant. Experiments were repeated three times.

Results

Determination of the intracellular PpIX levels in HCC cell lines

Our results showed that the intracellular PpIX level considerably increased in HCC cells treated with 1 mM 5-ALA for 4 h compared with the control group ($p < 0.01$). The level of intracellular PpIX in cells was 22,559.35 ± 2858.5 and 27,335.86 ± 2787.5 in Huh-7 and SNU-449 cells, respectively, compared with control cells. Therefore, the amount of intracellular PpIX level was significantly higher in SNU-449 cells compared to Huh-7 cells (Fig. 1).

Evaluation of the cytotoxic effects of 5-ALA/PDT on HCC cells

The WST-1 analysis was performed to determine the cytotoxic effect of 5-ALA on the cells after laser irradiation with 3, 6, 9, and 12 J/cm^2 as summarized in Fig. 2. After laser irradiation with 3, 6, 9, and 12 J/cm^2 in Huh-7 cells, the cell viability reduced to 79.56 ± 0.35%, 79.96 ± 1.22%, 65.00 ± 3.32%, and 71.85 ± 3.39%, respectively ($p < 0.01$; Fig. 2A). Furthermore, the viability of SNU-449 cells significantly reduced to 85.39 ± 2.11%, 77.53 ± 1.19%, 63.78 ± 1.87%, and 59.46 ± 1.55% at 3, 6, 9, and 12 J/cm^2 laser irradiation, respectively. As a result, 5-ALA treatment resulted in a significant cytotoxic effect on HCC cell lines. Additionally, the most cytotoxic effects of 5-ALA were observed at 12 J/cm^2 in SNU-449 cells.

Apoptotic effects of 5-ALA on HCC cell lines after laser irradiation

Annexin V analysis was performed to determine the apoptotic effect caused by laser radiation in HCC cells treated with 5-ALA, and the obtained findings were summarized in Fig. 3. The apoptotic death in the cells induced by 1 mM 5-ALA significantly increased after the laser irradiation at 9 and 12 J/cm^2 in HCC cells ($p < 0.01$) compared with the control. The total apoptotic cell death rates increased to 34.66 ± 1.04% and 26.24 ± 1.09, at 9 and 12 J/cm^2, respectively, in the Huh-7 cell line ($p < 0.01$). On the other hand, 34.79 ± 1.25% and 41.31 ± 1.40% of the total apoptotic cell death were detected at 9 and 12 J/cm^2, respectively, in SNU-449 cells. 1 mM 5-ALA treatment caused more apoptotic death in SNU-449 cells than Huh-7 cells at 12 J/cm^2 laser irradiation. For this reason, SNU-449 cells were more sensitive to 5-ALA-based PDT than Huh-7 cells.

The morphological changes in HCC cell lines after treatment with 5-ALA/PDT

The effect of 5-ALA-based PDT on HCC cell morphology was evaluated by AO/EB staining (Fig. 4). In general, DNA fragmentation, chromatin condensation, cell shrinkage, and nuclear fragmentation were observed in HCC cells treated with 1 mM 5-ALA after irradiation with 9 and 12 J/cm^2 compared to control groups. Additionally, more pronounced nuclear fragmentation was observed in SNU-449 cells compared to Huh-7 cells.

Discussion

In the current study, we assessed the anticancer activity of 5-ALA mediated PDT in HCC cells and analyzed the differences between two HCC cells in response to 5-ALA-PDT treatment in terms of HBV status. Our preliminary findings demonstrated that 5-ALA treatment inhibited the proliferation of HCC cells after irradiation with particularly 9 and 12 J/cm^2 through apoptosis. Additionally, SNU-449 cells were more sensitive to 5-ALA than Huh-7 cells due to HBV status and its molecular features. Huh-7 cell line is an epithelial-originated, aggressive, resistant, and heterogeneous group, whereas the SNU-449 cell line is a mesenchyme cell line [13]. Additionally, Huh-7 cells have a point mutation in the *p53* gene. p53 is a transcription factor that regulates the cell cycle and thus, p53 mutations are observed in different types of cancer. Furthermore, *CDKN2A* gene deletion is identified in SNU-449 cells. The *CDKN2A* codes p16 (INK4A) and p14 (ARF) proteins, which are the INK4 family member, and thus suppress the cell cycle [14–16].

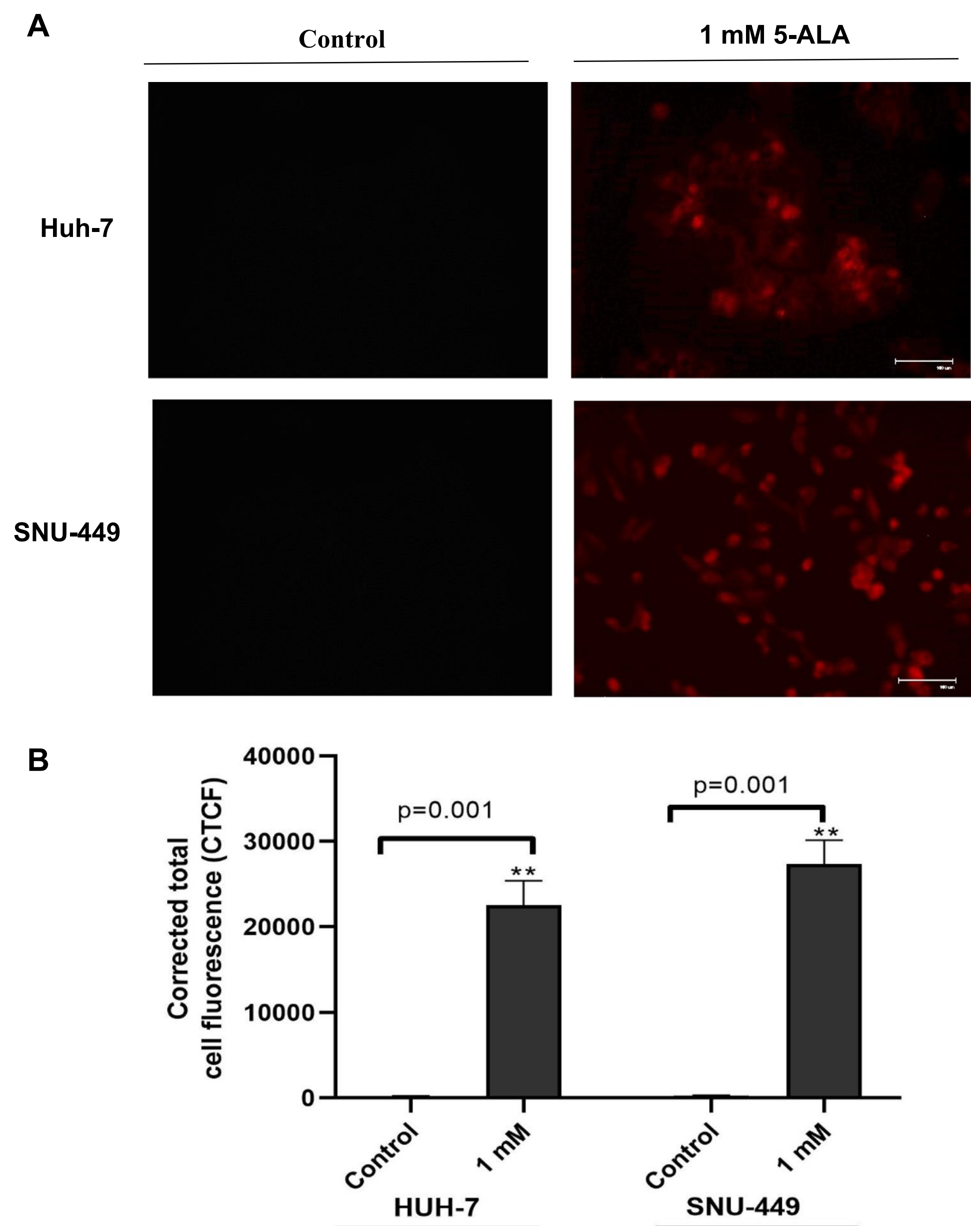

Fig. 1 Intracellular PpIX levels in 1 mM 5-ALA treated Huh-7 and SNU-449 cells after 4 h incubation. **A** Images of intracellular PpIX amount in HCC cells compared with control groups. **B** Statistical comparison of the level of intracellular PpIX fluorescence in the cells ($p<0.01$**) (The scale bar is 100 µm)

Therefore, these molecular features could affect the response of HCC cells to 5-ALA-mediated PDT.

PDT is based on PS and causes cell death in the presence of oxygen. Additionally, the wavelength used is dependent on the PS. In the literature, the therapeutic effects of 5-ALA/PDT on the treatment of different types of cancer (breast, prostate, pancreas, colon) have been investigated [17–20]. However, there is no study exploring the anticancer effects of 5-ALA/PDT on HCC cells in terms of HBV status. Therefore, we firstly investigated the intracellular PpIX accumulation and then irradiated HCC cells with a diode laser to assess the therapeutic potential of PDT in HCC treatment. In our study, the intracellular PpIX levels were significantly increased after treatment with 1 mM 5-ALA for 4 h. Additionally, the level of PpIX was higher in SNU-449 cells than Huh-7 cells. In our previous study, the intracellular level of PpIX was higher in MDA-MB-231 triple negative breast cancer cells than MCF-7 Luminal A breast cancer cells [21]. Additionally, Tsai et al. (2004) state that the intracellular PpIX level is lower in MCF-7/ADR-resistant cells than MCF-7 breast cancer cells [22]. Morito et al. (2019) note that the level of PpIX is different in the subtypes of breast cancer cells [23]. Therefore, the conversion of 5-ALA to PpIX could be different according to cell types and subtypes.

5-ALA-based PDT has been approved for clinical use in the USA and Europe to treat solar keratosis and skin cancer [24–26]. In this context, it is essential to determine the

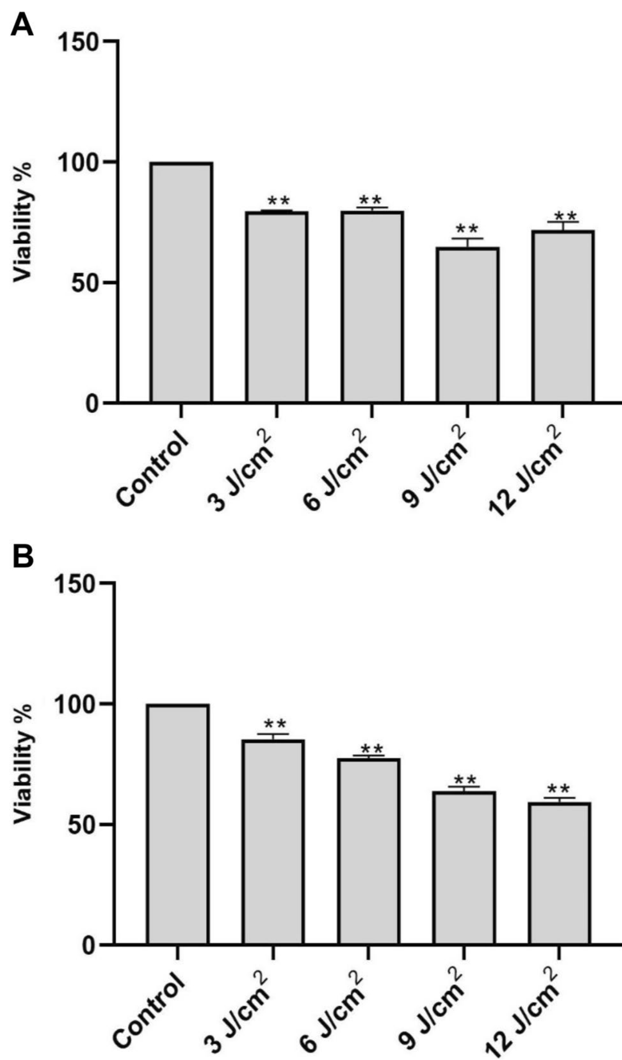

Fig. 2 Results of WST-1 analysis of viability percentages of **A** Huh-7 and **B** SNU-449 cells after laser irradiation with different power intensity ($p<0.01$**)

application protocol of PDT for each cancer type. In preclinical studies, the cytotoxic, apoptotic, and/or autophagic effects of 5-ALA/PDT on particularly different cancer cells are determined [21, 27–29]. Our previous findings show that the effects of 5-ALA on different sub-types of breast cancer are different [21]. According to WST-1 results, the proliferation of breast cancer cells is significantly decreased at 9 and 12 J/cm^2 laser irradiation and 1 mM 5-ALA treatment is more effective in MDA-MB-231 cells compared to MCF-7 cells [21]. In another study, 0.1–4 mM 5-ALA treatment at 5 J/cm^2 laser irradiation reduces the viability of HeLa cervical cancer cell, and the most effective concentration of 5-ALA is 1 mM for the treatment of cervical cancer [24]. In the study of Osaki et al. (2017), KLN205 lung cancer cells are treated with 0.6, 1.2, 2.5, and 5 mM 5-ALA for 4 h and irradiated with 630 nm laser (1.5 and 10 J/cm^2). They show that 5-ALA significantly reduces the viability of the cells and the efficacy of 5-ALA is higher in the presence of metformin [28]. Abo-Zheid et al. (2018) evaluate the cytotoxic effect of 0.5 and 1 mM 5-ALA on HepG2 and MCF-7 cells by irradiating a He–Ne laser for 4 min at 60 J/cm^2 power density using 633 nm wavelength. According to their findings, the survival rate is considerably decreased in both cell lines due to the increasing concentration of 5-ALA [29]. In our study, the viability of both HCC cell lines remarkably reduced after 6, 9, and 12 J/cm^2 laser irradiation ($p<0.01$). However, the response of SNU-449 cells to 5-ALA/PDT was higher than Huh-7 cells. Therefore, the response of HCC cells to 5-ALA mediated PDT was different, and these differences could be related to the different characteristics of the cells and HBV status.

Furthermore, several studies investigate the apoptotic effect of 5-ALA/PDT on different cancer types in the literature [21, 28]. As a result of stimulation with red (600–740 nm) and green light (450–580 nm), hydroxyl radicals cause apoptotic cell death by the production of ROS such as singlet oxygen, hydrogen peroxide, and superoxide formed by photochemical reaction [30, 31]. In our previous study, we found that 5-ALA-induced apoptotic death depends on the increasing power density and concentration of 5-ALA ($p<0.05$) [21]. In the study of Osaki et al. (2017), the combination of 5-ALA/PDT and metformin cause autophagic death as well as apoptosis in lung cancer cells [28]. Additionally, several studies demonstrate that 5-ALA treatment results in apoptosis in different types of cancer cells through increased the number of micronucleus in cells, the disrupted structure of the cell membrane, chromatin condensation, and the over-expression of Bax protein level [28, 29, 32]. Our findings showed that 5-ALA/PDT caused a significant apoptotic death in HCC cells and this death was more profound in SNU-449 cells compared to Huh-7 cells. Furthermore, 5-ALA-induced apoptosis was observed by AO/EB staining. Although the highest apoptotic effect of 5-ALA on Huh-7 cells was determined at 9 J/cm^2 laser irradiation, 5-ALA treatment induced more apoptosis in SNU-449 cells at 12 J/cm^2 laser irradiation. In this context, further studies should be performed to elucidate the molecular mechanism of apoptotic death induced by different power densities.

Diode lasers are used as the most practical method for PDT for many applications due to less costly than previously used lasers. Since the laser is monochromatic, all waves are parallel to each other and can apply high energy even to a minimal area and thus laser as a light source is more effective for PDT applications. Additionally, the laser provides a greater penetration depth compared to LEDs [33]. Therefore, we used a diode laser system in this study and its efficacy for different irradiation was analyzed. However, the cytotoxic and apoptotic effects of

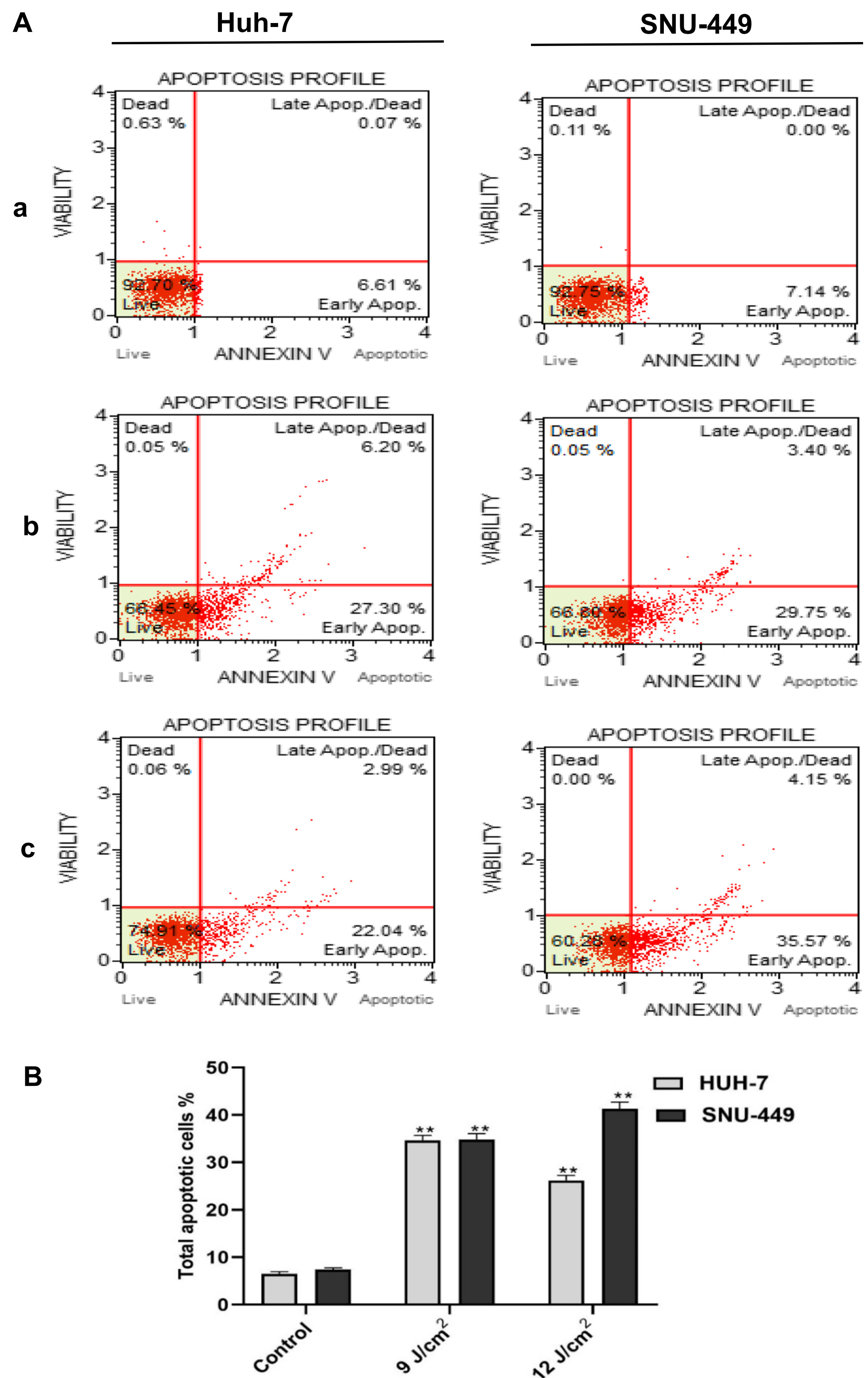

Fig. 3 5-ALA treatment resulted in apoptotic cell death. **A** The results of Annexin V analysis in Huh-7 and SNU-449 cell lines (a) Control, (b) 9 J/cm^2, and (c) 12 J/cm^2. **B** Statistical comparison of total apoptotic death rates in Huh-7 and SNU-449 cells ($p < 0.01$**)

5-ALA on HCC cells changed in different laser irradiation. Therefore, the efficacy of different light sources (LEDs, etc.) on 5-ALA based PDT at different irradiation needs to further investigations.

Conclusions

In conclusion, we, for the first time, explored the cytotoxic and apoptotic effects of 5-ALA/PDT on HCC cell lines with

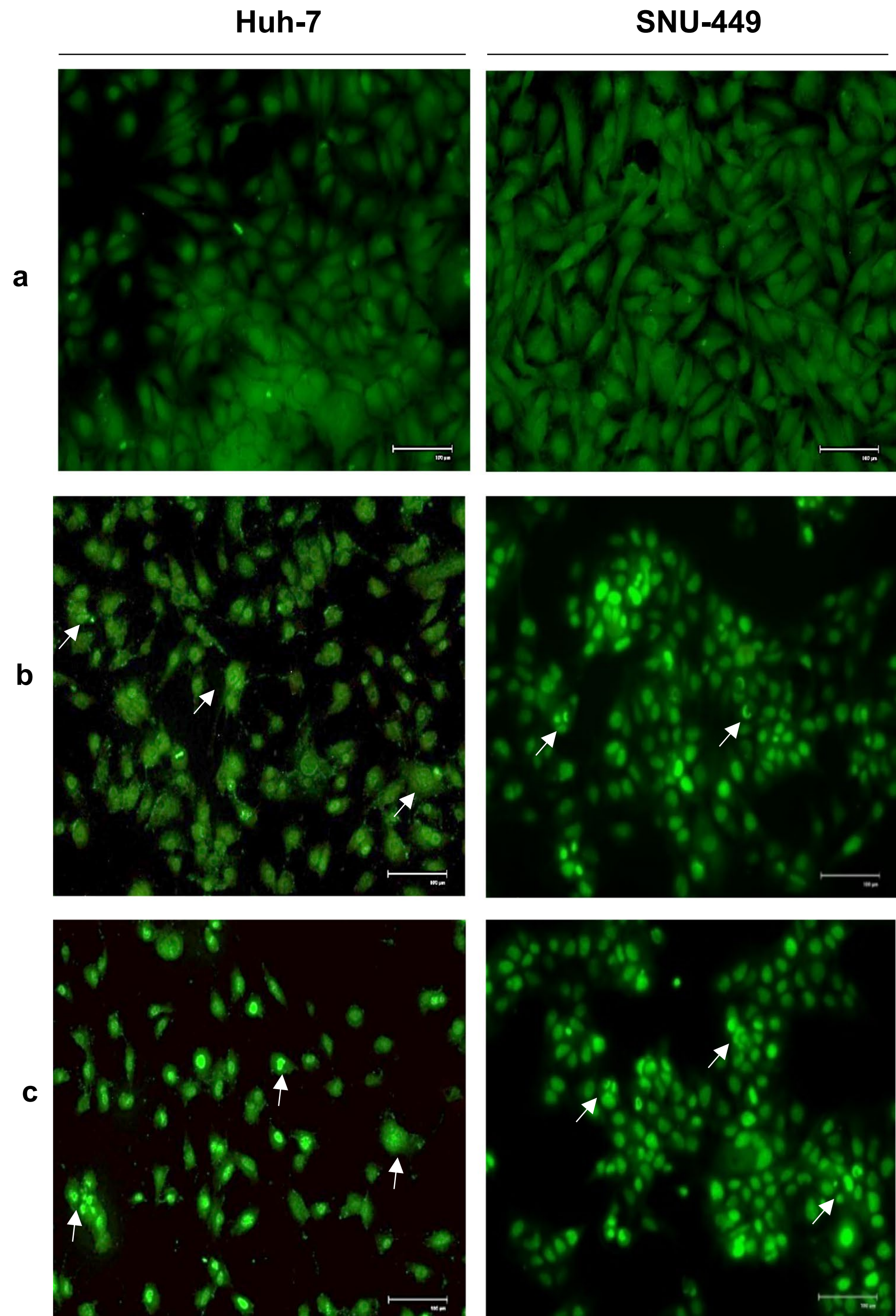

Fig. 4 The morphological changes of HCC cells after laser irradiation with different power intensity (arrows show DNA fragmentation and disruption of cell structure). **a** Control, **b** 9 J/cm^2, **c** 12 J/cm^2 (The scale bar is 100 µm)

different properties in vitro. According to the obtained data, 5-ALA treatment significantly suppressed the viability of the HCC cells and caused apoptotic death. Additionally, SNU-449 cells were more responsive to 5-ALA than Huh-7 cells. Our preliminary data can contribute to developing new 5-ALA/PDT-based treatment strategies in the treatment of HCC. However, further studies are required to elucidate the molecular mechanisms of cell death induced by 5-ALA in HCC cell lines and to determine the effects of different PS and light sources on the viability of HCC, in vitro and in vivo experiments.

Declarations

Conflict of interest The authors declare no competing interests.

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