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Original Article

Apoptotic effects of Photofrin-Diomed 630-PDT on SHEEC human esophageal squamous cancer cells

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Abstract: Photodynamic therapy (PDT) using photofrin-II is a clinically effective treatment for both non-neoplastic and neoplastic diseases. Herein, we performed an in vitro experiment to study the anti-tumor effect and mechanisms of photofrin-II mediated PDT for esophageal squamous cell carcinoma (ESCC) cell line, SHEEC. In this study, human ESCC cell line SHEEC and parental normal cell line SHEE were used. The anti-tumor effect of PDT was determined by evaluating cell viability using CCK-8 assay, apoptosis and generation of reactive oxygen species (ROS). PDT induced significant apoptosis in SHEEC and SHEE cells in a time- and photofrin-II dose-dependent manner. Furthermore, PDT treatment induced significant death of SHEEC, instead of SHEE cells. The apoptotic outcome was accompanied by concurrent generation of ROS. In summary, PDT shed light on therapy of ESCC, functioning as a useful tool for ESCC clinical treatment, providing a better understanding of Photofrin-Diomed 630-PDT in SHEEC cells.

Keywords: SHEE, SHEEC, PDT, photofrin-II, apoptosis, ROS

Introduction

Operation, chemotherapy and radio therapy remains the most common methods of treating cancer, however, due to significant side effects of these treatments, Photodynamic therapy (PDT) was used as an alternative treatment for cancer patients. PDT is a light-based oncological and non-malignant diseases treatment modality, in which visible light is used to activate a photosensitizer, a light-absorbing molecule. Namely, the tumor-targeting photosensitizing molecule is administered followed by a light of a corresponding wavelength irradiation so as to induce the generation of reactive oxygen species (ROS) which was proven to be responsible for the DNA damage and cell death [1]. Dougherty etc has reported the significant clinical efficacy of PDT, which was later approved as the first drug-device combination by US Food and Drug Administration [2, 3]. To date, various tumors including esophagus cancer [4], gastric cancer [5], skin caner [6], head and neck cancer [7], etc have been treated by PDT.

It has been established that PDT could induce apoptotic, necrotic and autophagy cell death. Wu etc showed that ALA-mediated PDT initiated apoptotic cell death via the up-regulation and activation of p38 mitogen activated protein kinase (MAPK), the stress-activated c-jun N-terminal kinases (JNK) and ERK [8]; Mohamed etc demonstrated the results based on the cytoplasmic organelles and the intranuclear localization extensively enhanced the efficacy of PDT with appropriate photosensitizer and light dose and support the idea that PDT can contribute to elimination of malignant cells by inducing apoptosis, which is of physiological significance [9]; H Wang etc reported that DTPP showed an efficient growth inhibition of MCF-7 during PDT treatment [10].

Porfimer sodium (Photofrin II) is a photosensitizer which distributes selectively to tumor tissues, and causes tumor cell death by combination with light irradiation. Porfimer sodium is activated by laser irradiation at 630 nm, which can reacts with tissue oxygen to produce highly reactive excited siglet oxygen ($1O_2$) [11]. It has been reported that photodynamic therapy is a safe and effective treatment for the palliation of obstructing and bleeding esophagus cancer [12], early unresectable lesions, palliation of locally advanced disease, and salvage therapy

for stent blockage or local tumor recurrence [13, 14], Barrett's esophagus [15] and even esophageal candidiasis [16]. Moreover, photofrin-mediated PDT has been suggested to allow for preservation of function and structure of the larynx without systemic toxicity [17], to induce apoptosis and inhibit survival of human pancreatic cancer cells [18], to treat T1 and T2 oral squamous cell carcinoma [19]. Yang etc has reported the photofrin-associated inhibition of EGFR in esophageal squamous cell carcinoma cell line CE48T [20]. However, the molecular mechanisms in esophageal cancer during PDT remain unclear. In this study, we used human ESCC cell line SHEEC and parental normal cell line SHEE to study the anti-tumor effect of PDT by evaluating cell viability using CCK-8 assay, apoptosis and generation of reactive oxygen species (ROS). It was found that PDT induced significant apoptosis in SHEEC and SHEE cells in a time- and photofrin-II dose-dependent manner. Furthermore, PDT treatment induced significant death of SHEEC, instead of SHEE cells. The apoptotic outcome was accompanied by concurrent generation of ROS. Overall, this study provided a better understanding of Photofrin-Diomed 630-PDT in SHEEC cells.

Materials and methods

Ethics statement

For tissue samples, written informed consent was obtained from patients. The procedures used in this study were approved by the Institutional Review Board of the Henan University of Science and Technology and was conformed to the Helsinki Declaration, and to local legislation.

Cell culture

SHEE human immortalized esophageal squamous cancer cells and the induced cancerous subline SHEEC were cultured in M199 culture medium (GIBCO, BRL Co. Ltd., USA), supplemented with 10% fetal ovine serum (FBS; Thermo Scientific Waltham, MA), gentamicin (50 mg/mL), penicillin (60 mg/mL) and streptomycin (100 mg/mL) at 37°C in a humidified incubator with 5% CO_2.

Photosensitizer and laser light delivery system

Photofrin-II was purchased from Sinclair Pharmaceuticals Co., Ltd (Toronto, Canada). The laser used in this study was a DIOMED-630 (DIOMED Co., Hertfordshire, England) laser system using a semiconductor laser irradiator. The details of the settings in the laser system as well as the optimal doses of photofrin and laser irradiation in vitro and in vivo were as described previously [21, 22].

Photofrin-II-mediated PDT in cell culture

SHEE and SHEEC cells were placed into 96-well plates at a concentration of 1×10^4 cells/well, incubated with photofrin-II (10 μg/ml) for 150 min, then were washed twice with PBS followed by incubation with M199 culture medium and finally cells were subjected to laser irradiation (wavelength, 630 nm; laser power, 25 mW/cm^2; time, 25 s) [21] and harvested for apoptotic rates evaluation. Images of cellular fluorescence were captured using MetaMorph software (Molecular Devices, MDS Analytical Technologies) and the average pixel intensities were determined using morphometric analysis.

Quenching studies

SHEE and SHEEC cells were placed into 96-well plates at a concentration of 1×10^4 cells/well, incubated with photofrin-II (10 μg/ml) for 150 min, then were washed twice with PBS followed by incubation with M199 culture medium supplemented with different levels of NaN_3, and subjected to laser irradiation (wavelength, 630 nm; laser power, 25 mW/cm^2; time, 50 s) [21].

Annexin V/propidium iodide double-staining flow cytometry

The FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen 556547) was used to assess cell apoptosis induced by PDT treatment. Cells were harvested at 150 min after treatment with photofrin with or without subsequent irradiation. To analyze phosphatidyl serine exposure using 5 μl Annexin V-FITC staining and loss of cell membrane integrity using 5 μl Propidium Iodide (PI) staining. And were stained with annexin V-FITC and propidium iodide (PI). These cells were analyzed with flow cytometer (Beckman Coulter-EPICS XL, USA). Unstained cells were used as negative controls. Data collected were analyzed using the Beckman Coulter FACSDiva software (Beckman Coulter). Cells were discriminated into four groups: viable cells (annexin V-/PI-), necrotic dead cells (annexin V-/PI+), early apoptotic cells (annexin

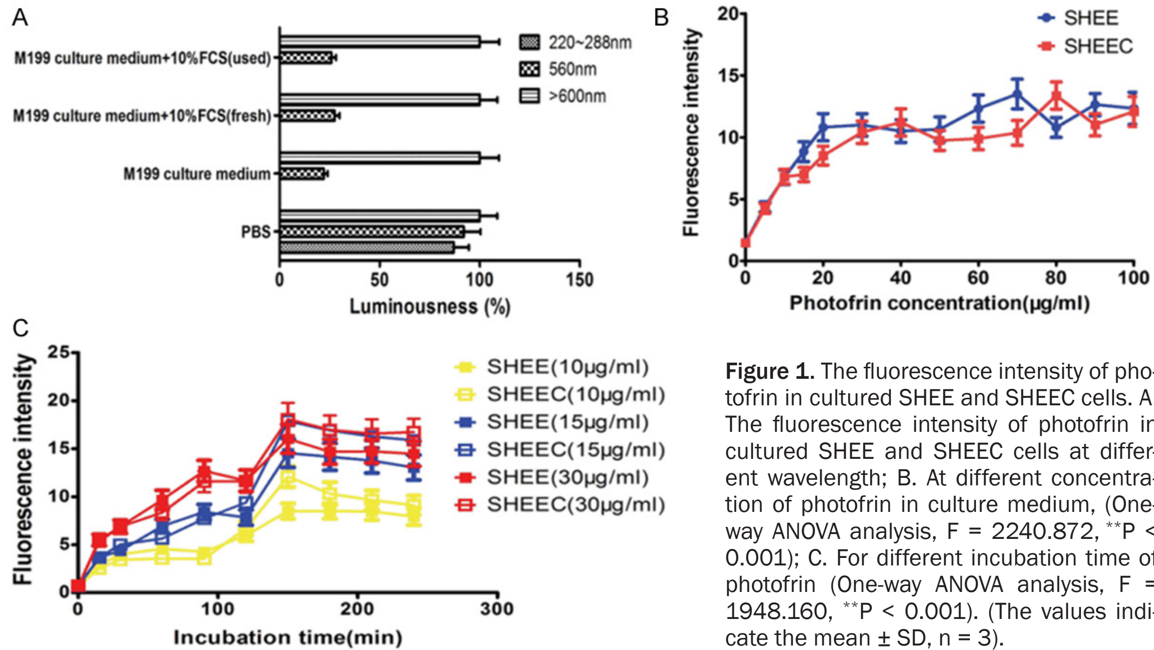

Figure 1. The fluorescence intensity of photofrin in cultured SHEE and SHEEC cells. A. The fluorescence intensity of photofrin in cultured SHEE and SHEEC cells at different wavelength; B. At different concentration of photofrin in culture medium, (One-way ANOVA analysis, F = 2240.872, $^{**}P < 0.001$); C. For different incubation time of photofrin (One-way ANOVA analysis, F = 1948.160, $^{**}P < 0.001$). (The values indicate the mean ± SD, n = 3).

V+/PI-) and late apoptotic cells (annexin V+/PI+). Data was analyzed with FlowJo version7.6.5 (TreeStar, Ashland, USA).

MTT assay

The viability of cells incubated with photofrin of different concentration and for different time were examined by MTT (3-2,5-diphenyl tetrazolium bromide) assay (Sigma, USA) as described previously [23, 24]. After the culture medium was pipetted out, 50 mL MTT (2 mg/mL) was added to each well and cells were then incubated for 4 hours at 37°C. Solubilize the cells with DMSO (100 mL/well) for another 10 min, thoroughly mixed them by vibration for 5 min, and absorbance was measured at 540 nm.

Cell viability assays

After 24 hrs of PDT treatment, the number of viable cells was quantified using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). A total of 10 μl of CCK-8 solution was added into each well, and cells were then incubated for 3.5 hr at 37°C in 5% CO_2. Absorbance was measured at 450 nm using a microplate reader, according to the manufacturer's instructions. Relative inhibitory rate of cell growth was calculated according to the formula: Relative inhibitory rate = (A2-A1)/A2×100%, A1 is the mean absorbance of transfected cells, and A2 is the mean absorbance value of control groups. All experiments were performed with five wells per experiment in triplicate.

Measurement of intracellular ROS levels

The induction of intracellular ROS during PDT was examined using an OxiSelect Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA), which uses the oxidation-sensitive fluorescent probe 29,79-dichlorodihydrofluorescein diacetate (DCFH-DA). This assay was performed by adding DCFH-DA to cells 4 h after PDT and quantifying intracellular ROS levels by detecting oxidized fluorescent 29,79-dichlorodihy-drofluorescein (DCF) using a fluorometric imaging plate reader (ARVOX5; PerkinElmer, Waltham, MA, USA) at 480/530 nm.

Soft agar colony-formation assays

2.5×10^4 cells of SHEEC cells were suspended in 0.67% agarose containing media with or without photofrin (30 mg/mL), with 1% agarose containing the medium per well on top of it. Then, the gel was laser irradiated 24 h after the gel formation, and cells were allowed to grow for another 2 weeks. Colonies were stained with 0.02% Giemsa Stain Solution (Santa Cruz Biotechnology, Inc Tokyo, Dallas, Texas, U.S.A), and the number and the size of colonies per high-power field were measured later.

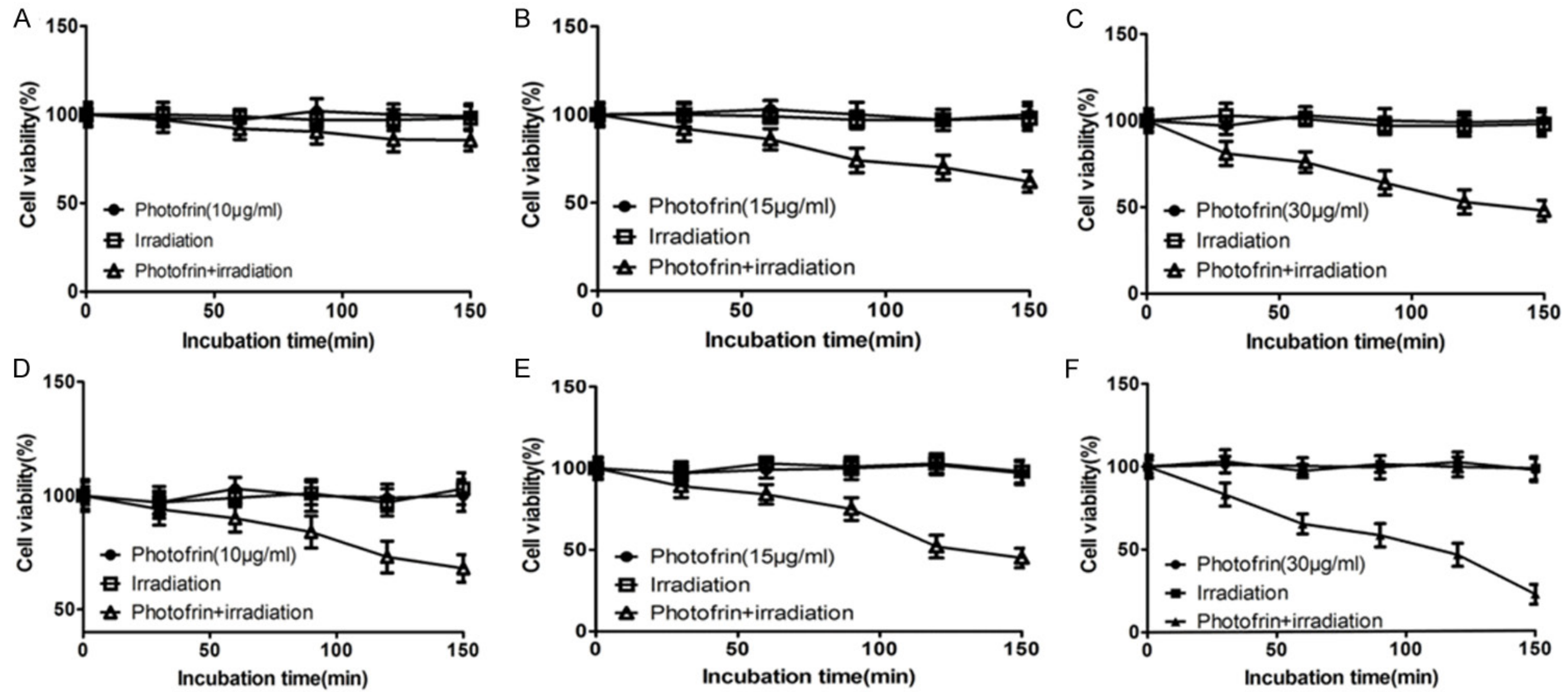

Figure 2. Cytotoxic effect of photofrin-mediated PDT on SHEE and SHEEC cells. Cell viability was assessed using the MTT assay. A-C: Cell viability of SHEE cells (One-way ANOVA analysis, A: F = 7.858, P = 0.005; B: F = 9.868, P = 0.002; C: F = 13.417, **P < 0.001); D-F: Cell viability of SHEEC cells (One-way ANOVA analysis, D: F = 8.141, P = 0.004; E: F = 8.273, P = 0.004; F: F = 11.043, P = 0.001). (The values indicate the mean ± SD, n = 3).

Statistical analyses

Statistical analyses of Quenching studies, apoptosis assay, MTT assay, Cell Viability Assays intracellular ROS levels, and Soft agar colony-formation assays were performed by One-Way Anova with Bonferroni post-test (comparing all groups) or Dunnett post-test (comparing all groups to a control group) using the SPSS statistics software (version 17; SPSS Inc., Chicago, IL, USA). Differences in values were considered as significant if the *P*-value was less than 0.05. All experiments were conducted at least 3 times and presented as Mean ± S.D. and were analyzed by a 2-tailed paired t-test.

Results

Measurement of photofrin in cultured SHEE and SHEEC cells

First, the absorption spectroscopy of culture medium was examined and 630nm was used in this study according to the results showed in **Figure 1A**. Then the uptake of photofrin by cultured SHEE and SHEEC cells was examined by the fluorescence spectrophotometer. As shown in **Figure 1**, when incubated for 120 min, photofrin was incorporated in SHEE and SHEEC cells in a dose-dependent manner; when incubated at specific concentration of photofrin, photofrin uptake peaked at 150 min.

Cytotoxic effect of photofrin on SHEE and SHEEC cells

To examine the cytotoxic effect of photofrin in SHEE and SHEEC cells, we examined the cell viability using a MTT assay after photofrin-mediated PDT. As shown in **Figure 2**, neither photofrin alone nor diode laser alone exhibited cytotoxicity in SHEE and SHEEC cells; however, photofrin incubation followed by laser irradiation induced an apparent time and dose-dependent cytotoxicity.

Induction of apoptosis by photofrin-mediated PDT in SHEE and SHEEC cells

Annexin V/Propidium iodide double-staining flow cytometry was performed after cells were treated with photofrin-mediated PDT. As shown in **Figure 3**, photofrin-mediated PDT induced significant apoptosis of SHEE and SHEEC cells. Moreover, the late apoptosis rates of SHEEC cells both in experimental groups and in control groups were significantly lower than that of SHEE cells.

Increased levels of intracellular ROS by photofrin-mediated PDT

Previous study showed that the induction of ROS was one of the key mechanisms by which PDT could exert cell-killing effect [1, 25]. To examine whether SHEEC cells treated with photofrin-mediated PDT display increased levels of intracellular ROS, we used the oxiSelect intracellular ROS assay kit to evaluate the intracellular ROS level. As shown in **Figure 4**, the DCF assay revealed that intracellular ROS levels were significantly elevated by P-PDT in a photofrin dose (**Figure 4A**) and time (**Figure 4B**)-dependent manner. NaN_3 is the quencher of $1O_2$ [26], which belongs to ROS and is the excited state of O_2. Pretreatment of NaN_3 resulted in the suppression of cell-killing effect in a dose-dependent manner (**Figure 4C**, **4D**).

Photofrin-mediated PDT inhibited anchorage-independent cell growth of SHEEC cells

Soft agar assay is an effective way to evaluate the anchorage-independent cell growth [27]. Hence, soft agar assay was used to examine whether photofrin-mediated PDT affects the anchorage-independent cell growth of SHEEC cells. As shown in **Figure 5**, neither photofrin alone nor irradiation alone affected the cell colony formation; however, photofrin-mediated PDT significantly suppressed the colony formation.

Discussion

In this study, we demonstrated that photofrin was incorporated in SHEE and SHEEC cells in a dose-dependent manner; when incubated at specific concentration of photofrin, photofrin uptake peaked at 150 min; photofrin incubation followed by laser irradiation induced an apparent time and dose-dependent cytotoxicity; the DCF assay revealed that intracellular ROS levels were significantly elevated by P-PDT in a photofrin dose and time-dependent manner; photofrin-mediated PDT significantly suppressed the colony formation. Namely, PDT shed light on therapy of ESCC, functioning as a useful tool for ESCC clinical treatment, provid-

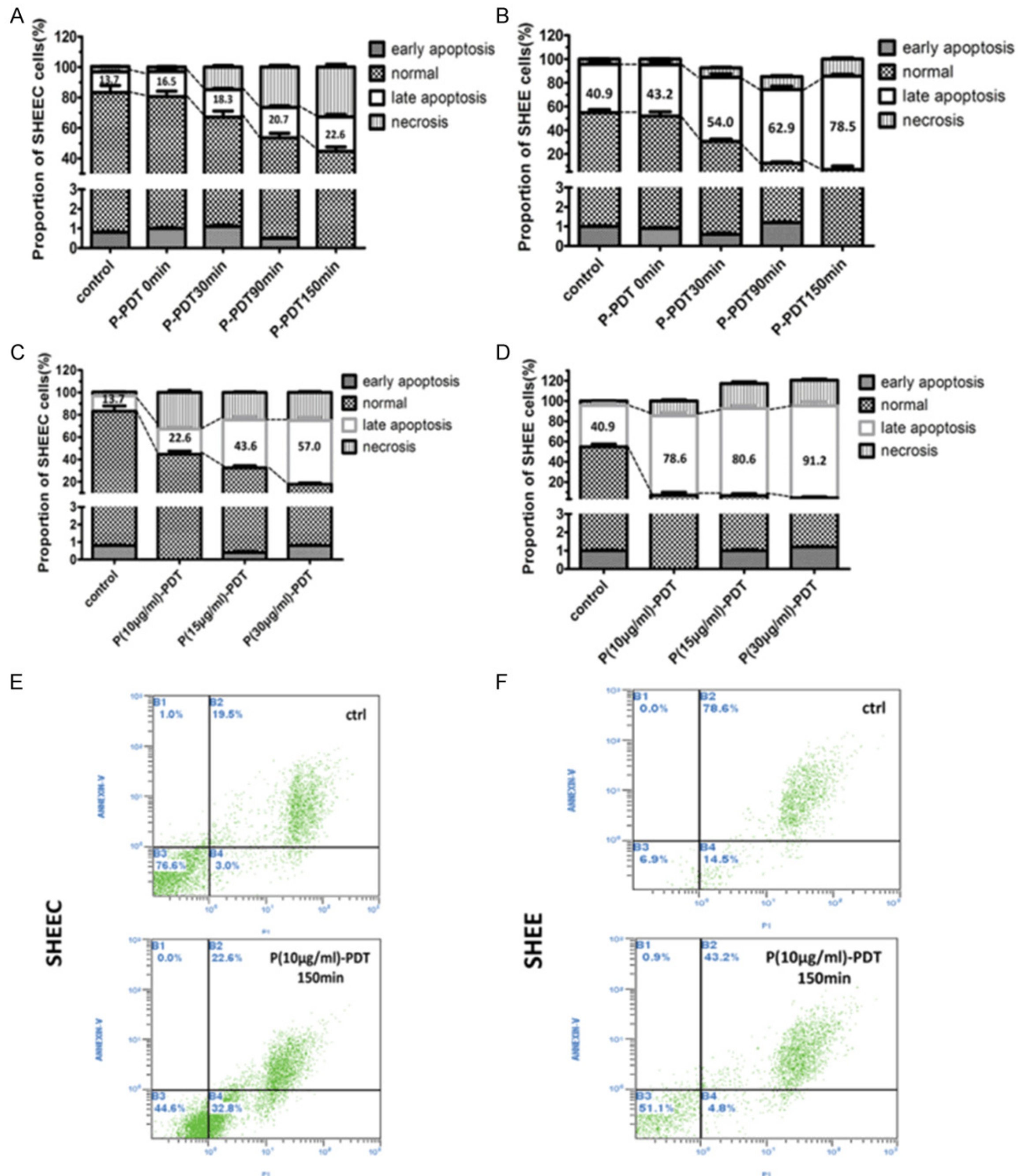

Figure 3. Induction of apoptosis in SHEE and SHEEC cells treated with photofrin-mediated PDT(P-PDT). Proportion rates of cells treated with P-PDT(photofrin 10 μg/ml) for different time, A. SHEEC cells, B. SHEE cells; Proportion rates of cells treated with P-PDT (photofrin 30 μg/ml) for 150 min, C. SHEEC cells, D. SHEE cells, E. Flow cytometric analysis of apoptosis in SHEEC cells, F. Flow cytometric analysis of apoptosis in SHEE cells. (One-way ANOVA analysis for proportion rates of late apoptosis, A. F = 13.776, **P < 0.001; B. F = 172.167, **P < 0.001; C. F = 276.763, **P < 0.001; D. F = 451.461, **P < 0.001).

ing a better understanding of Photofrin-Diomed 630-PDT in SHEEC cells.

Previous studies have showed that PDT was en effective way for suppressing the development and metastasis ability of tumors, and multiple photosensitizers have been used to perform PDT [1, 28, 29]. For example, Shinya et al used human ESCC cells, TE-5, TE-8, TE-10 and TE-11, to examine the cytotoxic effect of talaporfin

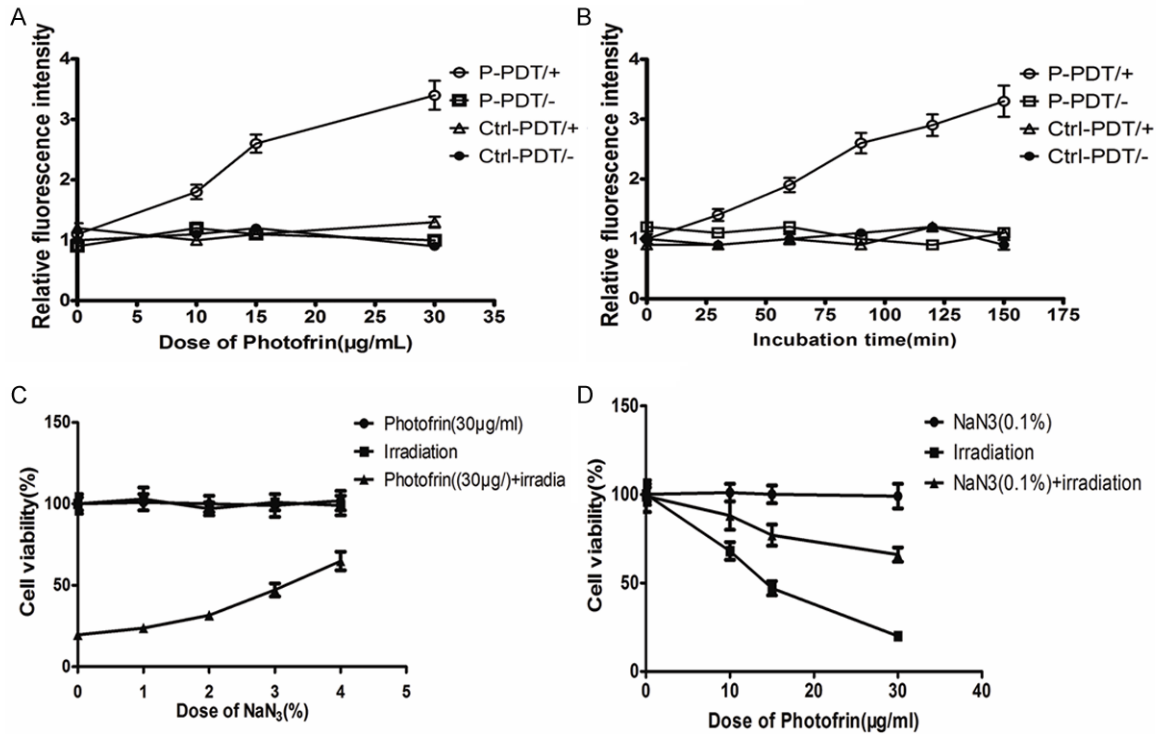

Figure 4. Induction of ROS in SHEEC cells treated with photofrin-mediated PDT (P-PDT). A. The relative fluorescence intensity of SHEEC cells at different concentration of photofrin (One-way ANOVA analysis, F = 5.156, *P = 0.0240); B. For different incubation time of photofrin (One-way ANOVA analysis, F =9.643, ***P = 0.0009). (The values indicate the mean ± SD, n = 3); C. Cell viability rate of SHEEC cells with the pretreatment different concentrations of NaN_3 at, 1: 0.0%, 2: 0.001%, 3: 0.01%, 4: 0.1% (One-way ANOVA analysis, F = 55.33, ***P < 0.0001). D. Cell viability rate of SHEEC cells with the pretreatment of NaN_3 when different dose of photofrin was used (One-way ANOVA analysis, F = 6.104, *P = 0.0358).

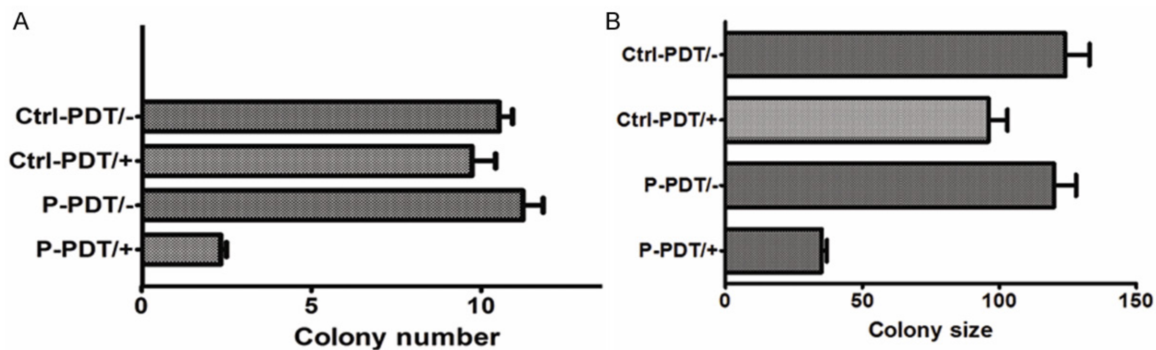

Figure 5. Inhibition of anchorage-independent cell growth in SHEEC cells treated with photofrin-mediated PDT (P-PDT). A. The colony number of SHEEC cells (One-way ANOVA analysis, F = 6.382, **P = 0.0013); B. The colony size of SHEEC cells (One-way ANOVA analysis, F = 7.643, **P = 0.0040). (The values indicate the mean ± SD, n = 3).

Sodium-Mediated PDT (t-PDT) and demonstrated that t-PDT induced potent cytotoxicity in ESCC cells independent of their differentiation grade or 5-FU resistance; moreover, t-PDT also induced robust apoptosis, perinuclear vacuolization, nuclear fragmentation and induction of annexin V-positive cells and this apoptotic response was accompanied by concurrent activation of ROS, and induction of DNA double-strand breakage [30]. CRISTINA et al have found that HEp-2 cells did not express β-integrin or FAK 12 h following PDT, that is, the PDT

reduces the adhesive ability of HEp-2 cells, inhibiting their metastatic potential; PDT using 5-aminolevulinic acid (ALA) is one of the promising treatments for malignant tumor because of its selectivity, low toxicity, rapid effect, and rapid clearance from the body [31]; Hypericin, a promising photosensitizer in the context of clinical photodynamic therapy due to its excellent photosensitizing properties and tumoritropic characteristics. Hypericin-PDT induced cytotoxicity elicits tumor cell death by various mechanisms including apoptosis, necrosis and autophagy-related cell death [32].

Further study displayed that specific signaling pathways were involved with the PDT efficacy [8, 33, 34]. Based on system biology models of the molecular interactions involved in the PDT processes previously established, and regarding a cellular decision-making system as a noisy communication channel, Ioannis used rate distortion theory to design a time dependent Blahut-Arimoto algorithm where the input was a stimulus vector composed of the time dependent concentrations of three PDT related cell death signaling molecules and the output is a cell fate decision [35].

Moreover, some molecules have been found to be useful to enhance the efficacy of PDT. 5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) catalyzes the first committed step of heme biosynthesis in animals. The erythroid-specific ALAS isozyme (ALAS2) is negatively regulated by heme at the level of mitochondrial import and, in its mature form, certain mutations of the murine ALAS2 active site loop result in increased production of protoporphyrin IX (PPIX), the precursor for heme. Light treatments revealed that ALAS2 expression results in an increase in cell death in comparison to aminolevulinic acid (ALA) treatment producing a similar amount of PPIX. The delivery of stable and highly active ALAS2 variants has the potential to expand and improve upon current PDT regimes [24]. Another study showed that the treatment of combination of inhibitors of FECH and ABCG2 could improve PPIX accumulation and PDT efficacy.

However, limited reports on the efficacy of this photofrin for the treatment of esophageal cancer have been published to date. Our results demonstrated that PDT shed light on therapy of ESCC, functioned as a useful tool for ESCC clinical treatment, and provided a better understanding of Photofrin-Diomed 630-PDT in SHEEC cells.

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Disclosure of conflict of interest

None.

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