Photodiagnosis and Photodynamic Therapy 29 (2020) 101554

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Differential in *vitro* sensitivity of oral precancerous and squamous cell carcinoma cell lines to 5-aminolevulinic acid-mediated photodynamic therapy

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ARTICLE INFO

Keywords:
Photodynamic therapy
Oral precancerous lesions
Oral squamous cell carcinoma
Aminolevulinic acid
Potential malignant diseases
Matrix metallopeptidase

ABSTRACT

Objectives: The clinical effect of 5-aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) may be correlated with the degree of dysplasia of cancer tissues, but much is still unknown regarding the differences in its effectiveness, especially in oral cancer and precancerous lesions. The aim of this study is to compare the effects of ALA-PDT on a human oral precancerous cell line (DOK) and an oral squamous cell carcinoma cell line (CAL-27).

Methods: First, we explored the dose- and time-dependent responses of DOK and CAL-27 cells to ALA-PDT. DOK and CAL-27 cells were incubated with various concentrations of ALA (from 0.25 to 2 mM), followed by PDT using laser irradiation at 635 nm. The resulting photocytotoxicity was assessed in both cell lines using MTT assays. Further, apoptosis was assessed using flow cytometry, reactive oxygen species (ROS) generation was evaluated with 2,7-dichlorofluorescein diacetate (DCFH2-DA), and the response to treatment was examined via RT-qPCR and Western blotting to measure the mRNA and protein expression levels of matrix metallopeptidase 2 (MMP-2) and MMP-9.

Results: ALA-PDT inhibited the proliferation of DOK and CAL-27 cells in a dose- and time-dependent manner. Dose-effect and inhibition-time relationships were also found. The rates of DOK and CAL-27 cell apoptosis when the ALA dose was 1 mM were 30.66 ± 3.10% and 75.40 ± 1.29%, respectively ($P < 0.01$). Following PDT, compared with DOK cells, the ROS level in CAL-27 cells was significantly increased and was correlated with an increase in the ALA concentration. Mechanistically, both the mRNA and protein expression levels of MMP-2 and MMP-9 were found to be regulated in both cell types after ALA-PDT.

Conclusion: ALA-PDT effectively killed DOK and CAL-27 cells in a dose- and time-dependent manner in *vitro*. However, under the same conditions, the susceptibilities of these cell lines to ALA-PDT were different. Further studies are necessary to confirm whether this difference is present in clinical oral cancer and precancerous lesions.

1. Introduction

Oral cancer is the most common malignant tumor of the oromaxillofacial region, and oral squamous cell carcinoma (OSCC) is the most common pathological type of oral cancer [1]. Although the incidence of oral cancer is not high, recent data combined from broad geographic areas indicate that approximately 160,000 cases of OSCC have been newly reported in Asia [2]. Oral precancerous lesions, by definition, are chronic conditions possessing great potential for malignant transformation. Most OSCC is preceded by a precancerous lesion, such as leukoplakia, erythroplakia, oral submucous fibrosis and oral lichen planus [3,4]. A systematic review of the literature by Warnakulasuriya et al. revealed that the overall malignant transformation rate of leukoplakia was 3.5% [5]. However, there is a paucity of information regarding the differences between oral precancerous lesions and OSCC.

Commonly used human cancer models include cancer cell lines and primary patient-derived tumor xenografts. Cancer cell lines are derived from primary patient tissue and have contributed tremendously to cancer research [6]. The “premalignant” oral mucosa cell line DOK (dysplastic oral keratinocyte) was described in 1992 and was derived from a piece of tongue showing epithelial dysplasia [7]. Identification of the molecular changes characteristic of epithelial dysplasia

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https://doi.org/10.1016/j.pdpdt.2019.08.036
Received 24 April 2019; Received in revised form 16 August 2019; Accepted 30 August 2019
Available online 31 August 2019

development and progression provides great insights into the mechanism of oral carcinogenesis. To date, the DOK cell line is most often used to study the multistage process of oral cancer development. Upon analyzing the differential expression patterns of genes in DOK cells, a previous study identified a number of specifically regulated genes that were enriched for many pathways associated with carcinogenesis and the DNA damage response [8]. In addition, understanding the differences between in *vitro* human premalignant and malignant human oral epithelial cell lines can provide an experimental basis for therapy [9]. Dil et al. investigated the function of aberrantly expressed, nuclear localized decorin in the DOK and SCC-25 lines. It is interesting to note that in the two cell lines, different effects on migration and invasion were found [10]. CAL 27 is one of the most frequently used cell lines in the field of OSCC studying, which was also established from poorly differentiated squamous cell carcinoma tissue at the middle of the tongue. CAL 27 cell line, compared with DOK, has a feature of tumorigenicity in athymic nude mice [11]. However, little information is available concerning the biological characteristics of the DOK cell line and oral squamous cell carcinoma cell lines.

Cancer treatment options have rapidly expanded owing to the introduction of tumor-targeted therapy. The application of surgery is limited since there are many factors to consider when choosing a therapy for tumors in the oral cavity and maxillofacial region. Photodynamic therapy (PDT) has shown efficacy and is one of the current treatment options for patients with oral precancerous lesions and/or OSCC. The main principle of PDT is that a photosensitizer (PS) selectively penetrates the targeted tissues, and when the PS is activated by a specific wavelength of visible light, the tumor cells can be killed through production of singlet oxygen and free radicals. Multiple retrospective studies have investigated the relative benefits of PDT, and these reports have supported the benefit, safety and efficacy of PDT in patients with oral cancer conditions [12,13]. A systematic meta-analysis of cohort studies assessing the clinical effect of PDT in early-stage OSCC patients showed no statistically significant difference in the complete response and recurrence between PDT and surgery [14]. We also previously discovered that topical ALA-PDT provides a strategic therapeutic advantage in oral precancerous lesions [15,16].

Despite successful treatment of oral cancer and precancerous lesions, the differences in sensitivity between oral precancerous and OSCC cells to PDT are rarely mentioned. The biological complexity of oral precancerous lesions and OSCC has increased the difficulty of designing a therapeutic regimen. Selection of the appropriate treatment programs should be based on the different responses to therapy among cells. Here, we report the results of an analysis of the differential susceptibility of DOK cells and CAL-27 cells to ALA-PDT. Our findings suggest that ALA-PDT inhibits cell proliferation in the two cell lines in a dose- and time-dependent manner. In addition, noticeable differences were observed in their susceptibility to ALA-PDT.

2. Materials and methods

2.1. Cell culture

DOK cells and CAL-27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibody for use as anin *vitro* model in our studies, as described previously [7,17]. The cells were incubated in 5% CO_2 at 37 ℃.

2.2. PDT procedure

ALA-PDT involved exposing cultured cells treated with a photosensitizer to laser irradiation. The ALA photosensitizer, purchased from Fudan-Zhangjiang Bio-Pharmaceutical Co., Ltd., Shanghai, China, was dissolved in 0.9% sodium chloride to form a 50 mM solution and was kept in the dark at −20 ˚C prior to use. Based on our previous findings, we performed assays using 0.25, 0.5, 0.75, 1, and 2 mM ALA, with an incubation time of 4 h [16]. The cells were exposed to laser irradiation using a He-Ne ion laser (LH-600 Leiyi Laser Technology Co., Ltd., Tianjin, China) at a wavelength of 635 nm and were further incubated for a specific time (2, 4, 6, 12, or 24 h) after ALA-PDT. A light power density of approximately 18 mW/cm^2, an illumination time of 50 s, and a light energy density of approximately 10 J/cm^2 were used. The detailed in *vitro* illumination system characteristics and data have been reported previously [18].

2.3. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

The effect of ALA-PDT on cell proliferation was measured using an MTT kit according to the manufacturer's instructions. The cells were inoculated into 96-well plates, treated with 20 µl MTT reagent at a concentration of 5 mg/ml and incubated for 4 h. After the culture supernatant was discarded, 10 µl of dimethyl sulfoxide was added to dissolve formazan crystals. The absorbance was measured using a microplate reader (BioTek ELX808 American) at 490/570 nm.

2.4. Cell apoptosis analysis

For the measurement of cell apoptosis of in *vitro* cultured DOK cells and CAL-27 cells, 1×10^6 cells were analyzed using an Annexin-V apoptosis detection kit. The cells were washed, fixed, and permeabilized according to the manufacturer's instructions. All of the samples were assayed and quantified using a Guava EasyCyte flow cytometer (Millipore American).

2.5. Reactive oxygen species (ROS) production

ROS formation was detected with 2,7-dichlorofluorescein diacetate (DCFH2-DA), a fluorescent probe, according to the instructions of an ROS assay kit. DOK cells and CAL-27 cells were incubated with 10 µM DCFH2-DA dissolved in serum-free medium at 37 ˚C for 30 min. The fluorescence emission at 525 nm was then measured after excitation at 488 nm using a microplate reader (BioTek ELX808 American), and the cells were subsequently photographed with a fluorescence microscope.

2.6. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from cells using TRIzol® reagent (Pufei, cat. no. 3101-100, Shanghai, China), and cDNA was prepared with M-MLV Reverse Transcriptase (Promega, cat. no. M1705, Madison, WI, USA). The cDNA generated was used as a template for RT-qPCR using SYBR Green Master Mixture (Takara, Otsu, Japan). The primer sequences and probe combinations were as follows: MMP-2, 5′−CCCCA AAACGGACAAAGAG-3′ (forward) and 5′-CTTCAGCACAAACAGGT TGC-3′ (reverse); MMP-9, 5′-GAACCAATCTCACCGACAGG-3′ (forward) and 5′-GCCACCCGAGTGTAACCATA-3′ (reverse). β-Actin was used as an internal reference, and the primer sequences were 5′-ACAGGAAGT CCCTTGCCATC-3′ (forward) and 5′-ACTGGTCTCAAGTCAGTGTAC AGG-3′ (reverse). The differences between samples were calculated using the 2-DCt values.

2.7. Western blotting assay

The cells were lysed in RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Inc.). Then, the protein concentration was measured with the BCA Protein Assay Kit (Beyotime, Shanghai, China). The total protein was separated via SDS-PAGE and was then electrically transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membranes were incubated with specific primary antibodies

at 4 °C overnight. Membranes were washed with TBST and incubated with secondary antibody for 1.5 h at room temperature. Finally, the membranes were incubated in Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) before visualization. β-Actin was used as the internal reference.

2.8. Statistical analysis

All data were analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) software. The data are presented as the means ± SDs or the numbers (percentages), and the experiments were performed independently in triplicate. Continuous variables were compared using a two-tailed *t*-test and the Mann–Whitney U test. A *P*-value less than 0.01 was considered to indicate a statistically significant difference.

3. Results

3.1. Cytotoxicity and photocytotoxicity

First, we analyzed the relationships between photocytotoxicity and the photosensitizer concentration in two cell lines. The cells were incubated with different doses of ALA (0.25, 0.5, 0.75, 1, or 2 mM) for 4 h and then excited with a laser. The images in Fig. 1A, B demonstrate that ALA-PDT inhibited the proliferation of DOK cells and CAL-27 cells in a dose- and time-dependent manner. The optimum inhibition efficiencies for both cell lines were obtained at 1 mM ALA at 12 h after ALA-PDT, and the viabilities of DOK cells and CAL-27 cells were approximately 34.07 ± 2.09% and 21.09 ± 2.78%, respectively ($P < 0.01$). However, it was also found that the photocytotoxicities of laser irradiation of the DOK cells and CAL-27 cells differed, and the difference between the photocytotoxicities appeared in a certain range. As shown in Fig. 1C, no obvious cell death was observed in the two cell lines after ALA-PDT at low concentrations. At doses of 0.25 and 0.5 mM, the viabilities of both cell lines decreased but remained above 60% after PDT. DOK cells and CAL-27 cells showed marked reductions in cell viability induced by PDT with increasing ALA concentrations from 0.75 to 2 mM. The decreasing trends gradually became nonsignificant at 12 to 24 h after PDT. Thus, CAL-27 cells had a higher sensitivity to ALA-PDT than DOK cells. The IC50 of ALA was decreased in a time-dependent manner (2 h: 4.05 vs 2.24 mM for DOK and CAL-27 cells, respectively; 4 h: 1.30 vs 1.26 mM for DOK and CAL-27 cells, respectively; 6 h: 0.95 vs 0.83 mM for DOK and CAL-27 cells, respectively; 12 h: 0.79 vs 0.61 mM for DOK and CAL-27 cells, respectively; 24 h: 0.81 vs 0.62 mM for DOK and CAL-27 cells, respectively) (Supplement Fig. 1).

Furthermore, we adopted an MTT assay to determine the cell viability of DOK cells and CAL-27 cells in the control, ALA (1 mM), laser irradiation (635 nm, 10 J/cm^2), and ALA-PDT groups. Compared with the control group, neither ALA nor laser irradiation individually induced an appreciable divergence in cell viability, whereas ALA-PDT induced obvious cytotoxicity in the two cell lines after 12-h treatments (two-tailed *t*-test, $P < 0.01$) (Fig. 1D).

3.2. Apoptosis rates of DOK and CAL-27 cells

To assess the influence of ALA-PDT on the apoptosis of DOK cells and CAL-27 cells, flow cytometry and Annexin-V staining were performed at 12 h after PDT. Compared with the control group, the apoptosis rates in both cell lines treated with ALA-PDT showed dose-dependent increases, evidenced by the ratios of apoptotic cells in the DOK cells and CAL-27 cells (Fig. 2A). Meanwhile, in the CAL-27 cells, a better apoptosis-inducing effect was observed. The proportions of apoptotic CAL-27 cells were 75.40 ± 1.29% and 80.03 ± 3.77% after PDT at doses of 1 and 2 mM ALA, respectively, while in DOK cells, the apoptosis rates were 30.66 ± 3.10% and 42.33 ± 1.92%, respectively ($P < 0.01$). As shown in Fig. 2B, the rates of cell apoptosis and secondary necrosis in the CAL-27 cells were significantly higher than those in the DOK cells. Of note, the main difference existed in the effect of PDT on the number of late apoptotic cells.

3.3. ROS level variations in DOK and CAL-27 cells

Based on the concepts and principles of PDT, ROS production is one of the most important indicators to identify the therapeutic effect [19,20]. To investigate whether differences in intracellular ROS levels existed in DOK cells and CAL-27 cells treated with ALA-PDT, we used a DCFH2-DA fluorescence staining assay kit to evaluate ROS generation. As shown in Fig. 3A and Table 1, the DCFH2-DA assay revealed that the ROS levels in CAL-27 cells were significantly increased by ALA-PDT in a photosensitizer dose- and time-dependent manner, whereas the levels in DOK cells increased slightly until the concentration of ALA was increased above 1 mM. At a concentration of 1 mM ALA, ALA-PDT elicited stronger DCFH2-DA fluorescence in CAL-27 cells than in DOK cells (Fig. 3B).

3.4. MMP-2 and MMP-9 protein and mRNA expression in DOK and CAL-27 cells

To investigate the possible mechanisms of ALA-PDT-induced killing of DOK cells and CAL-27 cells, we observed changes in the expression of MMP-2 and MMP-9 in both cell lines treated with 1 mM ALA-induced PDT at 12 h after laser irradiation. We found that the protein levels of MMP-9 obviously decreased in both cell lines at this concentration of ALA, while the levels of MMP-2 were significantly lower in treated CAL-27 cells than in control cells (Fig. 4A,B). However, changes in the MMP-2 levels in DOK cells were not obvious (Fig. 4B). Additionally, we performed qRT-PCR to determine the mRNA expression levels of MMP-2 and MMP-9, and the results support the above findings (Fig. 4C, D).

4. Discussion

Among the various factors determining its antitumor activity, the photosensitizer is doubtlessly the most significant component of PDT[21]. Accordingly, the curative effect of PDT is decided by the photosensitizer, ROS generation and light. Photosensitizers are reagents that produce ROS upon light illumination and are commonly used to induce oxidative stress during PDT. ALA is a nonporphyrin photosensitizer characterized by high selectivity for tumor cells and tissue permeability. As a precursor of the photosensitizer-protoporphyrin IX (PpIX), ALA can induce PpIX synthesis in malignant cells[22]. Moreover, ALA can rapidly induce PpIX synthesis, which makes it desirable to minimize the photocytotoxicity in normal tissues[23]. As previously reported, preclinical and clinical studies of ALA-PDT against oral precancerous lesions and oral cancer have shown positive and promising results [12,16,24,25]. A rational dosage regimen should be established on the basis of in *vitro* drug sensitivity tests. Rosin et al. found that oral cancer cells can exhibit resistance to PDT mediated by ALA[18]. The SCC9 cell line can survive 5-ALA-PDT administration by reducing PpIX synthesis and initiating signaling pathways related to cell proliferation and apoptosis. In addition, it is of theoretical and practical significance for the clinical application of ALA to thoroughly discuss and study the influence of PDT on different cell lines. It is recognized that sensitivity to PDT might differ between various cell types. Although the survival activity of both A375 cells (a human melanoma cell line) and A431 cells (a nonmelanoma skin carcinoma cell line) could be inhibited by ALA-PDT in a dose- and time-dependent manner, the optimum inhibition efficiencies and apoptotic rates of both cell lines were significantly different [26]. However, we found no study showing a difference in sensitivity of oral precancerous and squamous cell carcinoma cell lines to ALA-PDT in *vitro*. Here, we investigated the effect of ALA or laser irradiation alone on DOK cells and CAL-27 cells. The cell proliferation test results showed that ALA exhibits no cytotoxicity without laser

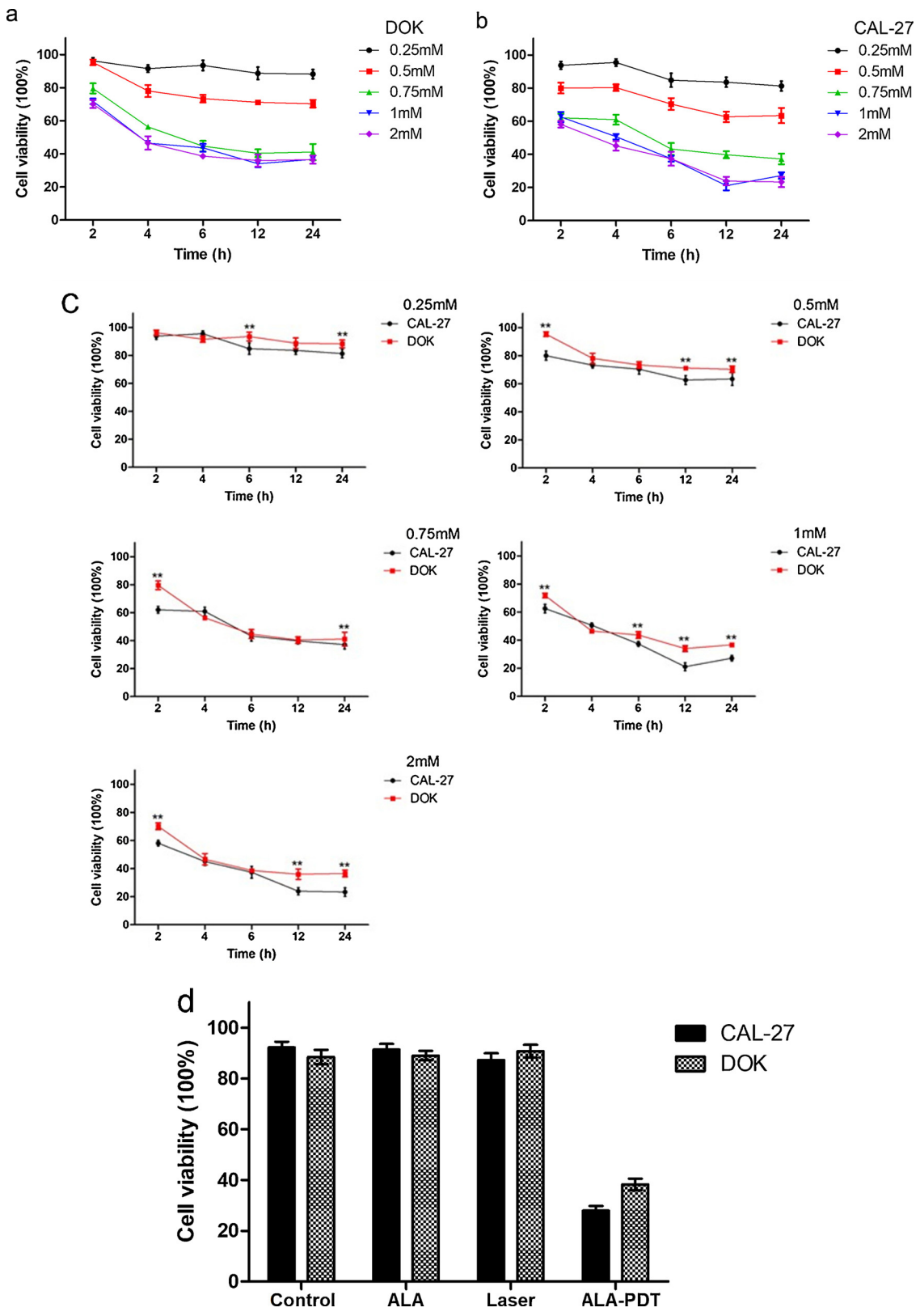

Fig. 1. Cell viability of (a) DOK cells and (b) CAL-27 cells determined by MTT assay after ALA-PDT. Two cell lines were incubated with 0.25–2 mM ALA for 4 h and then irradiated with 10 J/cm^2 light dose. The percentage of cell viability was shown in a dose- and time-dependent manner. (c) Comparisons of cell viability in two cell lines with ALA at the different concentrations. (d) The effect of 5-ALA, laser irradiation and ALA-PDT on both cells. All results are expressed as as mean ± SD of triplicate determinations from three independent experiments. Data were significant at $p < 0.01$ (**) for DOK cells in comparison to CAL-27 cells as recorded by two-tailed *t*-test.

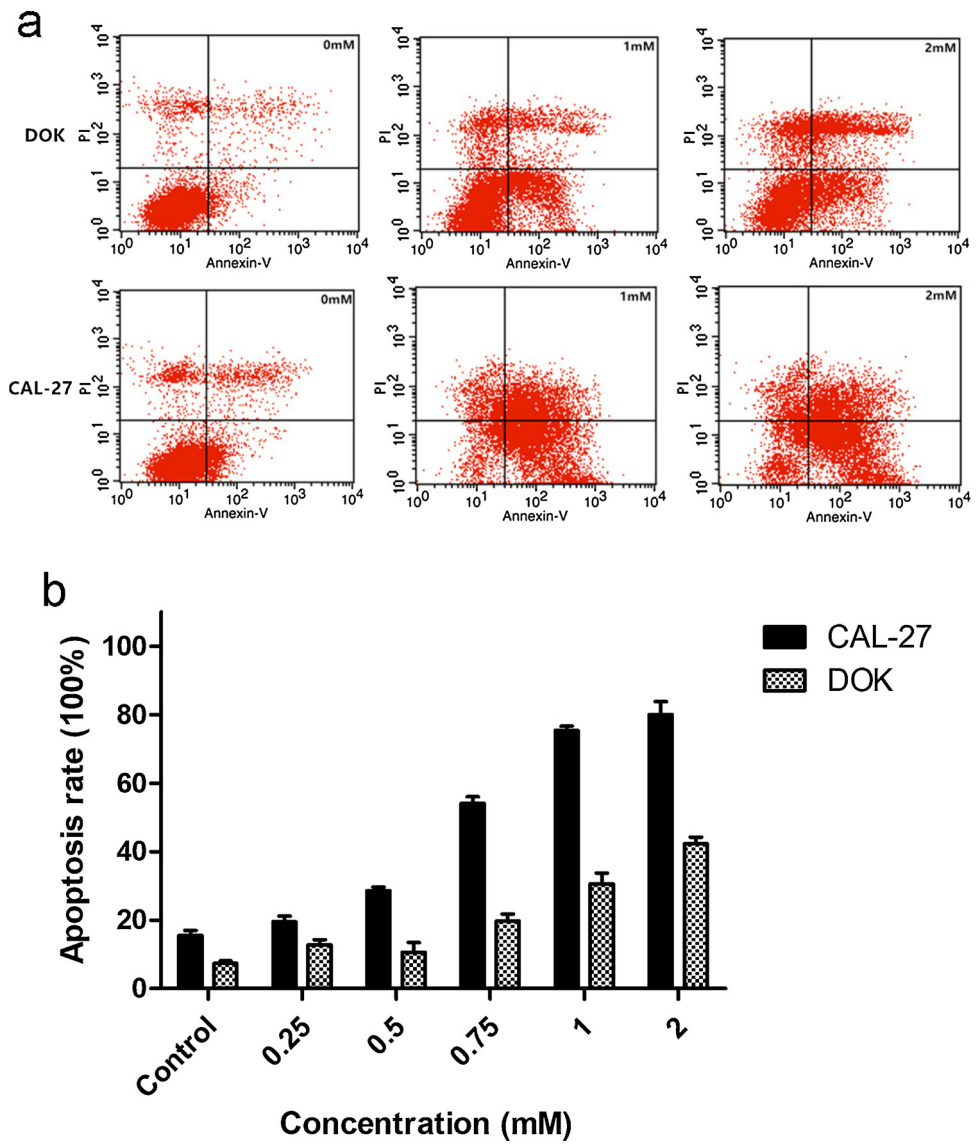

Fig. 2. Apoptosis assay using flow cytometry of DOK cells and CAL-27 cells after ALA-PDT treatment. Cells were treated with 0.25–2 mM ALA, and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining was performed after ALA-PDT. Representative flow cytometry figures and apoptosis rates of both cells are shown in a and b, respectively. In all above experiments, the data were performed independently in triplicate.

irradiation. Other preclinical studies using ALA have also described this finding in breast cancer and hepatocellular carcinoma cells [27,28].

Similar to DOK, CAL-27 is considered a representative cell line for OSCC studies and was established from tumor tissue obtained from a 56-year-old Caucasian male with tongue squamous cell carcinoma [29]. Our study is the first to investigate the difference in sensitivity to ALA-PDT between oral precancerous lesions and OSCC in *vitro*. Our previous study showed that topical ALA-PDT is effective in treating oral precancerous lesions, especially in the presence of dysplasia [16]. Therefore, we hypothesized that a potential link exists connecting the differences between oral precancerous lesions and oral cancer and their susceptibility to ALA-PDT. In this study, we found that ALA-PDT had a remarkable dose- and time-dependent inhibitory effect on the proliferation of both cell lines. In addition, the optimum inhibition efficiencies for DOK cells and CAL-27 cells were obtained with 1 mM ALA at 12 h after ALA-PDT treatment. However, the optimum ALA dose for oral precancerous lesions and OSCC needs to be further identified in clinical studies. Interestingly, the effects of PDT on DOK cells and CAL-27 cells appear to be similar but also display some differences. Both cell lines were cultured at the same density, but the inhibitory effect of ALA-PDT on CAL-27 cells was higher than that on DOK cells. Indeed, the findings are consistent with the pathobiological behavior of OSCC cells, which have a higher metabolic rate and proliferate faster than cells in precancerous lesions [30,31]. Based on the dose-response relationship between PDT and tissue damage in an animal model, Nauta et al. found that in *vivo* Photofrin-induced fluorescence signals in premalignant lesions and OSCC were increased with an increasing epithelial atypia index (EAI) [32].

PDT is usually defined as a powerful inducer of tumor cell apoptosis in many situations in which the apoptotic response of neoplastic cell lines can rapidly be initiated [33]. The fact that there are multiple forms of apoptosis that account for the death of tumor cells and that these individual forms of apoptosis can occur in different tissues makes assessing cell apoptosis complicated. In our study, the apoptosis rate was obviously increased in DOK and CAL-27 cells after ALA-PDT, indicating that the treatment can induce apoptosis in both cell lines. Flow cytometry results also showed that the primary difference between the DOK and CAL-27 cell apoptosis rates was in late-stage apoptosis, which suggests that the killing effect of ALA-PDT on both cell lines may be mediated through promotion of late apoptosis. Mitochondrial ROS

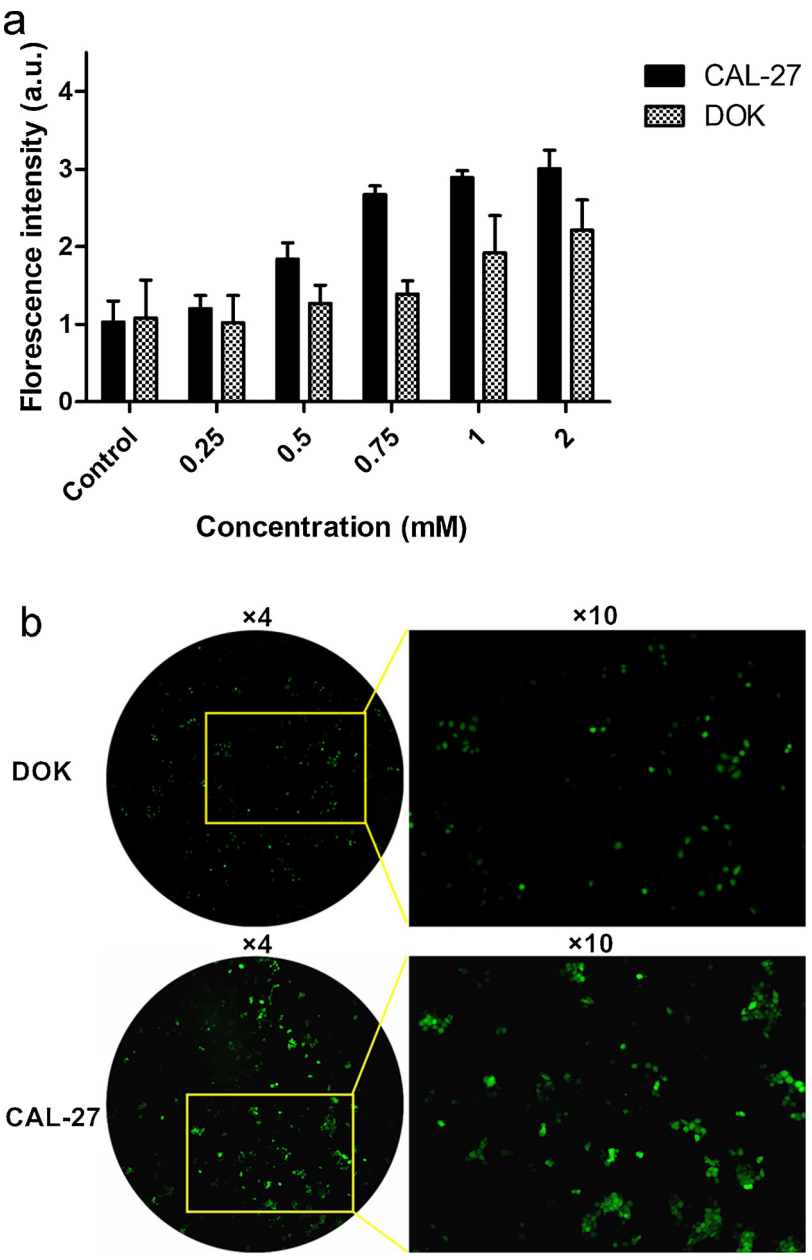

Fig. 3. Cellular ROS in DOK cells and CAL-27 cells after ALA-PDT treatment. (a) ROS levels of both cells treated with 0.25–2 mM ALA were measured by DCFH-DA fluorescence staining assay kit. (b) Representative DCF fluorescence figures were photographed by fluorescence microscope. In all above experiments, the data were performed independently in triplicate.

Table 1
DCFH2-DA Fluorescence intensity of DOK and CAL-27 cells after ALA-PDT (a.u.).

	$DCFH_2$-DA Fluorescence intensity (a.u.)					
	Control	0.25 mM	0.25 mM	0.75 mM	1 mM	2 mM
CAL-27	1.03 ± 0.27	1.20 ± 0.17	1.84 ± 0.21**	2.67 ± 0.11**	2.89 ± 0.09**	3.00 ± 0.24**
DOK	1.08 ± 0.49	1.02 ± 0.35	1.27 ± 0.23	1.39 ± 0.17	1.92 ± 0.48**	2.21 ± 0.39**

** $P < 0.01$ compare with that of respective control group.

production can be of tremendous importance for cell proliferation and apoptosis [34,35]. Recently, mitochondria-targeting therapeutic strategies have been considered as a novel idea for cancer treatment. As we know from previous research, ALA-induced PpIX is initially localized in the mitochondria [36]. Excessive mitochondrial ROS production may lead to cell death induced by oxidative stress; however, the responses differ under different scenarios and in different cell types [37]. We demonstrated that ROS levels were elevated in both cell lines. However, compared with DOK cells, ALA-PDT showed more influence on ROS production in CAL-27 cells. Thus, we assume that mitochondrial ROS production was induced by ALA-PDT, probably providing a clue to the reasons underlying the differential susceptibility of DOK cells and CAL-

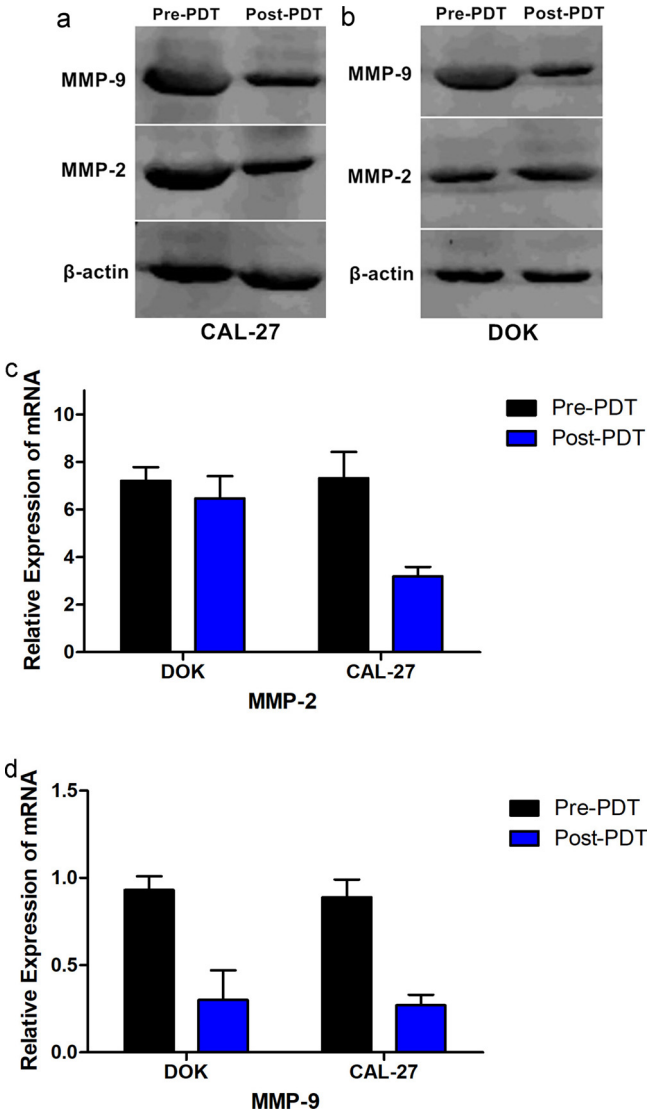

Fig. 4. The expression of MMP-2 and MMP-9 by western blotting and qRT-PCR in DOK cells and CAL-27 cells before and after treated with 1 mM of ALA induced PDT at 12 h after laser irradiation. The protein expression of MMP-2 and MMP-9 in CAL-27 cells (a) and DOK cells (b) by western blotting. β-actin was used as a loading control. The mRNA expression of MMP-2 (c) and MMP-9 (d) in both cells by the qRT-PCR. Histogram representing indicated the results of three independent experiments.

27 cells to ALA-PDT.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases involved in degrading extracellular matrix (ECM) components and are closely associated with many malignant processes, including cell growth, apoptosis, malignant transformation, and invasion, among others [38–40]. Normally, MMP activity and expression levels are tightly regulated at different stages. In many malignant epitheliogenic tumors, the disruption of the subendothelial basement membrane and invasion by malignant cells is generally considered the biological basis for tumorigenesis and progression. Previous studies have demonstrated that 23 MMPs are expressed in humans, and they are classified according to their architectural features and specificity for ECM components [41,42]. To date, some MMP collagenases, including MMP-2 and MMP-9, have been proven to contribute to the cleavage of ECM in the tissue microenvironment, which may promote cancer cell invasion. A fibronectin type IV collagen binding site is located in the catalytic domain of MMP-2 and MMP-9, and its degradation plays a key role in numerous phases of tumor development [43]. Overexpression of MMP-2 and MMP-9 has been identified in a large proportion of OSCC. A single study from India analyzed plasma and tissue levels of MMP-2 and MMP-9 in 60 untreated patients with OSCC and found significantly higher levels in malignant tissues than in adjacent normal tissues [44]. In 2010, these researchers further assessed the value of MMP-2 and MMP-9 and their inhibitors in the prediction of invasion and metastasis of OSCC [45]. MMP-2 and MMP-9 levels have been regarded as potential markers to determine the treatment and prognosis of patients with cancer. In particular, MMP-9 has proven to be a biomarker for the efficacy of photodynamic therapy in OSCC and leukoplakia, which is identical to our findings [46]. To investigate the role and possible mechanism of ALA-PDT, we analyzed the variations in MMP-2 and MMP-9 expression in DOK cells and CAL-27 cells via Western blotting and qRT-PCR. Our data revealed that following ALA-PDT, the levels of MMP-9 were significantly downregulated in both cell lines, while MMP-2 expression showed different trends. Jordan et al. found that MMP-9 mRNA was overexpressed (> 2-fold) in 29 of 34 (85%) dysplasias and in 15 of 15 (100%) OSCCs, whereas the overexpression of MMP-2 was found in only 32% and 47% of oral dysplasias and cancers, respectively. In comparison with MMP-2, MMP-9 showed a closer relationship with malignant transformation of oral dysplasia to oral cancer; thus, it might be a biological index of the malignancy degree of oral precancerous and cancer cells [47]. These data suggest a possible reason for the difference in sensitivity to ALA-PDT between DOK cells and CAL-27 cells.

A better understanding of the pathophysiological characteristics of oral precancerous and squamous cell carcinoma cells is essential for exploring the mechanism of ALA-PDT. It is well known that mitochondrial ROS formation plays a critical role in PDT-induced apoptosis. In the present study, although both cell lines exposed to ALA-PDT produced a higher level of ROS than control group cells, the treatment offered clear advantages in CAL-27 cells. In previous research, a connection between MMPs and PDT was found. In a head and neck squamous carcinoma cell line, 9-hydroxypheophorbide-mediated PDT could not only suppress cell migration and invasion but also trigger the downregulation of MMP-2 and MMP-9 via ROS-mediated inhibition of phosphorylation [48]. ROS also regulates the activity of via oxidation of the prodomain cysteine [49]. However, to date, the correlation between MMPs and ALA-PDT remains unclear but may have an important influence on the physiology of oral cancer and precancerous lesions. Regrettably, certain limitations of the present study should be acknowledged, although we focused on MMP-2 and MMP-9. For example, there is a lack of in *vivo* data to support our findings. Additionally, further studies should be devoted to determining how other related molecules and signaling pathways interact with each other in the process.

5. Conclusion

In summary, the current results confirmed, for the first time, that oral precancerous and OSCC cells have different sensitivities to ALA-PDT in *vitro* and revealed changes in MMP-2 and MMP-9 expression after the treatment. However, only a few of the physiological functions and possible mechanisms of ALA-PDT have been well elucidated in our research. Therefore, future work should focus on in *vivo* functions and critical mechanisms, which may provide better evidence to facilitate our understanding of the potential of ALA-PDT as a targeted therapy.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported by National Natural Science Foundation of China (Grant No 81771071), Non-profit Industry Research Specific Fund of National Health and Family Planning Commission of China

(Grant No 201502018), Program for New Clinical Techniques and Therapies of Peking University School and Hospital of Stomatology (PKUSSNCT-16A07), and Program of Peking University School and Hospital of Stomatology (PKUSS20170103).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pdpdt.2019.08.036.

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