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A Comparative Study on the Enhancement Efficacy of Specific and Non-specific Iron Chelators for Protoporphyrin Ⅸ Production and Photosensitization in HaCat Cells

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Summary: The iron chelators can be utilized in target cells to improve 5-aminolaevulinic acid (ALA)-based photodynamic therapy (PDT). The purpose of this study is to compare the effect of two kinds of iron chelators, desferrioxamine (DFO) and ethylenediaminetetraacetic acid (EDTA) on the enhancement of ALA-PDT. HaCat cells were cultured in medium containing 2.0 mmol/L of ALA and 0.5 mmol/L of DFO or EDTA. After 3-h incubation in the dark, the concentration of cellular protoporphyrin Ⅸ (PpⅨ) was detected by high performance liquid chromatography (HPLC), and the fluorescence of PpⅨ was observed at 630 nm emission under confocal laser scanning microscope. For PDT, HaCat cells were irradiated using 632.8 nm laser, and the fractions of apoptotic and necrotic cells were flow cytometrically assayed. Related differences in morphology and ultrastructure of HaCat cells were observed using optical microscope or transmission electron microscope. Compared to incubation with ALA alone, the addition of DFO or EDTA increased the concentration of cellular PpⅨ and the fluorescent density of PpⅨ, and also increased cell death ratio after PDT. PDT using ALA plus DFO produced the highest cellular PpⅨ level, greatest cell death ratio and most severe structural damage to the cells. It was concluded that both DFO and EDTA could enhance ALA-based PpⅨ production and PDT. Compared to the non-specific iron chelator of EDTA, the specific chelator, DFO, showed more potential for the enhancement.
Key words: aminolaevulinic acid; desferrioxamine; ethylenediaminetetraacetic acid; keratinocytes; photodynamic therapy; protoporphyrin Ⅸ

Photodynamic therapy (PDT) is a new treatment modality for various skin diseases based on the administration of photosensitizers that, together with light, kill the target cells[1, 2]. A widely used photosensitizer is 5-aminolevulinicacid (ALA), a natural precursor of heme, which can induce the accumulation of the photosensitizer protoporphyrin Ⅸ (PpⅨ)[3]. Most cells of the human body can transform ALA into porphyrins. After the application of ALA to human skin, the porphyrins accumulate mostly in sebaceous glands and the epidermis. However, neoplastic cells synthesize more porphyrins than normal cells do. The first step in natural heme biosynthesis is the formation of ALA from glycine and succinyl coenzyme A through the action of ALA synthase. Then a series of biochemical reactions leads to PpⅨ, and in the final step ferrochelatase catalyzes the incorporation of Fe(Ⅱ) into PpⅨ to form heme. Ferrochelatase converts PpⅨ into the heme by inserting ferrous iron into PpⅨ[4]. It has been proposed that, by chelating intracellular iron the availability of iron required by the enzyme ferrochelatase for insertion into PpⅨ in the formation of heme can be reduced, therefore reducing the conversion of PpⅨ to heme further increases the accumulation of PpⅨ in the cell.

The PpⅨ-induced PDT produces good clinical outcomes with excellent cosmetic results[5, 6]. Its efficacy for nodular or thick lesions, however, appears to be inferior to standard therapies unless repeated treatments are performed or lesions are pre-treated with physical protocols[7, 8]. Therefore, further enhancement of ALA-PDT is required and may be possible by employing exogenous materials to temporarily increase PpⅨ accumulation above the levels normally achieved by using ALA alone. Various adaptations to the standard treatment have been considered, including skin pre-treatment with the use of iron chelators.

Iron chelators can be divided into two categories based on their specificity of ions chelation. A non-specific chelator, such as ethylenediaminetetraacetic acid (EDTA)[9], binds to most metal divalent cation by enzymatic reactions and is used extensively as a sequestering agent. A specific iron chelator or selective iron chelating agent such as desferrioxamine (DFO), binds only to iron ions[10]. DFO has a great potential to enhance ALA-induced PpⅨ accumulation and PDT effect than EDTA, because of its greater affinity for iron[11]. It has been reported that both EDTA and DFO could enhance

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ALA-PDT effects *in vitro*[9, 12]. Therefore, the main goal of this study was to compare EDTA and DFO on promoting ALA-PDT in HaCat cells.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

PpⅨ, penicillin, streptomycin, trypsase, Hanks' balanced salt, Dulbecco's modified Eagle medium (DMEM) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, USA. Fetal calf serum (FCS) was bought from Sijiqing Bio-Engineering Materials Co. Ltd., China, and ALA hydrochloride was from Fudan-Zhangjiang Bio-Pharmaceutical Co. Ltd., China, for *in vitro* PDT experiments. The desferrioxamine mesilate for injection was produced by Novartis Pharma Schweiz AG, Switzerland. EDTA calcium disodium ($CaNa_2$-EDTA, $C_{10}H_{12}N_2O_8Na_2Ca{\cdot}2H_2O$) was procured from Wuhan Helian Chemicals & Instrument Co. Ltd., China. FITC-annexin Ⅴ/propidium iodide (PI) apoptosis kit was the product of MultiSciences Biotech Co. Ltd., China. All chemicals used in this study were of analytical grade.

1.2 HaCat Cell Culture

The HaCaT cells, a spontaneously transformed human keratinocyte cell line, were kindly provided by Dr. Liu Dong's laboratory at Tianjin Changzheng Hospital, China. Cells were maintained in monolayer cultures in DMEM supplemented with 10% FCS, 50 U/mL penicillin, 50 mg/mL streptomycin and nonessential amino acids. They were grown in 5% CO_2 at 37°C and passaged every 3–5 days as previously described[13]. Prior to the experimentation, freshly trypsinized cells were seeded into 12-well plates (flat bottom) at a density of 0.5×10^6 cells/mL and incubated for 72 h, which would reach an 80% confluence after that. Before conducting all measurements cell viability was assessed using the trypan blue exclusion method and was >98% for all experiments.

1.3 Grouping

HaCat cells were cultured in four different compositions of media that contained 2.0 mmol/L ALA alone, 2.0 mmol/L ALA plus 0.5 mmol/L EDTA, 2.0 mmol/L ALA plus 0.5 mmol/L DFO, or none for blank control. These cells were incubated in the dark at 37°C for 3 h. Each group had monolayer culture of HaCat cells growing on the cover glass. All test solutions were freshly prepared before each experiment by dissolving ALA, EDTA or DFO in modified DMEM. The pH of the solutions was checked and adjusted to physiological pH 7.4 using NaOH (0.5 mol/L) as necessary. These solutions were then sterilized with nitrocellulose filter (0.22 μm; Millipore) before being diluted to the final concentrations. After the addition of the test solutions, all procedures were carried out under reduced light levels. All experiments were performed in triplicate and repeated on two separate days, to give a total of six readings for each data point shown.

1.4 HPLC Analysis of PpⅨ

The cells were then washed twice in PBS and diluted with 0.5 mL of formic acid for 15 min at room temperature. The porphyrins in the cultured cells were extracted as described for fluorimetry and the pH adjusted to 5 or above with saturated sodium acetate[14]. The samples were then washed with an equal volume of distilled water and passed through a filter paper saturated in ethyl acetate. The porphyrins were analyzed by the high performance liquid chromatography-fluorescence detection (HPLC-FD), according to a modified method reported by Tunstall *et al*[14]. They were dried under a stream of nitrogen gas and stored in the dark at 4°C until required. The dry porphyrin samples were dissolved in 100 μL of methanol containing 10% HCl immediately before analysis with HPLC system (Dionex, USA). The reverse phase HPLC analysis of 20 μL reconstituted sample was performed using a Waters Nova Pak C18 analytical column (150 mm×3.9 mm). Solvent A was 10% acetonitrile/90% ammonium acetate (1.0 mol/L, pH 5.16), and Solvent B was 90% methanol/10% acetonitrile. The fluorescence was detected at 488 nm excitation and 630 nm emission. The porphyrin standards were dissolved in the pre-mentioned extraction solvent mixture. The PpⅨ concentration in the samples was determined by measuring the area under the curve and compared with standard curves. These experiments were repeated three times.

1.5 CLSM Detection of PpⅨ Fluorescence

The HaCat cells growing on cover glass were analyzed by confocal laser scanning microscope (CLSM) of TCS NT type (Leica Lasertechnik GmH, Germany). It was equipped with Argon (PMT approximately 600) and Krypton (PMT approximately 800) laser. The cells were fixed with 4% paraformaldehyde and then incubated with 0.1% sodium borohydride buffer. The coverslip with cells were transferred to a Mowiol (Colbiochem, Germany) drop on a glass slide and examined with CLSM. The filter was OG 590 for Dil and Rh-PE. The confocal analysis was performed in accordance with standard protocol[15, 16]. The PpⅨ in HaCat cells was excited using a 488 nm wavelength kripton–argon laser, and the fluorescence signals were collected by the filter at 630 nm. The final fluorescence images were constructed by photo-counting process.

1.6 Laser Irradiation

The HaCat cells were exposed to a 2-min irradiation with the laser light at a wavelength of 632.8 nm[17]. The high-power semiconductor laser system (Xingda Light-Electric Medical Equipment Co., China) was used, and the total fluence was 2.9 J/cm^2 at a density of 300 mW/cm^2. Thereafter, the culture medium was replaced by the DMEM supplemented with 10% FCS. The cells were cultured for another 12 h after the medium replacement. ALA-PDT was performed independently three times on HaCat cells to minimize intra-experimental variability.

1.7 Flow Cytometric Analysis

FITC-annexin Ⅴ/PI double staining was used in the flow cytometric detection of apoptosis and necrosis[18]. The annexin Ⅴ was a chimeric recombinant protein produced by fusing green fluorescent protein to the N-terminus of annexin Ⅴ. After PDT, the HaCat cells were rinsed in D-Hanks and trypsinized by 0.25% trypsase. They were harvested by a centrifugation of 800 g for 5 min and washed twice with ice-cold PBS. Then they were re-suspended in PBS buffer (pH 7.2, supplemented with 0.5% BSA) at a concentration of 1×10^6

cells/mL, and then 100 μL of cell suspension was added into 5-mL FCM tube. A total of 5 μL of annexin Ⅴ-FITC and 10 μL of 20 μg/mL PI were added and incubated for 15 min in the dark before the further addition of 400 μL of PBS. This mixture was incubated for 10 min at room temperature, and then the cellular fluorescence was measured by flow cytometry with a FACS-SCAN apparatus (Beckman-Coulter, USA). The signal was detected in FL1 and FL3 channels, and the quadrant markers were set on dotplots of unstained and stained cells. The apoptotic percentages of 10 000 cells were calculated using the software provided by the manufacturer.

1.8 Morphological Observation

After ALA-PDT, the HaCat cells growing on cover glass were fixed at room temperature for 30 min with a fresh-prepared 4% paraformaldehyde solution in PBS. The fixed samples were stained with hematoxylin and eosin, and then observed for morphological changes under the optical inverted microscope. For the electron microscopy, the HaCaT cells on cover slips were washed three times with PBS and then fixed with 2% glutaraldehyde for 1 h at 4°C. The samples were post-fixed with 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 2 h, and stained with uranyl acetate (5 mg/mL), dehydrated in acetone. Finally they were embedded in Epon 812. The sections were post-stained with uranyl acetate and lead hydroxide. The sub-cellular structure was observed under transmission electron microscope (Hitachi H600, Japan)[19]. Ultrathin sections were obtained by using ultramicrotome PowerTome-XL (RMC, USA).

1.9 Statistical Analysis

The data were obtained from three independent experiments and presented as $\bar{x}\pm s$. Comparisons were made by employing SPSS13.0 software for windows. A *P*-value of less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Quantitative Detection of Cellular PpⅨ

The cellular PpⅨ levels of blank control, ALA alone, ALA plus EDTA, and ALA plus DFO were 0.007±0.001, 0.368±0.031, 0.640±0.043 and 1.205±0.068 μg/L respectively. Statistically significant differences were found between any two of them ($P<0.01$). All groups (except for blank control) had substantial PpⅨ content. Compared to the group treated with ALA alone, PpⅨ levels were higher in any group treated with ALA plus iron chelator, especially in the DFO group ($P<0.01$).

2.2 PpIX Fluorescence

Fig. 1 shows the confocal fluorescence photomicrographs obtained from control and treated HaCat cells growing on cover glass. These results were in agreement with those of HPLC analysis for celluar PpⅨ, as shown above. The most intense red fluorescence was found in the cells treated with ALA plus DFO, although the group of ALA plus EDTA also showed increased PpⅨ fluorescence as compared to cells treated with ALA alone and blank control cells.

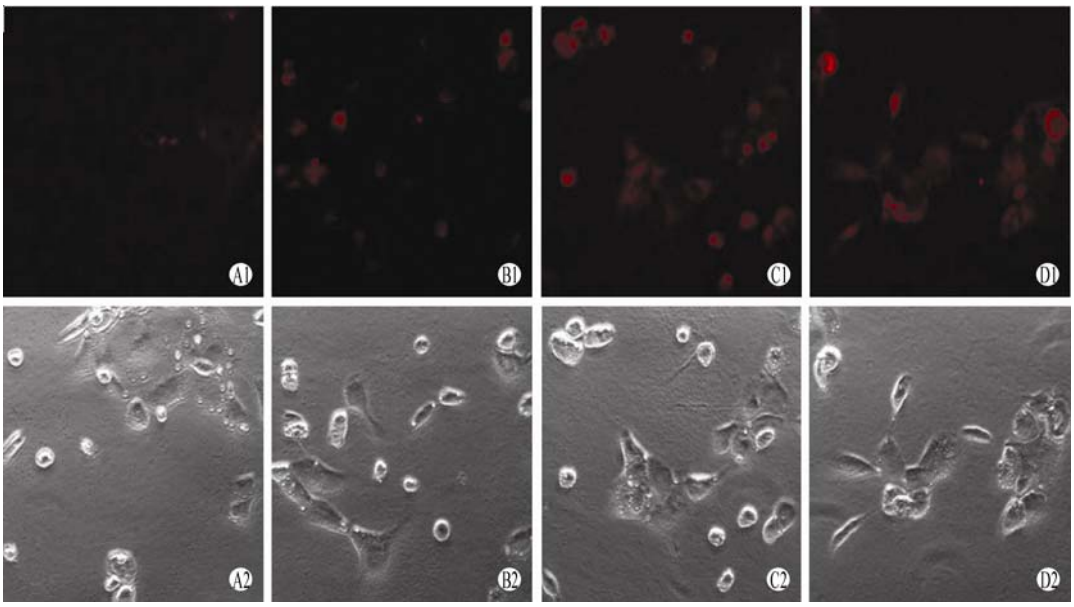

Fig. 1 CLSM images of PpIX fluorescence in HaCat cells
A1–D1: Fluorescent contours; A2–D2: primary appearances of the same cells
A: Blank; B: ALA; C: ALA plus EDTA; D: ALA plus DFO
Non-specific fluorescent dots were found among the black background of the cells for blank control (A1). The group of ALA plus DFO showed the strongest fluorescence (D1).

2.3 Apoptosis and Necrosis Ratios

After ALA-PDT, apoptotic and necrotic cells were detected in all the four groups. Their apoptosis ratios were 0.21%±0.02%, 1.46%±0.13%, 3.25%±0.32% and 5.87±0.49% respectively. The necrosis ratios were 0.08±0.02%, 0.10±0.02%, 9.24±0.75% and 14.50±0.91% in the same order (fig. 2). There were significant differences between the total cell death ratios of any two groups ($P<0.01$). Total death ratios in order from least to greatest were blank<ALA<ALA plus EDTA<ALA plus DFO.

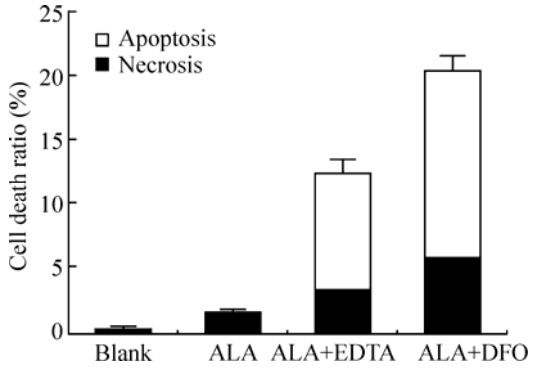

Fig. 2 The death ratios of HaCat cells after ALA-PDT
Compared to the cells of ALA group, the cells treated with ALA plus EDTA or DFO had higher ratios of death (P<0.01). A significant difference was found between the later two groups with addition of iron chelators (P<0.01).

2.4 Morphological Changes

Cells were morphologically assessed by using optical microscopy. The morphological changes were obvious in the groups of ALA alone or ALA in combination with iron chelators, including apoptotic changes in the nuclei, hypervacuolization of the cytoplasm, and destruction of the cytoplasmic membrane, which were conistent with previously reported findings[20, 21]. Moreover, the cytological damage seemed to be more severe in the cells treated with ALA plus DFO than that of any other groups (fig. 3).

Subcellular changes were further examined by using electron microscopy. The most significant damage was found in mitochondria, as reported before[19, 22]. Swelling occurred in the cellular organs such as mitochondria and endoplasmic reticula in most cells treated with ALA alone or ALA plus iron chelators. Some mitochondria had electron-dense materials inside in the cells treated with ALA plus EDTA or DFO. The ultrastructure of the cells in the control group showed no unchange.

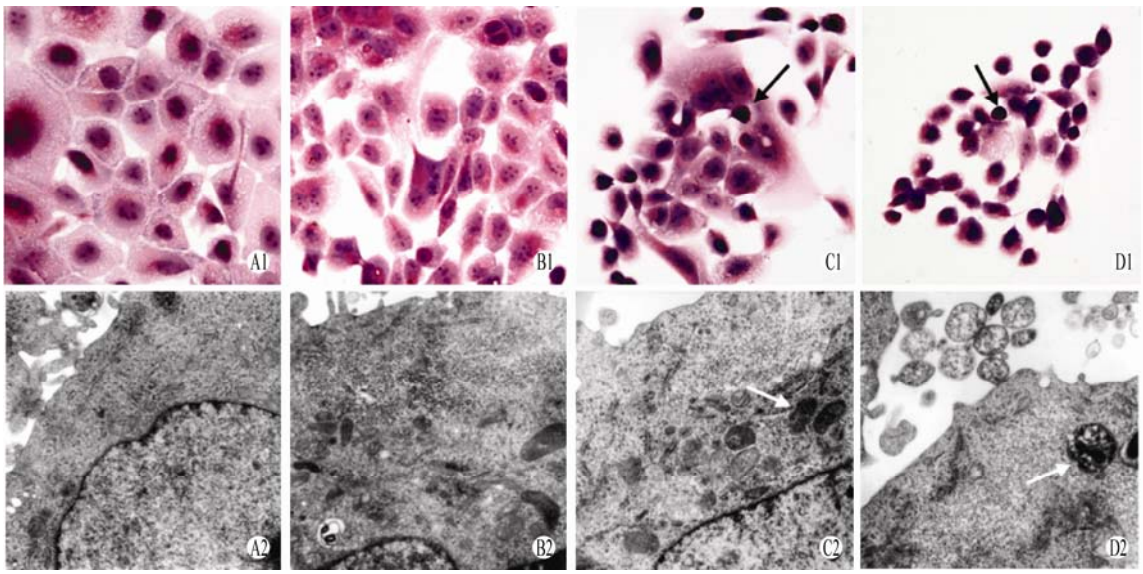

Fig. 3 Optical microscopic (A1–D1, ×400) and transmission electronic (A2–D2, ×5000) images of HaCat cells after PDT
A: Blank; B: ALA; C: ALA plus EDTA; D: ALA plus DFO
Cells detachment resulted in sparse appearances in the group of ALA alone or ALA plus EDTA/ DFO. Most cells showed smaller profiles, shorter pseudopods, shrunk nuclei or even karyopycnosis (black arrow), except for the cells as blank control. Ultrastructural analysis showed abnormal mitochondria and even dark material inside them (white arrow) in the cells treated with ALA alone or plus chelators.

3 DISCUSSION

The mechanism by which iron chelators work as PDT enhancers depends on the pathway of heme biosynthesis where PpⅨ is the immediate precursor of heme[23]. Therefore, it's very important to understand the effects of different iron chelators on PpⅨ production. It has been proved that $CaNa_2$-EDTA could increase the PpⅨ accumulation and photo-sensitisation in HEp-2 cells and the treatment depth in the cutaneous cancers[12]. However, the iron chelator DFO has even higher affinity and can bind iron with a large preference over other metal ions. It was found that the combined incubation of ALA and DFO could enhance the accumulation of PpⅨ on an epidermal equivalent of human skin[24]. Moreover, DFO itself generates PpⅨ in absence of ALA, and DFO in combination with light could photo-kill the cancer cells *in vitro*, at high concentration of 1.0 mmol/L or more[25].

The results in our study supported the previous work and showed that iron chelating agents were able to increase PpⅨ content and fluorescence in the presence of ALA. And, for the first time, we demonstrated the differences in enhancing PpⅨ accumulation mediated by EDTA or DFO. At the same concentration, ALA plus DFO produced higher cellular PpIX amount and more intense porphyrin fluorescence. The photodynamic reaction was detected by flow cytometric analysis of cells death, including apoptosis or necrosis. This study showed higher ratios of total cell death in the group with addition of DFO than that in the EDTA group. The mor-

phological damage of HaCat cells after PDT was in agreement with the results of PpⅨ detection and cell death analysis. The most severe destruction was found in HacCat cells treated with ALA plus DFO, including poor adherence, shrinkage, disappearance of pseudopods, karyopycnosis and so on. The mitochondria were traditionally considered the most probable PDT target. In our experiment, transmission electron microscopy exhibited that the mitochondria were found to be ultrastructurally abnormal in the cells treated with ALA alone or plus iron chelators. The relationship between subcellular destruction and exgenous factors applied on HaCat cells was similar to that observed under optical microscope. In the cells treated with ALA plus DFO, some mitochondria became darker and even had electron-dense materials inside. The organelles could presumably be converted into secondary lysosomes, abundant at later stage[22]. Also it was of interest that the destruction of some mitochondria could occur with others left undamaged. This might be due to the non-uniform photosensitization inside cytoplasm. DFO was shown to be significantly superior to EDTA in enhancing PpⅨ accumulation and consequent photodynamic reactions induced by ALA.

Two main mechanisms might be involved in the superiority of DFO to EDTA as enhancer in ALA-PDT. One is that DFO can selectively block heme biosynthesis as a more specific iron chelator. The second may be DFO's potential of inducing minimal PpⅨ without ALA. These possibilities require further study.

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