The oxidation Stress, mitochondrial-pathway Apoptosis and the antagonism effects of chrysophanol in SH-SY5Y Cells via DTPP induced photodynamic therapy

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Research Article

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Abstract

To investigate the susceptibility of SH-SY5Y cells to DTPP-based photodynamic therapy(PDT) and the interaction with chrysophanol. Cells were treated with increasing concentrations of DTPP between 2.5 to 20 μg·mL^{-1} and the DTPP -induced killing effects in tumor cells was quantified determined. PDT photocytotoxicity was measured by exposing the cells to a 630-nm irradiation for 1.2–9.6 J/cm^2. The photodynamic stress was assessed by superoxide dismutase, malondialdehyde, and total antioxidative capacity assays. The apoptosis pathway of SH-SY5Y cells after PDT was studied by JC-1 and caspase-9/Caspase-3 determination. The cell apoptosis cells stained with Annexin-V-FITC and propidium iodide were detected through flow cytometry. In the PDT process, a dose-dependent killing effect of DTPP concentrations and irradiation doses were found. The viability of SH-SY5Y cells treated with 10 μg·mL^{-1} DTPP rapidly decreased to 55%. Cells apoptosis is mediated by a mitochondrial -pathway with a total apoptosis rate of 33.8% with 10 μg·mL^{-1} of DTPP after illumination at 2.4 J/cm^2. Thus, our results demonstrated that an oxidation stress was produced by ROS in PDT and a non-reversible cells oxidative damage appeared via cells modulating the oxidative stress balance during the PDT response. Chr had an inhibitor effect on the capture of ROS and the destruction of cell membrane induced by PDT. In conclusion, neuroblastoma cell line SH-SY5Y cells were susceptible to DTPP-PDT, resulting in a mitochondrial apoptosis pathway. DTPP is a potential type I photosensitizers to revitalize photodynamic oncotherapy in SH-SY5Y cells. There is an antagonism effect of chrysophanol on PDT in SH-SY5Y neuroblastoma cells.

Introduction

Neuroblastoma represents a highly malignant and rare type of sympathetic solid tumor. It accounts for approximately 15% of all malignant tumors in pediatric and is associated with extremely low survival rates [1]. Multi-modality therapeutic interventions include milder chemotherapy and surgery followed by ASCT (autologous stem cell transplantation) and immunotherapy. However, survival rates remain very low. There is a crucial need for developing new and more effective compounds that are able to inhibit tumor growth and development for patients with NB. It was reported recently that neuroblastoma cell lines (GOTO, NB9, IMR32, and NB1) were susceptible to 5-ALA-PDT [2].

Photodynamic therapy (PDT) was discovered for a malignancy treatment by Prof. Hermann Von Tappeiner and groups over a century ago [3, 4]. PDT has been proven to be an effective treatment of various cancers and non-cancerous diseases such as age-related macular degeneration, bacterial infections and atherosclerosis. PDT is based on a photochemical reaction between photosensitizers (PS) and light. It is a two-stage procedure consisting of the tissue administration of a PS, followed by exposure to light, usually visible light with a specific wavelength (600–900 nm). This results in the formation of reactive oxide species (ROS) that causes phototoxic damage and disturbs proliferations of cancer cells or tissues and to kill target cells [5]. PDT is considered to be an effective method in clinical practice for tumor disease and tumor vascular supply [6]. In addition, fluorescence emitted by PS has

been used to guide tumor removal. A recent study reported the use of a combination of 5-ALA and Photofrin® for repetitive PDT after fluorescence-guided resection [7] had a improved survival rate without any added risks. Novel methods to improve selectivity and potency and new photosensitive molecules are constantly being discovered by researchers [8]. Second-generation PSs such as hematoporphyrin derivatives, have been widely used in clinical practice of cancer therapy, but there still exist some disadvantages, especially skin phototoxicity [9]. Our previous study has showed that DTPP has no cytotoxicity in any cell line and DTPP-induced PDT had high killing efficiency on LA795 cells [10, 11].

As the increasing of proportion of the elderly in the total population, much attention has been focused on many kinds of health care products, as “natural antioxidants”, that possess generally specific medically and hygienically beneficial activities. Among the wide range of natural antioxidants, anti-aging chrysophanol (chr) is abundant in our daily food and medicine. Chr is the main active ingredient of rhubarb rhi Radix et Rhizoma., Polygoni multiflorum Multiflori Radix. and Polygoni Cuspidati Rhizoma et Radix. Previous studies have demonstrated that chr has anti-proliferative effects on a variety of cancer cells, including human lung cancer cell, breast cancer cell, human malignant meningioma and etc [12]. In addition, chr has antioxidant activity, which can reduce the formation of intracellular ROS and cause losing of mitochondrial membrane potential, down-regulating the Bcl-2/Bax ratio and activate the apoptosis molecular of Caspase-3, 9 [12–15]. Many hypotheses have been suggested that the anti-inflammatory, neuro-protection, improvement of learning and cognitive dysfunction, protection of myocardium effects of chr may be related to its antioxidant activity and some has been evaluated by several studies [16]. Furthermore, chr has been proved to be able to scavenge superoxide anions, hydroxyl radical and hydrogen peroxide *in vitro* by chemical methods and also produce singlet oxygen and hydroxyl radicals when irradiated with a visible light at wavelength greater than 430 nm [17]. The underlying mechanisms of chemotherapeutic agents, ionizing radiation, and PDT on tumor cells are ROS induced tissue injury [18], and these ROS may be neutralized by an antioxidant defense system in the body, including CAT, SOD, and glutathione peroxidase (GSH), as well as other exogenous substances with antioxidant properties. Due to some controversial findings, the application of antioxidants in PDT is a controversial topic. On the one hand, some study suggested that antioxidants can protect healthy cells and tissues from free radicals damage without affecting any PDT treatment effectiveness; but on the other hand, some findings suggested that antioxidants reduced the PDT effectiveness of cancer treatment against malignant or tumor cells. Reports such as vitamin C inhibits the oxidation treatment of 5-ALA induced PDT of cancer cells [19]; T-butyl-4-hydroxyanisole (BHA) can improve the efficiency of PDT[20]; antioxidant has no significant improved effect on HL-60 cells as hypericin-mediated PDT [21]. At present, many cancer patients prefer to combine some traditional Chinese medicine as conservative treatment or adjuvant therapy. In general, Rhubarbs are regarded to be safe and common in proprietary Chinese medicines. It should be noticed that antioxidant may play a role in reducing the effectiveness of tumor therapy on tumor cells, although it reduces the damage on healthy body from cancer treatments.

In the study, the validity of DTPP- based PDT on SH-SY5Y cell lines, the inhibitor effects of chr on DTPP-PDT and the mechanism were investigated. For the study, new windows are open in the treatment of neuroblastoma field so-call photodynamic therapy that incorporates a nonpoisonous photosensitizer-

DTPP; and the data for the study and utilization of xanthraquinone compounds was accumulated in the clinical treatment of PDT.

Materials And Methods

Compounds and reagents

MTT (3-(4, 5)-Dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazo liumromide) assay kits (BOSTER biological technology, China), DMSO, Dulbecco's modified Eagle's F12 (DMEM-F12) cellular medium, fetal bovine serum (FBS) and penicillin and streptomycin (Hyclone, Germany), DMSO (Sangon biotech, China), ROS Molecular Probes, MDA Assay Kit , T-AOC, SOD Assay Kit and Caspase 3, 9 Assay Kit (Beyotime Institute of Biotechnology, China). The photosensitizing drug DTPP was provided and prepared according to the paper [9].

Cell lines

SH-SY5Y human neuroblastoma cells were purchased from the Institute of of Basic medicine Cell Center of Peking Union Medical College in China. The cells were cultured in DME F12 medium supplemented with 10% FBS, and withpenicillin 100 U/mL - streptomycin100 $\mu g \cdot mL^{-1}$.

Cell viability Assay

Briefly, cells were cultured in a 96-well flat-bottom culture plate with cell densities of 3×10^4 cells/well. After 24-h culture at 37 °C in a humidified 5% CO_2 incubator, the MTT solution ($5mg \cdot mL^{-1}$, 10 µL) was added to each sample, and incubated for another 4 h at 37 °C. The absorption densty (A) was measured at 570 nm using a UV-visible spectrophotometer (Thermo). The cell survival rate was calculated as follows:

$$Cell\ survival\ rate\ (\%) = \frac{A_{test} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

where A_{test} and $A_{control}$ are the absorption value at 570 nm of the cells in test group and cells in control group , respectively. A_{blank} is the absorption value at 570 nm of a blank cell plate.

Mitochondrion localization of DTPP

SH-SY5Y cells were seeded in a confocal laser scanning microscope dish at a concentration of 1×10^5 cells/well and left overnight at 37°. The following day, cells were added with 1 mL of DMEM-F12 medium containing 10 $\mu g \cdot mL^{-1}$ of DTPP and co-cultured for 24 h in the dark, then washed with cold PBS carefully and labeled with 1 mL Mito Tracker green and Hoechst 33342 fluorescent probes. Cells were observed using a confocal laser scanning microscope (Zeiss, Jena, Germany). Excitation wavelength was at 488, 350, and 630 nm for Mito Tracker, Hoechst 33342, and DTPP, respectively.

Killing effects and reaction type analysis of DTPP- PDT in SH-SY5Y cells

Light source, PS type and oxygen are the three factors affecting PDT. In the present experiment, dark toxicity of PS, PS concentrations, energy density and antioxidants were studied to influence the activity of PDT. DTPP was dissolved in serum free medium to get 10 $\mu g \cdot mL^{-1}$ solution and filtered to remove bacteria. SH-SY5Y cells were seeded in a 96-well culture plate with cell densities of 3×10^4 cells/well at 37℃ and with 5% CO_2 in the incubator. After the cells had reached 80% confluence, the medium was replaced with 100 μL of serum free medium solution with a concentration of 2.5, 5, 7.5, 10, 15, 20 $\mu g \cdot mL^{-1}$ of DTPP in the dark, respectively. After the cells were incubated for 24 hours, they were washed three times with cold PBS and expose to cell medium for another 2 h at 37℃ and with 5% CO_2 in the incubator. To study the toxic of DTPP, cells viability was determined directivity without further irradiation. To study the relationship of different concentrations of DTPP with PDT efficiencies, cells were irradiated with a 630-nm semiconductor laser with a power density of 20 mW/cm^2 and total energy density of 4.8 J/cm^2. To study the relationship of different Energy densities with PDT efficiencies, cells were irradiated for energy density of 1.2–9.6 J/cm^2 (1 to 8 min) with a power density of 20 mW/cm^2. The antioxidants sodium azide (SA, NaN_3) is single-oxygen quencher and D-mannitol (DM) is hydroxyl radical scavenger, they are used to study the reaction type (type I or type II) of PDT, firstly, cells were pre-exposed to 10 mM/L of sodium azide (SA) or 40 mM/L of D-mannitol (DM) for 2h, then the medium was replaced with in 1 mL medium containing 10 $\mu g \cdot mL^{-1}$ DTPP and co-incubation for 24 h as above. Cells were irradiated with an energy density of 0, 1.2, 2.4, 4.8 or 7.2 J/cm^2. After PDT, they were cultured for 24 h. All the previous cells viability was evaluated by MTT assay and cells without treatment as control. Every experiment was repeated four times.

Determination of ROS output by PDT in SH-SY5Y cells

A ROS Assay Kit fluorescent probe DCFH-DA is used to detect ROS. DCFH-DA does not have fluoresce itself and can pass through the cell membrane freely. After entering the cell, DCFH can be hydrolyzed by the cell esterase to generate DCFH. DCFH will not penetrate cell membranes, making it easy for probes to be loaded into cells. Intracellular ROS can oxidize non-fluorescent DCFH to produce fluorescent DCF. The level of intracellular ROS can be determined by measuring the fluorescence of DCF. SH-SY5Y cells were seeded in a 6-well plate with a density of 1.2×10^6/well, they were added with 10, 15 or 20 $\mu g \cdot mL^{-1}$ DTPP serum-free medium, respectively and cocultured for 24 hours. DCFH-DA was diluted The cell culture medium was replaced with 100 μL of 10 μmol/L.DCFH-DA to fully cover the cells and incubate for 20 minutes in a 37 °C cell incubator. The cells were washed three times with serum-free cell culture medium to fully remove the DCFH-DA not enter the cells. and Then cells were treated by PDT for 2.4 $J/cm^2$and the fluorescent value of DCF was determined once per 10 min from 60 to 120 min by an ultraviolet visible spectrophotometer (Therm) with fluorescence excitation wavelength of 488 nm and emission wavelength of 525 nm to study the dynamic changes of ROS along with time after PDT. Every experiment was repeated four times.

The oxidation and anti-oxidation balance changing in cells treated by PDT with DTPP

PDT causes oxidative damage to cells, and the molecular/biological structure of cells during death or apoptosis is altered by oxidative stress induced by PDT. The photo-oxidative changes by PDT may produce lipid peroxidation of tumor cells. Cells oxidative damage was induced via modulating the oxidative stress balance. The oxidation and anti-oxidation effects of cells after PDT treatment was assessed by the changes of intracellar malondialdehyde (MDA), Total antioxidative capacity (T-AOC) and SOD, which were tested by MDA. T-AOC and SOD assays, respectively. MDA is a major aldehydic metabolite of lipid peroxidation and has been used to reflect lipid peroxidation [22]. SH-SY5Y cells were seeded in a 96-well cell culture plate at a density of 3×10^4 per/well and divided into groups as following: Control group, Laser group (20 mW/cm^2, 2.4 J/cm^2), DTPP group and PDT damage groups (10 μg·mL^{-1}) (n=4). Cells had no intervention were set as control. PDT damage group: cells were cultured with 10.0 μg·mL^{-1} DTPP for 24 hours and then irradiated with a 630 nm-laser for total energy density of 2.4 J/cm^2 with a power density of 20 mW/cm^2. One hour later, the activities of MDA, T-AOC and SOD in SH-SY5Y cells were assessed using the corresponding commercial kits according to the manufacturer's protocols.

Mitochondrial membrane potential (MMP)

MMP changing with energy density

Mitochondrial membrane potential (MMP) was measured by JC-1, which can congregate selectively in the lower MMP cells and is green fluorescence but changes to red fluorescence when spread in normal cells. SH-SY5Y cells were cultured in a 24-well plate at a density of 3×10^5 cells per well and they were treated with a dose of 10 μg·mL^{-1} DTPP for 24h in dark. They were irradiation at 630 nm for energy density of 1.2, 2.4 and 4.8 J/cm^2, respectively (n=4). After 0.5 h, the cells were washed with PBS for three times and loaded with JC-1 probe as the standard program, the fluorescence of JC-1 in cells was observed by fluorescence microscope. JC-1 monomer: excitation wavelength was at 590 nm, emission wavelength was at 530nm; JC-1 polymer: Excitation wavelength was at 525 nm, Emission wavelength was at 590 nm.

MMP changing with time

SH-SY5Y cells were cultured in a 24-well plate with 3×10^4 cells in each well. After adherence, 10 μg·mL^{-1} DTPP was added avoiding light until the cells were adhered to the wall. Cells were treated with a dose of 10 μg·mL^{-1} DTPP for 24h in dark and irradiation at 630 nm for energy density of 2.4 J/cm^2 (n=4), the change of MMP was determined and compared by the ratio of green fluorescence from monomer JC-1 and red fluorescence from mitochondria at 0.5, 1, 2, 4 and 6 h after PDT (n=4), respectively. Ratio = green fluorescence/red fluorescence, when it is bigger, the MMP is lower.

Caspase- 3/ Caspase- 9

Caspase- 3 plays a critical role in the process of nuclear apoptosis, including chromatin condensention and DNA fragmentation. After cytochrome C is released from mitochondria, Caspase-9 can form a complex with cytochrome C and Apaf1 and be activated simultaneously. Activated Caspase-9 can further activate Caspase-3, thereby promoting the subsequent apoptosis signal; it is the most important enzyme with cell apoptosis. The experiment next consisted of five groups: Control group, Laser group (20 mW/cm^2, 2.4 J/cm^2), PDT damage group. The control group had no intervention. PDT damage group: cells were treated with 10.0 mg/L DTPP for 24 hours and then exposed to a energy density of 2.4J/cm^2 with a power density of 20 mW/cm^2. Levels of Caspase-3/ Caspase-9 of treated cells were tested by a assay of Caspase-3 or 9 follow the instructions at 4^{th} h、6^{th} h or 24^{th} h later, respectively (n=4).

Measurement of cell apoptosis ratio induced by DTPP-PDT

An Annexin V-FITC and pidium iodide (PI) double fluorescence staining assay was used for flow cytometry analysis. This method was based on Annexin- V can combine with Phosphatidyl serine on the cell membrane of apoptosis cells and PI can enter membrane damaged cells through cell membrane. SH-SY5Y cells were seeded in a 24-well cell plate with 3×10^4 cells in each well and cultured. Cells were co-cultured with 10 $\mu g \cdot mL^{-1}$ of DTPP for 24 h before illumination for a energy density of 1.2、4.8 or 7.2 J/cm^2, respectively with a power density of 20 mW/cm^2 (n=3). SH-SY5Y cells were collected and loaded with Annexin V-FITC and PI, successively 2 h after PDT to determine the apoptosis cells induced by DTPP-PDT. With cells without treatment, Laser only, DTPP only groups as controls. The cells were detected through the flow cytometry (BD) with an excitation wavelength of 488 nm.

Effect of chr on cell viability

Logarithmic growth phase cells were collected and seeded into a 96-well cell plate at a density of 1×10^5 cells per well. For the dose-time dependent experiment, they were treated with chr at a concentration of 6.25, 12.5, 25, 50, 100 μmol/mL, respectively; and for 12, 24, 48 h respectively, according to the results of the pre-experiment.

Effect of chr on ROS output in PDT

ROS output in SH-SY5Y cells was studied after using PDT treatment. SH-SY5Y cells were cultured in a 96-well plate (1×10^5 cells/well) and divied into five groups: Control group, PDT damage group (10 $\mu g \cdot mL^{-1}$ DTPP, 2.4 J/cm^2), SA+PDT, SOD+PDT, and Chr+ PDT group (6.25, 12.5, 25, 50, 100 $\mu g \cdot mL^{-1}$) (n=4). SA (50 mM/L), SOD (125 U/mL), or chr (6.25–100 μmol/mL) was pre-added to the cell reaction medium, respectively and incubated for 30 min before treated with 10.0 μg/mL DTPP for 24h and illumination for 2.4 J/cm^2. Half hour later, cells were washed and labeled with ROS molecular probe in situ at room temperature for 20 minutes and then incubated at 37°C for another 0.5 h. Finally, Cells were washed with medium three times and loaded with ROS probes. Finnnaly, the ROS was analyzed immediately by an ultraviolet visible spectrophotometer (Thermo) as previous experiment. Cells without treatment were as control. Cells with SA and SOD treatment were positive controls.

Effect of chr on cell survival and apoptosis rate in PDT

SH-SY5Y cells were cultivated in a 96-well plate (1×10^5 cells per well) and divided into six groups: Control group, light group, DTPP group, SOD+PDT, PDT damage group (10 $\mu g\cdot mL^{-1}$ DTPP, 2.4 J/cm^2), and chr+DTPP group (10, 15, 20 $\mu g\cdot mL^{-1}$). The control group had no intervention. Cells were pre-treated with 125 U/mL of SOD for 30 min before PDT was the positive group. In PDT damage group, cells were incubated with 10.0 $\mu g\cdot mL^{-1}$ of DTPP for 24 h and irradiated by a semi- conductor laser system for an energy density of 2.4 J/cm^2 with a power density of 20 mW/cm^2. In chr treated group, cells were incubated with 5, 10, or 20 μmol/mL chr, respectively at 37°C, 6 h later with 10.0 μg/mL DTPP for 24h before elimination by a 630 nm semi-conductor laser system. Cells were cultured for another 24 h and cell viability was tested by MTT assay and every sample has four apertures. Cells without treatment were as control, with Laser only and DTPP only treatment as negative control.

For cell apoptosis experiment, SH-SY5Y cells were cultivated in a 24-well plate with 3×10^4 cells in each well and co-cultured with 10 $\mu g\cdot mL^{-1}$ of DTPP for 24 h in the dark. The experiment groups and treatment method were the same as above. After PDT treatment, cells were cultured for another 2 h at 37°C, then collected and stained with FITC Annexin V and PI for FACS analysis. Each sample has three apertures.

Effect of chr on cell membrane integrity

PI was used to detect the effect of chr on cell membrane integrity after DTPP-PDT treatment. PI is a nuclear staining reagent that stains DNA and emits red fluorescence when embedded in double-stranded DNA. PI does not cross living cell membranes, but can cross damaged cell membranes to stain the nucleus. Fluorescence intensity of PI was measured by a Flow cytometry, which was a sign of membrane integrity and cell viability. SH-SY5Y cells were seeded into a 24-well culture plate, and the experiment was divided into six groups: Control group, light group, DTPP group, PDT damage group (10 $\mu g\cdot mL^{-1}$ DTPP, 2.4 J/cm^2), and chr+DTPP group (10, 15, 20 $\mu g\cdot mL^{-1}$). The control group did not receive any intervention. In PDT damage group, cells were incubated with 10 $\mu g\cdot mL^{-1}$ of DTPP for 24 h, and then irradiation was performed with an energy density of 2.4 J/cm^2. In the chr+PDT group, cells were pre-treated with 100 μL of chr solution at a concentration of 6.25, 12.5 and 25 μmol/mL, respectively for 6 h before DTPP addition and irradiation. Cells were cultured for another 4 h at 37°C after PDT. After the cells were digested, cells suspensions were centrifuged at 2000 r/min for 3 min and immobilized by 70% cold alcohol for 30 min, and then resuspended in 1mL PBS including 10 μM of PI at room temperature for 30 min, then reaction mixture was removed and washed by 180 μL PBS three times, the intensity of the red fluorescence was measured and analyzed by the Flow cytometry. The experiment was repeated 4 times.

Statistical Analysis

SPSS 20.0 software and one-way ANOVA were used to analyze the data. The differences between each group were analyzed by one-way ANOVA. Probability values less than 0.05 were considered statistically significant.

Results

Mitochondrion localization of DTPP

The mitochondrion localization of DTPP in SH-SY5Y cells was observed by a laser scanning microscopy (Fig. 1). Red fluorescence, green fluorescence was from the mitochondrion; yellow fluorescence was merged by red and green fluorescence. The result revealed that DTPP was located in mitochondria without localization in cell nucleus.

Killing effects analysis of DTPP-PDT in SH-SY5Y cells

When SH-SY5Y cells were co-cultured with a concentration range 2.5–20 μg·mL^{-1} of DTPP in dark for 24 h, the cell viability was higher than 98% (Fig. 2a). The data showed that dark toxicity of DTPP on SH-SY5Y cells was very low.With the same energy density of laser and the same power density, the results showed DTPP had a concentration-dependent killing effects on induced PDT in SH-SY5Y cells. The killing effects of DTPP-mediated PDT were significant when the DTPP concentration was higher than 5 μg·mL^{-1}; the survival rates of SH-SY5Y cells decreased with an increase of DTPP concentration in the range of 7.5 μg·mL^{-1}to 20 μg·mL^{-1}, and the value of survival rate decreased from 90.4% for the 7.5 μg·mL^{-1}-group to 74.7% for the 20 μg·mL^{-1}-group. From the data of Fig. 2b, given the same power density and the same dosage of 10 μg·mL^{-1} DTPP , the energy density-dependent killing effect was seen with energy density at a range of 1.2–9.6 J/cm^2; and the cell survival rates decreased from 98.6% to 55.0% .

SA and DM are used to determine the reaction type of PDT. The single-oxygen quencher of SA blocks type I reaction of PDT and hydroxyl radical scavenger of DM blocks type II reaction occurred during the process of PDT. The survival rates of PDT cells with an antioxidant were higher than that without any antioxidant (Fig. 2c). When the energy density increased from 1.2 to 3.6 J/cm^2, the survival rates increased by 10.01±2.31% and 15.20±3.21% after pre-blocked by SA or DM compared with PDT group. But when illuminated for lower than 0.6 J/cm^2 or higher than 7.2 J/cm^2, addition of SA or DM has not increased the survival rates. Therefore, SA had a significant antioxidant capacity than DM. Thus, both type I and type II reactions occurred simultaneously during the process of DTPP-PDT on SH-SY5Y cells, with type I as a predominating reaction.

Production of ROS and stress response

DTPP-PDT can lead to ROS appearing immediately in SH-SY5Y cells at 60 min (Fig 3a), and it get to a maximum value of 1.3 times of the control group (data no show). The output of ROS increased with the increase of DTPP concentrations at 10, 15 and 20 μg·mL^{-1}. The ROS output got the max value at 90 min after PDT and it sustained to 120 min. MDA, SOD and T-AOC in SH-SY5Y cells were determined at 1 h post PDT with DTPP of 2.5, 5, and 10 μg·mL^{-1} respectively (Fig 3b-d). SOD in cells after PDT illumination at 2.4 J/cm^2 were decreased (P<0.01) and MDA and T-AOC was increased (P < 0.01)

compared with the control group. However, the decreasing of SOD may be related to the consumption caused by its resistance to ROS, T-AOC increased for stress response, and MDA accumulation resulted from cell mortality damage.

MMP disappeared and Caspase-3 / Caspase-9 activity increased

Mitochondrion plays a central role in the process of apoptosis. In this experiment, cell apoptosis induced by DTPP-base PDT was also through mitochondria process. With the increase of energy density, the green fluorescence increased and the red fluorescence decreased, which indicated that the cells MMP decreased (Fig 4a). MMP decreased immediately at 30 min after PDT in SH-SY5Y cells (Fig 4b). From 0.5h to 1h after PDT, the MMP (△ ψm) decreased most obviously, the MMP got the lowest value at 1 h, the membrane potential was recovered a little at 2h, and then the membrane potential kept decreasing. With the disappearance of MMP, intracellular Caspase-3/9 increased (Fig 4c), these phenomena occurred simultaneously with the increase of ROS. After 2.4 J/cm^2 of PDT, the activity of Caspase-3 was significantly increased at 4h then decreased at 6 h and 12 h respectively. For the activity changes of Caspase-9, it gets highest at 6h, then decreased at 12h. PDT activated Caspase-3 firstly, then Caspase-3 triggered down Caspase-9 to an active state. The results showed that the apoptosis of SH-SY5Y cells was mainly induced by DTPP-PDT via a Mitochondrial/Caspase-9/Caspase-3 pathway.

Apoptosis ratio

Annexin V-FITC and PI double staining was employed to confirm cell apoptosis induced by DTPP-based PDT, FACS was performed. DTPP at a concentration of 10 μg·mL^{-1} was added and co-cultured for 24 h before illumination with a energy density of 2.4、4.8 or 7.2 J/cm^2, respectively. Annexin V^-/PI^-, V^+/PI^-, V^+/PI^+, V^-/PI^+cells accounted for viable, early apoptotic, late apoptotic and necrotic cells respectively. The total apoptosis rates, including early and late apoptotic were increased approximately to 13.1%, 17.0%, and 33.8% comparing with control group after illumination at 2.4, 4.8 and 7.2 J/cm^2, respectively. SH-SY5Y cells were treated for 7.2 J/cm^2 accounted for a 16.2% early apoptosis and 17.6% late apoptosis (Fig. 5).

Effect of chr on cell viability

SH-SY5Y cells were exposed to a concentration of 6.25–100 μmol/mL of chr, respectively. The cell cytotoxicitiy of chr was shown in Fig. 6a.When treated with 6.25-12.5 μmol/mL of chr for 12- 24 h, no toxicity was observed on SH-SY5Y cells in comparison with untreated control cells. However, the survival rates of cells decreased with the increase of chr in the range of 25-100 μmol/ml, with cell viability rates decreased from 84.8% to 72.0% when treated for 24 h. When incubation for 48 h, cell survival rates were reduced from 96.4% to 50.0% with increased chr concentrations from 12.5 to 100 μmol/mL.

Effect of chr on ROS output in PDT

The ROS output after pre-treated with chr and PDT was shown in Fig. 6b, the ROS output in the PDT group was higher than any other groups. When illuminated with 2.4 J/cm^2, the ROS outputs decreased significantly ($P < 0.05$) from 199.50±13 (PDT damage group) to 162.25±13 and 126.75±12 after pre-adding of SA and SOD, respectively. However, the ROS outputs decreased to 132.20±14 and 126.05±10 after adding 6.25 and 12.5 μmol/mL of chr, respectively. The ROS output for all chr antioxidant treated groups was significantly lower compared with PDT group ($P<0.05$), with a maximum ROS decreased rate of 54.0% when 50 μmol/mL of chr was added. However, the ROS output decreasing rate was only 8.35% when 100 μmol/mL of chr was added. The reason may be that 100 μmol/mL of chr destroyed cell membranes and large amounts of ROS fluorescent probes might enter cells when loading with ROS probes. Chr was an antagonistic substance /inhibitor of PDT indicating that chr was fighting against the oxidative damage caused by PDT with anti-type I reaction playing a major role.

Effect of chr on cell survival and apoptosis rate in PDT

The effect of chr on cell activity and apoptosis were shown in Fig. 6b.Cell survival rate was significantly decreased to 48.9±7.48% after PDT treatment. However, when SH-SY5Y cells were incubated with chr at a concentration of 6.25, 12, 25 μmol/mL, respectively for 6 h at 37°C before 2.4 J/cm^{2}PDT irradiation, a dose-dependent increase in cell survival rate were observed ($P<0.05$) compared with the PDT group. The apoptosis rates decreased to approximately 10% for the SOD positive control group and to 25.3%, 14.0%, and 9.1% for the cells chr of 6.25, 12, 25 μmol/mL was pre-treated, respectively. The early apoptosis rates decreased after PDT, but the late apoptosis rates were not decreased significantly for chr of 6.25, 12, 25 μmol/mL.

Effect of ch on cell membrane integrity

The effect of chr on cell membrane was shown in Fig. 7, compared with PDT group, the fluorescence intensity were decreased to various degrees after pre-treated with 6.25, 12.5 and 25 μmol/mLof chr, suggesting decrease of incompleted cell numbers. Chr has a certain antagonistic effect on the destruction of cell membrane by PDT and the cell membrane may be one of the targets of chr. It is speculated that chr itself is also responsible for the destruction of cells at the concentration of 100 μmol/mL.

Discussion

PDT has an active effect in cure of disease and solid cancers; the character of PSs, including chemical structure, charge and relative hydrophilicity or hydrophobicity is an important factors to determine the effect of PDT [23, 24]. With the improvement of PDT, it may be a new method to cure neuroblastoma. In this study, DTPP-mediated PDT has a PS- or energy density-dependent anti-tumor activity on SH-SY5Y cells *in vitro*. DTPP is a second-generation PS, with a character of a low toxicity and high efficiency; with a dosage of 5 μg·mL^{-1}as lowest effective concentration to activate PDT (Fig. 2). The power density directly related to oxygen consumption has an important effect on the cytotoxicity of PDT. It has been

reported that high power density reduces or even suppresses tumor growth, while low power density promotes tumor growth [25]. There is a critical level for power density, which can block the effect of PDT for an oxygen vacuum. Our present results demonstrated that the cell survival rates of SH-SY5Y cells decreased after PDT with the energy density increasing at the range of 1.2–7.2 J/cm^2. However, the killing effect at energy density of 7.2 J/cm^2 was similar to that at 9.6 J/cm^2, probably because the maximal killing of 50% of tumor cells occurred at 10 μg·mL^{-1} DTPP –based PDT with 7.2 J/cm^2. The PDT will get E_{max} when intracellular oxygen is running out. A PS is excited from the ground state (^{1}PS) to the excited state (^{1}PS*, ^{3}PS*) by the appropriate wavelength excitation light. ^{3}PS* leads to the formation of free radicals such as radicals and superoxide, which is the type I reaction induced by PDT; ^{3}PS* can transfer energy to molecular oxygen directly to generate ROS and 1^*O_2, which is the type II reaction. Although ROS can serve as a signaling molecule to activate protective signaling pathways, elevation of ROS (e.g., •O^{2-}, •OH) caused oxidative stress [6, 7]. Multiple signal cascades are activated in cancer cells exposed to photodynamic stress and transformed into adaptive or cell death responses according to the sub-cellular localization of the reactive ROS [22]. Furthermore, the targets of free-radical mediated damage are proteins, lipids and DNA [26]. Physiological homeostasis is essential for survival, but ROS produces an unbalance to oxidation stress. In the process DTPP-PDT, we found that both type I and type II reactions were involved, and type I reaction played a major role. Type II reactions were found in oxygen-rich environments and type I reactions were found in anoxic environments. In addition, emerging research suggests that type I PDT is superior to type II PDT in cancer treatment because it reduces the dependence on oxygen [22]. This study shows that neuroblastoma cell line SH-SY5Y cells were susceptible to DTPP-PDT, and DTPP is a potential type I PSs to revitalize photodynamic oncotherapy in SH-SY5Y cells (Fig. 2).

Oxidative stress is a negative effect produced by free radicals in the body and is considered to be an important factor leading to apoptosis, cancer, neurodegeneration, mitochondria and mitochondrial dysfunction play an important role in these diseases [28]. Oxidative stress might be inhibited by antioxidant enzyme SOD intercellular or extracellular to catalyze into less toxic hydrogen peroxide and oxygen [27]. In the study, PDT produces excessive ROS through oxidative damage to kill tumor cells, and SOD alleviated oxidative stress triggered by DTPP-PDT and alleviated the cell toxicity under PDT (Fig. 3b, Fig. 6b). In the process, cell lipid membranes also undergo oxidative damage. DTPP-base PDT produced cell damage by increasing ROS, decreasing SOD and increases the levels of T-AOC and MDA in cells (Fig. 3). Thus, our results demonstrated that an oxidation stress was produced by ROS in PDT and a non-reversible cells oxidative damage appeared via cells modulating the oxidative stress balance during the PDT response. DTPP-based PDT initially results in MMP disappeared and the release of cytochrome C from mitochondria into the cytolymph, and subsequently activates Caspase-9 and Caspases-3 to initiate a mitochondrial apoptosis pathway (Fig. 4). The mechanisms might involved of antioxidation inside and outside the cell and subcellular components such as mitochondria, lysosomes, rough endoplasmic reticulum, and cell nucleus may also be altered after PDT and therefore require further study.

Chr has multiple pharmacological effects, including neuroprotective effects and antioxidant properties, and is popular in diet and medicine [31]. Furthermore, the antitumor effects of chr on A549, H738, and

HUVEC cells were reversed by the ROS inhibitor NAC [31]. Chr inhibited proliferation and promoted apoptosis in colorectal cancer cells by activating the intrinsic mitochondrial apoptotic pathway by targeting decorin [30]. Chr has both a significant antioxidant activity and an anticancer effect; the interaction between chr and PDT has not been reported. In this study, the interaction of chr with DTPP-mediated PDT in SH-SY5Y neuroblastoma cells were evaluated. Our results demonstrated that 12.5 μmol/mL chr has no toxic on SH-SY5Y neuroblastoma cells, and cell proliferation was decreased with chr concentrations between 25 μmol/mL to 100 μmol/mL after incubated for 24 and 48 h (Fig. 3a) that was consisted with reports that chr can inhibit the growth of many types of cancers [29, 30]. Our results demonstrated chr showed strong antagonistic actions to PDT, or exhibited prevent effects against cell killing effect of PDT by scavenging ROS (Fig. 6b, c). However, chr at concentration of 6.26-50 μmol/mL (Fig. 6b) can decrease the ROS (e.g., $\bullet O^{2-}$, •OH) and at concentration of 6.26-25 μmol/mL decreased apoptosis ratio by PDT (Fig. 6c) and when using DTPP-PDT, which show a anti- apoptosis effect that was contradictory to the report that chr increases the production of ROS and can promote apoptosis in cancer cells by activating the intrinsic mitochondrial apoptotic pathway [28, 29]. The reason maybe that chr may produce lower dose of ROS as a signal to promote cell apoptosis but chr (6.26-50μmol/mL) can destroy the ROS (e.g., $\bullet O^{2-}$, •OH) when using DTPP-PDT to induced cell apoptosis process, the relationship of chr and ROS was bidirectional and the underlying mechanism needs further study. Chr had a certain blocking effect on cell membrane damage after PDT at 6.5–50 μmol/mL in a non-dose-dependent manner (Fig. 7), indicating that the cell membrane was a action target of chr.

In conclusion, the novel PS DTPP can induce effective PDT in type I manner to produce oxidative damage and kill SH-SY5Y cells. Chr has antagonistic effect on PDT effect and cell membrane was its targets, other mechanisms of action need to be further investigated. For the study, new windows are open in the treatment of neuroblastoma field so-call PDT that incorporates a nonpoisonous PS-DTPP and a database was establish about the effects of natural antioxidants on photodynamic effects to guide the diet of patients receiving PDT. And the application of PDT and interaction with chr need to be further certified in clinical.

Declarations

Author Contributions

Liqing Zheng and Jianhua Liu conceived and designed the experiments; Yixia Shen performed the experiments; Yong Hou analyzed the data; Zhao Wang and Li Ze contributed to the cell experiments; Liqing Zheng wrote the paper.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics and animals were not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

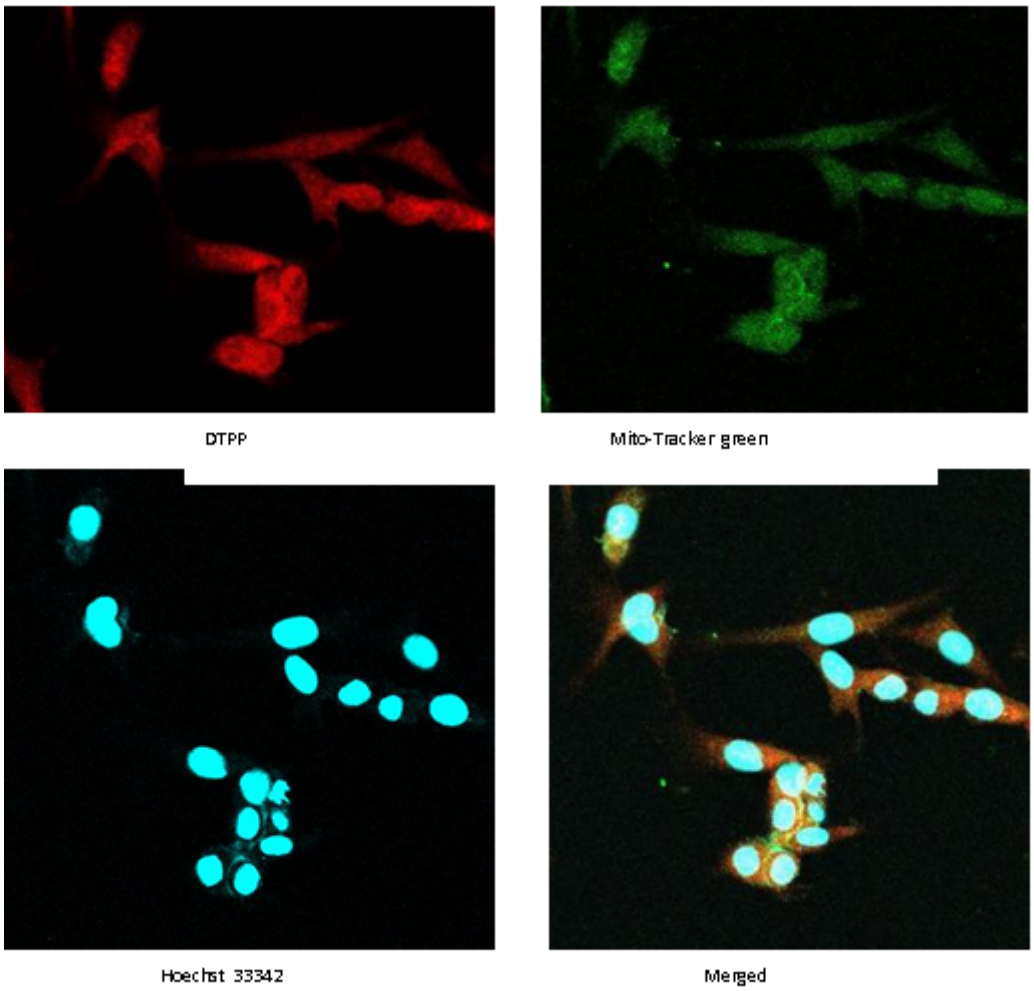

Figure 1

The mitochondrion location of DTPP in SH-SY5Y cells.

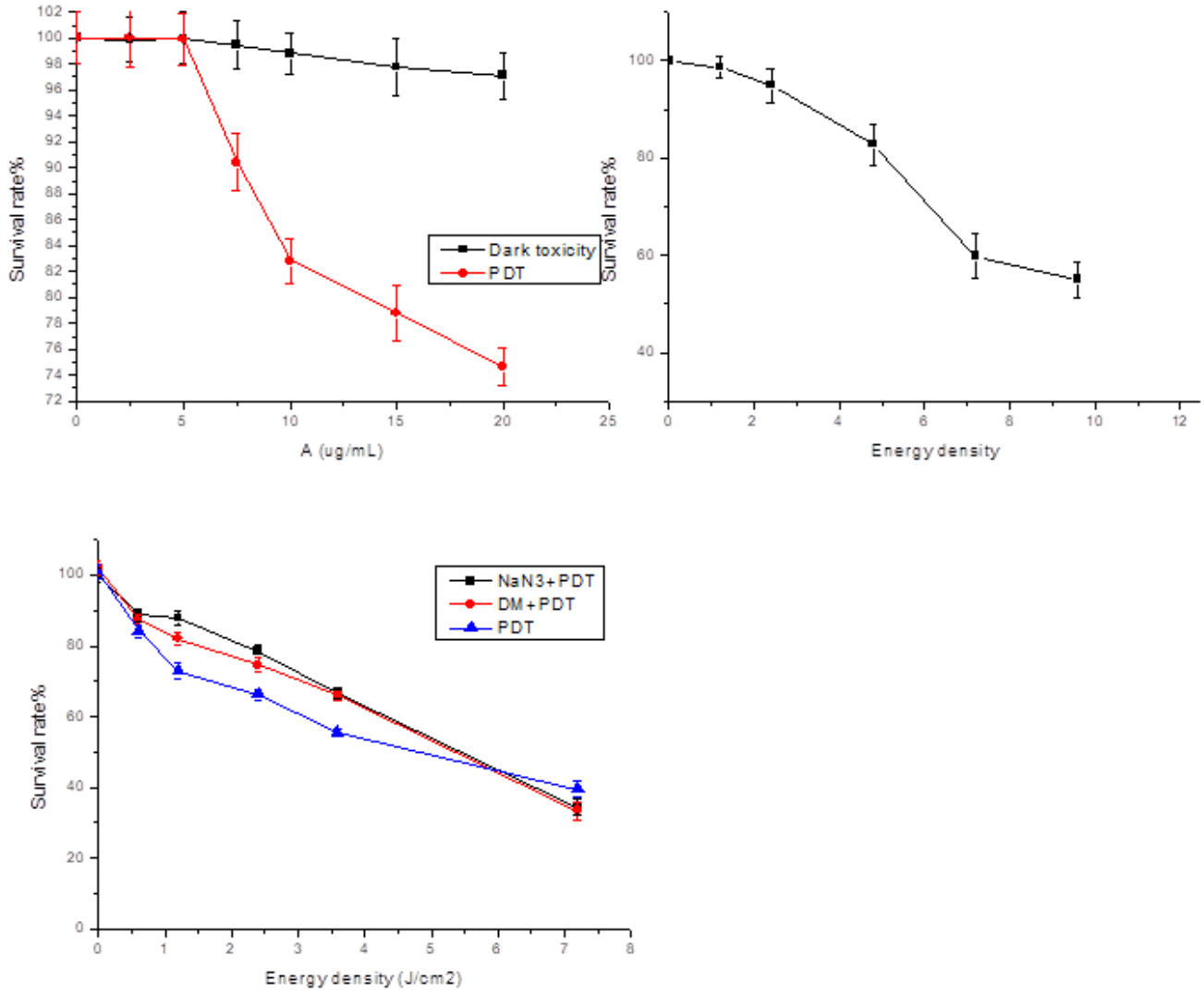

Figure 2

Effect of different factors on the PDT in SH-SY5Y cells. **a.** Dose of DTPP -survival rate relationship in PDT. **b** Energy density - survival rate relationship in PDT. **C.** Effects of SA, DM on the survival rates (*n=4*).

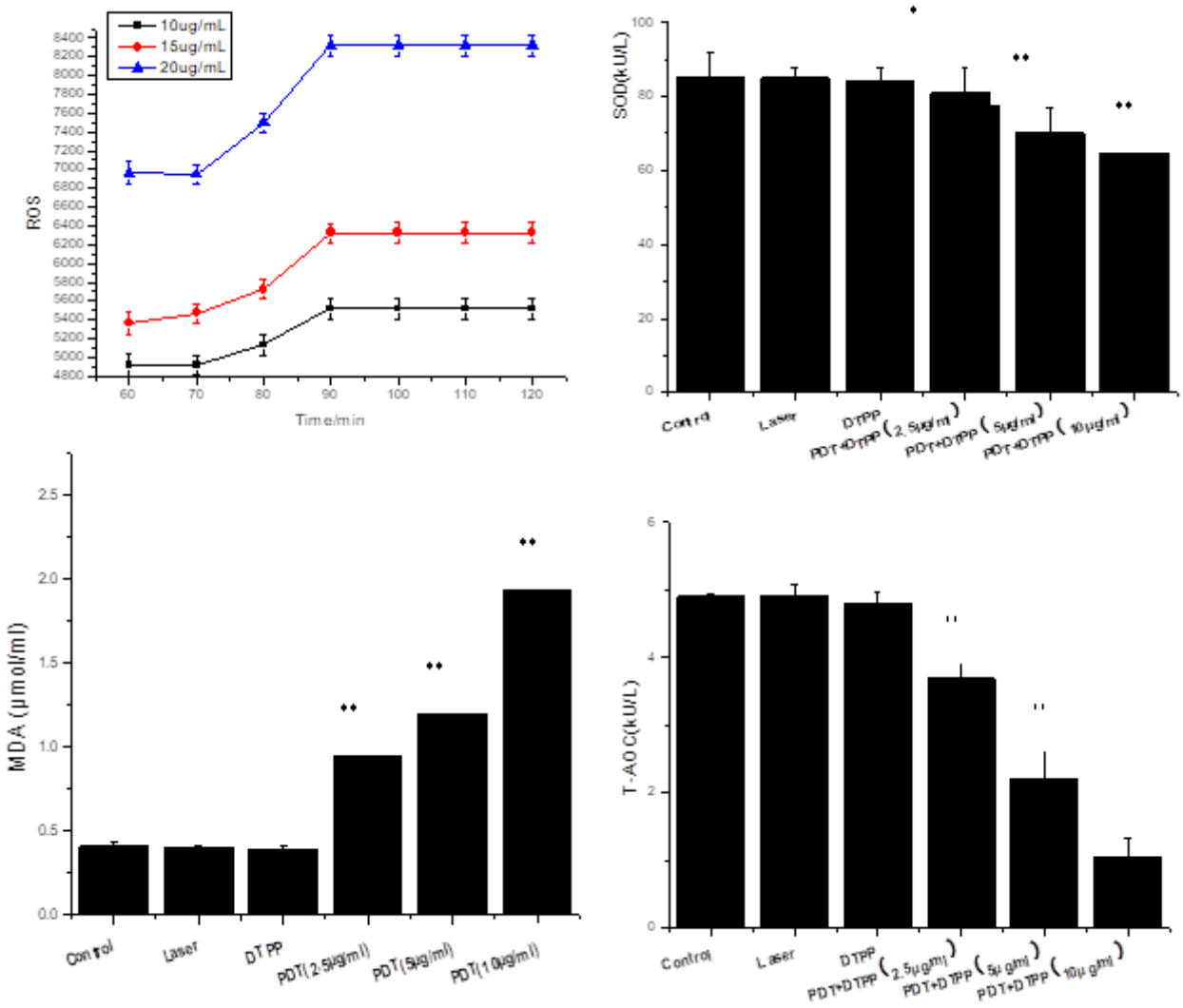

Figure 3

ROS, SOD, MDA and T-AOC in SH-SY5Y cells after PDT illumination. SH-SY5Y cells were treated with DTPP 2.5,5 or 10 μg·mL^{-1} and illuminated with 2.4 J/cm^2 *in vitro*. SOD, MDA and T-AOC products were determined at 1 h after PDT. (n=4,$^{*}P$<0.05, $^{**}P$<0.01 vs control group).

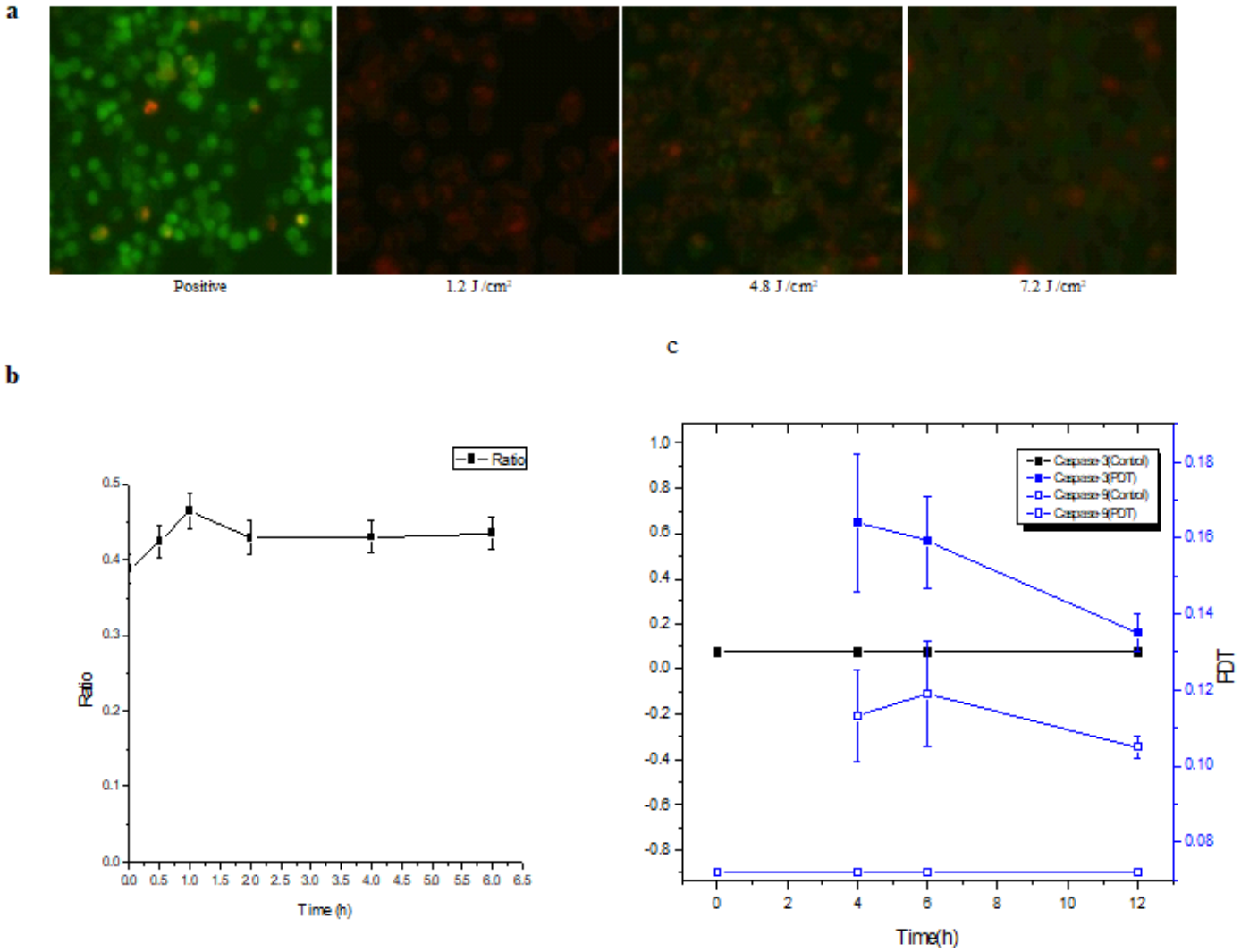

Figure 4

Changes in mitochondria membrane potential induced by DTPP based-PDT **a**. The fluorescence picture of JC-1 in SH-SY5Y cells. The cells were irradiation at 630 nm with energy density of 1.2, 4.8 and 7.2 J/cm^2, respectively. Cells of positive reagent treatment served as a positive control. **b.** MMP changing with time. **c**. The activities of Caspase-3 /9 in SH-SY5Y cells after PDT. Cells without treatment served as controls (n=4)

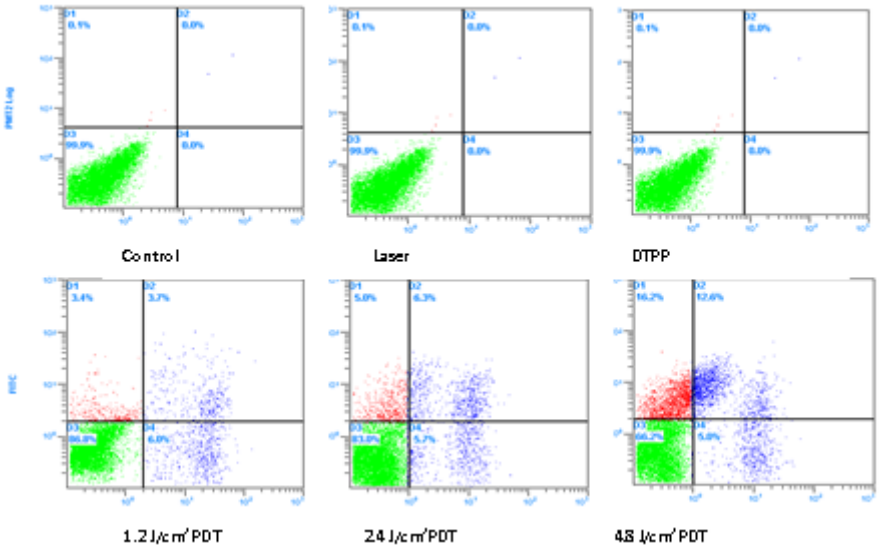

Figure 5

Cell apoptosis ratio after DTPP-based PDT. SH-SY5Y cells were exposed to 10 μg·mL^{-1} DTPP for 24 h and illuminated for a energy density of J/cm^2, respectively with a power density of 20 mW/cm^2 (n=3).

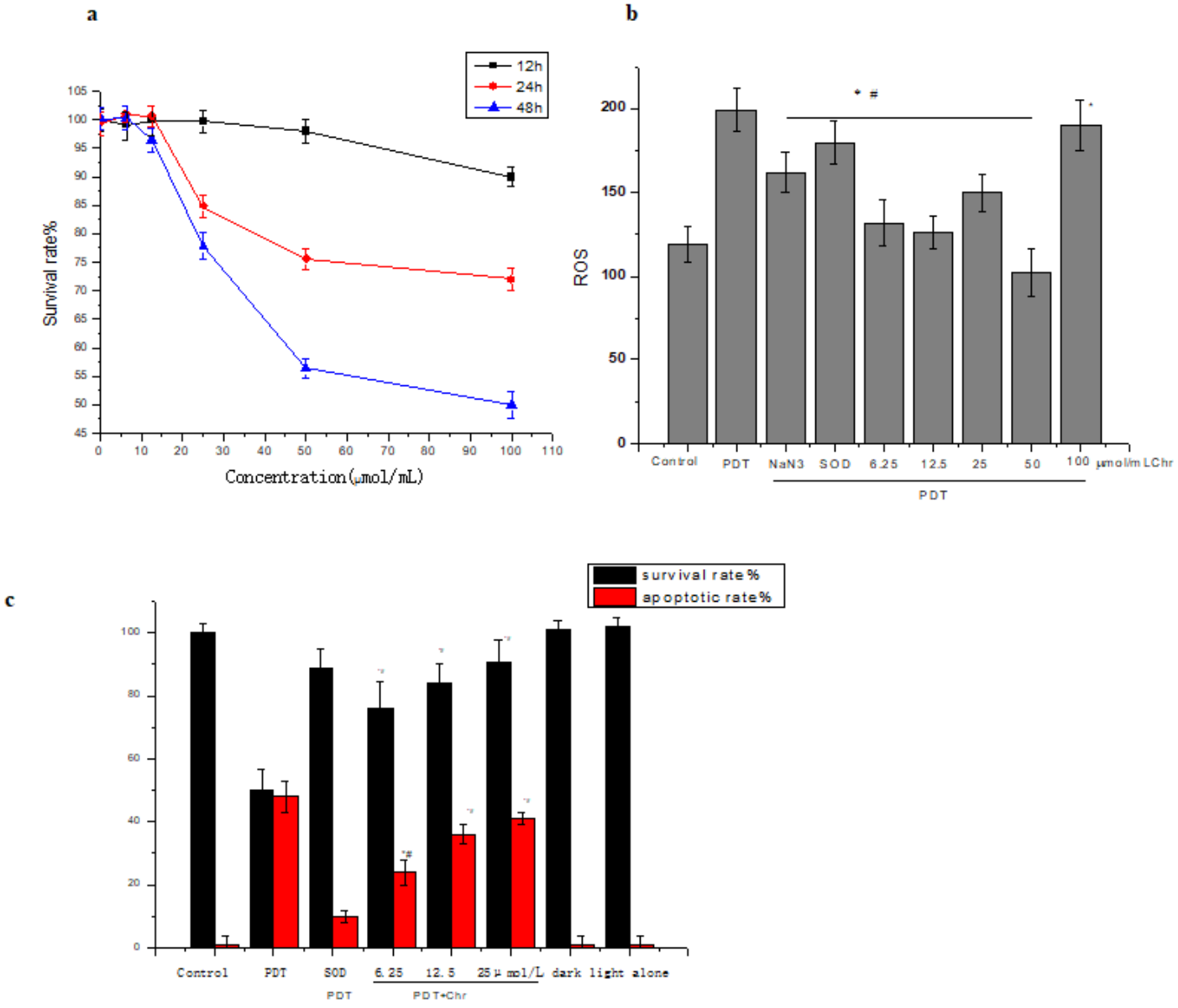

Figure 6

Effect of chr on cell viability and cell apoptosis ratio in PDT on SH-SY5Y cells. **a.** Effect of chr on cell viability. SH-SY5Y cells were incubated with 6.25, 12.5, 25, 50, and 100 μmol/mL of chr in FBS-rich DMEM-F12, respectively, for 12□24 and 48 h, respectively. Untreated SH-SY5Y cells were used as controls (n=4). **b.** Effect of chr on cell viability and cell apoptosis ratio in PDT (n=4). **c.** Effect of chr on ROS output induced by PDT in SH-SY5Y cells. ($^{*}P < 0.05$ *vs* control group, $^{\#}P < 0.05$ *vs* PDT group, n=4).

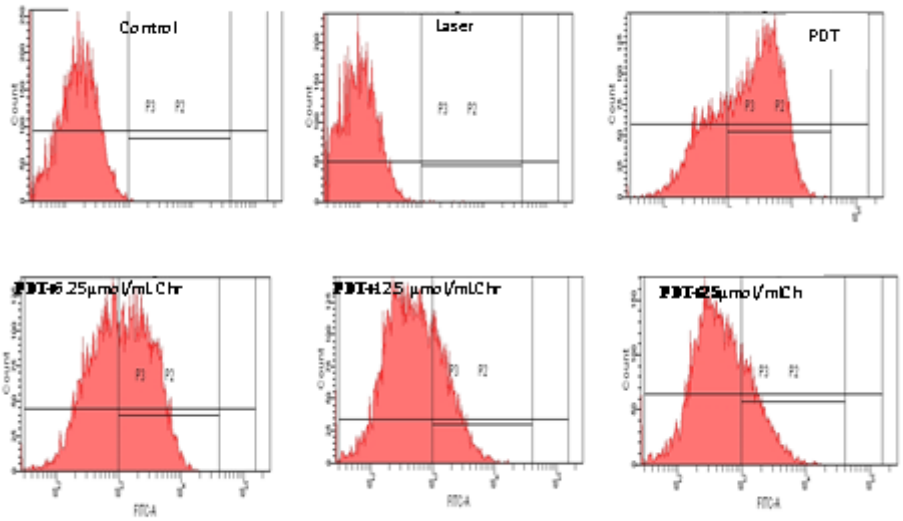

Figure 7

Effect of chr on cell membrane integrity after DTPP-PDT. Cells were incubated with 100 μL of 6.25, 12.5 and 25 μmol/mL chrysophanol solution for 6 h before irradiation and stained with PI at 37℃ for 4 h analyzed by Flow cytometry at room temperature.