CdSe/ZnS quantum dots induce photodynamic effects and cytotoxicity in pancreatic cancer cells

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Abstract

AIM: To investigate the photodynamic effect of CdSe/ZnS quantum dots (QDs) on pancreatic cancer cells and elucidate the probable mechanisms.

METHODS: The pancreatic cancer cell line SW1990 was treated with different concentrations of CdSe/ZnS QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L), with or without illumination. The viability of SW1990 cells was tested using the Cell Counting Kit-8 (CCK-8) assay. The ultrastructural changes of SW1990 cells were observed by transmission electron microscopy. Apoptosis was detected by nuclear staining and flow cytometry (FCM). Reactive oxygen species (ROS) were measured...
Pancreatic cancer is a malignant neoplasm with a very poor prognosis. The 5-year survival is < 5% and medium survival is about 6 mo\(^1\). Surgical resection is the first-choice treatment for pancreatic cancer, however, 80% of patients may already have locally advanced or metastatic cancer when diagnosed, and only 10%-15% are eligible for surgery\(^2\). The majority of pancreatic cancer patients have to undergo radiotherapy or chemotherapy, although the survival rates of these nonsurgical patients are similar. Thus, there is an urgent need to identify a novel effective therapy.

Photodynamic therapy (PDT) is an innovative method that utilizes a photosensitizing agent or photosensitizer (PS) followed by light exposure to treat various diseases. Reactive oxygen species (ROS) are generated when PSs are activated by illumination and subsequently destroy cancer cells\(^3\). In PDT, only cells in contact with the PS, light and oxygen are affected, thus PDT is more selective than conventional chemotherapy and radiotherapy\(^4\). Most PSs are based on a tetrapyrrole structure. The first PS used clinically for cancer therapy was hematoporphyrin derivative, a water-soluble mixture of porphyrins. As hematoporphyrin derivative is purified from porfimer sodium, it has some disadvantages, such as instability in aqueous solution, long-lasting skin photosensitivity, and weak absorption at the therapeutic wavelength of 630 nm\(^5\). 5-aminolevulinic acid is a second-generation photosensitizer (PS). It is a biosynthetic precursor of protoporphyrin and needs to be converted to protoporphyrin as an active PS\(^6\). However, its skin photosensitivity is still an unresolved problem\(^7\). Therefore, it is necessary to develop new PSs to confer survival benefits with fewer side effects.

Quantum dots (QDs) are colloidal semiconductors and mainly composed of group II–VI or group III–V elements\(^8\). QDs are of interest to many researchers due to their unique optical properties. QDs possess several characteristics such as large absorption spectra, narrow and symmetric emission bands, and a high molar extinction coefficient, which make them superior to conventional PSs in PDT\(^9,10\). Recently, many studies have shown the potential applications of QDs for PDT. With illumination, the QD conduction-band electron can be transferred to surrounding O\(_2\) and produce ROS, thus making QDs a potential PS for PDT\(^11,12\).

In this study, we prepared water-dispersible CdSe/ZnS QDs with an extensive absorption in the UV-visible region and a strong emission peaking at 560 nm. We investigated the photodynamic effects of CdSe/ZnS QDs on pancreatic cancer cells, and analyzed the possible molecular mechanism involved in this procedure.
He SJ et al. QDs induce apoptosis in tumor cells

University. Trioclylphosphine oxide, CdO and tetradecylphosphonic acid were heated to 180 °C under argon, exsiccated and exhausted under a vacuum. When the reaction temperature reached 330 °C, selenium precursor solution was added to trioclylphosphine and mixed together until the temperature decreased to 240 °C. ZnS stock solution was added along with dimethyl zinc solution and vigorously stirred until the molar ratio of Cd/Se: Zn/S reached 1:4. The mixture was cooled to room temperature and settled with anhydrous methanol, centrifuged (4000 rpm) and washed three times with anhydrous methanol to remove residua such as trioclylphosphine oxide and unreacted reagents. The precipitate was suspended in phosphate-buffered saline (PBS). The morphology of QDs was observed using a Morgagni 268(D) transmission electron microscope (FEI, Hillsboro, OR, United States). The UV-Vis spectra of the QDs suspensions were scanned within the wavelength range of 200-800 nm at 22 °C and automatically corrected for the suspension, using an Avantes UV-Vis spectrophotometer (Apeldoorn, The Netherlands).

Cell culture
The human pancreatic cancer cell line SW1990 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The SW1990 cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in humidified air containing 5% CO2 at 37 °C. Cells in the exponential growth phase were used in the following experiments.

Cytotoxicity assays
The cytotoxicity induced by QDs and PDT was determined using the Cell Counting Kit-8 (CCK-8) assay. The cells were seeded on a 96-well plate at 8 × 103 cells/well and incubated overnight before QDs were added. The cells were divided into two groups (A with illumination and B without illumination). The cells in each group were treated with different concentrations of QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L) for 1 h. After incubation, all cells were washed with PBS to remove excess QDs and fresh media were added. Cells in Group A were irradiated using ZF-20D Ultraviolet Analyzing Equipment at a wavelength of 365 nm and power of 19 mW cm-2, and then incubated in RPMI 1640 medium with 10% FBS for a further 24 h at 37 °C in a humidified 5% CO2 atmosphere. However, the medium in Group B was removed and replaced with RPMI 1640 medium with 10% FBS, and then the cells were incubated in humidified air containing 5% CO2 at 37 °C for a further 24 h. After 24 h incubation, CCK-8 dye (Dojindo Laboratories, Kamimashiki-gun, Kumamoto, Japan) was added to each well, and the 96-well plates were put into a constant temperature incubator (37 °C) for 1 h. The absorbance of the solution was measured at 450 nm using an ELISA reader (Thermo Fisher Scientific, MA, United States). Cell viability was calculated as a percentage of the treated samples relative to untreated controls.

Subcellular damage of QDs using transmission electron microscopy
Transmission electron microscopy (TEM) was used to investigate the intracellular localization and subcellular structural targets of QDs in SW1990 cells. The cells were seeded on six-well plates at 4 × 105 cells/well and cultured overnight. Twenty-four hours later, after treatment [A: normal SW1990 cells; B: CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination; C: CdSe/ZnS QDs (2 μmol/L, 3 h) and illumination (20 J/cm2)], the cells were collected and washed three times with cold PBS, pelleted using centrifugation (1000 rpm), and fixed in 2.5% glutaraldehyde for 2 h. Cell pellets were washed in PBS, postfixed with 1% osmium tetroxide, and dehydrated with an ascending series of alcohols. The specimens were cut into ultrathin sections (50-70 nm), placed onto copper grids, and stained with uranyl acetate and lead citrate for ultrastructural analysis using a JEM-1011EX transmission electron microscope (Jeol, Tokyo, Japan).

Apoptosis by flow cytometry
The Annexin V-FITC Apoptosis Detection Kit (BD Pharmaningen, San Jose, CA, United States) was used to detect QD-induced apoptosis of SW1990 cells. Cells (2 × 105) were seeded in six-well plates and allowed to adhere overnight. The cells were treated [A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) with illumination (20 J/cm2)], collected and washed twice with cold PBS. The cell pellets were resuspended in binding buffer, and incubated with staining solution [annexin V/propidium iodide (PI) = 1:1] in the dark for 15 min at room temperature. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States).

Measurement of ROS generation
SW1990 cells were seeded on six-well plates at 4 × 105 cells/well and exposed to QDs as for flow cytometry (FCM) after the cells adhered. After 24 h, the cells were rinsed with cold PBS and stained with 2',7'-dichlorofluorescin diacetate (H2DCFDA; Sigma-Aldrich, St. Louis, MO, United States) was used in serum-free medium. After incubation for 30 min at 37 °C in the dark, the cells were washed with serum-free medium three times and resuspended in cold PBS. The DCF fluorescence was observed by a fluorescence microscope (BD Biosciences) and the fluorescence intensity was measured by FCM (BD Biosciences, San Jose, CA, United States). To investigate the role of...
Statistical analysis
All experiments were performed in triplicate. The results were expressed as mean ± SD and analyzed by the Student's t test with SPSS version 13.0 (SPSS Inc., Chicago, IL, United States). Comparisons among multiple groups of data were analyzed by one-way analysis of variance. P < 0.05 was considered statistically significant.

RESULTS

Synthesis and characterization of QDs
QDs were synthesized as previously described. The TEM results showed that QDs were spherical particles with an average size of 5 nm. The peaks of QDs in the UV-Vis analysis showed that the absorbance of QDs was the highest in the UV part of the spectrum, and decreased exponentially when approaching higher wavelengths. The photoluminescence spectra demonstrated that QDs have highest luminescence in the visible part of the spectrum, especially at 560 nm (Figure 1).

Cytotoxicity of QDs
The CCK-8 assay was used to examine the viability of SW1990 cells after different treatments. Cell viability was decreased when the concentration of QDs increased (Figure 2A). Longer incubation time led to lower viability (Figure 2B). Cell viability showed a greater reduction with illumination (Figure 2C). QDs with illumination induced more cytotoxicity in SW1990 cells than QDs alone. More cell damage occurred when the light dose was higher. Illumination alone (10, 20 and 30 J/cm²) without QDs had limited effects on SW1990 cells (Figure 2C).

Immunoblotting
Proteins were resolved by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Anti-Bcl-2 (dilution 1:1000; Cell Signaling Technology, Danvers, MA, United States;), anti-Bax (dilution 1:1000; Cell Signaling Technology), anti-caspase 3 (dilution 1:1000; Cell Signaling Technology), anti-cleaved caspase-3 (dilution 1:1000; Cell Signaling Technology) and anti-β-actin (dilution 1:1000; Cell Signaling Technology) antibodies were used to detect their corresponding proteins followed by anti-rabbit or anti-mouse IgG secondary antibodies (dilution 1:1000; Cell Signaling Technology). Image acquisition was performed with the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, United States). The optical densities of the protein bands were measured by GS710 Densitometer and analyzed with Quantity One image analysis software (Bio-Rad Laboratories).

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ROS production in the cytotoxicity of QD-PDT, SW1990 cells were preincubated with 5 mmol/L N-acetylcysteine (NAC) (Sigma-Aldrich), a ROS scavenger, for 1 h before treatment. Cell viability was evaluated by the CCK-8 assay.

Transcriptional analysis of time course in response to QD-PDT
Real-time RT-PCR was performed using 7500/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) to analyze the apoptosis-related mRNA expression of QD-PDT treated SW1990 cells, such as Bax, Bcl-2 and caspase-3. The primers used in the real-time RT-PCR assay were Bax forward (5’-GAAGAAGATGGGCTGAGG-3’); Bax reverse (5’-TGCCCAGAGAGGTATT-3’); Bcl-2 forward (5’-CCGGATCACATTCTGGAAG-3’); Bcl-2 reverse (5’-AGGCAGAGAATGGAAGT-3’); Caspase-3 forward (5’-AGATGGTTTGAGCCTGAGGA-3’); Caspase-3 reverse (5’-CAGTCGCTATGGGAAGAT-3’); and GAPDH forward (5’-TGACCACCAACTGCCTAG-3’); GAPDH reverse (5’-GGATGCAGGGATGATGTT-3’).

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The QD-PDT-induced subcellular damage of SW1990 cells was detected by TEM (Figure 3). Under normal conditions, SW1990 cells had a round shape
and well-structured mitochondria in the cytoplasm. The cell nucleus was round or class round in the middle of cytoplasm. Nevertheless, after treatment with QDs and illumination, SW1990 cells were significantly damaged. Vacuoles and irregularly sized mitochondria appeared. Organelle degeneration, and chromatin condensation and aggregation at the periphery of the nucleus were observed (Figure 3B and C). The main difference between treatment with 1.5 and 2 μmol/L was the percentage of apoptotic and dead cells, thus the latter induced more cell death.

The percentage of apoptotic and necrotic cells was analyzed by fluorescence microscopy and FCM. SW1990 cells were stained with PI and Hoechst 33342. There were more apoptotic bodies in Group D and several cells were even stained with red fluorescence (Figure 4). FCM indicated that the percentage of apoptotic cells was higher in Group D, however, the percentage of necrotic cells remained at a low level.
Measurement of ROS generation
ROS generation was determined by DCF fluorescence in SW1990 cells. The intracellular ROS content in SW1990 cells treated with light (20 J/cm$^2$) was increased. However, ROS formation significantly increased in SW1990 cells with QDs (1.5 μmol/L, 3 h) and PDT (1.5 μmol/L, 3 h, 20 J/cm$^2$), especially in cells with PDT (Figure 6A and B). These results indicated that illumination enhanced ROS generation in SW1990 cells treated with QDs.

Expression of mRNA and protein
The mRNA expression level of Bax, Bcl-2 and caspase-3 was measured by RT-PCR. The expression level of each gene was normalized to GAPDH. The mRNA expression level of Bax and caspase-3 increased significantly as compared to control cells, while the level of Bcl-2 decreased (Figures 7 and 8). The protein expression level of these three genes was consistent with corresponding mRNA expression.

Effects of ROS and caspase inhibitors on QD-induced PDT
Pretreatment with an antioxidant (NAC), markedly restored cell viability of SW1990 cells after QD-PDT treatment (Figure 6C), which verified the role of ROS in QD-PDT-induced cytotoxicity. To demonstrate the role of apoptosis in QD-PDT, the pan-caspase inhibitor Z-VAD-FMK was added to the cell culture 1 h before treatment. Inhibition of caspase activation by Z-VAD-FMK abrogated QD-PDT-induced cell death (Figure 9).

DISCUSSION
PDT has been widely used clinically to treat a wide range of malignant cancers, such as esophageal and skin cancer. PDT consisted of two parts: administration of a PS and exposure to light to activate the agent$^{[13,14]}$. In this study, we synthesized QDs with a CdSe core and ZnS shell and demonstrated the possible QD-induced PDT effects on pancreatic cancer cells.

Selection of an appropriate light wavelength was important. Blue light resulted in inefficient tissue penetration, unlike red and infrared radiation. The range of 600-1200 nm was considered the optical window for tissue penetration. However, only light < 800 nm could generate $^{1}\text{O}_2$, and light > 800 nm could not provide sufficient energy to initiate photosensitization$^{[15]}$. Thus, there is no ideal single light source for all PDT reactions, even with the same PS. In this study, we selected 365 nm as our illumination wavelength, which happened to be the appropriate excitation wavelength for CdSe/ZnS QDs.

It is reported that cadmium induces ROS generation and triggers apoptosis via a caspase-dependent pathway$^{[16,17]}$. Recently, some studies have shown that
Figure 5  Apoptosis and necrosis were observed in SW1990 cells treated by CdSe/ZnS quantum dots with illumination. A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm²); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm²). FCM of SW1990 cells showed that SW1990 cells had a higher apoptosis rate (53.2%) in Group D than in Groups A, B and C. QDs: Quantum dots.
CdSe-core QDs induce cell death by releasing free Cd\(^{2+}\) from the CdSe lattice, and this effect could be impeded by the addition of a coating such as ZnS\(^{18}\). Here, we synthesized water-soluble CdSe/ZnS QDs. A ZnS coating made the QDs more biocompatible with cells. However, it effectively reduced ROS generation. QDs are generally used as bioimaging probes for tracing and immunostaining cells\(^{19,20}\). In this study, QDs were used as photosensitizers in PDT of the pancreatic cancer cell line SW1990. QDs with ZnS coating showed less cytotoxicity in the dark, even when incubated with cells for 12 h at a concentration of 1.5 \(\mu\)mol/L.

Figure 6 Reactive oxygen species generation was detected after treatment of CdSe/ZnS quantum dots with illumination. A: Fluorescent images of ROS in SW1990 cells (Bar: 200 \(\mu\)m); B: Relative ROS level measured by FCM; C: Cell viability of SW1990 cells by CCK-8 assay. Control: normal SW1990 cells; Light: SW1990 cells with illumination (20 J/cm\(^2\)); QDs: SW1990 cells treated with CdSe/ZnS QDs (1.5 \(\mu\)mol/L, 3 h); PDT: SW1990 cells treated with CdSe/ZnS QDs (1.5 \(\mu\)mol/L, 3 h) and illumination (20 J/cm\(^2\)), NAC: N-acetylcysteine, a ROS scavenger, 5 mmol/L NAC was added to the cell culture for 1 h before treatment. QDs: Quantum dots; ROS: Reactive oxygen species.

Figure 7 Changes in mRNA expression levels of Bax, Bcl-2 and caspase-3 following CdSe/ZnS quantum dots with illumination. A: Normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm\(^2\)); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 \(\mu\)mol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 \(\mu\)mol/L, 3 h) and illumination (20 J/cm\(^2\)). \(b\) vs group A. QDs: Quantum dots.
However, when irradiated by UV, the cytotoxicity of QDs was apparent. Cell viability was decreased when the concentration of QDs increased and incubation time with QDs and light dose increased, which was similar to other studies[21]. TEM, fluorescence microscopy, and FCM illustrated the ability of QDs to generate PDT.
During PDT, ROS generation increased, as reported by Waterhouse et al\(^{25}\), who suggested that the mitochondrion was a vital organelle in programmed cell death and could be mediated by many regulatory factors of apoptosis. To determine whether ROS were increased in QD-induced PDT, we used a probe to detect intracellular ROS variation. Surprisingly, even when coated with ZnS, QDs still generated ROS after illumination, which was statistically significant compared with the control, light and QDs groups. Inhibition of ROS generation with NAC attenuated the cytotoxicity of QD-induced PDT of pancreatic cancer cells, thus ROS were important in this procedure.

To investigate the molecular mechanism of QD-induced PDT, we chose three representative proteins (Bcl-2, Bax and caspase-3) to identify their connection with QD-induced PDT. In this study, we observed apoptosis during QD-induced PDT. Apoptosis has been widely studied and is believed to be triggered by several signals, including a series of proteins\(^{26,27}\). The Bcl-2 family of proteins constitutes a central checkpoint\(^{28}\). Bax and Bcl-2 are two members of the Bcl-2 family and function as regulatory proteins\(^{26,27}\). In this study, we found that QDs increased Bax expression and decreased Bcl-2 expression at the mRNA and protein levels. Several studies have clearly defined Bax as a proapoptotic protein and Bcl-2 as an antiapoptotic protein\(^{28}\). In our study, Group D (cells with PDT) showed higher expression of Bax and lower expression of Bcl-2, which to some degree explained the greater apoptosis and necrosis in this group. These results were consistent with other studies\(^{29,30}\). Caspase-3 is a member of the cysteine-aspartic acid protease family. As an executioner, caspase-3 is practically inactive until it is cleaved by an initiator caspase when apoptotic signaling events occur. Caspase-3 can be activated in apoptotic cells through extrinsic or intrinsic pathways\(^{31-34}\). In this study, cleaved caspase-3 was observed after cells were treated with QDs and illumination. To confirm that apoptosis was involved in the QD-induced PDT effects on pancreatic cancer cells, Z-VAD-FMK was used to restore cell survival and indeed promote cell survival. These results indicated that Bcl-2, Bax and caspase-3 participated in the process of QD-PDT-induced apoptosis. Specifically, QD-PDT downregulated Bcl-2, upregulated Bax, and facilitated caspase-3 cleavage, thus promoting the killing of pancreatic cancer cells.

In summary, this study showed that QDs could be potential PSs for PDT to treat pancreatic cancer by inhibiting SW1990 cell proliferation and inducing apoptosis through ROS generation. QD-PDT may induce apoptosis through ROS-, caspase-3-mediated apoptotic pathways, with upregulation of apoptosis signaling molecules such as Bax and downregulation of Bcl-2. These findings provide a new application for PDT in pancreatic cancer. However, more preclinical and clinical trials should be undertaken before further clinical application.
He SJ et al. QDs induce apoptosis in tumor cells

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