Nanoplatform based on carbon nanoparticles loaded with doxorubicin enhances apoptosis by generating reactive oxygen species for effective cancer therapy

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Abstract. At present, due to its wide application and relatively low cost, chemotherapy remains a clinically important cancer treatment option; however, a number of chemotherapeutic drugs have important limitations, such as lack of specificity, high toxicity and side effects, and multi-drug resistance. The emergence of nanocarriers has removed numerous clinical application limitations of certain antitumor chemotherapy drugs and has been widely used in the treatment of tumors with nanodrugs. The present study used carbon nanoparticles (CNPs) as a nanocarrier for doxorubicin (DOX) to form the novel nanomedicine delivery system (CNPs@DOX)was demonstrated by UV-vis and fluorescence spectrophotometry, ζ potential and TEM characterization experiments. The results confirmed the successful preparation of CNPs@DOX nanoparticles with a particle size of 96±17 nm, a wide range of absorption and a negatively charged surface. Furthermore, CNPs@DOX produced more reactive oxygen species and induced apoptosis, and thus exhibited higher cytotoxicity than DOX, which is a small molecule anticancer drug without a nanocarrier delivery system.. The present study provides a strategy for the treatment of tumors with nanomedicine.

Introduction

Cancer is a worldwide public health issue and one of the major contributors to the global burden of disease (1). According to the Global Cancer Observatory 2020 database, ~10 million people worldwide die from cancer each year (2). Given the ageing population, the number of cancer deaths worldwide is expected to continue to rise, creating a significant public health burden (3,4). Current cancer treatment options include surgery, anticancer drugs, radiation therapy and immunotherapy, alone or in combination (5). Chemotherapy has been a clinically important cancer treatment option due to its wide application and relatively low cost. However, numerous chemotherapeutic drugs have important limitations, such as lack of specificity, toxic side effects, poor water solubility, low bioavailability and multi-drug resistance (6-8). For example, doxorubicin (DOX) is an antitumor chemotherapy drug that has been widely used in the clinic and has shown powerful therapeutic effects against several cancer types, including breast cancer (9), malignant lymphoma (10), acute leukemia (11) and lung cancer (12,13). However, due to the lack of tumor specificity and serious side effects, such as myelosuppression and cardiac toxicity, its clinical application is severely limited (14).

To solve the clinical problems of chemotherapy drugs, drug delivery systems based on nanocarriers have been widely developed, which have clear advantages in cancer therapy. Firstly, due to the enhanced permeability and retention (EPR) of the tumor, the nanosystem has an increased inherent capacity to accumulate at the tumor site rather than in normal tissue (15,16). Secondly, blood vessels in tumor tissue have a larger aperture compared with healthy tissue, leading to the preferential accumulation of nanodrugs in the tumor, improving the therapeutic effect and reducing systemic toxicity (17). At present, due to their excellent physical and chemical properties, rich functional groups (such as amino, hydroxyl and carboxyl groups), large surface area and good biocompatibility, carbon-based nanomaterials, including carbon nanoparticles, carbon nanotubes, graphene and its derivatives, have aroused great interest in biomedical applications such as drug delivery, bioimaging and therapy (18-20).

In present study, UV-vis and fluorescence spectrophotometry, ζ potential and TEM characterization experiments were used to verify whether the nanodrug delivery system was successfully prepared. Cytotoxicity assay, intracellular ROS detection and apoptosis assay were performed to evaluate the toxicity and killing mechanism of the nanomedicine delivery

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system on cancer cells. This study aims to provide a potential strategy for the therapy of tumors with nanomedicine.

Materials and methods

Materials. CNPs were synthesized based on a previously reported method (21). DOX was purchased from Merck KGaA. Other chemical reagents for the experiment were bought from Sinopharm Chemical Reagent Co., Ltd., unless otherwise specified.

Material synthesis. CNPs were prepared using heat treatment. In brief, the carbon source [citric acid, 20% (w/v)] and the surface modifier [reduced glutathione (GSH), 20% (w/v)] were dissolved in deionized water and heated in an oil bath (130˚C) for 10 min. After the reaction, the solution was cooled to room temperature and the pH was adjusted to 7.4. The solution was then further dialyzed using a dialysis bag [molecular weight cut-off (MWCO)=3,500 Da] to remove free citric acid and GSH. Finally, the solution was filtered to retain the liquid, and CNPs were obtained.

DOX solution (800 mg/l; 5 ml) was slowly added to the CNP solution at room temperature and magnetically stirred for 2 h. After the mixed solution was allowed to stand for 2 h, the free DOX was removed by dialysis through the dialysis bag (MWCO=14 kDa). The obtained CNPs@DOX was stored at room temperature for characterization and experimentation.

Characterization methods. A total of 10 μl CNP (100 mg/l) and CNPs@DOX (100 mg/l) solutions were dripped onto carbon-coated 400-mesh copper grids by pipette gun and air-dried for 24 h to prepare the sample for transmission electron microscopy (TEM; Hitachi, Ltd.) observation. The ζ potentials of aqueous CNPs and CNPs@DOX were measured using dynamic light scattering (Malvern Instruments, Ltd.). The ultraviolet (UV)-visible (vis) absorption and fluorescence spectra were measured using a UV spectrophotometer (Hitachi, Ltd.) and fluorescence spectrophotometer (Hitachi, Ltd.), respectively.

DOX loading study in CNPs@DOX. DOX solutions (300, 400, 500, 800 and 1,000 mg/l) were added to CNP (2,000 mg/l) solution to synthesize CNPs@DOX. By measuring the absorbance of DOX at 480 nm in aqueous solution, the standard calibration curve of DOX was obtained and the drug loading efficiency (DLE) of CNPs@DOX was calculated (22). The DLE was calculated as follows: DLE (%)=[(amount of DOX-amount of free DOX)/(amount of DOX)] x100.

Cell culture. Mouse breast cancer 4T1 and human breast cancer MCF7 cell lines were purchased from the Cell Bank of Chinese Academy of Sciences. DMEM and FBS were purchased from Gibco; Thermo Fisher Scientific, Inc. Cells were cultured using DMEM supplemented with 10% FBS (37˚C; 5% CO_2 atmosphere).

Cytotoxicity evaluation. The cytotoxicity of CNPs@DOX and DOX were evaluated using the 4T1 and MCF7 cell lines. The cells were seeded in 96-well plates ($5x10^3$ cells per well) and incubated in DMEM containing different concentrations (0, 0.1, 1, 2.5, 5 and 10 mg/l) of CNPs@DOX or DOX for 24 h at 37˚C. The original medium was aspirated and the cells were incubated for 1 h with fresh medium containing 10 μl Cell Counting Kit-8 reagent (Dojindo Laboratories, Inc.). Finally, the absorbance [optical density (OD)] was measured at 450 nm with a microplate reader to calculate cell viability as follows: Cell viability (%)=[($OD_{treated}$-OD_{blank})/($OD_{control}$-OD_{blank})] x100, where $OD_{treated}$, $OD_{control}$ and OD_{blank} were the absorbance values of the sample wells.

Cell apoptosis. The 4T1 cells were cultured in 6-well plates ($1x10^5$ cells per well) for 12 h at 37˚C, and when cell proliferation reached 60-70%, the original medium was sucked out and the cells continued to be cultured with fresh medium containing CNPs@DOX solution (5 mg/l DOX) and DOX solution (5 mg/l) for 24 h at 37˚C. The cells were then trypsinized, washed with PBS and collected ($1x10^6$ cells). An annexin V-FITC/PI Apoptosis detection Kit (BD Biosciences) was used to stain apoptotic cells by mixing 100 μl cell suspension, 5 μl annexin V-FITC and 5 μl PI. The cells were incubated for 15 min at 37˚C and 200 μl binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.50 mM $CaCl_2$) was added to each suspension. Finally, apoptosis was measured using a flow cytometer (FACSCalibur™; BD Biosciences) and analyzed by BD FACSDiva™ Software v9.0.

ROS detection in vitro. Intracellular ROS changes were detected using the fluorescent probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). Specifically, the 4T1 cells ($1x10^5$ cells) were cultured with media containing CNPs@DOX solution (2.5 mg/l DOX) and DOX solution (2.5 mg/l) for 24 h at 37˚C, and then incubated with the ROS Assay Kit (S0033S, Beyotime Biotechnology) according to the manufacturer's instructions. Finally, confocal laser scanning microscopy (CLSM; Olympus Corporation) was used to detect intracellular ROS production.

Statistical analysis. The data are expressed as the mean ± standard deviation. GraphPad Prism 8 software (Dotmatics) was used for statistical analysis. The unpaired t-test or one-way analysis of variance with Tukey's post hoc test was used to compare the differences between the experimental groups. Drug loading efficiency was calculated using a standard calibration curve. $P<0.05$ was considered to indicate a statistically significant difference.

Results and discussion

Characterization of CNPs@DOX. The particle sizes of CNPs and CNPs@DOX were assessed using TEM. The average particle size of CNPs in the aqueous solution was 79±14 nm, and the average particle size of CNPs combined with DOX to form the nanomedical drug delivery system CNPs@DOX was 96±17 nm. CNPs@DOX, as a nanomedicine, can target tumor tissue through the EPR effect, whereas DOX, as a small molecule drug, does not have this targeting ability. Therefore, CNPs@DOX is conducive to the accumulation of DOX at the tumor site through the EPR effect, thereby exerting antitumor effects (Fig. 1).

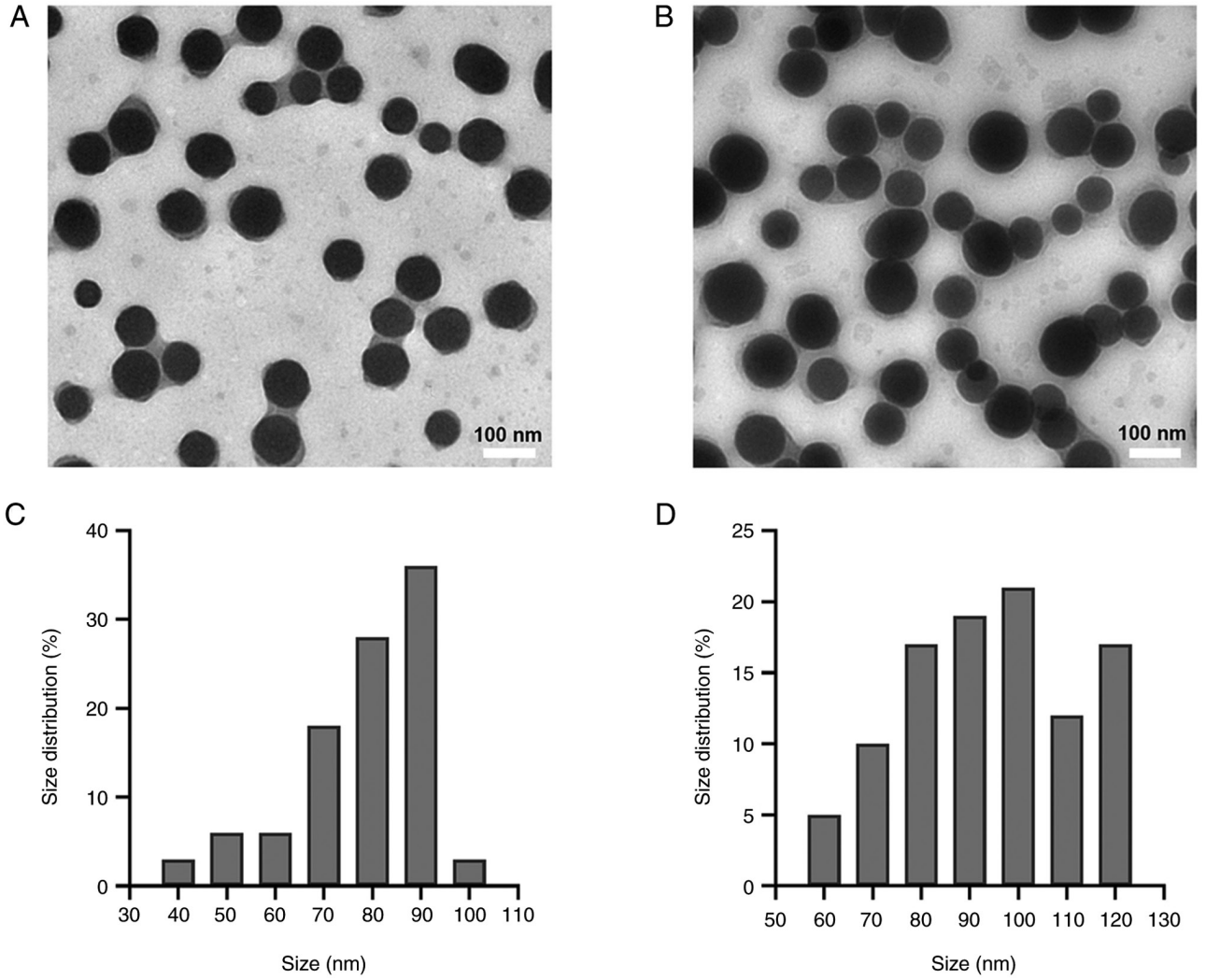

Figure 1. Material characterization. Transmission electron microscopy images of (A) CNPs and (B) CNPs@DOX. Particle size distribution of (C) CNPs and (D) CNPs@DOX. CNPs, carbon nanoparticles; DOX, doxorubicin.

UV-vis spectra demonstrated notable absorbance of CNPs at 374 nm and of DOX at 480 nm; however, CNPs@DOX demonstrated strong absorbance at 518 nm (Fig. 2A), which was different from that of both CNPs and DOX due to the interaction between the CNPs and the DOX. Moreover, fluorescence spectroscopy revealed an emission peak at 471 nm for CNPs, at 592 nm for DOX and at 468 nm for CNPs@DOX (Fig. 2B).

The ζ potential of the CNPs was ~-33.2 mV, and the introduction of DOX was associated with a marked increase in the ζ potential to ~-15.6 mV (Fig. 2C; Table I), which indicates the successful preparation of CNPs@DOX. It has been reported that the negative charge on the surface of nanoparticles makes them more conducive to travelling within blood circulation (23,24).

The aforementioned results confirmed successful preparation of CNPs@DOX nanoparticles with a particle size of 96±17 nm, a wide range of absorption and a negatively charged surface.

Table I. ζ potentials of CNPs and CNPs@DOX in aqueous solution.

Group	ζ potential, mV
CNPs	-33.2±4.3
CNPs@DOX	-15.6±3.5

Data are presented as the mean ± standard deviation. CNPs, carbon nanoparticles; DOX, doxorubicin.

DOX loading study in CNPs@DOX. To assess the DOX loading rate in CNPs@DOX, DOX solutions of different concentrations (300, 400, 500, 800 and 1,000 mg/l) were added to CNPs (2,000 mg/l) solution to synthesize CNPs@DOX. The DLE was calculated using standard calibration curves of DOX (Fig. S1). The results demonstrated that the DLE reached ≥77.52% when DOX solution was added at 800 mg/l (Fig. 2D). All experiments in the present study used CNPs@DOX, synthesized by adding 800 mg/l DOX solution (DLE=77.52%).

Cytotoxicity evaluation. The cytotoxicity assays of free DOX and CNPs@DOX demonstrated notable dose-dependent cell

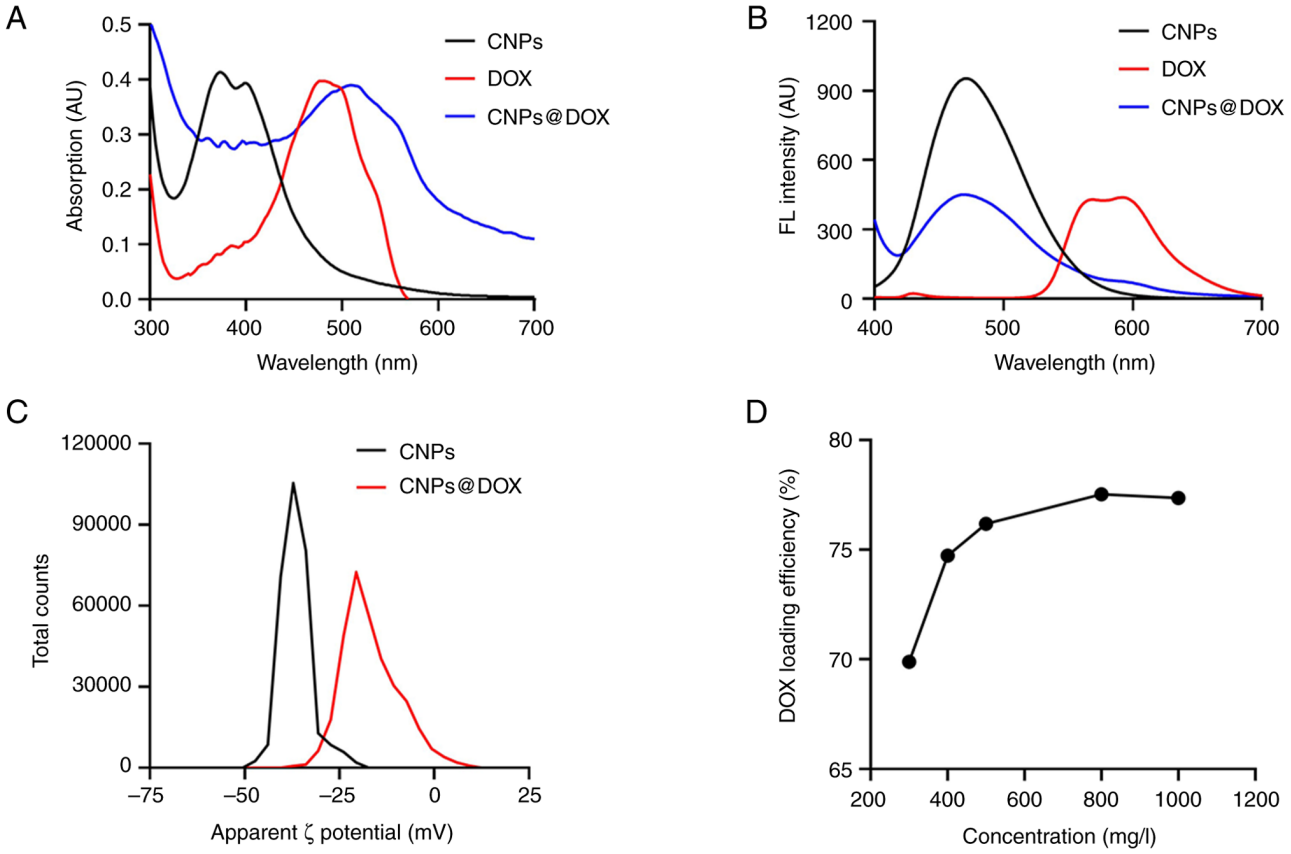

Figure 2. Material characterizations and drug loading efficiency. (A) Ultraviolet-visible spectra of CNPs, DOX and CNPs@DOX. (B) FL spectra of CNPs, DOX and CNPs@DOX. (C) ζ potentials of CNPs and CNPs@DOX in aqueous solution. (D) Drug loading efficiency of CNPs@DOX at different concentrations of DOX. CNPs, carbon nanoparticles; DOX, doxorubicin; FL, fluorescence.

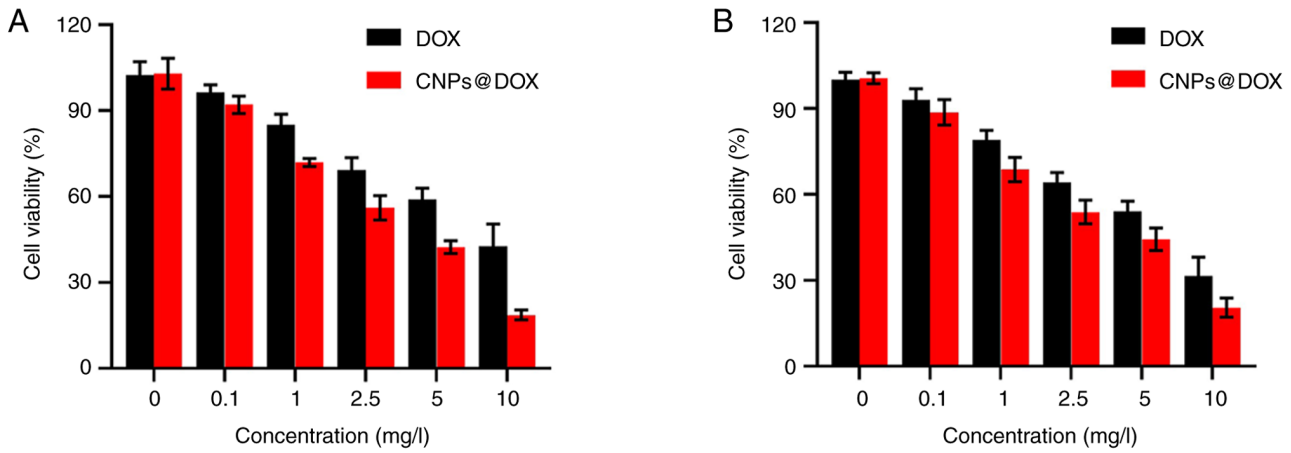

Figure 3. Cytotoxicity evaluation. Cytotoxicity after 24 h of (A) 4T1 and (B) MCF7 cells incubated with DOX and CNPs@DOX at different concentrations (0, 0.1, 1, 2.5, 5 and 10 mg/l). CNPs, carbon nanoparticles; DOX, doxorubicin.

viability inhibition in both the 4T1 and MCF7 cells at 24 h (Fig. 3). In particular, when the drug concentration was 5 or 10 mg/l, the inhibitory effect of CNPs@DOX on the viability of 4T1 cells was significantly greater than of DOX (Fig. 3A). When the drug concentration was 10 mg/l, the inhibitory effect of CNPs@DOX on the viability of MCF7 cells was markedly greater than that of DOX (Fig. 3B). Nanomaterials have shown great potential in encapsulating and transporting drugs within tumor cells, penetrating cell membranes and releasing drugs via EPR effects (25), increasing drug accumulation within tumor cells but decreasing drug accumulation in normal cells (26). Therefore, CNPs@DOX, as a nanomaterial, may accelerate the internalization of drugs by cells, causing DOX to act on nuclei faster and kill tumor cells more effectively (27,28).

Cell apoptosis. The cytotoxic effects of DOX on tumor cells are mainly exerted via two mechanisms (29): One is to

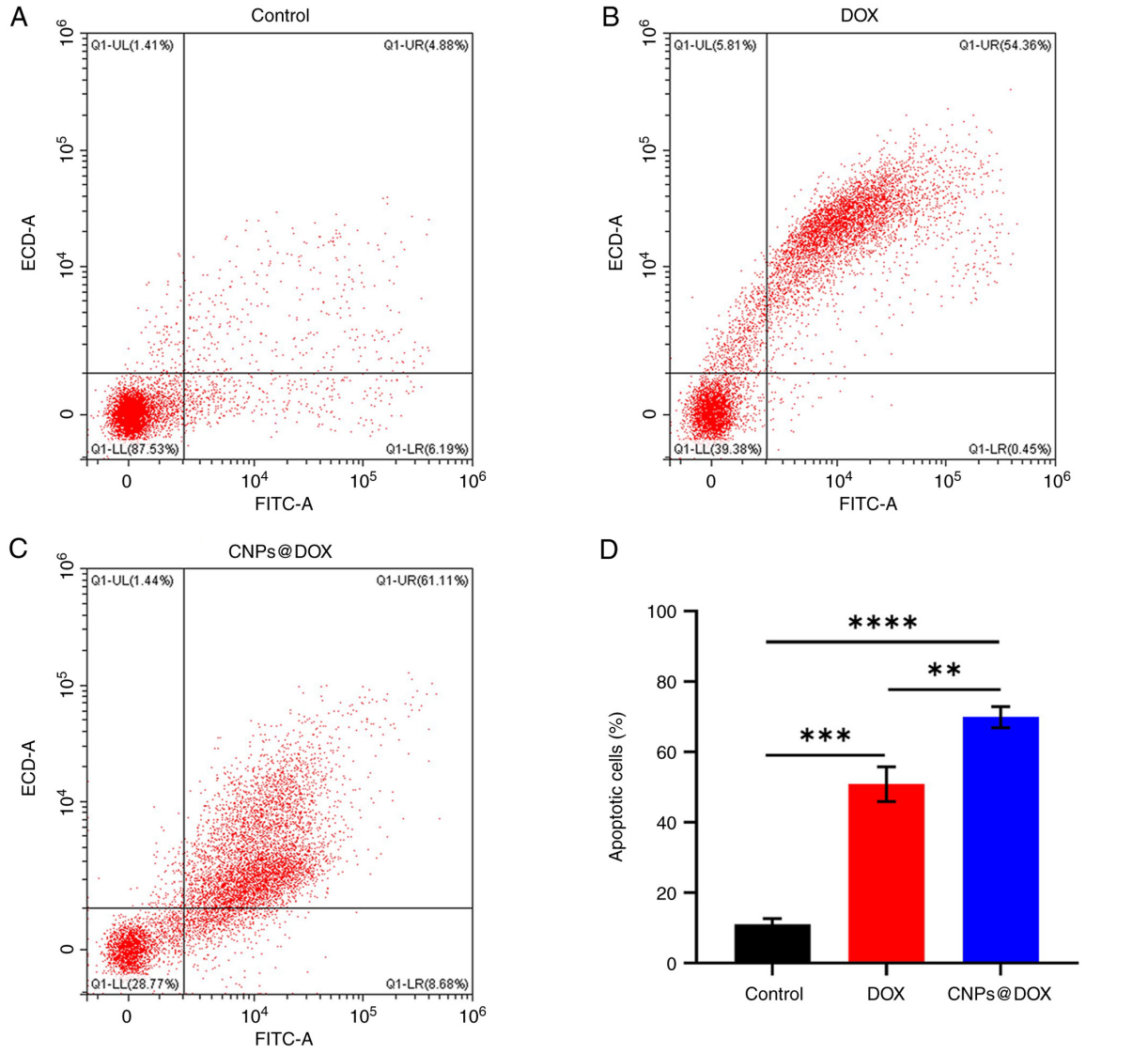

Figure 4. Cell apoptosis. Analysis of the apoptosis of 4T1 cells after different treatments for 24 h: (A) Control, (B) DOX (5 mg/l) and (C) CNPs@DOX (5 mg/l). The upper left quadrant represents cell debris due to mechanical damage; the lower left quadrant indicates normal cells; the upper right quadrant indicates late apoptotic cells; and the lower right quadrant indicates early apoptotic cells. (D) Quantification of the percentage of apoptotic 4T1 cells after different treatments. **P<0.01; ***P<0.001; ****P<0.0001. CNP, carbon nanoparticle; DOX, doxorubicin.

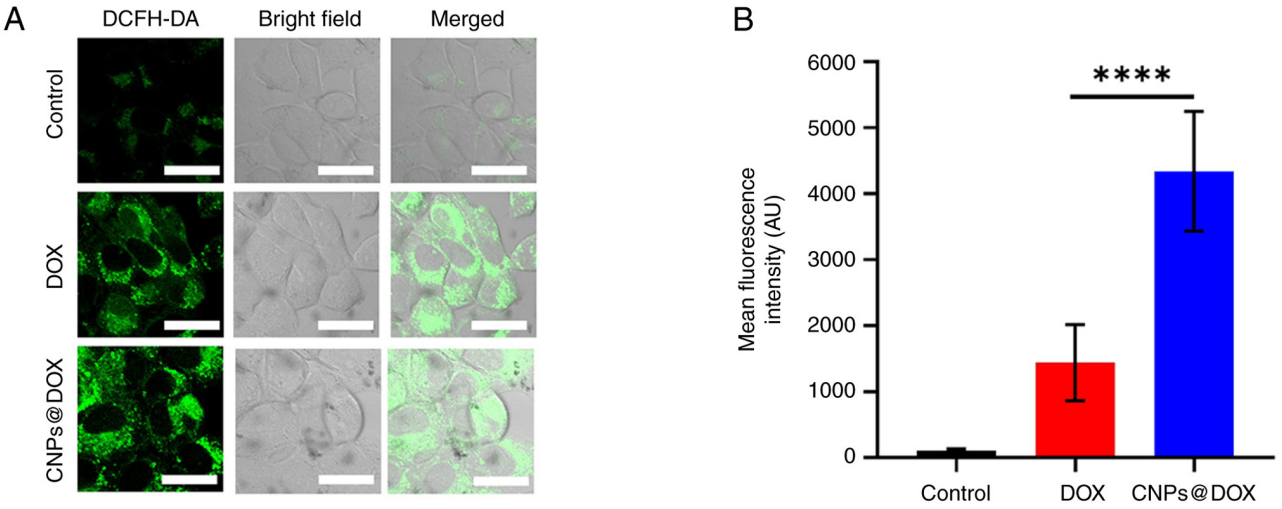

Figure 5. Intracellular ROS assay. (A) Fluorescence images of ROS produced after different treatments of 4T1 cells by confocal laser scanning microscopy. Scale bar, 20 μm. (B) Semi-quantitative fluorescence analysis of ROS produced. ****P<0.0001. ROS, reactive oxygen species; CNPs, carbon nanoparticles; DOX, doxorubicin, DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate.

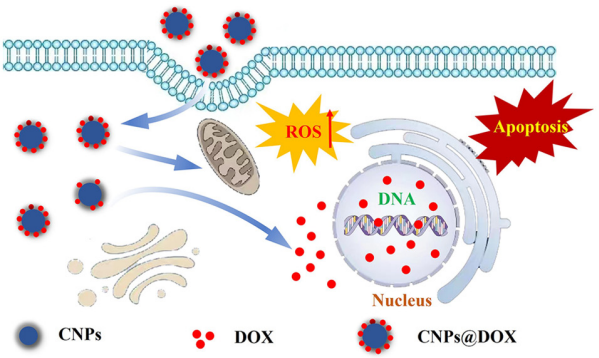

Figure 6. Schematic illustration of cell apoptosis induction by CNPs@DOX. CNPs, carbon nanoparticles; DOX, doxorubicin; ROS, reactive oxygen species.

insert G-C base pairs into the DNA sequence to induce cell apoptosis by inhibiting DNA replication (30); and the other is that DOX acts as an electron acceptor in the redox reaction and is oxidized into semi-quinone free radicals, which causes oxidative damage to cell membranes, protein and DNA through the generation of ROS, thus inducing the apoptosis of cancer cells (31). The present study evaluated the effect of CNPs@DOX and DOX on the apoptosis of 4T1 cells. The results demonstrated that the rates of apoptosis of 4T1 cells in the DOX and CNPs@DOX groups were 50.85±4.91 and 69.89±2.99%, respectively, which were significantly higher than the rate of 11.02±1.64% in the control group (Fig. 4). In addition, the apoptosis rate of the CNPs@DOX group was also significantly higher than that of DOX group, indicating that CNPs@DOX significantly increased the apoptosis of the 4T1 cells, and the drugs delivered by CNPs could serve an antitumor role by promoting cell apoptosis.

Intracellular ROS assay. ROS are metabolic by-products of cellular aerobic respiration and serve an important role in cell signaling and homeostasis (32). Different ROS control diverse aspects of cell behavior from signaling to death, and dysregulation of ROS production and ROS limitation pathways are common features of cancer cells (33). Previous studies have reported that chemotherapy drugs can promote apoptosis and inhibit tumor growth by increasing ROS levels in tumor cells (34,35). The present study used the DCFH-DA fluorescent probe to detect intracellular ROS production and CLSM to detect DCF fluorescence to determine intracellular ROS levels. The results demonstrated that green fluorescence was notably enhanced after the 4T1 cells were incubated with DOX and CNPs@DOX, whilst almost no green fluorescence was detected in the control group (Fig. 5A). In addition, semi-quantitative fluorescence results further indicated that the amount of ROS produced in the CNPs@DOX group was significantly higher than that in the DOX group (Fig. 5B). These results indicate that CNPs@DOX can increase intracellular ROS levels, thereby promoting tumor cell apoptosis and inhibiting tumor growth.

Limitations. The present study only demonstrated that CNPs@DOX had a good killing effect on tumor cells at the cellular level and failed to carry out experiments at the animal level to further explore the anti-tumor effect of CNPs@DOX, which is the limitation of this study.

Conclusion. The present study used CNPs as a nanocarrier for DOX to prepare a novel nanomedicine delivery system, CNPs@DOX (Fig. 6). Through UV-vis and fluorescence spectrophotometry, ζ potential and TEM characterization experiments, the results obtained indicated that the nano-drug delivery system CNPs@DOX, with a drug loading rate of 77.52% and particle size of 96±17 nm, was successfully prepared. Furthermore, *in vitro* experiments demonstrated that CNPs@DOX could promote tumor cell apoptosis by increasing intracellular ROS levels, which should have good antitumor effects and great clinical application potential in tumor therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YLi and JZ wrote the original draft of the manuscript, and created figures, tables and visual representations of the data. CW, YLa, HF and QW analyzed and interpreted the data. YLi, JZ and XJ conceived and designed the study. ZL, JC and XZ performed the experiments. YLi, JZ and XJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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