Increased chemosensitivity and radiosensitivity of human breast cancer cell lines treated with novel functionalized single-walled carbon nanotubes

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Abstract. Hypoxia is a major cause of treatment resistance in breast cancer. Single-walled carbon nanotubes (SWCNTs) exhibit unique properties that make them promising candidates for breast cancer treatment. In the present study, a new functionalized single-walled carbon nanotube carrying oxygen was synthesized; it was determined whether this material could increase chemosensitivity and radiosensitivity of human breast cancer cell lines, and the underlying mechanisms were investigated. MDA-MB-231 cells growing in folic acid (FA) free medium, MDA-MB-231 cells growing in medium containing FA and ZR-75-1 cells were treated with chemotherapy drugs or radiotherapy with or without tombarthite-modified-FA-chitosan (R-O_2-FA-CHI)-SWCNTs under hypoxic conditions, and the cell viability was determined by water-soluble tetrazolium salts-1 assay. The cell surviving fractions were determined by colony forming assay. Cell apoptosis induction was monitored by flow cytometry. Expression of B-cell lymphoma 2 (Bcl-2), survivin, hypoxia-inducible factor 1-α (HIF-1α), multidrug resistance-associated protein 1 (MRP-1), P-glycoprotein (P-gp), RAD51 and Ku80 was monitored by western blotting. The novel synthesized R-O_2-FA-CHI-SWCNTs were able to significantly enhance the chemosensitivity and radiosensitivity of human breast cancer cell lines and the material exhibited its expected function by downregulating the expression of Bcl-2, survivin, HIF-1α, P-gp, MRP-1, RAD51 and Ku80.

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Introduction

Chemotherapy and radiotherapy are common treatment approaches for breast cancer (1). Although the technology underlying chemotherapy and radiotherapy has been developed, resistance to chemotherapy and radiotherapy remains an issue (2). An important characteristic of breast cancer is the presence of hypoxia (3). For example, the median partial pressure of oxygen (PO_2) level measured in breast cancer was 10 mmHg, while the median PO_2 level was 65 mmHg in normal breast tissue (4). The hypoxic microenvironment in tumors is frequently caused by imbalance of oxygen demand and supply, and the malfunctioning of the blood vessels feeding tumors (5). This hypoxic condition makes tumor cells initiate various adaptation processes (6). In addition, various clinical trials have demonstrated that the presence of hypoxia is a major cause of treatment failure (7-11). The hypoxia-tolerant cancer cells are resistant to chemotherapy and radiotherapy (6).

Resistance to chemotherapy and radiotherapy may be overcome by a variety of measures demonstrated by experiments. Increasing oxygen pressure within the tumor [for example, administration of red blood cells (12) and hyperbaric oxygenation (13)], and administration of chemoradiotherapy sensitization agents [for example, nitroimidazoles, including nimorazole (14)] are among the approaches. However, the

effectiveness of such measures remains controversial, and no clear benefits of these measures have been demonstrated (13). In particular, when increasing the oxygen pressure within the tumor, the oxygen content needs to be controlled within a small range or it cannot sensitize the tumor to radiotherapy (15). Furthermore, in a previous study, the radiotherapy sensitizer tirapazamine did not improve the treatment outcome and increased the incidence of adverse events (16).

Carbon nanotubes (CNTs) have been exploited in several biomedical applications (17,18). CNTs have been proposed to exhibit the potential to cross the cell membrane (19) and effectively transport molecules into the cytoplasm (20). Both single-walled (SW) CNTs and multi-walled nanotubes are being considered as effective nanocarriers for drug transportation (21).

Considering the characteristics of CNTs, the present study speculated that SWCNTs may serve as a nanocarrier for delivery of O_2, with the aim of alleviating the hypoxic conditions of cancer lesions and increasing sensitivity to chemotherapy and radiotherapy in tumor cells.

Despite the application of SWCNTs as O_2 delivery vehicles, tombarthite, a mineral containing 17 chemical elements, was used to increase the dispersion of SWCNTs in order to reduce the toxicity (22,23). Folic acid (FA) was also used, in an attempt to make the material selectively accumulate in cancer lesions.

In the present study, the synthesis of the novel oxygen-carrying tombarthite-modified FA-conjugated chitosan (R-O_2-FA-CHI)-SWCNT nanocarrier for targeted delivery of O_2 was described, and the chemoradiotherapy sensitizing properties of this novel material were investigated *in vitro*. The potential underlying mechanisms involved were also assessed.

Materials and methods

Purification of the SWCNTs and preparation of R-O_2-SWCNTs

Purification of the SWCNTs. The purification of the SWCNTs with purity >95%, a length of 0.5-2 μm and a diameter of 10-20 nm (Nanjing XianFeng Nano Material Technology Co., Ltd., Nanjing, China) was performed by the School of Mechanical and Power Engineering, Shanghai Jiaotong University (Shanghai, China). The SWCNTs (100 mg) were added to a mixture of 98% H_2SO_4 and 78% HNO_3 (v/v, 3:1, 50 ml) in a conical flask and sonicated at room temperature for 2 h. The SWCNTs were washed with ultrapure water and dried. Subsequently, 300 ml deionized water was added to the conical flask, causing the SWCNT to sink and three layers to form. The upper layer was a clear liquid and was removed after 6 min. The residual turbid solution (middle layer) was filtered through a microporous membrane (0.45 μm; Beijing Xinwei Technology Group Co., Ltd., Beijing, China), and was then washed with ultrapure water to neutral pH. The material was dried at 60˚C in a baking oven.

Preparation of R-O_2-SWCNTs. The purified SWCNTs were immersed in tombarthite modifier liquid (m/v, 2 mg/ml) containing 1.5% of the tombarthite element lanthanum, 96% ethyl alcohol, 0.2% EDTA, 0.8% NH_4Cl, 0.5% HNO_3 and 1% carbamide, for 2 h, followed by sonication for 1-3 h. Subsequently, the product was filtered through a microporous filtration membrane (0.45 μm) and dried.

Production of R-O_2-SWCNTs. The R-O_2-SWCNTs were placed in the sample chamber of a vacuum heating system and heated to 300-400˚C and vacuumized for 5 h, to the vacuum degree of 10^{-3} Pa. A total of 9 MPa pure oxygen was pumped into the sample chamber following cooling to room temperature, while maintaining the vacuum status. The R-O_2-SWCNTs were obtained when the pressure in the sample chamber remained stable for 2-3 h.

Preparation of R-O_2-FA-CHI-SWCNTs

Conjugation of CHI. The R-O_2-SWCNTs (20 mg) were sonicated in CHI (Shanghai YuanYe Biotechnology Co., Ltd., Shanghai, China) solution (40 mg in 0.05 mol/l acetic acid) for 20 min and subsequently stirred at room temperature for 16 h. The modified SWCNTs were collected and filtered through a 10 KDa molecular weight cut-off membrane (Shanghai Lvniao Biocompany, Shanghai, China) to remove excess chitosan, and subsequently washed with ultrapure water several times.

Conjugation of FA. FA (Shanghai YuanYe Biotechnology Co., Ltd.) and N,N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC; Shanghai YuanYe Biotechnology Co., Ltd.) were added to a solution of R-O_2-CHI-SWCNTs in PBS at pH 7.4, with final concentrations of 1.5 mg/ml FA, 1.2 mg/ml EDC and 1 mg/ml CHI-SWCNTs. The mixture was allowed to react overnight at room temperature in the dark. The solution was subsequently dialyzed three times to ensure complete excess removal of unconjugated FA and EDC, and subsequently dried at 30˚C. In this way, R-O_2-FA-CHI-SWCNTs were obtained. Ultrapure water was used as a solvent in the experiment.

Characterization of R-O_2-FA-CHI-SWCNT. X-ray photoelectron spectroscopy (XPS) to characterize the newly synthesized material was performed on a PHI Quantera II Scanning XPS Microprobe with an aluminium anode (Physical Electronics, Inc., Chanhassen, MN, USA). Low resolution survey scans (pass energy, 280 eV; time per step, 100 ms) were performed to determine the elemental composition of the CNTs, and high resolution element scans (pass energy, 26 eV; time per step, 500 ms) were conducted to the obtain bonding information of each element. The UV-visible absorption spectral measurements were performed on the Lambda 17 UV-vis 8500 spectrometer (PerkinElmer, Inc., Waltham, MA, USA) with a 1-cm pathlength quartz cuvette. Stock solution (10 mg/ml) of each sample was prepared in water. The spectra were recorded at 20°C.

Cell lines and cell culture. Human breast cancer cell lines MDA-MB-231 and ZR-75-1 were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in an environment of 5% CO_2 at 37˚C in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1%

100 U penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 U streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated under normoxic (21% O_2 and 5% CO_2) or hypoxic (1% O_2 and 5% CO_2) conditions. When each cell type had grown to 80% confluence, the cells were washed three times with PBS (10 mM sodium phosphate buffer, pH 7.2; GE Healthcare Life Sciences, Logan, UT, USA), and incubated at 37˚C with 0.25% trypsin-EDTA until they dissociated from the flask, centrifuged at 177 x *g* for 5 min at room temperature and resuspended in fresh media. The resuspended pellet was passaged at a ratio of 1:3 into a new flask.

Treatment with chemotherapy drugs and irradiation procedure. A total of five frequently-used chemotherapy drugs in clinical practice of treating breast cancer: 5-fluorouracil, epirubicin, pirarubicin, paclitaxel, docetaxel and carboplatin, were used at the given concentrations offered by the pharmaceutical department of XinHua Hospital (Shanghai, China; 3 mg/ml 5-fluorouracil; 0.5 mg/ml epirubicin; 0.08 mg/ml pirarubicin; 0.46 mg/ml paclitaxel; and 1.0 mg/ml carboplatin). Irradiation was performed at room temperature with single doses of X-rays ranging from 2 to 8 Gy, using a linear accelerator with 6 MeV photons/100 cm focus-surface distance, with a dose rate of 2.0 Gy/min.

Cell viability assay. MDA-MB-231 and ZR-75-1 cells were seeded into 96-well culture plates (Corning Incorporated, Corning, NY, USA) at 5,000 cells/well. After culturing overnight, the cells were washed with FBS-free RPMI-1640. MDA-MB-231 and ZR-75-1 cells were cultured with ordinary medium or FA-free medium and divided into the following groups: Blank control group, R-O_2-FA-CHI-SWCNTs-treated group, chemotherapy-group and R-O_2-FA-CHI-SWCNTs-chemotherapy group under hypoxic conditions. The control cells were incubated with ultrapure water instead of drug. The treatment group cells were incubated with R-O_2-FA-CHI-SWCNTs, chemotherapy drugs or R-O_2-FA-CHI-SWCNTs plus chemotherapy drugs for 48 h at 37˚C.

Subsequently, the cells were washed three times with PBS and FBS-free RPMI-1640 (100 μl) was used to substitute the culture medium. A total of 10 μl water-soluble tetrazolium salts-1 (WST-1) reagent (Roche Diagnostics, Indianapolis, IN, USA) was added to each well and incubated for an additional 2.5 h at 37˚C. The plates were read at 450 nM using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed independently at least three times.

Colony forming assay. The survival and proliferation potential of cells treated with R-O_2-FA-CHI-SWCNTs and/or ionizing radiation was assessed by colony forming assay. Initially, exponentially growing cells in 6-well plates were irradiated (0, 2, 4, 6 or 8 Gy) following incubation with or without R-O_2-FA-CHI-SWCNTs under hypoxic conditions for 48 h at 37˚C. Following irradiation, the cells were washed with PBS twice and trypsinized, suspended in complete medium, counted, diluted serially to appropriate densities and re-plated in new 6-well culture plates, allowing the formation of macroscopic colonies. Following incubation at 37˚C for 14-21 days, cells were fixed with methanol, and stained with Giemsa. Colonies containing >50 cells were counted. The plating efficiency (PE) and surviving fraction (SF) were calculated as follows: PE (%) = (colony number / inoculating cell number) x 100; SF (%) = PE (tested group) / PE (0-Gy group) x 100. The cell-survival curve was plotted with GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA), using the linear-quadratic formula $SF = \exp[-(\alpha D + \beta D^2)]$, where α and β describe survival curve characteristics that classify cellular response to radiation, and D indicates the dose of radiation. The sensitization enhancement ratio (SER) was calculated as follows: SER = SF2 (tested group) / SF2 (0 Gy group).

Cell apoptosis assay. MDA-MB-231 and ZR-75-1 cells were seeded into 6-well dishes (Corning Incorporated) overnight. For the chemotherapy experiment, cells were treated with chemotherapy drugs at the given concentration, R-O_2-FA-CHI-SWCNTs or chemotherapy drugs and R-O_2-FA-CHI-SWCNTs for 48 h under hypoxic conditions at 37˚C. For the radiotherapy experiment, cells were incubated in hypoxic conditions with or without R-O_2-FA-CHI-SWCNTs for 48 h at 37˚C, and treated with radiotherapy (4 Gy), followed by incubation for another 24 h at 37˚C.

Cells were collected and double-stained for cell apoptosis and death detection. The apoptotic cells were stained by Annexin V, while the necrotic cells were stained with propidium iodide (PI). The Annexin V and PI staining was carried out by using an Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The cells were suspended in binding buffer with a cell concentration of ~10^6 cells/ml after they were harvested and washed with cold PBS buffer. Subsequently, 5 μl Annexin V and 1 μl PI were added into a 100 μl suspension of cells, and stained for 15 min at room temperature. Following staining, 400 μl binding buffer was added into the above 100 μl cell suspension. Finally, the stained cells were analyzed by fluorescence-activated cell sorting (BD LSR II; BD Biosciences).

Protein extraction and western blot analysis. Total protein extraction was performed as described below. Cells were washed twice with ice-cold PBS. Total cell lysate was prepared using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) (v/v, 100:1). Subsequently, lysate was centrifuged at 177 x *g* for 10 min at 4˚C to collect the supernatant. Protein lysate concentrations were determined by the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology).

Western blotting reagents (Beyotime Institute of Biotechnology) were used as received. To quantify protein levels, equal amounts of protein (40 μg) were subjected to 10 or 15% SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane, which was then exposed to 5% non-fat dried milk in TBS containing 0.1% Tween 20 (0.1% TBST) for 1 h at room temperature, and incubated overnight at 4˚C with antibodies against B-cell lymphoma 2 (Bcl-2; 1:500; E17; Abcam, Cambridge, UK), hypoxia-inducible factor 1-α (HIF-1α; 1:2,000; EP1215Y; Abcam), survivin (1:5,000; EP2880Y; Abcam), Ku80 (1:1,000; EPR3468; Abcam),

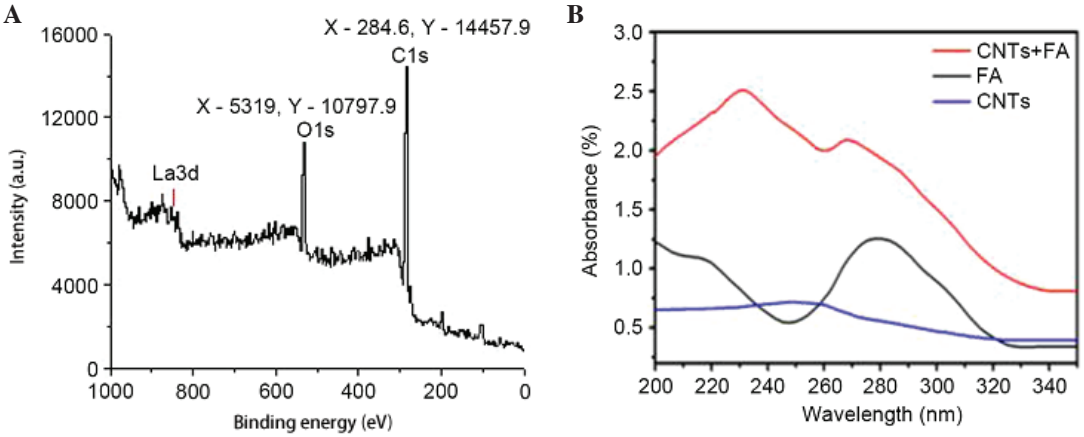

Figure 1. (A) X-ray photoelectron spectroscopy analysis of solutions of R-O_2-SWCNTs. (B) Ultraviolet-visible spectra of solutions of R-O_2-SWCNTs, free FA, and R-O_2-FA-CHI-SWCNTs. R-O_2-SWCNTs, tombarthite-modified-single-walled carbon nanotubes; FA, folic acid; CHI, chitosan.

P-glycoprotein (P-gp; 1:1,000; EPR10363; Abcam), multidrug resistance-associated protein 1 (MRP-1; 1:500; MRPm5; Abcam), RAD51 (1:10,000; EPR4030 (3); Abcam) and β-actin (1:1,000; AC-74; Beyotime Institute of Biotechnology). The membranes were subsequently washed with 0.1% TBST prior to incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:1,000; A0208; Beyotime Institute of Biotechnology) or -mouse (1:1,000, A0216; Beyotime Institute of Biotechnology) secondary antibodies. Immune complexes were detected with chemiluminescence reagents and measured using ECL Plus (Bio-Rad Laboratories, Inc., Hercules, CA). Band intensities were quantified using ImageJ software, version 2.1.4.7 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS version 20 (IBM SPSS, Armonk, NY, USA). Data are presented as the mean ± standard deviation of the results from three or four independent experiments. Statistical comparisons were performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Synthesis of R-O_2-FA-CHI-SWCNTs. The starting materials were oxygen R-O_2-SWCNTs, which were synthesized by the School of Mechanical and Power Engineering, Shanghai Jiaotong University. Fig. 1A shows the results of XPS analysis which confirmed the conjugation with O_2 and R. Subsequently, R-O_2-SWCNTs were functionalized with CHI and FA. The final product was denoted as R-O_2-FA-CHI-SWCNT. As shown in Fig. 1B, the conjugation with FA was confirmed by examining the ultraviolet visible (UV-vis) absorption spectra. The absorbance peaks at 280 nm in UV-vis spectra corresponded to the characteristic peaks of FA.

Cytotoxicity of R-O_2-FA-CHI-SWCNTs. The toxicity of SWCNTs was has been widely discussed and disputed in published articles (24-26). In the present study, the effect of R-O_2-FA-CHI-SWCNTs on the proliferation of MDA-MB-231 and ZR-75-1 cells was determined by WST-1 assay. Fig. 2 shows the results of WST-1 assays on the proliferation ratio of MDA-MB-231 and ZR-75-1 cells following treatment with R-O_2-FA-CHI-SWCNTs at concentrations from 5-500 μg/ml for 48 h. No significant inhibition

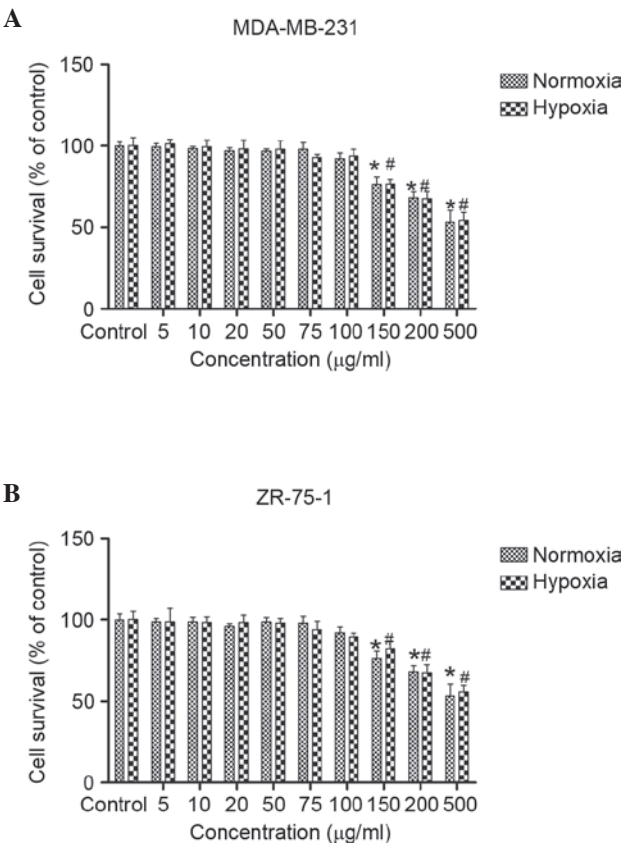

Figure 2. Effects of R-O_2-FA-CHI-SWCNTs at various doses on the viability of MDA-MB-231 and ZR-75-1 cells under normoxic and hypoxic conditions. (A) MDA-MB-231 cells were incubated with R-O_2-FA-CHI-SWCNTs at concentrations from 5-500 μg/ml for 48 h. The number of viable cells was measured by WST-1 assay. *P<0.05 vs. control group in normoxic conditions. #P<0.05 vs. control group in hypoxic conditions. (B) ZR-75-1 cells were incubated with treatment R-O_2-FA-CHI-SWCNTs at concentrations from 5-500 μg/ml for 48 h. The number of viable cells was measured by WST-1 assay. *P<0.05 vs. control group in normoxic conditions. #P<0.05 vs. control group in hypoxic conditions. Data are presented as the mean ± standard deviation. R-O_2-SWCNTs, tombarthite-modified-single-walled carbon nanotubes; FA, folic acid; CHI, chitosan; WST-1, water-soluble tetrazolium salts-1.

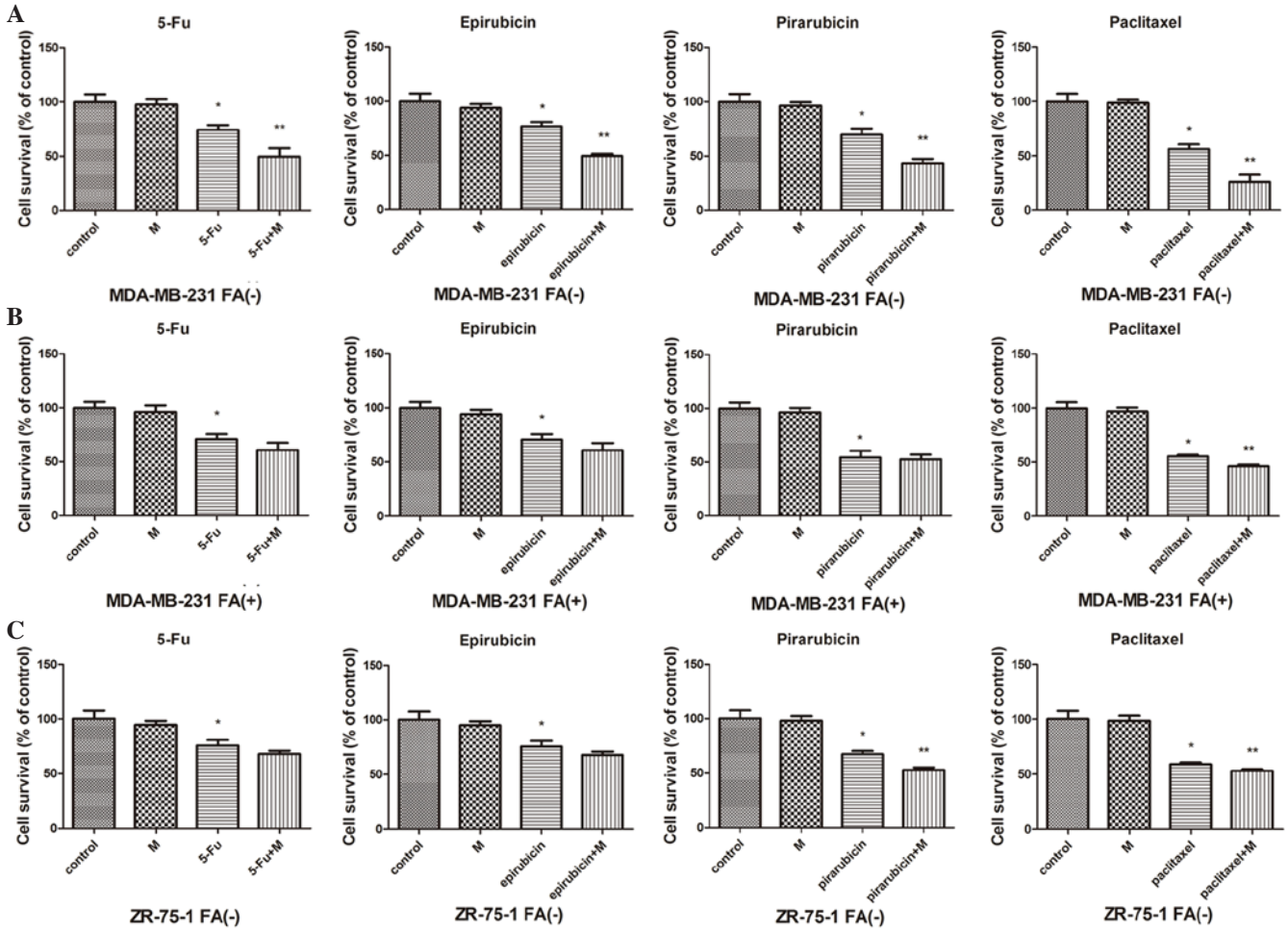

Figure 3. Cell survival rates of MDA-MB-231 and ZR-75-1 cells cultured with or without FA. The cells were divided into four groups: Control, M, chemotherapeutic drug (5-Fu, pirarubicin, epirubicin or paclitaxel) and chemotherapeutic drug + M groups. Cell survival was determined using water-soluble tetrazolium salts-1 assays. (A) FR-positive MDA-MB-231 cells cultured in FA-free medium. (B) MDA-MB-231 cells cultured in FA-containing medium. (C) FR-negative ZR-75-1 cells. Each value represents the mean ± standard deviation (n=3). *P<0.05 vs. control group. **P<0.05 vs. chemotherapy group. R-O_2-FA-CHI-SWCNTs is denoted as M in the figure. R-O_2-SWCNTs, tombarthite-modified-single-walled carbon nanotubes; FA, folic acid; FR, FA receptor; CHI, chitosan; 5-Fu, fluorouracil.

of cell proliferation was observed when the concentration of R-O_2-FA-CHI-SWCNTs was <100 μg/ml, indicating no clear cellular toxicity of the novel functionalized CNTs below a certain dose. However, the cell viability was significantly inhibited by R-O_2-FA-CHI-SWCNTs when the concentration of the material was >150 μg/ml (P<0.05). The toxic effect of the synthesized material at various doses indicated a concentration threshold of viability inhibition at 100-150 μg/ml. Accordingly, 100 μg/ml concentration of R-O_2-FA-CHI-SWCNTs was used for further experiments to identify the chemotherapy and radiotherapy sensitizing effect.

R-O_2-FA-CHI-SWCNTs increase the sensitivity of cells to chemotherapy. In the present study, the anticancer effects of chemotherapy or chemotherapy and R-O_2-FA-CHI-SWCNTs combined treatment were determined by the WST-1 assay on two human breast carcinoma cell lines: MDA-MB-231 (which harbor FA receptor) and ZR-75-1 (which are FA receptor negative). As shown in Fig. 3, treatment with R-O_2-FA-CHI-SWCNTs and chemotherapy reduced the survival of MDA-MB-231 cells in FA-free medium significantly compared to chemotherapy alone cells (P<0.05). In MDA-MB-231 cells in medium containing FA, treatment with R-O_2-FA-CHI-SWCNTs and paclitaxel caused a reduction of cell survival significantly compared to treatment with paclitaxel alone (P<0.05), and caused a modest but not significant reduction in the other chemotherapy drug groups. In ZR-75-1 cells, a significant reduction in cell survival caused by adding R-O_2-FA-CHI-SWCNTs was observed in the pirarubicin and paclitaxel groups (P<0.05); however, the effect of R-O_2-FA-CHI-SWCNTs was modest but not significant in the remaining two chemotherapy drug groups.

An Annexin V-fluorescein isothiocyanate (FITC)/PI experiment was performed to further investigate the effect of sensitization from R-O_2-FA-CHI-SWCNTs in hypoxic conditions. As shown in Fig. 4, the apoptotic rate was 39.0±1.31% when treated alone with carboplatin, and that increased to 52.5±2.21% when treated with carboplatin and R-O_2-FA-CHI-SWCNTs in combination. As R-O_2-FA-CHI-SWCNT treatment alone had no effect on apoptosis in MDA-MB-231 cells, it appears that R-O_2-FA-CHI-SWCNTs sensitizes the breast cancer cell lines to chemotherapy.

Table I. Radiation response variables of MDA-MB-231 and ZR-75-1 cells following combined treatment with M.

Cell line treatment	α	β	α/β	SER
MDA-MB-231 FA (-)				
IR	0.03823	0.01486	2.57	
IR+M	0.08692	0.02028	4.29	1.11
MDA-MB-231 FA (+)				
IR	0.03234	0.01372	2.36	
IR+M	0.04842	0.01857	2.61	1.02
ZR-75-1				
IR	0.02944	0.01281	2.30	
IR+M	0.04587	0.01587	2.89	1.03

SER, sensitization enhancement ratio; FA, folic acid; IR, irradiation; M, tombarthite-modified-folic acid-chitosan-single-walled carbon nanotubes; α, dsDNA break caused by a single hit (irreparable); β, dsDNA break caused by a double hit (repairable).

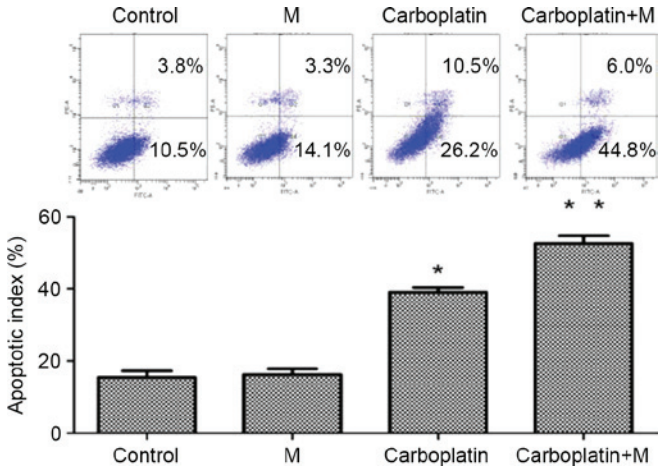

Figure 4. Cell apoptosis and death detected by flow cytometry. Untreated MDA-MB-231 cells under hypoxic conditions served as a control group. MDA-MB-231 cells were incubated with 100 μg/ml R-O_2-FA-CHI-SWCNTs under hypoxic conditions. MDA-MB-231 cells were incubated with carboplatin under hypoxic conditions. MDA-MB-231 cells were incubated with carboplatin plus 100 μg/ml R-O_2-FA-CHI-SWCNTs under hypoxic conditions. The populations in quadrants 2 and 4 indicate the percentage of necrotic and early-apoptotic cells, respectively. R-O_2-FA-CHI-SWCNTs are denoted as M in the figure. Data in the graph are presented as the mean ± standard deviation. R-O_2-FA-CHI-SWCNTs, tombarthite-modified-folic acid-chitosan-single-walled carbon nanotubes. *P<0.05, **P<0.01.

R-O_2-FA-CHI-SWCNTs increase the sensitivity of cells to radiotherapy. To investigate whether R-O_2-FA-CHI-SWCNTs modulated the response of MDA-MB-231 and ZR-75-1 cells to radiation, 100 μg/ml R-O_2-FA-CHI-SWCNTs was added to cells 30 min prior to irradiation. As shown in Fig. 5, pre-incubation with R-O_2-FA-CHI-SWCNTs reduced clonogenic survival in comparison to irradiated controls. As shown in Table I, the SER values for MDA-MB-231 cells in FA-free medium was 1.11; the SER values for MDA-MB-231 cells in medium containing FA was 1.02, and the SER values for ZR-75-1 cells was 1.03.

To further assess the effect of R-O_2-FA-CHI-SWCNTs on the radiotherapy sensitivity of MDA-MB-231 cell lines, an

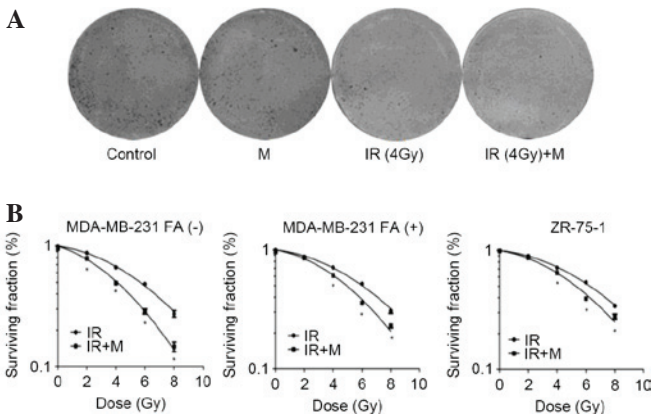

Figure 5. (A) MDA-MB-231 cells in FA-free medium were treated with R-O_2-FA-CHI-SWCNTs, irradiation (4 Gy) or a combination of R-O_2-FA-CHI-SWCNTs and irradiation (4 Gy), respectively. Control cells were untreated. Colony forming assays for these four groups are shown. (B) Survival curves with SF normalized to the plating efficiency were fitted according to the linear quadratic equation: SF=exp[-(αD+βD2)]. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, vs. irradiation treated cells. R-O_2-FA-CHI-SWCNTs are denoted as M in the figure. FA, folic acid; R-O_2-SWCNTs, tombarthite-modified-single-walled carbon nanotubes; CHI, chitosan; SF, surviving fractions; D, dose; IR, irradiation.

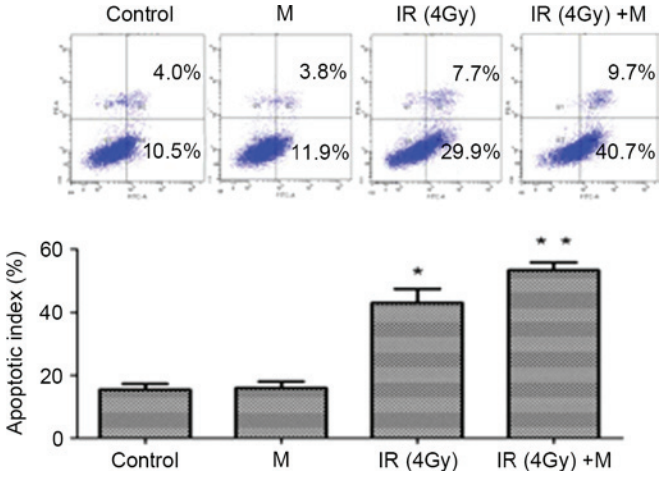

Figure 6. Cell apoptosis and death detected by flow cytometry. Untreated MDA-MB-231 cells under hypoxic conditions served as a control group. MDA-MB-231 cells were incubated with 100 μg/ml R-O_2-FA-CHI-SWCNTs under hypoxic conditions. MDA-MB-231 cells were irridiated with 4 Gy under hypoxic conditions. MDA-MB-231 cells were irradiated with 4 Gy plus 100 μg/ml R-O_2-FA-CHI-SWCNTs under hypoxic conditions. The populations in quadrants 2 and 4 indicate the percent of necrotic and early-apoptotic cells. R-O_2-FA-CHI-SWCNTs are denoted as M in the figure. R-O_2-FA-CHI-SWCNTs, tombarthite-modified-folic acid-chitosan-single-walled carbon nanotubes; IR, irradiation. *P<0.05, **P<0.01.

Annexin V-FITC/PI experiment was performed. As shown in Fig. 6, the apoptotic rate of MDA-MB-23 cells was 43.1±4.36% at an irradiation dose of 4 Gy. R-O_2-FA-CHI-SWCNTs increased the apoptotic rate of irradiated (4 Gy) MDA-MB-231 cells to 53.4±2.41% (P<0.05).

Effect of R-O_2-FA-CHI-SWCNTs on proteins associated with apoptosis, and chemotherapy and radiotherapy sensitivity in MDA-MB-231 cells. To investigate the proteins involved in the sensitizing effect of R-O_2-FA-CHI-SWCNTs administrated

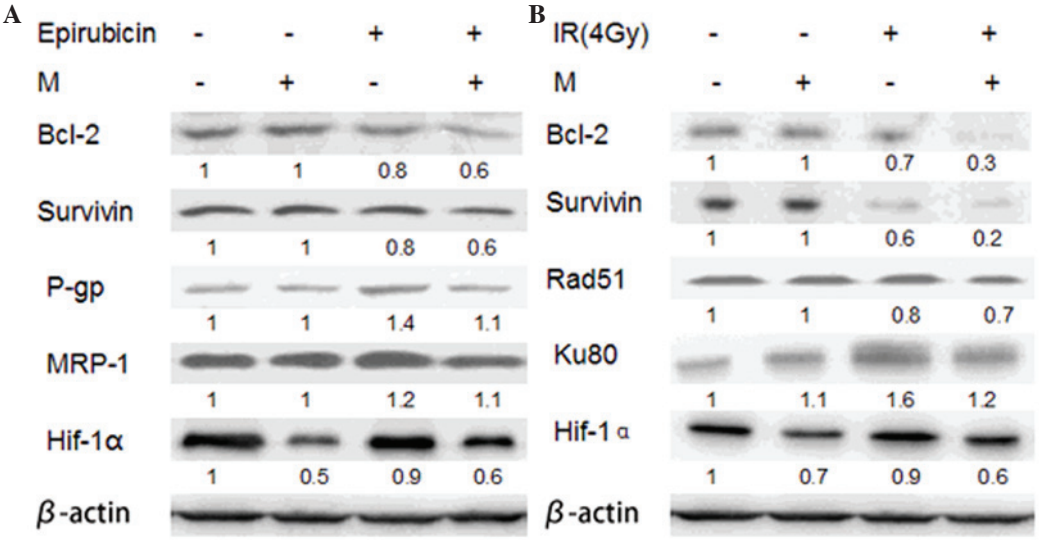

Figure 7. (A) Epirubicin group, western blot analysis was performed against Bcl-2, survivin, P-gp, MRP-1 and HIF-1α. β-actin was used as a loading control. (B) Radiotherapy group, western blot analysis was performed against Bcl-2, survivin, RAD51, Ku80 and HIF-1α. β-actin was used as a loading control. M, tombarthite-modified-folic acid-chitosan-single-walled carbon nanotubes; IR, irradiation; Bcl-2, B-cell lymphoma 2; P-gp, P-glycoprotein; MRP-1, multidrug resistance-associated protein 1; Hif-1α, Hypoxia-inducible factor 1-α.

in combination with epirubicin or radiotherapy (4 Gy) on MDA-MB-231 cells, the expression of certain proteins were determined by western blotting.

As illustrated in Fig. 7, treatment with R-O_2-FA-CHI-SWCNTs in combination with epirubicin or radiotherapy resulted in a decrease in Bcl-2 and survivin levels, which may be partially responsible for the apoptotic tendency, and a decrease of HIF-1α levels, which indicated the amelioration of hypoxic status. The levels of P-gp and MRP-1, which are associated with chemoresistance, and the expression of RAD51 and Ku80, which are associated with radioresistance, was downregulated.

Discussion

Breast cancer poses a major challenge to the health of women worldwide. The modalities of treatment include surgery, chemotherapy, and hormonal and radiation therapies (1). A significant cause of breast cancer treatment failure is the cancer cells becoming resistant to drugs or radiation, leading to progression of the tumor into an invasive and metastatic phenotype (27). The inability of chemotherapy or radiotherapy to totally eradicate tumors may be due to the presence of hypoxic cells (28-30). Therefore, oxygen is extremely important for effective anticancer chemotherapy and radiotherapy.

The utilization of nanomedicine in oncology has drawn attention in recent years, particularly in the field of breast cancer research (31-33). Efforts have been made to apply nanomedicine in cancer treatment to improve the efficiency of anticancer regimens (17,18,34,35). In the present study, SWCNTs were used as the carrier of oxygen, which was expected to increase the sensitivity of cancer cells to chemotherapy and radiotherapy.

FA receptor (FR) is a glycosylphosphatidylinositol-linked membrane glycoprotein and is overexpressed on the surfaces of numerous cancer cells, including breast cancer cells; it is almost absent in the majority of normal tissues (36-38). FA is internalized into the cytoplasm via FR-mediated endocytosis (37-39). Functionalization of R-O_2-SWCNTs with FA may target this receptor specifically. In the present study, a novel oxygen carrier based on single-walled carbon nanotubes was successfully synthesized, which was subsequently shown to have characteristics of targeted and good dispersibility (40).

The WST-1 assay and cell colony formation assay in the present experiment revealed that administration of R-O_2-FA-CHI-SWCNTs plus chemotherapy or radiotherapy yielded increased inhibition of cell proliferation compared with chemotherapy or radiotherapy alone, and the inhibition was more marked and the sensitivity enhancement rate was the highest in MDA-MB-231 cells in FA-free medium. As ZR-75-1 cells are FR-negative, and MDA-MB-231 cells are FR-positive, FA in the medium binds with FR, so the FR was partly blocked and could not bind with the synthesized material. Therefore, it may be assumed that R-O_2-FA-CHI-SWCNTs release O_2, which spreads to the cancer cells through free diffusion. The enhanced inhibition of cell proliferation in MDA-MB-231 cells in FA-free medium suggests that R-O_2-FA-CHI-SWCNTs are able to supply oxygen to cancer cells through the free diffusion of oxygen, as well as the binding of FA to FR.

Flow cytometry in the present study confirmed that R-O_2-FA-CHI-SWCNTs plus chemotherapy or radiotherapy induced an increased rate of cell apoptosis compared to chemotherapy or radiotherapy alone.

Bcl-2 is an anti-apoptotic protein, and has a significant role in regulating cell apoptosis (41,42). Bcl-2 interacts indirectly with Bcl-2-like protein 4 and prevents caspase activation, including caspase-9 (43,44). Overexpression of Bcl-2 is a significant pathway of resistance in treating cancer with chemotherapy and radiotherapy (45). Survivin is an inhibitor of apoptosis protein, and is poorly expressed in normal breast tissue and overexpressed in neoplastic breast tissue (46). Therefore, downregulation of Bcl-2 and survivin may be a potential strategy for breast cancer therapy. In

the present study, under hypoxic conditions, the addition of R-O_2-FA-CHI-SWCNTs may have inhibited Bcl-2 and survivin, which demonstrates its effect of increasing sensitivity to chemoradiotherapy.

Under hypoxic conditions, breast cancer cells must adapt to exist in the microenvironment, and the expression of HIF-1α is elevated (47,48). In the present experiment, the administration of R-O_2-FA-CHI-SWCNTs downregulated the expression of HIF-1α, which indicated that the hypoxic conditions and chemoradiotherapy resistance were alleviated. Furthermore, the decrease of MRP-1 and P-gp expression caused by adding R-O_2-FA-CHI-SWCNTs may potentially reduce tumor resistance to chemotherapy.

In radiotherapy, DNA damage is created by direct ionization from radiation, or is induced by interaction with oxygen centered radicals that are formed by the ionization of water surrounding DNA (49-51). Unrepaired DNA double-strand breaks (DSBs) may lead to fatal changes, including chromosomal aberrations (52). In the absence of molecular oxygen, the damage is more repairable, as oxygen is able to react with the broken ends of DNA, making them less easily repaired by a cell (53,54). There are two major signaling pathways to repair potentially lethal DNA DSBs: Homologous recombination (HR) and nonhomologous DNA end joining (NHEJ) (55). RAD51 is a central player in the HR-mediated repair of DSBs (56), and Ku80 is one of the important factors that can function to mediate NHEJ (56). Based on the results of the present study, which identified decreased levels of RAD51 and Ku80 proteins, it may be hypothesized that radiosensitivity is increased with R-O_2-FA-CHI-SWCNTs administration, and the radiosensitizing effect may involve impairment of DSBs repair.

In conclusion, the present study successfully synthesized a novel functionalized SWCNT, which was denoted as R-O_2-FA-CHI-SWCNTs, and the results of the present study suggest that administration of R-O_2-FA-CHI-SWCNTs sensitizes human breast cancer cells to chemotherapy and radiotherapy by alleviating hypoxic conditions, leading to increased rates of proliferation inhibition and apoptosis of breast cancer cells. Apoptosis associated proteins (Bcl-2 and survivin), proteins indicating chemosensitivity (MRP-1 and P-gp), hypoxia associated protein HIF-1α and proteins involved in DSB repair in radiotherapy (RAD51 and Ku80) were all downregulated. Animal experiments are required in order to confirm the results of the present study. Further experiments in different cell lines are required to investigate if the novel synthesized R-O_2-FA-CHI-SWCNTs are an effective chemoradiotherapy sensitizer.

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