PO_2-based biodosimetry evaluation using an EPR technique acts as a sensitive index for chemotherapy

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Abstract. The partial pressure of oxygen (PO_2) in the tumor microenvironment directly affects tumor sensitivity to chemotherapy. In the present study, a lithium phthalocyanine probe was implanted into MCF-7 human breast cancer cells, followed by transplant of the cells into nude mice. The present study used an electron paramagnetic resonance (EPR) oximetry measuring technique to dynamically monitor PO_2 in the tumor microenvironment prior to and following chemotherapy, and aimed to determine the precise time window in which the microenvironmental PO_2 peaked following chemotherapy. The results indicated that PO_2 was significantly higher in breast cancer compared with control ($P<0.05$). Following four cycles of chemotherapy, the activity of NADH dehydrogenase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase in the mitochondria of cells was significantly reduced when compared with their activity prior to chemotherapy ($P<0.05$). Regional blood flow in tumor tissues undergoing chemotherapy was significantly lower than that prior to chemotherapy ($P<0.05$). The rate of cellular apoptosis in the PO_2 peak-based chemotherapy group was significantly greater than that in the conventional chemotherapy group after two and four cycles of chemotherapy ($P<0.05$). Tumor volume in the PO_2 peak-based chemotherapy group was significantly reduced compared with that in the 0.9% NaCl solution control and the conventional chemotherapy groups after four cycles of chemotherapy ($P<0.05$). The tumor inhibitory rate of the experimental group was significantly higher than that of the conventional chemotherapy group ($P<0.01$). In conclusion, the present study may provide guidance for the development of effective strategies depending on tumor-maximal response to chemotherapy in an oxygen-rich environment. Additionally, the present study aimed to establish a foundation for a clinical noninvasive assessment intended to guide treatment and formulate individual regimens, in order to improve cancer therapeutics, sensitivity monitoring and curative effect estimation.

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Introduction

Malignant tumors are a major class of diseases that threaten human health and life. Chemotherapy is an important component in many comprehensive therapeutic regimens currently available for various types of cancer (1). Insensitivity or resistance of tumor cells to chemotherapeutic agents is a common occurrence and often leads to treatment failure (2). Additionally, hypoxia is a key feature of the solid tumor microenvironment (3). It is well known that hypoxic tumor microenvironments are associated with a higher invasive ability and the occurrence of distal metastases, that they can increase the resistance of tumor cells to chemotherapy, and may result in reduced chemotherapeutic efficacy (4,5). Therefore, tumor chemoresistance under hypoxic microenvironments has become a popular research topic.

Frequently, studies pertaining to tumor resistance to chemotherapy focus on tumor cell intrinsic factors, including the elevated expression of multiple drug resistance-associated proteins, or the activation of cell injury repair-associated genes (6,7). It has been demonstrated that tumor microenvironments serve a critical role in the mediation of acquired resistance (8,9). The antitumor effects of numerous chemotherapeutic agents primarily depend on the partial pressure of oxygen (PO_2) of the tumor microenvironment (8,9). Tumor-cell hypoxia leads to increased production of nucleophiles, including glutathione. Tumor cells then compete with the alkylating group for common target DNA, resulting in reduced sensitivity to chemotherapy (10,11). Under aerobic environments, the semiquinone free radicals of doxorubicin activate the generation of superoxide compounds (12). The superoxide compounds produce hydrogen peroxide through the disproportionation reaction and are degraded to oxygen free radicals, which cause oxidative stress injury and DNA splitting to the cell membranes (13). It has been reported that the hypoxic environment promotes pancreatic cancer cell proliferation, induces hypoxia-inducible factor-1α (HIF-1α) expression and increases gemcitabine resistance (9).

Additionally, the survial time of pancreatic cancer is shorter in patients with a strong HIF-1α expression compared with that in those with a weak HIF-1α expression (9). A previous study revealed that the survival time of patients with PO_2 values >0.33 mmHg was longer than that in those with PO_2 values <0.33 mmHg (P=0.05) (14). Additionally, single factor analysis and multivariate analysis revealed that the tumor PO_2 was the only significant prognostic factor in 41 patients with squamous cell carcinoma of the head and neck (14).

The measurement of tumor oxygen tension using a PO_2 microelectrode in tumor models revealed that the PO_2 in tumor microenvironments was significantly lower compared with the controls following treatment with multiple chemotherapeutic drugs (15,16). The cytotoxic effects of chemotherapeutic drugs for breast cancer are affected by the PO_2 in tumor microenvironments (17,18).

Currently, techniques used to directly measure intracellular and tissue PO_2, inlcude tumor vessel analysis, determination of tumor metabolism, determination of DNA damage in tumor cells, measurement of intra-tumor oxygen tension, detection of radioactive hypoxia markers or endogenous biological hypoxia markers, Oswald's magnetic resonance imaging (MRI) method, single-photon emission computed tomography (SPECT) and positron emission tomography (PET) (19). However, all of these techniques have limitations, including invasiveness, narrow measurement range and low temporal and spatial resolution (20). The oxygen electrode method has previously been considered to be the 'gold standard' for the detection of tumor oxygen partial pressure (21). However, the oxygen electrode method is only suitable for superficial tumors, and cannot be adapted for long-term monitoring due to its invasive nature, which limits its applications in a clinical setting (21). The electron paramagnetic resonance (EPR) technique provides a possibility to achieve non-invasive, real-time and accurate detection of dynamic changes of PO_2 in solid tumor microenvironments during the growth and treatment of solid tumors (22). The EPR technique provides important technical support for the *in vivo* measurement of PO_2 in physiological and pathological tissues and organs, and it has been widely applied in basic and clinical studies (23,24). Our previous study preliminarily measured the tumor PO_2 in mice transplanted with human breast cancer cells using the EPR technique, and validated hypoxia as a characteristic of breast cancer tumors in animal models. Hypoxia within the tumor microenvironment is an important factor in chemotherapy resistance (4,5). Therefore, measurement of the dynamic changes of the PO_2 during tumor physiology and treatment using the EPR technique may provide scientific evidence for identifying the oxygen-enriched environment and selecting an optimal time window for treatment.

The present study was designed in a nude mouse model for transplantation with the human breast cancer cell line, MCF-7. The biological characteristics of PO_2 in tumor microenvironments were investigated after chemotherapy was administered using the EPR technique. The present study also aimed to select the optimum time window to conduct anti-tumor therapy. The following were hypothesized: i) The EPR technique may be used to monitor changes in PO_2 in tumor microenvironments during the growth and treatment of transplanted tumors derived from the human breast cancer cell line, MCF-7, in nude mice; ii) determination of the timing of the peak PO_2 in tumor microenvironments following chemotherapy would facilitate the identification of the time window to achieve the maximal chemotherapy efficacy in an oxygen-enriched environment; and iii) the present study may provide a safer, simpler, and more effective approach for tumor treatment, monitoring of the sensitivity of the chemotherapeutic drugs, and assessment of chemotherapeutic efficacy. Therefore, the present study may provide novel strategies for personalized chemotherapy of cancer.

Materials and methods

Cells and animals. The breast cancer cell line, MCF-7, was purchased from the American Model Culture Preservation (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 5% CO_2 and 37˚C. 100 BALB/c nu/nu 4-week old female nude mice, weighing 16-20 g were purchased from the Experimental Animal Center of Medical University of Chongqing [Chongqing, China; experimental animal production license number, SCXK (Yu) 2012-0001]. All nude mice were quarantined for 1 week prior to the start of the experiment. Mice were housed in an animal facility maintained on a 12/12 h light/dark cycle, at a constant temperature of 23±1˚C and relative humidity of 44±5%, and were given free access to water and food. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the present study was approved by the Ethics Committee of Medical University of Chongqing (Chongqing, China). The mice were sacrificed by exsanguination under deep isoflurane anesthesia (1.5%).

Establishment of animal tumor models with human breast cancer cells and trial grouping. The nude mice were randomly assigned into a control group and an experimental group (20 mice/group). Human MCF-7 cells in the log-phase of growth were prepared for cell suspension. Cell suspension (0.2 ml; $1x10^7$ cells) were subcutaneously injected into the nude mice as experimental group or with saline (0.2 ml) as a control group, and a lithium phthalocyanine (LiPc) probe (gifted by Professor He Guanglong, the Ohio State University, Columbus, OH, USA) was implanted following tumor formation (2 weeks after transplantation; tumor size, ≥3 mm) in all groups. No other treatment was administered. Normal breast tissues of nude mice served as negative controls. The treatment protocol for the experimental group was as follows: Mice in the doxorubicin (AMQUAR, Shanghai, China)-treatment group were subcutaneously injected with $1x10^7$ MCF-7 cells, and an LiPc probe was implanted following tumor formation (2 weeks after transplantation; tumor size, ≥3 mm). Doxorubicin was administered by intraperitoneal injection at a dose of 2 mg/kg when the tumor PO_2 peaked following transplantation. The mice in the experimental group were further divided into the PO_2 peak-based chemotherapy group and the conventional chemotherapy group (10 mice/group). Mice in the PO_2 peak-based chemotherapy group were intraperitoneally injected with

doxorubicin when the tumor PO_2 had peaked. A total of 4 cycles of chemotherapy; were intraperitoneally injected into mice with doxorubicin in the conventional chemotherapy group as control once a week; mice in the normal saline group received an equal volume of normal saline. In the present study, mice were anesthetized with an intraperitoneal injection of 55 mg/kg ketamine and a combination of tiotropium (15 mg/kg) for EPR measurement. During the anesthesia, the mice were closely and regularly (every 15 min during anesthesia) monitored for deep pain recognition (firm toe pinch for pedal reflex response), major hemodynamic and other vital parameters, such as mucous membranes (to be pink and moist) and respiratory rate (regular and between 80 and 200), in order to adjust anesthetic delivery as appropriate to maintain surgical plane.

In vivo measurement of PO_2 in transplanted tumor microenvironments using the EPR technique. For the measurement of tumor tissue PO_2 *in vivo*, ~10 μg of LiPc was loaded in a 27-gauge needle and implanted in the tumor tissue following tumor exposure. The mouse was then transferred to the L-band EPR spectrometer (Magnettech GmbH, Berlin, Germany), and after 30 min equilibration of the probe with the surrounding tissue, the following parameters of EPR spectra were collected: frequency (1.1 GHz), microwave power (16 mW) and modulation amplitude (0.045 G). The sensitivity of the probe to tissue PO_2 is 5.8 mG/mm Hg.

Apoptosis of transplanted tumor cells determined using flow cytometry. A total of 10 mice from each group were sacrificed by exsanguination under deep isoflurane anesthesia (1.5%). The mice treated with doxorubicin or 0.9% NaCl were sacrificed at the following time points: Prior to treatment and 2 and 4 times following chemotherapy. The tumor tissues were collected using a mechanical dispersion method and enzyme assisted digestion method: Tissues were cut into small pieces, washed with cold PBS, and then trypsinized to produce single cells as described previously (25). After filtration through mesh, quantitative detection was performed using a Cyan FACScan and Summit version 4.3 software (DakoCytomation, Agilent Technologies, Inc., Santa Clara, CA, USA). The single-cell suspension was adjusted to a concentration of $1x10^5/mm^3$, washed once with incubation buffer (10 mmol/l HEPES/NaOH, pH 7.4, 140 mmol/l NaCl, 5 mmol/l $CaCl_2$; BD Pharmingen, BD Biosciences, Franklin Lakes, NJ, USA), and centrifuged at 300 x g at 4˚C for 5 min. To quantitatively analyze the rate of apoptosis of the cells, Annexin V-propidium iodide (PI) flow cytometry was performed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were re-suspended in 100 μl labeling solution (FITC-Annexin V; BD Biosciences), and incubated at room temperature in darkness for 10-15 min. Following centrifugation, the sediment cells were washed once with incubation buffer, and incubated in fluorescence (SA-FLOUS; BD Biosciences) solution at 4˚C for 20 min in the dark, with occasional agitation. Apoptosis of the transplanted tumor cells was detected by flow cytometry. For the flow cytometry analysis, the excitation wavelength was 488 nm. The fluorescein isothiocyanate (FITC) fluorescence was detected with a flow cytometer at an emission wavelength of 515 nm, and a wavelength 660 nm was used to detect the propidium iodide (PI; BD Biosciences), and quantitative detection was performed using Summit version 4.3 software (Beckman Coulter, Inc., Brea, CA, USA).

Measurement of transplanted tumor size and observation of general conditions of nude mice. The length (a) and width (b) of the tumor were measured using a vernier caliper, and the transplanted tumor volume (V) was calculated using the following formula: V = ab2/2. The inhibitory rate of the transplanted tumor growth caused by drug treatment was estimated by using the following formula: Inhibitory rate (%) = (1 - the transplanted tumor volume in the treatment group/the transplanted tumor volume in the negative control group) x100%. Additionally, 6 mice in each group were sacrificed after tumor formation, prior to treatment with doxorubicin, and 2 and 4 cycles of chemotherapy. Compared with the effects of mediation based on PO_2 and clinically routine mediation chemotherapy regimen, the volume of tumor in different group at the time points of 2 and 4 cycles of chemotherapy were measured; the inhibitory rate was calculated after the 4th cycle of chemotherapy.

Evaluation of local tumor blood flow using a laser Doppler perfusion imaging system. In order to measure regional blood flow (RBF) (ml/min), an optical surface suction probe with a diameter of 2 mm (P10d; Moor Instruments. Ltd., Axminster, UK) was placed on the surface of the tumor. The probe was connected to a laser Doppler tissue perfusion monitor (Moor Instruments, Ltd.) and the blood flow under the probe was monitored according to the manufacturer's protocol.

Determination of NADH dehydrogenase, succinate-cytochrome c reductase (SCR) and cytochrome c oxidase (CcO) activities. Tumor tissue samples were homogenized in ice-cold HEPES buffer (3 mmol/l; pH 7.2) containing sucrose (0.25 mol/l), EGTA (0.5 mmol/l) and protease-inhibitor cocktail (1:40; Roche Diagnostics, Basel, Switzerland). NADH dehydrogenase activity was measured in the presence of Tris-HCl buffer (20 mmol/l; pH 8.0) containing NADH (150 μmol/l) and coenzyme Q1 (100 μmol/l) (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The activity of SCR (or the super complex containing complex II and complex III) was measured in the presence of phosphate buffer (50 mmol/l; pH 7.4), containing EDTA (0.3 mmol/l; pH 7.93), KCN (100 μmol/l), succinate (19.8 mmol/l; pH 8.0; Sigma-Aldrich; Merck KGaA) and ferricytochrome *c* (50 μmol/l; Sigma-Aldrich; Merck Millipore). CcO activity was measured in the presence of phosphate buffer (50 mmol/l, pH 7.4) and reduced cytochrome *c* (60 μmol/l; Sigma-Aldrich; Merck Millipore). The extinction coefficients, ε550 nm=18.5 mmol/l·cm for cytochrome *c* and ε340 nm=6.22 mmol/l·cm for NADH dehydrogenase, were used for activity calculation. The activities were normalized to protein concentrations as nanomoles per milligram protein per minute and measured using spectrophotometery. Protein concentration of the tissue homogenate was measured by BCA assay (Pierce; Thermo Fisher Scientific, Inc.) (26).

Statistical analyses. Two-way analysis of variance (ANOVA) was used for data analysis of PO_2 and blood flow. One-way

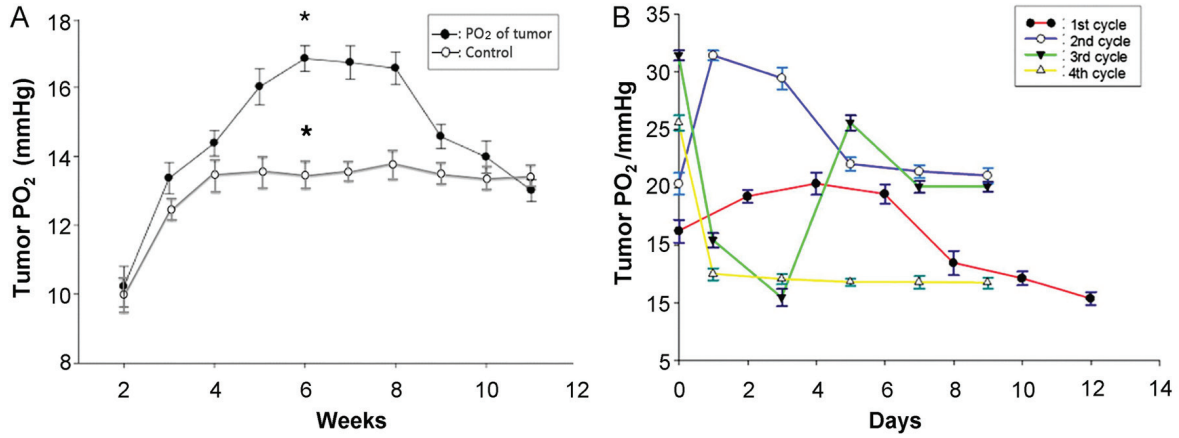

Figure 1. *In vivo* measurement of tumor PO_2 using EPR. (A) Changes in PO_2 in the tumor microenvironments during the growth of transplanted tumors derived from human breast cancer cells. (B) Monitoring of dynamic changes in PO_2 in tumor microenvironments in each cycle of doxorubicin (2 mg/kg) chemotherapy. PO_2, partial pressure of oxygen; EPR, electron paramagnetic resonance (*P<0.05 vs. Control).

ANOVA was used for enzyme activity analysis; results were expressed as mean ± standard error of the mean (SEM). Multigroup comparisons of the means were carried out by one-way ANOVA test with post hoc contrasts by Student-Newman-Keuls test. P<0.05, was considered to indicate a statistically significant difference.

Results

Changes in PO_2 in tumor microenvironments during growth of transplanted tumors derived from human breast cancer cells. After tumors (size ≥3 mm) formed 2 weeks after transplantation, a probe was implanted into the tumors, and changes in PO_2 were monitored using an EPR imaging system weekly. The PO_2 in tumor microenvironments was 10.14±0.31 mmHg 2 weeks after transplantation when the transplanted cells formed tumors, and the PO_2 increased with increased in tumor sizes. The PO_2 reached the peak (16.82±0.84 mmHg) 4 weeks after implantation. The peak PO_2 was significantly higher in the tumor group than the control group (13.71±0.41 mmHg, P<0.05). The PO_2 gradually decreased after 8 weeks (Fig. 1A).

Monitoring of changes in PO_2 in tumor microenvironments at various points in a chemotherapeutic course. According to the pattern of natural tumor growth, the peak PO_2 was evident 6 weeks after implantation (A0), when the PO_2 in the treatment group following administration of doxorubicin gradually increased until 4 days after administration (A4; 20.3±0.3 mmHg; Fig. 1B). Subsequently, the PO_2 gradually decreased. The second cycle of chemotherapy was administered by intraperitoneal injection of doxorubicin at the A4 time point (the first presence of the peak PO_2), and PO_2 peaked again 1 day after injection with doxorubicin, being 31.1±0.8 mmHg (Fig. 1B), The third cycle of chemotherapy was performed at B1 timing when the peak PO_2 emerged, and continuous measurement of tumor PO_2 revealed that the PO_2 decreased 3 days after injection, and that it increased and reached a peak 5 days after injection (C5; 26.1±0.4 mmHg). PO_2 was significantly reduced relative to that prior to administration of doxorubicin. The fourth cycle of chemotherapy was given at the emergence of peak PO_2 after the third cycle of chemotherapy. The PO_2 rapidly reduced to 10.8±0.6 mmHg one day after chemotherapy, then remained at a low level.

The association between oxygen consumption and oxygen supply was analyzed by measuring the mitochondrial activity and local blood flow of tumor in different chemotherapy cycles, and further analyzed the causes of the change of tumor PO_2 in different stages of chemotherapy. Following the initial round of chemotherapy, the mitochondrial activity of NADH dehydrogenase, SCR and CcO significantly decreased in tumor cells compared with that prior to chemotherapy (1.98±0.05 vs. 2.57±0.09 nmol/mg protein^{-1} min^{-1}, 2.10±0.14 vs. 3.39±0.19 nmol/mg protein^{-1} min^{-1}, and 2.80±0.12 vs. 3.34±0.27 nmol/mg protein^{-1} min^{-1}; P<0.05, P<0.01, P<0.05, respectively; Fig. 2A). Mitochondrial function was detected 1 day after the 2nd round of chemotherapy. The mitochondrial activity of NADH dehydrogenase, SCR and CcO in tumor cells was significantly reduced compared with that prior to administration (1.98±0.05 vs. 1.42±0.10 nmol/mg protein^{-1} min^{-1}, 2.10±0.14 vs. 1.55±0.03 nmol/mg protein^{-1} min^{-1}, and 2.80±0.12 vs. 1.68±0.12 nmol/mg protein^{-1} min^{-1}, respectively; P<0.01; Fig. 2B). After 5 days of the third administration of chemotherapy, the mitochondrial activity of NADH dehydrogenase, SCR and CcO significantly reduced compared with that pre-administration tissue (1.03±0.05 vs. 1.60±0.11, nmol/mg protein^{-1} min^{-1}, 1.41±0.04 vs. 1.55±0.03 nmol/mg protein^{-1} min^{-1}, and 1.09±0.08 vs. 1.68±0.12, respectively; P<0.05; Fig. 2C). The mitochondrial activity of NADH dehydrogenase after the 4th round of chemotherapy, SCR and CcO was significantly reduced compared with that prior to chemotherapy (1.03±0.05 vs. 1.14±0.03 nmol/mg protein^{-1} min^{-1}, 1.14±0.04 vs. 1.07±0.07 nmol/mg protein^{-1} min^{-1}, and 1.09±0.08 vs. 1.18±0.06 nmol/mg protein^{-1} min^{-1}, respectively; P>0.05; Fig. 2D).

Compared to the change of activity of mitochondrial activity, the RBF of each cycle of chemotherapy was detected at the same time point. The first chemotherapy the RBF was not significantly different pre- and post-administration (11.72±0.31 vs. 11.32±0.22, P>0.05; Fig. 3A).

The 2nd cycle of chemotherapy, the RBF was not significantly different from that prior to administration (11.44±0.39 vs. 11.72±0.31, P>0.05, Fig. 3B). The RBF was significantly

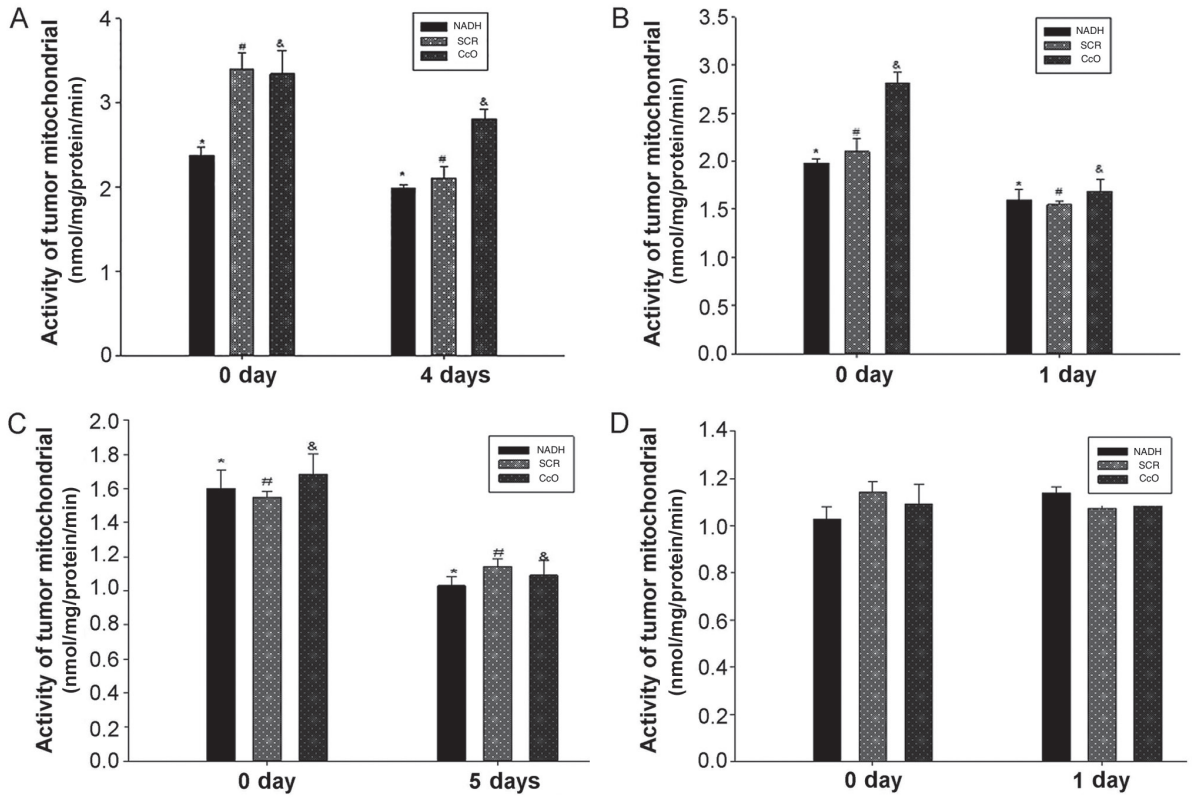

Figure 2. Mitochondrial activity at various stages of chemotherapy. (A) Four days after the first administration of chemotherapy (*P<0.05, $^{\#}$P<0.01, $^{\&}$P<0.05 vs. 0 day). (B) One day after the second round of chemotherapy was administered ($^{*,\#,\&}$P<0.01 vs. 0 day). (C) Five days after the third round of chemotherapy was administered ($^{*,\#,\&}$P<0.05 vs. 0 day). (D) One day after the fourth round of chemotherapy was administered, there was no statistical difference observed between the three groups. SCR, succinate-cytochrome *c* reductase; CcO, cytochrome c oxidase.

decreased in comparison with that prior to administration (8.77±0.12 vs. 11.44±0.39; P<0.05; Fig. 3C) after 3rd chemotherapy. The RBF was significantly reduced compared with that prior to chemotherapy (4.27±0.14 vs. 8.77±0.12; P<0.01; Fig. 3D) after the 4th cycle of chemotherapy.

Apoptosis of transplanted tumor cells. The apoptotic rate of transplanted tumor cells was measured at different time points: prior to chemotherapy (T0), after the 2nd round of chemotherapy (T2) and after the 4th round of chemotherapy (T4). The apoptotic rate of transplanted tumor cells in the PO_2 peak-based chemotherapy group was significantly higher than that in the conventional chemotherapy group after the 2nd cycle of chemotherapy (42.6 vs. 19.6%, P<0.05). The apoptotic rate of tumor cells following 4 cycles of PO_2 peak-based chemotherapy was significantly higher than that in the conventional chemotherapy group (60.6 vs. 47.2%, P<0.05). This suggests that hypoxia reduced the doxorubicin-induced apoptosis of transplanted tumor cells (Fig. 4).

Transplanted tumor growth in nude mice. The volume and inhibitory rate of the tumors from each group were measured and compared with that of each group prior to chemotherapy. The results revealed that there was no significant difference prior to chemotherapy between the PO_2 peak chemotherapy tumor volume (437.9+121.20 mm^3), the conventional chemotherapy group tumor volume (445.10+110.40 mm^3), and the 0.9% NaCl solution control group tumor volume (451.30+107.82 mm^3; P>0.05) prior to chemotherapy. The tumor in the PO_2 group was significantly reduced compared with that in the 0.9% NaCl solution control group after 4 cycles chemotherapy (1,220.75+148.91 vs. 2,512.55+201.22 mm^3; P<0.01). It was also significantly reduced compared with the conventional chemotherapy group (1,220.75+148.91 vs. 1,788.42+172.30 mm^3; P<0.05). The tumor inhibitory rate of the xenograft tumor group was significantly higher than that in the conventional chemotherapy group (51.43 vs. 28.82%; P<0.01) (data not shown).

Discussion

In the present study, the EPR technique was employed to accurately monitor PO_2 during tumor growth, and to determine when the PO_2 peaked, indicating a time window for increased efficacy of chemotherapy to inhibit tumor growth. Additionally, a mechanism was characterized by which change in PO_2 can be used to investigate change in the oxygen balance, mitochondrial activity (oxygen consumption) and local blood flow supply in tumors. Therefore, the data reveal a novel strategy for optimal administration of chemotherapy for individual patients with cancer.

As an essential environmental condition for solid tumor development, hypoxia serves a role in the resistance of solid tumors to radiotherapy and chemotherapy. Hypoxia has been demonstrated to be a prognostic marker in patients with tumors. Therefore, numerous strategies have been proposed to overcome tumor hypoxia (27,28). A precise understanding of the changes in oxygen enrichment/hypoxia during the

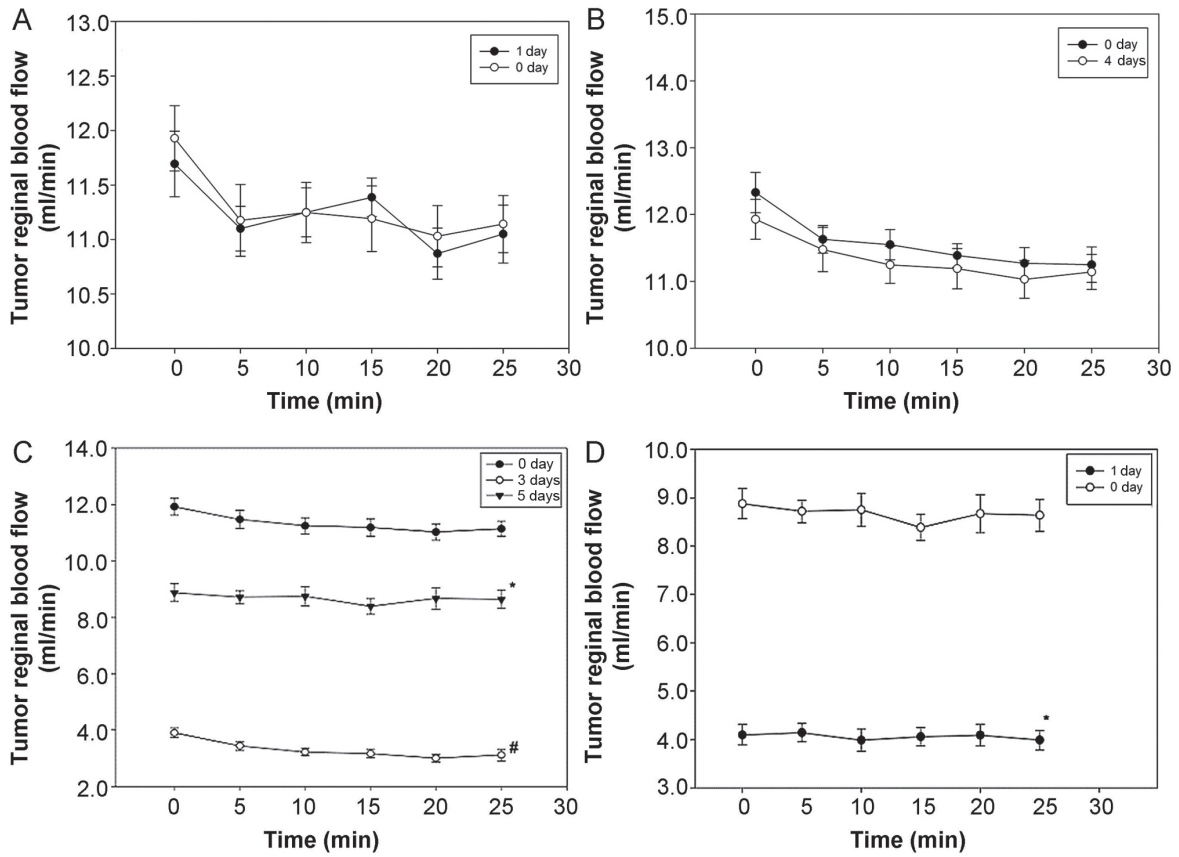

Figure 3. Measurements of regional blood flow (ml/min) using a Doppler flow meter. Blood flow was measured at 0, 3, 4 and 5 days. (A) RBF measured at the peak time of PO_2 in the first cycle of chemotherapy. (B) RBF measured at the peak time of PO_2 in the second cycle of chemotherapy. (C) RBF measured at the times of peak and trough PO_2 in the third cycle of chemotherapy. (D) RBF measured at the peak PO_2 time in the fourth cycle of chemotherapy. ($^*P<0.05$ and $^\#P<0.01$ vs. 0 days). RBF, regional blood flow; PO_2, partial pressure of oxygen.

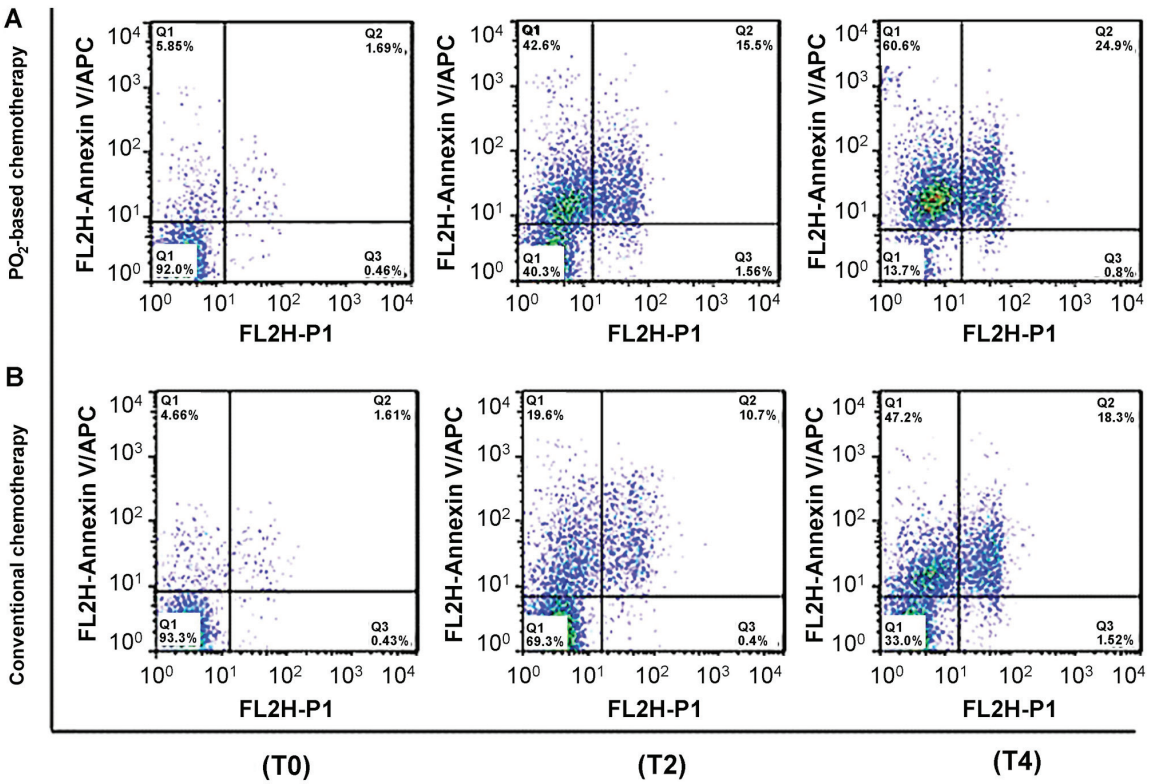

Figure 4. Comparison of apoptotic rates of transpalnted tumor cells between two groups by flow cytometry. (A) PO_2 peak-based chemotherapy regiment groups and (B) conventional chemotherapy regiment groups. PO_2, partial pressure of oxygen; T0, pre-chemotherapy; T2, second chemotherpay cycle; T4, fourth chemotherapy cycle.

treatment of solid tumors is of great importance to select a time window for maximum chemotherapeutic efficacy, to alter hypoxic tumor environments by administration of oxygen, and to select effective cytotoxic drugs (2,3). Seeing as an oxygen-enriched tumor environment increases sensitivity to chemotherapeutic agents, the purpose of the present study was to monitor tumor PO_2 pre- and post-chemotherapy using the EPR imaging system, to develop a chemotherapy administration pattern under an oxygen-enriched tumor environment, with an increased chemotherapy-sensitivity.

In the present study, an LiPc probe was implanted into nude mice after tumor formation (2 weeks after the transplantation of human breast cancer cells), and tumor PO_2 was continuously monitored. The findings revealed that the tumor PO_2 increased at an early stage of tumor growth, and reached its peak at 6 weeks following transplantation, followed by a gradual decline. The peak tumor PO_2 may be associated with fast vessel growth relative to tumor growth. Measurement of local tumor blood flow with a laser Doppler perfusion imaging system revealed that at the time of peak PO_2, the relative tumor blood flow at the corresponding time point was significantly increased compared with that at initial tumor formation. The following gradual reduction in tumor PO_2 may be explained by the following reasons: i) When solid tumors grow to a certain volume, blood vessels provide oxygen and nutrients required to support further tumor growth. It is estimated that the renewal rate of tumor cells is twice that of endothelial cells; therefore, the growth of blood vessels cannot meet the requirements of tumor growth, leading to ischemia, hypoxia and necrosis. ii) The tumor vessel density gradually increases in early tumor formation, however, it declines at later stages. The distance between the neighboring capillaries gradually increases, followed by tumor hypoxia and central necrosis. iii) The length of new blood vessels per unit volume of tumor is often reduced with tumor growth, while the lumen diameter increases, resulting in gradual reduction in surface area of the new blood vessels, which decreases the metabolic exchange rate required for tumor growth (29).

The PO_2 in tumor microenvironments alters the cytotoxic effects of doxorubicin. Under aerobic environments, the semiquinone free radicals of doxorubicin cause oxidative stress injury and mitochondrial dysfunction (30,31). In the present study, the first cycle of doxorubicin chemotherapy was administered when PO_2 was evident during natural tumor growth, and the following changes in tumor PO_2 were monitored.

The second cycle of chemotherapy was performed 4 days after the first cycle of chemotherapy when the peak PO_2 emerged, peak PO_2 was observed 1 day after the second cycle of chemotherapy and 5 days after the third cycle of chemotherapy were administered. However, the tumor PO_2 rapidly decreased following the fourth administration, and no fourth peak PO_2 was observed. The mitochondrial activity in transplanted tumor cells was significantly reduced in the first and second cycles of chemotherapy. However, there were no significant changes in local tumor blood flow, suggesting that early-stage chemotherapy did not affect tumor vessels.

The rapid reduction in tumor PO_2 in the third cycle of chemotherapy may be attributed to an insufficient oxygen supply caused by drug damage to tumor blood vessels. Subsequently, however, after 2 weeks the tumor PO_2 peaked again. suggesting that tumor blood vessels were regenerating (32). Further reduction in mitochondrial activity indicated the possibility of tumor cell damage. Determination of mitochondrial activity in the fourth cycle of chemotherapy revealed no significant difference in mitochondrial activity pre- and post- chemotherapy, which indicated that chemotherapy had achieved maximal efficacy against the tumor cells.

Flow cytometry revealed that the novel chemotherapeutic strategy achieved a higher apoptotic rate of transplanted tumor cells than the routine chemotherapy strategy (Fig. 4). Together with the results regarding tumor volume and inhibitory rate of transplanted tumors, this suggested that the novel chemotherapeutic strategy may significantly increase chemotherapy sensitivity, more effectively inhibit tumor growth and proliferation, and inhibit tumor progression.

The EPR technique is used for *in vivo* measurement of PO_2 in physiological and pathological tissues and organs and has been widely applied in basic and clinical studies (23,24). In the present study, the EPR technique was used for non-invasive, real-time and accurate monitoring of tumor growth for 9 weeks, and a method for long-term monitoring of PO_2 in tumor microenvironments was established. Additionally, the present study elucidated a therapeutic window (the optimal time to administer chemotherapy based on changes in PO_2 in tumor microenvironments). The present study provided novel strategies and approaches for clinical individual chemotherapy, notably for the neo-adjuvant chemotherapy of tumors.

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Availability of data and materials

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

Authors' contributions

MC conceived and designed the study. YL and SX conducted the experiments. YL performed the statistical analysis. MC reviewed and gave final approval of the version to be published. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Medical University of Chongqing (Chongqing, China).

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no competing interests.

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