Radiosensitization by Inhibiting Complex I activity in Human Hepatoma HepG2 cells to X-ray Radiation

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The purpose of this study is to investigate the influence of mitochondrial respiratory chain complex I inhibition on the radiosensitivity of HepG2 cells. The complex I inhibitor rotenone was used to inhibit complex I activity on HepG2 cells before X-ray irradiation. The cytotoxicity of rotenone was analyzed by MTT assay at various doses. Rotenone induced dissipation of mitochondrial membrane potential and increase of intracellular ROS production were observed. Intracellular ATP production level was determined using luciferin-luciferase assay kit. We further analyzed cell survival and cell cycle distribution of a combined treatment which HepG2 cells underwent 0.5 μM rotenone pretreatment firstly and irradiated with different doses of X-ray radiation afterwards. Our results suggest rotenone pretreatment prior to X-ray irradiation could induce a sensitizing effect on HepG2 cells by enhancing X-ray radiation induced proliferation inhibition and cell apoptosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide.1) The highest incidence rates of HCC were reported in the developing country of East Asia. Surgery and radiotherapy are two commonly used treatment modalities for HCC. Surgery resection offers a good therapeutic effect and low risk of complication, but it is limited to cases without concomitant liver cirrhosis or other chronic diseases.2,3) Conventional radiation such as X-ray radiation and γ radiation are low-LET radiation, which have a restrained efficacy due to the radio-resistance of cancer cell. Therefore, research about the radiosensitivity and improvement of X-ray radiation therapy is of medical importance.

As a typical ionizing radiation, X-ray radiation induces both direct and indirect effects in exposed cells. The direct biological effect is referred to interaction between photon and DNA molecule, whereas the indirect biological effect is mediated by the reactive oxygen species (ROS) produced by radiolysis and subsequent reaction.4) Radiation generated ROS is a common mediator of oxidative injuries of DNA, biomembrane and other important cellular structures. These oxidative injuries result in dysfunction of cell organelles and ultimately lead to cell death or apoptosis.5,6) But intracellular antioxidants and free radical scavenger enzyme posed a natural defense system which will buffer sudden increase of ROS and sustain a stable intracellular redox-state to prevent further oxidative injuries. Thus depletion of intracellular reducing force and perturbation of redox-state may provide radio-sensitizing effect and lower threshold for X-ray radiation induced cell death and apoptosis. Previous research reported this is a potential strategy for overcoming radio-resistance in various carcinomas.7–9)

Mitochondria are the most important bioenergetic site in eukaryocyte as well as central regulator of apoptosis.10) Previous research indicate mitochondria are also major endogenous reactive oxygen species source.11) Respiratory chain complex I has been identified as a major contributor of mitochondrial generated ROS, but under physiological condition the electron leakage from complex I only results in a small amount of ROS.12) However, complex I dysfunction or inhibition has been found resulted in enhanced ROS generation and cell apoptosis which lead to heart disease and neurodegenerative diseases.13) Previous research reported the classic complex I inhibitor rotenone could specifically bind to the ubiquinone binding site of Complex I and elevate intracellular ROS level by disrupting electron flow between FeS cluster and ubiquinone.14,15) In vitro experiment also suggests due to this trait rotenone showed certain anti-cancer effect16) and synergetic effect when combined with other oxidative
stress inducing drugs.  

In the present study, we investigated the complex I inhibition effect of rotenone on HepG2 cell and tested our hypothesis that complex I inhibition could increase intracellular ROS generation and may provide a radio-sensitizing effect.

**MATERIALS AND METHODS**

**Agents and chemicals**

DMEM medium and antibiotics were bought from GIBCO company (USA). The fetal calf serum was bought from Minhai company (China). Rotenone (Cat number: R8875), DCFH-DA (Cat number: D6883) and other unmentioned agents were all bought from Sigma-Aldrich. Rotenone powder was dissolved with 99.7% DMSO and diluted with DMEM medium to a concentration of 10 μM. In all tests, the control group was treated with DMSO which equals to the rotenone vehicle concentration.

**Cell culture and X-ray irradiation**

Human hepatoma HepG2 cell line was obtained from ATCC and grown in DMEM supplemented with 10% fetal calf serum and antibiotics (penicillin 100 unit/ml, streptomycin 100 μg/ml). X-ray irradiation was performed with a cabinet X-ray irradiation system (100 kvp, 3 mA, beryllium window thickness 0.76 mm, Faxitron X-ray corporation, USA) at the Institute of Modern Physics, Chinese Academy of Sciences (CAS). The dose rate was set to about 1.3 Gy/min. The film-to-source distance was set to 30.5 cm. Radiation field was rendered uniform by adjusting the film-to-source distance.

**Analysis of cell cycle**

Cell cycle status was assessed by PI staining and flow cytometry. Briefly, cells were resuspended in 1 ml of fluorescence stain solution (PI at 0.05 mg/ml in 0.1 mg/ml RNase and 0.1% Triton X-100) and incubated at room temperature for 30 min in dark. Twenty thousand events were measured per sample using flow cytometry (FACSCalibur, BD, USA). Cells at G1/S (2N) and G2/M phase (4N) were quantified using cell cycle analysis software (Flowjo 7.6). The subG1 fraction was determined and considered as cell death and apoptosis.

**Measurement of ROS production**

Measurement of intracellular ROS production was performed using the peroxide-sensitive fluorescent probe DCFH-DA (2',7'-dichlorofluorescein diacetate) described previously. Briefly, HepG2 cells were incubated under standard conditions with 0.5 μM rotenone for 24 h. At different time point (1 h, 12 h, 24 h), cells were washed once with PBS and harvested by trypsinization. Cells were resuspended in DMEM without phenol red and incubated with DCFH-DA (10 μM) in dark for 30 min at 37°C. After incubation, DCF fluorescence intensity was measured immediately with flow cytometry (FACSCalibur, BD, USA). In each analysis 20,000 events were recorded. The FL-1 mean value was measured and considered as an indicator of intracellular ROS level.

**Determination of intracellular ATP production**

Intracellular ATP production measurement was performed using a commercially available luciferin-luciferase assay kit (Nanjin Jianchen, China). Briefly, HepG2 cells were treated with 0.5 μM rotenone for 24 h, after treatment cells were washed once with ice-cold PBS and lysed with somatic cell ATP-releasing reagent on ice for 5 minutes. The supernatant was collected by centrifugation at 12,000 × g, 4°C. Then the supernatant, luciferin substrate and luciferase enzyme were mixed according to manufacturer’s instruction. The bioluminescence value of mixture was assessed with a microplate reader (Infinite M200, TECAN, Switzerland).

**Cytotoxicity assay**

The cytotoxicity of rotenone was quantified by measurement of the reduction of MTT. Briefly, HepG2 cells were seeded in 96-well plates at a density of 1 × 10^4 cells per well and incubated at 37°C overnight. Then cells were incubated with various concentrations of rotenone for 24, 48 and 72 hours. At the end of each treatment, 20 μl MTT (0.2 mg/ml) was added into 96-well plates directly and incubated for 2 hours sequentially. After the incubation, the medium was aspirated off and 150 μl DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 490 nm using microplate reader. All data were expressed as percentage of the control group.

**Measurement of mitochondrial membrane potential**

Mitochondrial membrane potential (MMP) was measured using fluorescent probe rhodamine123. Briefly, HepG2 cells were seeded into 96-well plates at a density of 1 × 10^4 cells per well and treated with various concentrations of rotenone for 24, 48 and 72 hours. At each end of treatment period, cells were washed three times with no-phenol red DMEM after all medium was removed and incubated with serum-free medium containing 10 μM rhodamine123 at 37°C for 30 min. Then cells were washed free of extracellular rhodamine123 with no-phenol red DMEM for three times. The fluorescent intensity value was assessed with Infinite M200 microplate reader (TECAN, Switzerland). All data were expressed as percentage of the control group.

**Cell survival analysis**

The sulforhodamine B (SRB) colorimetric assay was used for cell density determination after rotenone pretreatment and irradiation. All procedure was performed following Nature Protocoll. SRB assay is a method that used to determine cell density based on the measurement of cellular protein content, so it is not likely to be affected by changes of metabolism induced by rotenone compared with MTT assay.
Briefly, HepG2 cells were seeded into 12-well plate at a density of $2 \times 10^4$ and incubated overnight for adhesion. The three test groups of HepG2 were treated with rotenone, X-ray irradiation and a combined modality of rotenone pretreatment and different doses of X-ray irradiation respectively, whereas the control group was treated with 0.01% DMSO. After irradiation cells were transfected to incubator immediately and continuously cultured for 120 h. After the incubation period, cells were fixed with 10% trichloroacetic acid and stained for 30 min. Then the excess dye was removed by washing repeatedly with 1% acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using microplate reader. All data were expressed as percentage of the control group.

Statistical analysis
All data in figures and tables are given as mean $\pm$ SD from three replicates at least. The significance testing was performed using student's t-test (*: $P < 0.05$, **: $P < 0.01$ versus control group).

RESULTS

Cytotoxicity of rotenone on HepG2 cell line
According to our observation, rotenone induced a proliferation inhibition and HepG2 cell population decrease in a seemingly dose- and time- dependent manner (Fig. 1). Incubation with rotenone for 24 hours induced a decrease of cell viability by 5%, 6%, 15%, 19% at a concentration of 0.5 $\mu$M, 1 $\mu$M, 2 $\mu$M, 5 $\mu$M respectively. Longer rotenone exposure progressively decreased the cell viability and caused a serious inhibition of proliferation on HepG2. The reduction caused by 5 $\mu$M rotenone amounted to about 36% while lower concentrations induced reductions were within 20% after 72 h treatment. Incubation with 0.1 $\mu$M rotenone induced no significant effect compared with control group through whole treatment period. Therefore, 24 h exposure of 0.5 $\mu$M rotenone was chosen to be used in our experiment for complex I inhibition.

Rotenone induced dissipation of mitochondrial membrane potential
Our results indicate that rotenone treatment led to a dose-dependent depolarization of mitochondrial membrane potential in HepG2 cells. While 24 h rotenone treatment only caused a moderate decrease of cell population, the MMP of HepG2 cell decreased by nearly 10%, 24%, 37%, 40% and 50% at each test concentrations respectively (Fig. 2). Long term treatment resulted in a total collapse of MMP. After 72 h rotenone exposure most test results of MMP decreased below 40% compared with control group.

Rotenone induced reduction of intracellular ATP production
In our study, rotenone pretreatment only led to a moderate reduction of intracellular ATP production by 11% after 24 hours treatment (Fig. 3). Inhibition of complex I activity would attenuate aerobic respiration and ultimately lead to an

![Fig. 1. Rotenone induced proliferation inhibition of HepG2.](image1)

![Fig. 2. Rotenone induced depolarization of Mitochondrial membrane potential.](image2)

![Fig. 3. Rotenone induced decrease of ATP production.](image3)
intracellular ATP production decrease. So we presume the detected reduction of intracellular ATP production is a partial reflection of complex I function compromise.

**Rotenone induced generation of intracellular ROS**

To analyze whether incubation with 0.5 \( \mu \)M rotenone affect the intracellular ROS level of HepG2, fluorescent probe DCFH-DA was used to measure the ROS production at 1 h, 12 h and 24 h after rotenone treatment. Our results showed (Fig. 4), after 1 h rotenone incubation the intracellular ROS level elevated to about 120% compared with control group. The ROS production accumulated progressively and reached the peak at 12 h after incubation. But after 24 h rotenone incubation, the ROS level declined to a relative lower level which was slightly higher above the control group.

**Rotenone treatment results in G2/M cell cycle arrest**

Previous research reported that at low concentration rotenone would only block electron flow at complex I site, but at higher concentration rotenone could induce a dose-dependent cell cycle arrest predominantly at the G2/M phase. In order to investigate this effect, we analyzed the cell cycle of HepG2 treated with 0.5 \( \mu \)M, 1 \( \mu \)M and 2 \( \mu \)M rotenone for 12 hours and 24 hours. We found rotenone induced a dose-dependent G2/M cell cycle arrest and increase of subG1 fraction during the 24 h treatment. As Fig. 5 showed, 0.5 \( \mu \)M rotenone treatment led to a minor increase of subG1 fraction (6.32%) and an obvious G2/M arrest (29.37%) simultaneously at 24 h time point.

**Rotenone pretreatment enhances the radiosensitivity of HepG2 cells to X-ray radiation**

To investigate the radiosensitizing effect of rotenone pretreatment on HepG2 cell, a 5-day cell survival after X-ray irradiation was assessed with SRB assay. As our results showed, rotenone pretreatment improved X-ray irradiation induced cell proliferation inhibition and population decrease very effectively at 2 Gy and 4 Gy dose point. Compared with radiation group, the survival fraction of combined treatment group decreased about 22.66% and 12.84% more at 2 Gy and 4 Gy dose point. But at relative higher dose points (6 Gy, 8 Gy, 10 Gy), the difference between these two groups diminished slightly. In our experiment, the 5-day survival fraction of rotenone treatment group was about 92.63% ± 1.34% (data not shown in Fig. 6), so we presume rotenone treatment at a concentration of 0.5 \( \mu \)M only results in sublethal damage to HepG2 cells. Whereas such a low dose rotenone pretreatment may enhance the radiosensitivity of HepG2 cell to relative low dose X-ray radiation, but with higher dose delivery this effect may be not obvious.

![Fig. 4. Rotenone induced increase intracellular ROS generation.](Image)
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Rotenone pretreatment potentiate X-ray irradiation induced apoptosis

To analyze whether rotenone pretreatment affects radiation induced cell apoptosis, we quantified the proportion of apoptotic cells at 12 h and 24 h after rotenone treatment, X-ray irradiation and the combined modality of two treatments. The cell cycle analysis of 6 different groups (control, 0.5 μM rotenone treated, 4 Gy irradiation alone, 8 Gy irradiation alone, 4 Gy irradiation after 0.5 μM rotenone pretreatment, 8 Gy irradiation after 0.5 μM rotenone pretreatment) are illustrated in Fig. 7. As the results showed, 0.5 μM rotenone treatment only resulted in a small fraction of cell apoptosis at both time points (8.9%, 10.75%) after been replaced with fresh medium (no drug). Furthermore, after 24 h been released from rotenone treatment cell cycle distribution almost restored which indicates the complex I inhibition and G2/M arrest are reversible. To our observation, X-ray irradiation alone led to a seemingly time- and dose-dependent G2/M arrest on HepG2 cells in the 24 hours after irradiation, only minor fractions of SubG1 phase were detected in these two groups, so we presume X-ray irradiation may not lead to a detectable apoptosis in HepG2 during this period. But in the two groups which were treated with the combined modality we found more obvious changes of SubG1 fraction which indicates an increasing apoptosis in cells at both time points. So we presume rotenone pretreatment provided a radiosensitizing effect by facilitating HepG2 cells to X-ray irradiation induced apoptosis.

**DISCUSSION**

X-ray radiation is the most frequently used radiation in diagnose and curative treatment which has a good availability and relative low cost. But compared with High-LET radiations (carbon ion or other heavy ion radiation), conventional X-ray radiation has a lower biological effectiveness and more scattered dose distribution which lead to a necessity of delivery of higher radiation dose to reach same tumoricidal effect. However, high dose radiation might simultaneously raise the risk of damaging nearby normal tissue and secondary cancer incidence. Moreover, X-ray radiation has a higher oxygen enhancement rate which limits the efficacy of X-ray radiotherapy to various hypoxic tumor tissue. Therefore, the use of radiosensitizers in combination with X-ray radiation is aimed to increase the therapeutic efficacy and lower radiation dose to protect the neighboring normal tissue.

On large extent, X-ray irradiation induced damage is mediated by ROS produced from the primary radiolysis and subsequent reaction. But cellular anti-oxidant defense system including intracellular antioxidants like thiol molecules and ROS scavenger enzymes (catalase, peroxidases, SOD) pose an obstacle against radiation induced oxidative injuries.
and further cell apoptosis. Accordingly, radiosensitizers usually target intracellular redox, ROS generation and related signaling pathway.

Compared with normal cells, cancer cells are usually under higher oxidative stress which is presumed closely related to the dysfunction of oxidative phosphorylation of cancer mitochondria. Though this intrinsic oxidative stress may promote an adaptive up-regulation of antioxidant capacity which contributes to drug resistance, there is research also suggesting that introducing additional ROS insults may selectively enhance cancer cell killing by oxidative stress-generating agents through stress overload whereas normal cells can maintain redox homeostasis by adaptive response. Endogenous ROS originated from electron leakage of respiratory chain complex was previously acknowledged as an important role in neurodegenerative diseases. Recent studies revealed compounds interfere with respiratory chain may promote leakage of electrons and increase intracellular ROS production. Thus we presume inhibition of respiratory chain complex I may provide radiosensitizing effect by elevating ROS generation and eliciting mitochondrial oxidative stress. In our experiment, rotenone would induce an obvious mitochondrial membrane depolarization, intracellular ATP reduction and ROS generation at a moderate dose (0.5 μM). The combined modality of rotenone pretreatment and X-ray irradiation would induce a significant cell apoptosis in the 24 hours after irradiation and lead to a larger cell population decrease 5-day afterwards. To our speculation, this radiosensitizing effect may be attributed to following aspects. First, rotenone induced ROS generation may cause oxidative stress which leads to a depletion of intracellular antioxidants and exhaustion of ROS scavenging agents. Further cell apoptosis is induced. Second, the inhibition of respiratory chain complex I by rotenone may promote leakage of electrons and ROS generation, which is further enhanced by X-ray irradiation. Thus the combined modality of rotenone pretreatment and X-ray irradiation would induce a significant cell apoptosis in the 24 hours after irradiation and lead to a larger cell population decrease 5-day afterwards. To our speculation, this radiosensitizing effect may be attributed to following aspects. First, rotenone induced ROS generation may cause oxidative stress which leads to a depletion of intracellular antioxidants and exhaustion of ROS scavenging agents.

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**Fig. 7.** (A) Cell cycle analysis of 12 h after rotenone pretreatment and X-ray irradiation. (B) Cell cycle analysis of 24 h after rotenone pretreatment and X-ray irradiation.
ger enzymes, hence this effect may further potentiate radiation induced oxidative injuries. Second, previous research reported cells during G2/M period are more sensitive to ionizing radiation. So rotenone induced G2/M cell cycle arrest may also contribute to the radiosensitization effect. But there are reports suggesting rotenone-induced G2/M cell cycle arrest reflects disruption of microtubule assembly, so this point remains to be testified.

In summary, we demonstrate rotenone pretreatment could modestly enhance the radiosensitivity of human hepatoma HepG2 cells to X-ray radiation. This radiosensitizing effect might be mediated by rotenone induced intracellular ROS generation and mitochondrial oxidative stress, which further lead to a significant increase of X-ray radiation induced apoptosis. Our study suggests that mitochondrial respiratory chain complex I may be a potential target to improve X-ray radiation therapy for enhanced cancerous cell killing, but the underlying mechanism remains to be illustrated.

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REFERENCES