Role of Mitochondrial Electron Transport Chain Dysfunction in Cr(VI)-Induced Cytotoxicity in L-02 Hepatocytes

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Key Words
Hexavalent chromium [Cr(VI)] • Mitochondrial electron transport chain (ETC) • Mitochondrial respiratory chain complex (MRCC) • ATP, reactive oxygen species (ROS) • L-02 hepatocytes

Abstract

Background: Hexavalent chromium [Cr(VI)] and its compounds (e.g. chromates), which are extensively used in numerous industrial processes including leather tanning and steel manufacturing, are considered as priority pollutants. There is growing evidence supporting that Cr(VI) could be a human carcinogen that induces primary liver cancer after oral exposure, and this sheds light on the importance of the investigation of Cr(VI)-induced hepatotoxicity. Although it is known that mitochondria are major targets for heavy metals, the mechanisms of electron transfer chain (ETC) dysfunction involved in Cr(VI)-induced cytotoxicity are unclear.

Methods: In the present study, by using mitochondrial respiratory chain complex (MRCC) I inhibitor rotenone (ROT) and its substrates glutamate/malate (Glu/Mal), MRCC III inhibitor antimycin A (AA) and its substrate coenzyme Q (CoQ), and the antioxidant Vitamin C (Vit C), we aimed to elucidate the role of mitochondrial ETC dysfunction in Cr(VI)-induced cytotoxicity.

Results: We found that Cr(VI) targeted and inhibited MRCC I and III to induce ETC dysfunction, which played an important role in Cr(VI)-induced cytotoxicity.

Conclusion: Our current data provides novel important insight into the mechanisms of mitochondrial ETC dysfunction in Cr(VI)-induced cytotoxicity in the hepatocytes, and we will be poised to develop new methods in the prevention and treatment of liver diseases involving mitochondrial ETC dysfunction for the occupational exposure population.
Introduction

Chromium (Cr) in the environment primarily exists in two oxidized valence states: trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)]. Cr(III) is less-toxic and non-carcinogenic due to its poor membrane permeability and is known as essential micronutrients that involved in physiological functions [1]. Cr(VI) and its compounds (e.g. chromates), which are extensively used in numerous industrial processes including leather tanning, electroplating, and steel manufacturing, are considered as priority pollutants [2]. Cr(VI) accumulates and then persists at bronchial bifurcation sites after inhalation and is recognized as well-established human lung carcinogens by the World Health Organization (WHO) [3]. Although the potential health effect of Cr(VI) when it is orally ingested has long been debated, there is growing evidence supporting that Cr(VI) could be a human carcinogen after oral exposure. It is reported that rats and mice exposed to Cr(VI)-contaminated drinking water developed intestinal tumors [4]. Furthermore, a recent ecological mortality study revealed that the standardized mortality ratios (SMRs) for primary liver cancer were significantly high in the region where water was contaminated with Cr(VI) [5], which shed light on the importance of the investigation of Cr(VI)-induced hepatotoxicity for the protection of Cr(VI) occupational exposure populations.

Electron transfer chain (ETC), which locates at mitochondrial inner membrane, plays a central role in energy metabolism for its involvement in ATP production. ETC contains five mitochondrial respiratory chain complexes (MRCC) including MRCC I (nicotinamide adenine dinucleotide (NADH) dehydrogenase), MRCC II (succinate dehydrogenase), MRCC III (cytochrome c reductase/cytochrome bc1 complex), MRCC IV (cytochrome c oxidase), and MRCC V (mitochondrial F1Fo ATP synthase). It has been confirmed that MRCC I and III are the major sites that responsible for reactive oxygen species (ROS) generation [6]. Numerous studies have demonstrated that Cr(VI) exposure could induce oxidative stress that characterized by significant increased ROS levels [7-9], thus we inferred that ETC played a role in Cr(VI)-induced cytotoxicity.

It is well known that mitochondria are major targets for heavy metals, but the mechanisms of the disturbance of mitochondrial function induced by Cr(VI) are still not well demonstrated. The present work was made with the aim to elucidate the role of mitochondrial ETC dysfunction in Cr(VI)-induced cytotoxicity. Although ATP depletion, oxidative stress and apoptosis have been linked to Cr(VI)-induced cytotoxicity, additional scientific data is required to further understand the molecular and cellular mechanisms and to demonstrate the harmful effects caused by Cr(VI). The present work provides valuable information and evidence on the effect of mitochondrial ETC dysfunction on Cr(VI)-induced cytotoxicity in the hepatocytes through assays that determine the correlation between indexes of mitochondrial oxygen consumption rate , ATP depletion, ROS accumulation, and apoptosis.

Materials and Methods

Materials

Human L-02 hepatocyte line was provided by Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. Rotenone (ROT), Antimycin A (AA), Vitamin C (Vit C), Glutamate (Glu), Malate (Mal), Coenzyme Q (CoQ), ATP, Potassium dichromate (K₂Cr₂O₇), 3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyl tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 culture medium, fetal bovine serum (FBS), and trypsin were obtained from Solarbio (Beijing, China). All chemicals and solvents were of analytical grade or the best pharmaceutical grade.

Cell culture

L-02 hepatocytes were grown in RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and antibiotics (50 units/ml penicillin plus 50 μg/ml streptomycin), and cultured at 37°C.
under a humidified atmosphere of 5% CO\textsubscript{2}. The medium was changed every day and the cells were passaged every three days using trypsin.

**Measurement of cell survival rate**

MTT assay, which reflects the mitochondrial function of cells was performed to evaluate cell viability as previously described [10]. Briefly, L-02 hepatocytes in exponential growth were seeded at a density of 1×10\textsuperscript{4} cells/well in the 96-well plate. The chemicals of indicated final concentrations were added to the cultures. Control cells and medium controls without cells received DMSO without the chemicals. Triplicates were used for each concentration. The hepatocytes were incubated at 37°C in 5% CO\textsubscript{2} saturated atmosphere for indicated time period and then were washed twice with phosphate-buffered saline (PBS, pH 7.4). Cells were treated with 5 μl 5 mg/ml MTT solution for additional 4 h at 37°C, and then were lysed in PBS containing 20% Sodium dodecyl sulfate (SDS) and 50% N,N-dimethylformamide (pH 4.5). MTT conversion was quantified by a multiwell ELISA reader Versamax (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

**Mitochondrial preparation and protein determination**

Mitochondria were prepared as previously described [11] with some modifications. All solution and equipments should be pre-cooled to 4°C and keep on ice during the mitochondria isolation process. L-02 hepatocytes pellets were collected after chemical treatment, washed twice with ice-cold PBS, and then spun down at 750 × g for 5 min at 4°C. The packed cell volume was determined and five volumes of buffer A (250 mM sucrose, 20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM KCl, 1.5 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5) were added. The cells were incubated on ice for 2 min and then were homogenized with syringe (20-30 times, confirm >90% cells breakage has occurred). The homogenates were centrifuged twice at 750 × g (10 min, 4°C). The mitochondrial pellets were obtained by spinning the supernatants at 10,000 × g (15 min, 4°C), and then were re-suspended in buffer A. The protein concentrations of each treatment group were estimated using Coomassie Brilliant Blue (G-250) by the method of Bradford [12].

**Measurement of mitochondrial respiration**

Mitochondrial respiration was measured with a Clark-type oxygen electrode (Rank Brothers Ltd, Cambridge, England) according to the previous method [13]. Briefly, the mitochondrial suspension (1 mg/ml) were added to the buffer consisting of 130 mM KCl, 2 mM K\textsubscript{2}HPO\textsubscript{4}, 3 mM HEPES, 2 mM MgCl\textsubscript{2}, 1 mM EGTA. Then 5 mM glutamate-malate was added to the mixture. Then the oxygen consumption was measured and recorded.

State 4 respiration (ATP independent respiration) was monitored after addition of 2 μg/ml oligomycin in the absence of ADP. Subsequently, state 3 respiration (phosphorylating respiration) was measured after the addition of 2.0 μl ADP (27 mM) to determine the maximal rate of coupled ATP synthesis. The respiratory control ratio (RCR) was calculated as the ratio between state 3 and state 4 respiratory rates (State 3/State 4).

**Measurement of the activity of MRCC I-IV**

The mitochondrial respiratory chain enzyme activities (MRCC I-IV) were assayed with Mitochondrial Respiratory Chain Complexes Activity Assay Kits purchased from Genmed Scientifics (shanghai, China) and were quantified using an UV-9100 spectrophotometer. All enzyme activities assays were performed at 30°C except for complex IV at 25°C. The mitochondria were freeze-thawed for several rounds in the hypotonic media containing 25 mM potassium phosphate and 5 mM MgCl\textsubscript{2}. MRCC I (Nicotinamide adenine dinucleotide (NADH) CoQ oxidoreductase) activity which expressed as nmol oxidized NADH/min/mg prot was measured with or without ROT following the rate of the oxidation of NADH (100 μM) at 340 nm. MRCC II (succinate: 2,6-Dichloroindophenol (DCIP) oxireductase) activity which expressed as nmol reduced DCIP/min/mg prot was initiated by the addition of 50 μM decylubiquinone, was measured following the rate of reduction of 2, 6-Dichlorophenolindophenol (DCP) at 600 nm. MRCC III (ubiquinol: cytochrome c reductase) activity which expressed as nmol reduced cytochrome c/min/mg prot was measured following the rate of reduction of cytochrome c at 550 nm with or without specific inhibitor of ubiquinol cytochrome c reductase antimycin A. MRCC IV (cytochrome c oxidase) activity which expressed as nmol oxidized cytochrome c/min/mg prot was determined by measuring the oxidation of cytochrome c at 550 nm. All measurements were performed in triplicate.
Measurement of ATP levels

Intracellular ATP levels were determined in cell lysates using the luciferase-based ATP Assay Kit purchased from Nanjing Jiancheng Biotech Company (Nanjing, China) according to the manufacturer’s instructions. Briefly, the hepatocytes were seeded in the 6-well plates at the density of 10^5 cells/ml and were allowed to attach overnight. After the chemical treatment for indicated concentration and indicated time period, the cells were washed twice with PBS before treated with lysis buffer supplied with kit. The specific ATP working solution was added to each sample and incubated for 5 min before the cellular ATP levels can be determined using a luminescent plate reader. Results were normalized to the total protein level that determined by the method of Bradford.

Measurement of ROS production

Intracellular ROS levels were determined by staining with fluoroprobe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes, USA). Briefly, after treatment, 1x10^6 cells of each group were washed with PBS twice, loaded with 0.5 ml PBS containing CM-H2DCFDA (10 µM), and incubated for 40 min in the darkness. Then the cells were analyzed by flow cytometry using an excitation wavelength of 488 nm and emission wavelength of 535 nm. The amount of ROS level was considered to be directly proportional to the fluorescence intensity of 2', 7'-dichlorofluorescein (DCF), the oxidized product of CM-H2DCFDA.

Measurement of Caspase-3 activity

Caspase-3 activity was detected using caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, China). Briefly, L-02 hepatocytes were harvested and homogenized with lysis buffer (50 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), and 10 mM ethyleneglycoltetraacetic acid, PH=7.4). After centrifugation (13,000 rpm×5 min), the duplicate sets of supernatants were added with 100 µM caspase-3 substrate Ac-DEVD-pNA (final concentration). The samples were incubated at 37°C for 2 h and caspase-3 activity were obtained by measuring the absorbance of the cleavage of the colorimetric substrate at 405 nm.

Flow cytometry analysis for apoptotic cells

The apoptotic L-02 hepatocytes were measured by staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V (0.5 µg/ml final concentration)/propidium iodide (PI, 1 µg/ml final concentration) and determining with flow cytometer according to the manufacturer’s instructions. The percentages of cells undergoing apoptosis were analyzed by CELLQuest software and all experiments were performed three times independently.

Statistical analysis

The significance of differences between groups was assessed using one-way analysis of variance (ANOVA) (SPSS17.0). The acceptance level of significance was P<0.05. The results are expressed as mean values ± SD (Standard Deviation) for all the quantitative analyses represented in the figures. Linear correlation analysis was performed to calculate the correlation coefficient (r).

Results

Cr(VI) induced mitochondrial ETC dysfunction in L-02 hepatocytes

With respect to the cytotoxicity of Cr(VI) to human L-02 hepatocytes, the MTT assay demonstrated the effect of various concentrations (0-100 µM) and treatment time (6, 8, 12, 24 h) of Cr(VI) on the cell survival rate of hepatocytes as shown in Figure 1A. The MTT assay result revealed both concentration and treatment time-dependent loss of cell survival rate. We further asked whether mitochondrial respiration was affected in the hepatocytes in the presence of different concentrations of Cr(VI) for 12 h. The 12 hr time-point was chosen due to the observation that Cr(VI) showed the moderate cytotoxicity compared with other treatment time-points. State 3 (coupled to ADP phosphorylation) and State 4 (after ADP phosphorylation) respiration was used to assess the effect of Cr(VI) on the mitochondrial respiratory capacity. As shown in Figure 1B, State 3 respiration was decreased in a concentration-dependent manner which demonstrated significant
Impairment of mitochondrial electron transport and respiratory capacity. On the other hand, state 4 respiration was not affected. The RCR, measured as the ratio between rates of respiration in State 3 and State 4, was thus concentration-dependently reduced by Cr(VI).

The activities of MRCC were examined in mitochondrial preparations containing both the matrix and membrane fractions. There were no significant differences in the activities of MRCC II and MRCC IV \((p > 0.05)\) (Fig. 1C). In contrast, MRCC I and MRCC III showed significant decrease in the treatment groups compared with control groups \((p < 0.05)\) which indicating that MRCC I and MRCC III may be the main targets of Cr(VI) in the electron transfer chain. We inferred that Cr(VI) targets MRCC I and MRCC III to induce mitochondrial ETC dysfunction and cytotoxicity, and we used MRCC I inhibitor ROT and MRCC III inhibitor AA to confirm our hypothesis.

Different concentrations of ROT (0-20 µM) and AA (0-8 µg/ml) were exposed to hepatocytes. The result of MTT assay revealed that ROT and AA only decrease cell survival rate at high concentrations (Fig. 2A). We chose the concentration of 5 µM for ROT and 1 µg/ml for AA at which the survival rate was not significantly affected for the following experiments. Vit C is a widely used antioxidant which appears to protect the mitochondria from extensive damage thus saving the cell. When using different concentrations of Vit C (0-3 mM) to treat the cells, we found that the hepatocytes survival rate was first increased and then decreased (Fig. 2B), and we chose the concentration of 2 mM at which Vit C showed the best protective effect. We also chose the Cr(VI) treatment concentration of 25 µM at which the hepatocytes survival rate was between 65% to 70% for the following experiments.

**Cr(VI) inhibited mitochondrial oxygen consumption in L-02 hepatocytes**

Mitochondria account for most of cellular oxygen consumption and mitochondrial respiration plays the central role in energy production and cell survival. Incubation of
hepatocytes with ROT, AA and Cr(VI) induced significant decrease of oxygen consumption compared with control (p<0.05). The combination treatment of Cr(VI) with AA induced lower oxygen consumption compared with that of either Cr(VI) or AA alone treatment. The combination treatment of Cr(VI) with ROT resulted in the lowest oxygen consumption rate compared with other groups which indicating that MRCC I is the main target site in the ETC.
after Cr(VI) exposure (Fig. 3A). As shown in Figure 3B, Vit C rescued the inhibition of oxygen consumption rate caused by Cr(VI) and by the combination of Cr(VI) with ROT or AA, which is just as we expected. The curves of the recorded oxygen concentration data were shown in Figure 3C. Good agreement was found between the oxygen consumption rates of different treatment groups shown in histograms in Figure 3A&B and the curves in Figure 3C, indicating that the relationship between the oxygen consumption rate and oxygen concentration can be well expressed using Clark-type oxygen electrode. Each curve in Figure 3C was the primary oxygen trace from a Clark electrode showing a drop (labeled on the curve) in oxygen concentration in mitochondrial suspensions from different treatment groups. The slope of the curve represented the direct measure of the total mitochondrial oxygen consumption rate. The combination treatment of Cr(VI) with ROT revealed the sharpest drop of oxygen consumption and the use of Vit C inhibited the drop caused by Cr(VI) and other groups, which were consistent with the results in Figure 3A and 3B, respectively.

**Cr(VI) decreased ATP cellular content and induced oxidative stress in L-02 hepatocytes**

As the final and the most important step of cellular respiration, ETC is also where the majority of ATP is produced. To assess whether the dysfunction of ETC was accompanied by the reduction in cellular ATP levels, we measured ATP levels in different treatment groups. In the absence of Vit C treatment, ROT and AA alone treatments for 12 h showed no obvious change of ATP levels compared with control (p>0.05), while Cr(VI) alone and the combination treatments induced the reduction of cellular ATP content, especially in

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**Fig. 4.** Cr(VI) decreased ATP cellular content and induced oxidative stress in L-02 hepatocytes. L-02 hepatocytes were treated with ROT (5µM), AA (1 µg/ml), Cr(VI) (25 µM), the combination of Cr(VI) with ROT or AA in the presence or absence of Vit C (2 mM) for 12 h. (A) Intracellular ATP levels were determined in cell lysates using the luciferase-based ATP Assay Kit. (B-C) ROS levels were measure using fluoroprobe CM-H2DCFDA and the cells and the cellular ROS levels that were considered to be directly proportional to the fluorescence intensity, were quantitated by flow cytometry (B) and were detected with the fluorescence microscope (C). Data represent mean ± SD. *p<0.05, **p<0.01 compared with the group without Vit C treatment. *p<0.05, compared with the control group (no chemical treated).
the group of Cr(VI) plus ROT ($p<0.05$). The levels of ATP were markedly higher when the cells were co-treated with Vit C indicating that Vit C rescued ATP depletion. Co-treatment of ROT with Vit C showed the highest ATP level (Fig. 4A). Electron transport chain is the main site for ROS generation, thus we were not surprised to find that Cr(VI) and the combination treatments induced increased ROS levels in the absence of Vit C, and Vit C reduced ROS levels of the different treatment groups to the same level of the control group as shown in Figure 4B (the values of DCF fluorescence) and 4C (the fluorescence signals detected under microscope). The combination treatment of Cr(VI) with ROT showed the highest ROS level. It is well known that MRCC I inhibitor ROT induces cytotoxicity through enhancing mitochondrial ROS production [14], and we have confirmed in our previous study that Cr(VI) also targets MRCC I to induce the accumulation of ROS [15].

**Cr(VI) induced apoptosis in L-02 hepatocytes**

Caspase-3 is the major protease in apoptosis and we further verified whether caspase-3 activation was involved in Cr(VI)-induced cytotoxicity. We found that caspase-3 was activated in all treatment groups in the absence of Vit C and the highest activity occurred in the combination group of Cr(VI) plus ROT, suggesting that the cells were dying by a caspase-dependent pathway. Vit C inhibited caspase-3 activation in all treatment groups (Fig. 5A).
The hepatocytes were treated with different chemicals for 12 h, after which apoptosis was determined by Annexin V/PI analysis. As shown in Figure 5B, Cr(VI) increased the percentages of both early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells. The combination treatments of Cr(VI) plus ROT and Cr(VI) plus AA induced more early apoptosis than late apoptosis. Vit C protected the hepatocytes from both early and late apoptosis. The apoptosis rates of different chemical treatment groups were shown in Figure 5C, and the results were consistent with both Figure 5B and 5A.

Enhancing ETC function could prevent the cytotoxicity induced by Cr(VI)

We also confirmed that the cytotoxicity induced by Cr(VI) can be prevented by enhancing the mitochondrial ETC function. It is clear that the addition of mitochondrial ETC substrates such as glutamate/malate (Glu/Mal) can enhance ETC function by stimulating the accumulation of ATP. Since we found that Cr(VI) targeted and inhibited MRCC I and III to induce ETC dysfunction, we used MRCC I substrates Glu/Mal and MRCC III substrate coenzyme Q (CoQ) to enhance ETC function. As shown in Figure 6A-6C and just as we expected, the addition of Glu/Mal increased oxygen consumption rate and inhibited Cr(VI)-induced ATP depletion and caspase-3 activation, while the addition of CoQ showed slight inhibitory effect on Cr(VI)-induced caspase-3 activation and no effect on ATP depletion. The combination treatment of Glu/Mal plus CoQ revealed better inhibitory effect than Glu/Mal alone treatment. The addition of ATP can also rescue Cr(VI)-induced apoptosis in the hepatocytes because we found that the activation of caspase-3 was significantly inhibited in the combination treatment of Cr(VI) with ATP group (p<0.05) compared with Cr(VI) alone treatment group (Fig. 6D).

The correlation between the indexes of mitochondrial ETC dysfunction and Cr(VI)-induced cytotoxicity

After the treatments of Cr(VI) alone and the combination of Cr(VI) plus the inhibitors of ETC (ROT, AA), we performed the correlation analysis among indexes of mitochondrial ETC dysfunction (mitochondrial oxygen consumption rate and ATP content) and Cr(VI)-induced cytotoxicity. The correlation analysis showed a significant correlation between the indexes of mitochondrial ETC dysfunction and Cr(VI)-induced cytotoxicity. The results indicated that the correlation coefficient between the indexes of mitochondrial ETC dysfunction and Cr(VI)-induced cytotoxicity was significantly higher in the combination treatment group than in the Cr(VI) alone treatment group.
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Table 1. Correlation coefficients between indexes of cytotoxicity caused by Cr(VI) and mitochondrial ETC dysfunction in the hepatocytes. Note: *P=0.000, compared to control group.

| Index                      | Cytotoxicity caused by Cr(VI) | ETC dysfunction |
|---------------------------|------------------------------|-----------------|
| Apoptosis Rate            | 1.000                        |                 |
| Caspase-3 activity        | 0.972                        | 1.000           |
| ROS                       | 0.969                        | 0.952           | 1.000 |
| ATP                       | -0.072                       | -0.070          | -0.060 | 1.000 |
| Oxygen Consumption Rate   | -0.094                       | -0.901          | -0.072 | 0.920 | 1.000 |

cytotoxicity (apoptosis rate of L-02 hepatocytes, caspase-3 activity, ROS level). As shown in Table 1, the results revealed that the apoptosis rate of L-02 hepatocytes and caspase-3 activity, ROS level were positively correlated, and the correlation coefficients were 0.972 and 0.969, respectively. The apoptosis rate of L-02 hepatocytes and ATP content, mitochondrial oxygen consumption rate were negatively correlated with the correlation coefficients of -0.872 and -0.894, respectively. Caspase-3 activity and ROS level was positively correlated with the correlation coefficient of 0.952. Caspase-3 activity and ATP content, mitochondrial oxygen consumption rate were negatively correlated with the correlation coefficients of -0.970 and -0.901, respectively. ROS level and ATP content, mitochondrial oxygen consumption rate were negatively correlated with the correlation coefficients of -0.960 and -0.872, respectively. ATP content and oxygen consumption rate was positively correlated with the correlation coefficient of 0.920. Pairwise comparisons of the above indexes revealed statistical significance after the test of significance of correlation coefficient (p=0.000 for all comparisons).

Discussion

Inhibition of mitochondrial respiration, defects in mitochondrial energy metabolism such as ATP depletion, and increased ROS production are observed in most liver disease induced by toxicants. While many of the causative factors of hepatotoxicity have been identified, it is widely accepted that mitochondrial dysfunction is the key causative factor in liver disease [16], and the molecular mechanisms responsible for mitochondrial ETC dysfunction and the development of liver disease following toxicant exposure remain poorly understood. Being under the control of both nuclear and mitochondrial genomes, ETC remains to be fully understood. The mitochondrial ETC is the key component for cellular energy production, and it also contributes to mitochondrial dysfunction during various stressed conditions [17]. While being required for oxidative phosphorylation, oxygen also serves as the essential substrate for the production of ROS, which is implicated in toxicants-induced cytotoxicity. During the oxidative phosphorylation, electrons gain from reduced substrates are transferred to O₂ on ETC through electron transporters including MRCCI, III and IV, which in turn generate proton gradient to produce ATP. ETC is the main component of energy transformation in mitochondria and ROS are side products of electron transport on ETC. It is believed that ROS generation starts with the formation of superoxide radical (O₂⁻) which is the result of interaction between molecular oxygen (O₂) and free radicals such as semiquinone (Q⁻) [18]. When released and accumulated in excess under certain stress conditions such as hypoxia, aging process and toxicant exposure, ROS may directly damage the cells and induce cytotoxicity [19]. Although the previous researches have focused on the electron transport and coupled ROS production, the important details of the related mechanisms are still not understood. Currently it is remain controversial regarding the relative contribution of various sites on ETC to overall cellular ROS production and the related factors which may alter this contribution [20]. Although much is known of the functional role of ROS, much less is known about the specific molecular sites of electron leak and free
radicals generation in the electron transport system [21]. In the present study we thought that Cr(VI) may induce ROS accumulation by increasing the electron leakage at the sites of MRCC I and III and then lead to the formation of superoxide (O$_2^-$) through one-electron reduction of oxygen, which is consistent with previous report [22].

State 3/State 4 respiration, especially RCR, are strongly influenced by almost every functional aspect of oxidative phosphorylation, making them good and sensitive indicators of mitochondrial ETC dysfunction. ADP addition corresponds to state 3 respiration by initiating ATP synthesis coupled to proton reentry across the membrane, while ADP exhaustion leads to the reduction of respiratory rate and corresponds to state 4 respiration. State 3 respiration is considered to be the active state and state 4 respiration represents the resting or basal state of mitochondrial respiration, while in the present study there was obvious decrease in state 3 respiration but no significant change in state 4 respiration after different concentrations of Cr(VI) exposure indicating the cellular compensation effect may play a role in maintaining mitochondrial respiratory function. The functional consequence of the decrease in state 3 respiration could include ATP depletion thus lead to energy metabolism disorder [23].

The mitochondrial pathway and the death receptor pathway are two major cellular apoptosis pathways that have been most commonly identified and studied [24]. Caspase-3 is recognized as the key component involved in the underlying mechanisms of apoptosis [25] and the activation of which is thought to be the important and final step in most of the apoptosis pathways. Others have identified the electron flux on ETC as the key modulator of apoptosis caused by toxicants [26]. In current study we investigated the role of ETC defects in regulating apoptotic cell death and found that Cr(VI)-induced apoptosis in the hepatocytes depends on the loss of specific enzymatic activities. MRCC I is a large L-shaped mitochondrial inner membrane-bound enzyme. MRCC I plays an important role in the respiratory electron transfer system by coupling the transfer of electrons from NADH to ubiquinone to the creation of the proton gradient across the mitochondrial membrane that is necessary for ATP synthesis. And in this study we confirmed that MRCC I is the main target of Cr(VI) to induce mitochondrial ETC dysfunction and cytotoxicity.

To our surprise, the previous study reported that blockade of electron transport at complex III with AA protects mitochondria during ischemia [27], thus MRCC III can be recognized as a specific region in the ETC leading to mitochondrial damage during ischemia. They also confirmed that blockade of electron transport with ROT preserves respiration through cytochrome oxidase during ischemia. In our current study neither ROT nor AA showed any protective effect but aggravated Cr(VI)-induced cytotoxicity. Although it is clear that ROT induces apoptosis through enhancing mitochondrial ROS production [14], we will have to go further in this part because we are still unable to answer under which circumstance ROT will show protective effect.

Our data showed that Cr(VI)-induced mitochondrial respiration inhibition, ATP depletion and ROS accumulation in cultured L-02 hepatocytes could be prevented by Vit C, indicating that antioxidants can protect against Cr(VI)-induced cytotoxicity. Enhancing the ETC function by the addition of MRCC I substrates Glu/Mal and MRCC III substrate CoQ rescued Cr(VI)-induced inhibition of oxygen consumption rate, depletion of ATP levels and apoptosis. Collectively, our results suggest a possible link between mitochondrial ETC dysfunction and cytotoxicity caused by Cr(VI). Despite great advances in mitochondrial ETC dysfunction and the related cytotoxicity effects including energy metabolism disorder, oxidative stress and apoptosis caused by toxicants such as Cr(VI), there are clearly more questions than answers in this field. The exposure of Cr(VI) and its compounds (e.g. chromates) can occur as a result of several industrial applications including chromate pigments, stainless steel machining and welding, chrome plating, and others. Although it is confirmed that oral ingestion of Cr(VI) through drinking water could induce primary liver cancer [5], the related molecular mechanism are unclear. It is known that ROS has recently come to the forefront as important signaling molecules that play a central role in modulating Ca$^{2+}$ and other signalings [28], which is contradictory with what we have long believed that ROS are dangerous waste product of mitochondrial respiration. Oxidative stress induced by
mitochondrial ROS accumulation in response to ETC dysfunction [29] may in fact contribute to tumorigenesis [30]. Although we and others have confirmed that MRCC I on ETC is the main site of ROS production and ROS plays key role in toxicants-induced cytotoxicity, our knowledge of how ROS alters the surrounding cellular microenvironment and what impact these alterations will have on spontaneous tumors rates is lacking.

Our current data provides important new light on the issue regarding the role of mitochondrial ETC dysfunction in Cr(VI)-induced mitochondrial respiration inhibition, energy metabolism disorder and ROS accumulation in the cultured L-02 hepatocytes. Once we understand the interplay between ETC dysfunction and cytotoxicity induced by Cr(VI) and other hepatotoxic agents, we will be poised to develop the prevention and treatment methods for liver diseases involving mitochondrial ETC dysfunction for the occupational exposure population. We also suggest that a careful analysis of the association between mitochondrial ETC dysfunction and liver disease conditions should be performed for better understanding of disease pathogenesis and effectively developing adequate therapeutic strategies.

**Disclosure Statement**

The authors have no conflicts of interest to declare in relation to this article.

**Acknowledgements**

The authors thank all of the individuals in this laboratory for their ideas, suggestions and hard work. This research was financially supported by Chinese National Natural Science Foundation (NO. 81302456 and NO. 81172701), Specialized Research Fund for the Doctoral Program of Higher Education (NO. 20130162120076), China Postdoctoral Science Foundation (NO. 2013M542144) and Postdoctoral Science Foundation of Central South University (NO. 126645).

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