Effect of Hexavalent Chromium on Electron Leakage of Respiratory Chain in Mitochondria Isolated from Rat Liver

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Key Words
Hepatocyte • Mitochondria • Electron leak • GSH

Abstract
Background/Aims: In the present study, we explored reactive oxygen species (ROS) production in mitochondria, the mechanism of hexavalent chromium (Cr(VI)) hepatotoxicity, and the role of protection by GSH. Methods: Intact mitochondria were isolated from rat liver tissues and mitochondrial basal respiratory rates of NADH and FADH$_2$ respiratory chains were determined. Mitochondria were treated with Cr(VI), GSH and several complex inhibitors. Mitochondria energized by glutamate/malate were separately or jointly treated with Rotenone (Rot), diphenyleneiodonium (DPI) and antimycinA (Ant), while mitochondria energized by succinate were separately or jointly treated with Rot, DPI, thienyl trifluoroacetone (TFA) and Ant. Results: Cr(VI) concentration-dependently induced ROS production in the NADH and FADH$_2$ respiratory chains of mitochondrial electron leakage induced by Cr(VI) from NADH respiratory chain were mainly from ubiquinone binding sites of complex I and complex III. Conclusion: Treatment with 50μM Cr(VI) enhances forward movement of electrons through FADH$_2$, respiratory chain and leaking through the ubiquinone binding site of complex III. Moreover, the protective effect of GSH on liver mitochondria electron leakage is through removing excess H$_2$O$_2$ and reducing total ROS.

Introduction
Hexavalent chromium (Cr(VI)) is a well-known highly toxic metal and environmental pollutant, and was categorized as human carcinogen by IARC (International Agency for
Research on Cancer) in 1990. Industrial sources of Cr(VI) include plating, electroplating, leather tanning, cooling tower blowdown, and anodizing baths, etc [1]. It has been reported that Cr(VI) would cause hepatocyte apoptosis, characterized by reactive oxygen species (ROS) generation [2]. Excess reactive oxygen species (ROS) production is the primary cause of mitochondrial function damage and energy metabolism disorder, leading to hepatocyte apoptosis.

Generally, the possible sources of ROS production is the mitochondrial electron transport chain in hepatocyte [3, 4]. When exogenous toxins attack the mitochondrial respiratory chain, they would selectively inhibit mitochondrial complexes, resulting in blockage of normal electron transport passway and electron accumulation at the ubiquinone site. This would cause electron leakage from specific sites [5]. O$_2^-$ would be reduced to superoxide anion radical (O$_2^-$) by electron leaked from mitochondrial respiratory chain, which was further reduced to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase (MnSOD) [4]. Then H$_2$O$_2$ would generate hydroxyl radical (•OH) by reduction reaction. O$_2^-$, H$_2$O$_2$ and •OH are referred as ROS, collectively.

Mitochondria contain several ROS production sites. Determination of the production site is based on using inhibitors of electron transport. For example, if rotenone (complex I inhibitor) could increase ROS production in cells, endogenous ROS production would be from complex I. Moreover, it is well known that GSH in liver mitochondria (mtGSH) plays an important role in clearing away H$_2$O$_2$ and preventing hepatocyte mitochondria from impairment of respiratory function and dysfunction of energy metabolism [6, 7]. Mitochondria electron transport chain and the role of GSH in scavenging ROS are illustrated in Figure 1.

Previous studies strongly suggest that ROS produced in mitochondria plays a critical role in Cr(VI)-induced hepatocyte apoptosis. However, electron leakage of mitochondrial respiratory chain induced by Cr(VI) still remains unclear. In the present study, after treating isolated rat mitochondria with Cr(VI), we used glutamate/malate and succinate as substrates and a wide variety of complex inhibitors to explore the ROS production sites in mitochondria, the mechanism of Cr(VI) hepatotoxicity, and the role of GSH protection.

### Materials and Methods

**Materials**

Rotenone (Rot), diphenyleneiodonium (DPI), thenoyltrifluoroacetone (TTFA), antimycinA (Ant), L-glutamine, L-Malic acid and sodium succinate were purchased from Sigma (St. Louis, MO, USA).

**Mitochondrial isolation**

Rats received anesthesia by chloralic hydras after an overnight fast. The rat liver was removed for immediate mitochondrial isolation. Liver mitochondria were isolated by standard differential centrifugation at 4°C as previously described with modifications [8]. Liver excised from rat were weighed and carefully homogenated in isolation buffer (70mM sucrose, 210mM mannitol, 5mM Hepes, 1mM EGTA, 1%BSA, PH=7.4). Tissue debris and nuclei were separated from liver homogenate by centrifugation at 1000×g for 10 min. The supernatant solution was collected and centrifuged at 9000×g for 10 min. The mitochondria pellet was washed once with isolation buffer and re-suspended in respiratory buffer as mitochondria suspension (75mM sucrose, 50mM KCl, 30mM Tris-HCl, 2mM KH$_2$PO$_4$, 2mM MgCl$_2$, 10μM EGTA, PH=7.4). Isolated mitochondria were used immediately for measurement of respiration rate and ROS and superoxide formation.

**Mitochondrial protein measurements**

Mitochondrial protein concentrations were measured using a BCA protein quantification kit (Abcam, SE USA) with bovine serum albumin used as concentration standards. Standards and the samples were measured in duplicates and the mean values were used to calculate the protein concentrations.
Measurement of mitochondrial purity and functionality

Mitochondria re-suspended in respiratory buffer were stored on the ice for 2.5 hours, then we measured purity and functionality of mitochondria isolated with our method. We detected mitochondrial purity by detection of succinate dehydrogenase (SDH) activity in liver homogenate, supernatant solution and mitochondria suspension, respectively with SDH kit (BIOTANG Inc., Waltham, MA 02452 USA). The mitochondria suspension were fixed overnight with 2.5% glutaraldehyde, postfixed with 2% osmid acid, dehydrated, and embedded with epoxy resin. Thin sections were observed with a Hitachi H-600 transmission electron microscope. Mitochondrial ultra-structure was observed by electron microscope (magnification ×10,000 and ×30000, respectively). Then respiratory function of mitochondria was measured with Clark-type oxygen electrode. One mg of mitochondria protein was incubated in 2 ml of respiratory buffer preheated to 25°C. After 2min later, 5mm succinate was added as substrate. Then 3min later, state 3 respiration was initiated by adding 2um ADP, and oxygen consumption was measured as the slope of oxygen consumption curves. The respiratory control ratio (RCR) were calculated as the rate of ADP-stimulated oxygen consumption (State 3 respiratory) divided by the rate of oxygen consumption (state 4 respiratory) when all the ADP has been phosphorylated.

Measurement of mitochondrial basal respiratory rate

Mitochondrial basal respiratory rate of the NADH and FADH₂ respiratory chain were measured using substrate-stimulated O₂ consumption recorded with Clark-type oxygen electrode. Mitochondrial suspensions (1 mg of protein) were diluted to a total volume of 2ml in reaction buffer preheated to 25°C. Then 10mM glutamate/5mM malate or 5mM succinate were used as substrate 2 min later, respectively. Respiration rate was measured by the slope of oxygen consumption curves after substrates were added as nmol O₂/min/mg prot.

Measurement of ROS formation

Mitochondria (0.5mg/ml) were energized with complex I (10mM glutamate/ 5mM malate) and complex II (5mM succinate) substrates respectively. Mitochondria suspension were treated with Cr(VI) at 0μM, 12.5μM, 25μM, 50μM, and 100μM. Liver mitochondria exhibited a concentration-dependent increase in ROS production. Considering feasible mitochondria activity and distinct ROS production, we choose 50μM Cr(VI) as the final experimental concentration. ROS production in mitochondria co-incubated with 50μM Cr(VI) was measured with addition of specific inhibitors of respiratory complexes and 200μM glutathione as the following: Complex I inhibitors, 6.35μM Rot, 10μM DPI; Complex II inhibitor, 20μM TTFA; Complex III inhibitor, 3.75μM Ant. ROS production was assessed with 10μM 2’,7’-dichlorofluorescein-diacetate

Fig. 1. Electron transport illustration chart of respiratory chain of hepatocyte mitochondria.
Measurement of $\text{O}_2^-$ production and rate of electron leakage

Mitochondria were used under similar experimental conditions as mentioned above to measure $\text{O}_2^-$ production using 30μM dihydroethidium (DHE) fluorescent probes. $\text{O}_2^-$-mediated formation of fluorescent ethidium was recorded at $\text{Ex}_{\text{Eth}}/\text{Em}_{\text{Eth}}$. The rate of electron leakage could be calculated as the rate of $\text{O}_2^-$ production /2×rate of $\text{O}_2$ consumption [9].

Statistical analysis

All data was expressed as mean±SEM. Statistical comparisons of basal respiratory rates of the mitochondrial respiratory chain were done using t test. Comparisons among multiple treatments were done with analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Mitochondrial purity and functionality

Succinate dehydrogenase is tightly bound to the inner mitochondrial membrane and easily precipitated along with mitochondria. The activities of succinate dehydrogenase as marker enzymes of mitochondria, in mitochondria suspension were significantly higher than that in liver homogenate (Fig. 2A), which revealed that mitochondria were relatively pure.
Mitochondrial ultra-structure observation showed that mitochondria were relatively pure and morphologically intact and dense with no signs of swelling and damage (Fig. 2B, 2C). Mitochondrial respiratory control ratios (RCR) were higher than 3, which was indicative of tightly coupled mitochondria (Fig. 2D) [10].
Fig. 5. Effect of complex I inhibitors and complex III inhibitors on NADH respiratory chain in liver mitochondria. (A)(B) Effect of complex I inhibitors (ROT and DPI) on ROS production and electron leak rate from mitochondrial NADH respiratory chain induced by Cr(VI); (C)(D) Effect of complex III inhibitors (Ant) on ROS production and electron leak rate from mitochondrial NADH respiratory chain induced by Cr(VI). The concentrations of Cr(VI), Rot, DPI and Ant were 50μM, 6.35μM, 10μM and 3.75μM, respectively. *P<0.05, n=6.

Table 2. Effect of complex I inhibitors on NADH respiratory chain in liver mitochondria. Note: *P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI were 50μM, 6.35μM and 10μM, respectively.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>ROS content (FU/mgprot)</th>
<th>O2•− content (FU/mgprot)</th>
<th>O2•− content (nmol/min/mgprot)</th>
<th>Electron leak rate (%)</th>
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<td>0.137±0.035</td>
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<td>Rot</td>
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<td>Rot+DPI</td>
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<td>Cr(VI)</td>
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<td>0.30±0.115</td>
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<td>Cr(VI)+Rot</td>
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<td>7.27±0.525*</td>
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<td>23.13±1.843*</td>
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<td>Cr(VI)+DPI</td>
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<td>5.26±0.192*</td>
<td>0.95±0.045*</td>
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<td>17.63±3.873</td>
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<td>Cr(VI)+Rot+DPI</td>
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<td>6.19±0.078*</td>
<td>1.12±0.137*</td>
<td>0.39±0.050*</td>
<td>20.86±2.690*</td>
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**Basal respiration rate of NADH and FADH2 respiratory chain**

O2 consumption of glutamate/malate-energized and succinate-energized liver mitochondria was measured as basal respiratory rate of NADH and FADH2 respiratory chain (Fig. 3A, 3B). The basal respiratory rate of FADH2 respiratory chain was much greater than that of NADH respiratory chain (Fig. 3C, Table 1).

**ROS production of mitochondria induced by Cr(VI) of different concentrations**

Cr(VI) could induce the increase of ROS production in NADH and FADH2 respiratory chain in liver mitochondria in a concentration-dependent manner (Fig. 4).
Table 3. Effect of complex III inhibitors on NADH respiratory chain in liver mitochondria. Note: *P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI and Ant were 50μM, 6.35μM, 10μM and 3.75μM, respectively.

Table 4. Effect of complex I inhibitors on FADH₂ respiratory chain in liver mitochondria. Note: *P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI were 50μM, 6.35μM and 10μM, respectively.

Table 5. Effect of complex II inhibitors on FADH₂ respiratory chain in liver mitochondria. Note: *P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentrations of Cr(VI), Rot, DPI and TTFA were 50μM, 6.35μM, 10μM and 20μM, respectively.

Effect of complex inhibitors on NADH respiratory chain in liver mitochondria treated with Cr(VI)

In glutamate/malate-energized mitochondria, ROS production increased in the Rot group and the Rot+DPI group and decreased in the DPI group. In mitochondria treated with Cr(VI), ROS production increased significantly in the Cr(VI)+Rot group, the Cr(VI)+Rot+DPI group, and the Cr(VI)+DPI group (Fig. 5A, 5B), Table 2. Ant could significantly increase ROS production and electron leakage in mitochondria treated with or without Cr(VI) (Fig. 5C, 5D), Table 3.
Fig. 6. Effect of complex I inhibitors and complex III inhibitors on FADH$_2$ respiratory chain in liver mitochondria. (A) (B) Effect of complex I inhibitors (ROT and DPI) on ROS production and electron leak rate from mitochondrial FADH$_2$ respiratory chain induced by Cr(VI); (C)(D) Effect of complex II inhibitors (TTFA) on ROS production and electron leak rate from mitochondrial FADH$_2$ respiratory chain induced by Cr(VI); (E)(F) Effect of complex III inhibitors (Ant) on ROS production and electron leak rate from mitochondrial FADH$_2$ respiratory chain induced by Cr(VI). The concentration of Cr(VI), Rot, DPI, TTFA and Ant were 50µM, 6.35µM, 10µM, 20µM and 3.75µM, respectively. *P<0.05, n=6.

Table 6. Effect of complex III inhibitors on FADH$_2$ respiratory chain in liver mitochondria. Note: *P<0.05 vs the control group; *P<0.05 vs the Cr(VI) group. The concentration of Cr(VI), Rot, DPI, TTFA and Ant were 50µM, 6.35µM, 10µM, 20µM and 3.75µM, respectively.

Effect of complex inhibitors on FADH$_2$ respiratory chain in liver mitochondria treated with Cr(VI)

In succinate-energized liver mitochondria, ROS production and electron leakage decreased significantly in the Rot group, the DPI group and the Rot+DPI group. However, in mitochondria treated with Cr(VI), no significant difference was observed among the...
Fig. 7. Effect of GSH on electron leakage from mitochondrial NADH and FADH₂ respiratory chain induced by Cr(VI). (A) (B) Effect of GSH on ROS production and electron leakage from mitochondrial NADH respiratory chain induced by Cr(VI). (C) (D) Effect of GSH on ROS production and electron leakage from mitochondrial FADH₂ respiratory chain induced by Cr(VI). The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50μM, 200μM, 6.35μM, 10μM, 20μM and 3.75μM, respectively. *P<0.05, n=6.

<table>
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<th>Groups</th>
<th>N</th>
<th>ROS content (FU/mgprot)</th>
<th>electron leak rate (%)</th>
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<td></td>
<td></td>
<td>Cr(VI)</td>
<td>Cr(VI)+GSH</td>
</tr>
<tr>
<td>control</td>
<td>6</td>
<td>4.452±0.119</td>
<td>1.712±0.060</td>
</tr>
<tr>
<td>Rot</td>
<td>6</td>
<td>7.237±0.525</td>
<td>5.057±0.072 #</td>
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<tr>
<td>DPI</td>
<td>6</td>
<td>5.205±0.192</td>
<td>4.143±0.131 #</td>
</tr>
<tr>
<td>Ant</td>
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<td>5.684±0.192</td>
<td>4.285±0.122 #</td>
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<tr>
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<td>4.025±0.092 #</td>
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<tr>
<td>Rot+Ant</td>
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<td>7.580±0.272</td>
<td>5.866±0.163 #</td>
</tr>
<tr>
<td>DPI+Ant</td>
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<td>6.298±0.184</td>
<td>4.053±0.142 #</td>
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Table 7. Effect of GSH on electron leakage from mitochondrial NADH respiratory chain induced by Cr(VI)

Note: *P<0.05 vs the Cr(VI) group. The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50μM, 200μM, 6.35μM, 10μM, 20μM and 3.75μM, respectively.

Cr(VI)+Rot group, the Cr(VI)+DPI group and the Cr(VI)+Rot+DPI group (Fig. 6A, 6B, Table 4). Then we investigated the effect of TTFA, specific inhibitor of complex II, on FADH₂ respiratory chain. TTFA could increase ROS production and electron leakage in mitochondria treated with Cr(VI) with or without Rot, which was reversed by DPI (Fig. 6C, 6D, Table 5). The effect of Ant on FADH₂ respiratory chain was similar to that on NADH respiratory chain. ROS production and electron leakage in groups treated with Ant were higher than those not treated with Ant (Fig. 6E, 6F, Table 6).
Effect of GSH on electron leakage from liver mitochondrial respiratory chain

Effect of GSH on electron leakage from liver mitochondrial respiratory chain induced by Cr(VI). The concentration of Cr(VI), GSH, Rot, DPI, TTFA and Ant were 50 μM, 200 μM, 6.35 μM, 10 μM, 20 μM and 3.75 μM, respectively.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>ROS content (FU/mgprot)</th>
<th>electron leak rate (%)</th>
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<td>control</td>
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<td>36.326±0.357</td>
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<tr>
<td>Rot</td>
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<td>30.909±0.695</td>
</tr>
<tr>
<td>DPI</td>
<td>6</td>
<td>35.409±0.501</td>
<td>23.204±1.459</td>
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<tr>
<td>TTFA</td>
<td>6</td>
<td>42.653±0.978</td>
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<tr>
<td>Ant</td>
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<td>TTFA+Ant</td>
<td>6</td>
<td>47.715±2.015</td>
<td>28.617±0.520</td>
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Table 8. Effect of GSH on electron leakage from mitochondrial FADH2 respiratory chain induced by Cr(VI). Note: *P<0.05 vs the Cr(VI) group.
higher basal respiratory rate of FADH$_2$ respiratory chain. In glutamate/malate-energized mitochondria, ROS production increased in the Rot group. It is suggested that in mitochondria under normal physiological conditions, Rot inhibit electron driving from iron-sulfur center of complex I to ubiquinone and enhance their leakage from NADH respiratory chain [11]. ROS generation decreased in the DPI group, indicated that DPI would act on FMN site and block electron moving into the reduction center of complex I and then decrease electron leakage. These results were similar with others’ studies [22]. However, when mitochondria were treated with Cr(VI), the results were different. ROS content and electron leakage increased in the Rot+Cr(VI) group, but not changed in the DPI+Cr(VI) group. The results suggested that the ubiquinone binding site, but not the FMN site, is the main site of ROS production in NADH respiratory chain. Moreover, Ant could increase ROS production and electron leakage in mitochondria treated with Cr(VI), which suggests that Ant could inhibit electron moving from Cyt bH to ubiquinone, accumulate semiquinone and increase electron leakage [23, 24].

With regard to mitochondrial FADH$_2$ respiratory chain, it is generally acknowledged that complex II could not release electron by itself and electron could leak from complex I when they move backward from complex II to complex I [25], or leak from the ubiquinone binding site of complex III if they move forward along respiratory chain [26]. In succinate-energized mitochondria without Cr(VI) treatment, ROS production and electron leakage decreased in the Rot group and DPI group, and increased in the TTFA group and the Ant group. It is suggested that when electron generated from succinate moves backward from complex II to complex I, Rot would inhibit this process and DPI protects the FMN site of complex I to reduce electron leakage [18]. On the contrary, TTFA could block electron moving backward from complex II to ubiquinone, while Ant acts on the ubiquinone binding site of complex III, which leads to electron leakage and excess ROS generation.

As far as succinate-energized mitochondria treated with Cr(VI) are concerned, compared with the Cr(VI) group, ROS content and electron leakage in the Rot+Cr(VI) group and the DPI+Cr(VI) group showed no significant difference. ROS generation in Cr(VI)-treated mitochondria was irrelevant with electron moving backward from complex II to complex I. Then ROS content and electron leakage increased in the Cr(VI)+TTFA group compared with the Cr(VI) group. It is seemingly found that co-treatment of Cr(VI) and TTFA could promote electron leaked from the ubiquinone binding site of complex II, however, ROS content and electron leakage in the Cr(VI)+Rot+TTFA group and the Cr(VI)+DPI+TTFA group were significantly lower than those in the Cr(VI)+TTFA group. It is rational to postulate that the increase of electron leakage in the Cr(VI)+TTFA group was actually due to the backflow of electron from complex II to complex I. Furthermore, after Ant inhibits the ubiquinone binding site of complex III in mitochondria exposed to Cr(VI), ROS content and electron leakage increased significantly. The result above suggested that Cr(VI) could induce electron leakage from FADH$_2$ respiratory chain in liver mitochondria through the ubiquinone binding site of complex III, other than enhancing electron backflow from complex II to complex I or leaking from the ubiquinone binding site of complex II.

GSH, a critical endogenous antioxidant in mitochondria, could transform H$_2$O$_2$ into H$_2$O under the effect of glutathione peroxidase and transform itself into oxidized glutathione disulfide (GSSG), which could then transform into glutathione by glutathione reductase. Excess H$_2$O$_2$ could be cleared during this cycle [27]. Cederbaum et al. found that exogenous S-adenosyl-L-methionine (SAM) supplement, as a precursor of GSH synthesis, could protect liver mitochondria from oxidative damage [28, 29]. In our study, protective effects of exogenous GSH supplement on NADH and FADH$_2$ respiratory chain of liver mitochondria were similar. GSH could clear over excess ROS production in liver mitochondria induced by Cr(VI) in both NADH and FADH$_2$ respiratory chains, but no significant protective effect could be observed on electron leakage. It is indicated that GSH do exhibit protective effects on liver mitochondria by the elimination of excess H$_2$O$_2$ and ROS, but could not decrease electron leakage from mitochondrial respiratory chain.

In summary, our study demonstrated the sites of Cr(VI)-induced ROS production and the protective effect of GSH in mitochondrial NADH and FADH$_2$ respiratory chain. Cr(VI)
induced the increase of electron leakage from ubiquinone binding sites of complex I and complex III in NADH respiratory chain. While in succinate-energized mitochondria, Cr(VI) induced electron leakage from the ubiquinone binding site of complex III when the electron moved forward in the respiratory chain. GSH can alleviate Cr(VI)-induced liver mitochondrial oxidative damage through effective removal of excess H₂O₂ and total ROS content, rather than the improvement of mitochondrial respiration function to reduce electron leakage.

Acknowledgements

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