Cr(VI)-induced overactive mitophagy contributes to mitochondrial loss and cytotoxicity in L02 hepatocytes

Yujing Zhang¹, Huanfeng Bian², Yu Ma¹, Yuanyuan Xiao¹, Fang Xiao¹*

¹Department of Health Toxicology, Xiangya School of Public Health, Central South University, Changsha 410078, PR China

²Shajing Health Inspection Institute of Baoan District, Shenzhen 518104, PR China

Correspondence: Fang Xiao (fangxiao@csu.edu.cn)

Abstract

Hexavalent chromium [Cr(VI)] has aroused the main interest of environmental health researchers due to its high toxicity. Liver is the main target organ of Cr(VI), and the purpose of this study was to explore whether mitophagy contributes to Cr(VI)-induced hepatotoxicity and to demonstrate the potential mechanisms. Cr(VI) exposure induced mitochondrial loss, energy metabolism disorders and cell apoptosis, which were associated with the occurrence of excessive mitophagy characterized by the increased number of green fluorescent protein-microtubule-associated protein light chain 3 (GFP-LC3) puncta and lysosomal colocalization with mitochondria. In addition, the suppression of mitophagy by autophagy-related 5 (ATG5) siRNA can effectively inhibit Cr(VI)-induced mitochondrial loss and cytotoxicity. In summary, we reached the conclusion that Cr(VI)-induced overactive mitophagy contributes to mitochondrial loss and cytotoxicity in L02 hepatocytes, which will further reveal the possible mechanisms of Cr(VI)-induced hepatotoxicity, and provide a new experimental basis for the study of the health hazard effects of chromium.
Keywords

Hexavalent chromium [Cr(VI)]; mitophagy; mitochondrial loss; cytotoxicity; L02 hepatocytes

Introduction

Hexavalent chromium [Cr(VI)] has aroused the main interest of environmental health researchers, who believe that past exposures will affect community health [1]. This toxic substance enters the domestic environment when the facilities discharge polluted air, which then contaminates the nearby soil and water. The main non-occupational exposure occurs by drinking the polluted water. At present, water contamination caused by Cr(VI) is a worldwide problem, making this an issue of great public health significance. An ecological mortality study conducted in Greece revealed that the standardized mortality ratio (SMR) for primary liver cancer was statistically significantly elevated due to the water contamination with Cr(VI) [2]. Study on Cr(VI)-induced hepatotoxicity is the hot topic in the field of toxicology. At present, a large number of studies [3, 4] have confirmed that Cr(VI) can cause liver injury, and the mechanism involved have also been clarified.

Mitochondria are not only "stabilizers" for maintaining normal physiological functions of cells, but also "effectors" for external stimuli acting on cells [5]. It is known that mitochondria are involved in the regulation of important biological processes including reactive oxygen species (ROS) production, intracellular calcium level stabilization, cell apoptosis, and autophagy cascade signaling initiation [6]. Mitochondrial loss caused by various factor will have a serious impact on cells, resulting in energy metabolism disorders, the cellular environment imbalance, and even death [7]. The heavy metals with hepatotoxicity, such as cadmium, also exhibit mitochondrial toxicity. It has been confirmed that cadmium exposure caused mitochondrial loss in hepatocytes, which is manifested by the decrease of both mitochondrial DNA copy number and the expression of respiratory chain components, thus aggravating the energy metabolism disorder and causing cell death [8]. It is not clear whether Cr(VI) causes mitochondrial loss.
Autophagy is a non-selective kinetic process of mass degradation of cytoplasmic macromolecules and organelles in lysosomes, which plays an important role in the degradation of many intracellular contents such as peroxisome, endoplasmic reticulum and mitochondria [9]. Autophagy is a dynamic biological process in which the cell encapsulates the target to be degraded into autophagosomes (APs) through the cup-shaped double membrane structure of unknown origin, and then transports it to the lysosomal cavity to form autolysosomes (ALs) and degrades it. Since ALs are composed of single membrane, it is difficult to distinguish them from APs with double membrane structure under electron microscope, so they are collectively called autophagic vacuoles (AVS) [10]. Correspondingly, the special process of clearing mitochondria from cells by autophagy is called "mitophagy" [11]. Mitophagy is involved in many pathological processes, such as infection, cancer, aging and neurodegenerative diseases, and is closely related to cell differentiation, survival and homeostasis [12]. Under physiological conditions, mitophagy plays an advantageous role in clearing damaged mitochondria and ensuring mitochondrial quality [13]; however, the abnormal and persistent activation of mitophagy can stimulate the degradation of mitochondria, leading to energy metabolism disorders and cell death [14].

In this study, we explored whether Cr(VI) causes mitochondrial loss and its related mechanisms, and we also proposed that Cr(VI)-induced hepatotoxicity may arise from mitochondria loss due to abnormal mitophagy, which will further reveal the possible mechanisms of Cr(VI)-induced hepatotoxicity, and provide a new experimental basis for the study of the health hazard effects of chromium.

Materials and methods

Antibodies

Antibodies against microtubule-associated protein 1 light chain 3 (LC3) (18725-1-AP), and transcription factor A, mitochondrial (TFAM) (19998-1-AP) were purchased from Proteintech Group Inc. (Wuhan, China). NADH dehydrogenase subunit I (ND1) (DF4214), cytochrome c
oxidase subunit IV isoform 1 (COX4I1) (AF5468), and autophagy-related 5 (ATG5) (DF6010) antibodies were obtained from Affinity Biosciences (Cincinnati, OH, USA). Sequestosome 1 (SQSTM1/p62) (A19700), peroxisome proliferator-activated receptor gamma, and coactivator 1 alpha (PPARGC1A) (A17089) antibodies were purchased from ABclonal Technology (Wuhan, China). Antibody against β-actin (70-ab008-040) was obtained from MultiSciences Biotech Co. (Hangzhou, China).

**Cell culture**

Human L02 hepatocytes were obtained from the Experimental Central of Xiangya Hospital of Central South University and grown in RPMI 1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), which was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO2.

**RNA interference of ATG5**

L02 hepatocytes were seeded onto 6-well plates and cultured overnight. The cells were then transfected with siRNA targeting ATG5 (siB160225095638-1-5) or negative control (siB06525141910-1-5) duplexes using lipofectamine 3000 (Invitrogen Corporation, Carlsbad, CA) according to manufacturer’s protocol. The transfected cells were changed with complete medium 4-6 h later. These siRNA sequences were designed and synthesized by RiboBio (Guangzhou, China).

**Measurement of mitochondrial respiratory chain complexes (MRCCs) activities**

The mitochondrial enzyme activities of MRCC I (nicotinamide adenine dinucleotide hydride (NADH)-ubiquinone reductase), II (succinate dehydrogenase), III (ubiquinol: cytochrome c (Cyt c) reductase), IV (Cyt c oxidase), and V (ATP synthase) were assayed as previously described using commercial kits [15].

**Quantitative real-time PCR (qPCR) analysis**

The total RNA was isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA)
after chemicals treatment. The cDNAs were synthesized using the RT-PCR kit (Takara, Japan). The following primers were used: ATG5, 5'-GTGCTTCGATGATGTGTGGTT-3' (F), 5'-TGCAATAGCTAGCTTGCCAAA-3' (R); ACTB, 5'-CACCAGGCGTGATGTT-3' (F), 5'-CTCAAAATGATCTGG GTCAT-3' (R). qPCR was carried out using the Light Cycler®Nano SYBR Green I Master on a Light Cycler® Nano System according to the manufacturer's instructions. The mRNA levels were calculated using the 2^{\Delta\Delta CT} comparative CT method normalized to ACTB mRNA.

**Mitochondrial DNA analyses**

Total DNA was extracted from the treated cells using the DNA extraction kit (NEP002-1, Dingguo, China) according to the manufacturer's instructions. The relative mtDNA copy number was then detected by RT-PCR. The primers used: 5'-TACGCAAGGTTCCAACG-3' (F) and 5'-GGTGATGCGGATGTTG-3' (R) for ND1; 5'-TAGAAACCGTCTGAACATCC-3' (F) and 5'-ATGATTAGGGCGCTGA-3' (R) for COX4I1; 5'-GTTACTGCCTG TGGGGCAA-3' (F) and 5'-CAAAGGTTGGGTACGGT-3' (R) for β-globin. The PCR reactions were performed using a Light Cycler® Nano System. The PCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. A no-template control and three serial-dilution points (in 5-fold steps) of the DNA sample in every reactions were performed in triplicate. The relative mtDNA copy number was calculated as described for the relative RNA quantification.

**Western blotting analysis for protein levels**

For total protein extraction, the cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology, China, P0013B). The protein concentrations were determined using the Enhanced BCA Protein Assay Reagent (Beyotime Institute of Biotechnology, China, P0010), and equal amounts of each sample were boiled with 1× SDS-PAGE sample buffer for 10 min, separated on 5%-12% SDS-PAGE gels, and transferred onto NC membranes (PALL, USA, 66485). After blocking with 5% nonfat dried milk, the membranes were incubated with the appropriate antibodies. Finally, the protein bands were visualized using the enhanced
chemiluminescence kit (Thermo, USA, 34095). Densitometric analysis of the bands was performed using Image J software (National Institutes of Health, USA).

**ROS level analysis**

The intracellular ROS accumulation was detected by Reactive Oxygen Species Assay Kit. Treated L02 hepatocytes were washed by cold PBS and loaded with 10 μM DCFH-DA (Beyotime Institute of Biotechnology, China, S0033) at 37°C for 40 min. Then, the cells were washed with PBS and incubated with Hoechst33258 (1 mg/mL) (Beyotime Institute of Biotechnology, China, C1017) for 15 min in the dark at 37°C. Green fluorescence were observed using microscope (Olympus, Tokyo, Japan). ROS level was quantitated using flow cytometry.

**Determination of mitochondria mass**

The L02 hepatocytes were seeded in a 96-well plate at a final density of 0.5 × 10⁴ cells/well, and then were exposed to 5 μM 10-N-nonyl-acridine orange (NAO) (Invitrogen, A1372) for 30 min. The hepatocytes were washed twice with PBS. The fluorescence was then measured at the emission of 530 nm and the excitation of 485 nm using the Microplate Reader. The cellular fluorescence intensity was expressed as percentage of the control (% of control).

**Detection of mitochondrial respiration**

Mitochondrial respiration was determined using a Clark-type oxygen electrode (Rank Brothers Ltd, Cambridge, England). Briefly, the mitochondrial suspension (1 mg/ml) were added with the buffer (2 mM K₂HPO₄, 130 mM KCl, 3 mM HEPES, 2 mM MgCl₂, 1 mM EGTA), followed by the addition of 5 mM glutamate-malate. State 4 respiration (ATP independent respiration) was monitored in the absence of ADP after the addition of 2 μg/ml oligomycin, and state 3 respiration (phosphorylating respiration) was measured after the addition of 2 μl ADP (27 mM) to examine maximal rate of coupled ATP synthesis. The respiratory control ratio (RCR) was calculated as the ratio between state 3/state 4 respiration.

**ATP content detection**
The ATP level was determined using an ATP Determination Kit (Beyotime Institute of Biotechnology, China, S0026). 1×10^6 cells were lysed with 200 μl lysis buffer and then centrifugated at 12,000xg for 5 min at 4 °C, and the supernatant was transferred to a new tube. 100 μl ATP detection buffer was added to 100 μl samples, and then the luminescence of samples was measured in a luminescence plate reader with the integration time of 10 s. The ATP standard curve was prepared from a known amount (0.01-10 μM).

**Caspase activity assay**

The caspase 3/9 Activity Assay Kit (Beyotime Institute of Biotechnology, China, C1116, C1158) was used to detect of caspase-3/9 activities. The standard curve between caspase activities (0, 10, 20, 50, 100 and 200 μM) and absorbance was established. In brief, L02 hepatocytes were seed onto the 60 mm plates and given indicated treatments. Then, the cells were collected and incubated with Ac-DEVD-pNA (2mM) (for Caspase-3 activity) or Ac-LEHD-pNA(2mM) (for Caspase-9 activity) for 1 h at 37 °C in the dark. The absorbance was recorded at 405 nm. Caspase activities of the measured samples were extrapolated from the caspase activity standard curve.

**Detection of cell apoptosis**

The commercial Annexin V-FITC Apoptosis Detection Kit (Invitrogen Corporation, Carlsbad, CA, V13241) was used. The cells were treated with different chemicals and re-suspended in 100 μl of 1×binding buffer containing 5 μl Annexin-V-FITC and 1 μl propidium iodide (PI) for 30 min. Then 400 μl binding buffer was added to stop staining. The flow cytometric analysis was performed. Data analysis was performed using Flowjo 7.6 software.

**Transmission electron microscope (TEM) analysis**

For the TEM analysis, the L02 hepatocytes were treated with different concentrations of Cr(VI) (0, 8, 16 μM) for 24 h, washed twice with PBS and then fixed with 2.5% glutaraldehyde. The hepatocytes were post-fixed in 2% osmium tetroxide, embedded and stained with uranyl acetate/lead citrate. The observation was done using a Hitachi HT-7700 electron microscope (Japan) to detect autophagic vacuoles (AVs), as previously described.
Confocal laser microscopy

To assess autophagy, $1 \times 10^5$ L02 hepatocytes were seeded onto 6-well plate and transfected with green fluorescent protein-LC3 (GFP-LC3) plasmid (Jikai, China, GVAP01689345) for 24 h after incubation overnight. The cells were then incubated with the primary antibody of TOMM20 (Abcam, UK, ab56783) at 1:100 dilution overnight at 4 °C, followed by secondary antibody incubation for 1 h at room temperature. After incubation, the cells were stained with Hoechst33342 (1 mg/mL) (Beyotime Institute of Biotechnology, China, C1022) for 30 min in the dark at 37 °C. The colocalization of GFP-LC3 positive autophagosomes (APs) with the mitochondria was observed by Leica TCS SP5 II confocal spectral microscope.

Fluorescent confocal images of the colocalization of LysoTracker Red (Beyotime Institute of Biotechnology, China, C1046) with mitochondria were used to visualize mitochondria-containing autolysosome (AL) formation. In brief, the L02 hepatocytes were seeded onto 6-well plate and treated with Cr(VI) for 24 h after overnight incubation. After treatment, the cells were washed by PBS for 2 times before stained with LysoTracker Red at 1:15000 dilution for 45 min at 37 °C in dark. Then, the cells were washed by PBS and stained with MitoTracker Green (Beyotime Institute of Biotechnology, China, C1048) to locate mitochondria for another 45 min at 37 °C. Cells were rinsed with PBS and observed using confocal spectral microscope.

Statistics analysis

All results were presented as the mean ± SD from three independent experiments unless otherwise indicated. Difference of the data from different groups was performed using one-way analysis of variance. The Student t-test was used to evaluate the significant differences between the experimental values of the 2 samples being compared, and $p < 0.05$ was considered to be statistically significant.

Results
**Cr(VI) exposure induced mitochondrial loss**

Evidence suggested that mitochondria are the most vulnerable target of major environmental pollutants such as heavy metals [17]. We first determined the activities of MRCCs to confirm whether Cr(VI) exposure (0, 8, 16 μM) could induce mitochondrial inhibition. As shown in Figure 1A, the activities of MRCC I, II, and IV were decreased after Cr(VI) treatment in a dose-dependent manner. mtDNA copy number is closely related to the quality and quantity of mitochondria. mtDNA is involved in encoding 13 proteins closely related to mitochondrial function, and the maintenance of its quantity and quality plays a very important role in the function of mitochondria. We designed primers for ND1 (MRCC I subunit) and COX4I1 (MRCC IV subunit) genes encoded by mtDNA and analyzed the DNA extracted from cells by qPCR. The copy number of mtDNA can be reflected by the expression amount of the two genes. The result in Figure 1B showed that the copy number of mtDNA decreased significantly after Cr(VI) treatment. ND1 and COX4I1 are the core proteins in mitochondrial respiratory chain that control ATP production, and are closely related to the hydrogen and electron transfer reactions. The result in Figure 1C & 1D showed that the levels of the two proteins were decreased along with the increase of Cr(VI) exposure. The inhibition of MRCCs means that electrons can't pass through the respiratory chain smoothly, and the accumulation of leakage electrons will cause the burst generation of ROS. As shown in Figure 1E, the green fluorescence increased in a dose-dependent manner. The quantitative result of ROS level was shown in Figure 1F. Mitochondrial quality decline is one of the core indicators of mitochondrial loss. Figure 1G shows a significant reduction in mitochondrial mass after exposure to Cr(VI). After treatment with the highest dose of Cr(VI) for 24 h, the quality of mitochondria decreased by 40%.
Figure 1. Cr(VI) exposure induced mitochondrial loss. L02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8, 16 μM) for 24 h. (A) The activities of MRCCs were determined using the commercial kits. (B) The mRNA expressions of mtDNA-encoded genes ND1 and COX4I1 were analyzed by qPCR. (C) The protein levels of ND1 and COX4I1 were determined using western blotting analysis. (D) Densitometric analysis of the bands was performed using Image J software. (E) The intracellular ROS accumulation was detected by Reactive Oxygen Species Assay Kit. (F) ROS level was quantitated using flow cytometry. (G) Mitochondrial mass was determined using NAO. *p<0.05, compared with control group.
Cr(VI) exposure induced energy metabolism disorders and cell death in L02 hepatocytes

We examined whether mitochondrial respiration was affected in Cr(VI)-treated hepatocytes.

The effect of Cr(VI) on the mitochondrial respiratory capacity was evaluated by determining State 3 (coupled to ADP phosphorylation) and State 4 (after ADP phosphorylation) respiration. As shown in Figure 2A, State 3 respiration was decreased in a dose-dependent manner, indicating the impairment of mitochondrial electron transport and respiratory capacity. State 4 respiration was not affected by Cr(VI) treatment. The RCR, the ratio between rates of respiration in State 3 and State 4, was concentration-dependently reduced (Figure 2B). When mitochondria are lost or their functions are impaired, ATP consumption exceeds the production, resulting in the decrease of ATP content. Using luciferase method, we found that ATP level in L02 hepatocytes decreased in a dose-dependent manner after Cr(VI) exposure (Figure 2C). Cr(VI)-induced cell apoptosis was further confirmed by the increased activities of caspase-3 and caspase-9 (Figure 2D), and by the Annexin V/PI double staining results in Figure 2E.

*p<0.05, compared with Cr(VI) (8 μM) group.
Figure 2. Cr(VI) exposure induced energy metabolism disorders and cell death in L02 hepatocytes. (A) State 3 respiration was measured after the addition of Glu/Mal, and State 4 respiration was measured after the addition of oligomycin in the absence of ADP. (B) RCR was calculated as ratios of state 3 respiration/state 4 respiration. (C) Intracellular ATP level was detected using the commercial kit. (D) The activities of caspase-3 and caspase-9 were assayed using the caspase 3/9 Activity Assay Kit. (E) Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit. *p<0.05, compared with control group; #p<0.05, compared with Cr(VI) (8 μM) group.

Cr(VI) triggered mitophagy in the hepatocytes

The autophagic vacuoles (AVs) including autophagosomes (APs) and autolysosomes (ALs) that observed by transmission electron microscopy (TEM) are the gold indicators of autophagy. Compared with the control group, we found that Cr(VI) treatment significantly
increased the number of AVs in the hepatocytes in a dose-dependent manner (Figure 3A). The average number of AVs was shown in Figure 3B. LC3 is an important cytoskeleton protein in cells, and the transformation of Lc3-I to LC3-II is a key event that indicates the occurrence of autophagy. As shown in Figure 3C & Figure 3D, Cr(VI) significantly increased the expression of LC3-II.

Figure 3. Cr(VI) triggered mitophagy in the hepatocytes. (A) L02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8, 16 μM) for 24 h. TEM analysis was used to detect AVs. (B) The quantitative result of AVs observed under TEM. (C) Western blotting analysis of LC3 expression. (D) The protein bands were quantitated using Image J software.

*p<0.05, compared with control group; #p<0.05, compared with Cr(VI) (8 μM) group.

Mitophagy is a specific autophagy process that represents an important way to control the quality of mitochondria. Mitochondria are selectively wrapped into APs to form APs containing mitochondria, which combine with lysosomes to form ALs containing...
mitochondria, so as to achieve the purpose of degradation of mitochondria. We measured the number of APs containing mitochondria by transfecting GFP-LC3 plasmid into L02 hepatocytes and co-localizing it with TOMM20-labeled mitochondria. Using laser confocal three-dimensional reconstruction technique, we found that compared with the control group, the number of positive cells containing APs that engulfed mitochondria was increased when exposed to Cr(VI) (Figure 4A). The percentage of cells positive for APs was shown in Figure 4B. We also measured the number of ALs containing mitochondria by co-localizing LysoTracker-labeled mitochondria and MitoTracker-labeled lysosomes, and found that the number of positive cells containing ALs that engulfed mitochondria was also increased after Cr(VI) treatment (Figure 4C). The percentage of cells positive for ALs was shown in Figure 4D.
Figure 4. Cr(VI) triggered mitophagy in the hepatocytes. (A) The hepatocytes were exposed to PBS or Cr(VI) (16 μM) for 24 h. The number of APs containing mitochondria was examined by transfecting GFP-LC3 plasmid into L02 hepatocytes and co-localizing it with TOMM20-labeled mitochondria. (B) The percentage of cells positive for APs was calculated. (C) The number of ALs containing mitochondria was determined by co-localizing LysoTracker-labeled mitochondria and MitoTracker-labeled lysosomes. The pictures were taken under confocal laser microscopy. (D) The percentage of cells positive for ALs was shown. *p<0.05, compared with control group.
Cr(VI) affected the autophagic flow of the hepatocytes by increasing APs formation

The increase of APs containing mitochondria may be due to the enhancement of autophagic activity or the impairment of the degradation pathway of APs. We then examined how Cr(VI) affected the autophagic flow of the hepatocytes. Firstly, we detected the change of the level of SQSTM1/p62 protein, which was selectively incorporated into APs through direct binding with LC3, and effectively degraded by autophagic pathway. As shown in Figure 5A & Figure 5B, we observed a dose-dependent decrease in the level of SQSTM1 protein, which confirmed the existence of autophagy flow in the cells treated with Cr(VI). Then, we used the vacuolar-type H+-ATPase inhibitor Bafilomycin A1 (BafA1), which can induce the fusion disorder of APs and lysosomes, leading to the accumulation of APs. In the presence of 10 nm Baf A1, the percentage of GFP-LC3 positive APs in the cells treated with Cr(VI) increased significantly (Figure 5C & Figure 5D), suggesting that the accumulation of APs was due to the increase of APs formation, rather than the inhibition of the fusion process of APs and lysosomes. Another reason for mitochondrial loss is the impairment of mitochondrial biosynthesis. Mitochondrial biosynthesis is mainly regulated by PPARGC1A and TFAM, which are responsible for the synthesis of mitochondrial respiratory chain proteins encoded by mtDNA, such as complex IV subunit. However, we found that the protein levels of PPARGC1A and its downstream target TFAM were up-regulated after Cr(VI) treatment (Figure 5A & Figure 5B), suggesting that the Cr(VI)-induced mitochondrial loss was not due to the impairment of mitochondrial biosynthesis.
Figure 5. Cr(VI) affected the autophagic flow of the hepatocytes by increasing APs formation. (A) L02 hepatocytes were exposed to different concentrations of Cr(VI) (0, 8, 16 μM) for 24 h. The protein levels of SQSTM1, PPARGC1A, and TFAM were determined by western blotting. (B) The protein bands were quantitated using Image J software. (C) The hepatocytes were treated by Cr(VI) (16 μM) with or without the presence of 10 nm Baf A1 for 24 h. The GFP-LC3 positive APs were observed. (D) GFP-LC3 positive APs number (% of control) was calculated. *p<0.05, compared with control group; #p<0.05, compared with Cr(VI) (8 μM) group; &p<0.05, compared with Cr(VI) alone treatment group.

The excessive mitophagy promoted Cr(VI)-induced mitochondrial loss and cytotoxicity

In order to clarify whether mitochondrial autophagy was involved in Cr(VI)-induced mitochondrial loss and hepatotoxicity, we used ATG5 siRNA to inhibit mitochondrial autophagy. We designed three ATG5 siRNAs named ATG5-si1, -si2, and -si3, and found
ATG5-si1 was the most effective one. ATG5-si1 can successfully inhibit the gene (Figure 6A) and protein (Figure 6B & 6C) expression of ATG5. ATG5 siRNA significantly inhibited the increase of mitochondrial APs after Cr(VI) exposure (Figure 6D). The percentage of cells positive for APs was shown in Figure 6E.

Figure 6. The excessive mitophagy promoted Cr(VI)-induced mitochondrial loss and cytotoxicity. The hepatocytes were transfected with ATG5 siRNAs (-1, -2, -3) or control...
siRNA and were treated with PBS or Cr(VI) (16 μM) for 24 h. (A) mRNA levels of ATG5 were determined using qPCR. (B) The protein levels of ATG5 were assayed using western blotting. (C) The protein bands were quantitated using Image J software. (D) Mitochondrial APs were examined by transfecting GFP-LC3 plasmid into the hepatocytes and co-locating it with TOMM20-labeled mitochondria. (E) The percentage of cells positive for APs was calculated. *p<0.05, compared with con-si group; *p<0.05, compared with con-si plus Cr(VI) (16 μM) treatment group.

At the same time, ATG5 siRNA treatment effectively prevented Cr(VI)-induced mitochondrial loss, inhibited the reduction of mitochondrial DNA copy number (Figure 7A), and partially alleviated the decrease of ATP level (Figure 7B) and the increase of caspases activities (Figure 7C). The above results indicated that restricting the abnormal mitochondrial autophagy can prevent the mitochondria loss and the cell death induced by Cr(VI). In order to evaluate the effect of ATG5 interference on Cr(VI)-induced hepatotoxicity, we tested the effects of ATG5 siRNA on Cr(VI)-treated hepatocytes after 24, 36 and 48h respectively, and found that ATG5 interference can significantly reverse the cytotoxic effect of Cr(VI), such as the increase of caspases activities (Figure 7D), the collapse of MMP (Figure 7E), and the enhancement of apoptosis (Figure 7F) after 24h, but it has no significant effect on the toxic effect after 48h. This finding confirmed that when Cr(VI) induced severe liver injury, even inhibition of excessive mitochondrial autophagy could not reverse the hepatotoxicity, suggesting that ATG5 interference can only prevent the early liver toxicity of Cr(VI).
Figure 7. The excessive mitophagy promoted Cr(VI)-induced mitochondrial loss and cytotoxicity. (A) The mRNA levels of ND1 and COX4I1 which indicated mitochondrial DNA copy number were determined using qPCR. (B) Intracellular ATP level was detected using the commercial kit. (C) The activities of caspase-3 and caspase-9 were assayed using the commercial kit. (D) The effects of ATG5 siRNA on Cr(VI)-treated hepatocytes after 24, 36 and 48h were evaluated. The activities of caspases were then determined. (E) The change of MMP was detected by JC-1 staining using flow cytometer. (F) Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit. *p<0.05, compared with con-si group; *p<0.05, compared with con-si plus Cr(VI) (16 μM) treatment group.

Discussion

Mitochondria play an essential role in various cellular pathways including ATP generation, calcium homeostasis, apoptosis regulation and nucleotide synthesis. Mitochondria are known
as semi-autonomous organelles since they have their own DNA (mtDNA), which encodes for 13 essential subunits of MRCCI, III, IV, and V [18]. Mitochondrial quality control, which plays an important role in the stability of mitochondrial function, is crucial for cell fate determination, and mitophagy is one of several processes to maintain mitochondrial quality.

Once mitochondrial quality control is disrupted, dysfunctional mitochondria with inhibited ATP production and enhanced ROS production accumulate, affecting cell fate. Hence, mitochondrial dysfunction definitely leads to cell dysfunction including cell death, which contributes to the pathogenesis of various diseases [19]. ROS, which are composed of a group of highly reactive molecules, participate in a variety of intracellular activities including cellular growth, proliferation, aging and apoptosis in regular conditions [20]. Mitochondria are known as the main intracellular sources of ROS. We have demonstrated in our previous research that MRCC I is the main site of ROS generation in mitochondria, and also the main reduction site of Cr(VI) after entering mitochondria [15]. The over accumulation of ROS may cause mitochondrial depolarization, triggering mitophagy, a mechanism that plays an important role in eliminating ROS by inhibiting oxidative stress [21]. ROS-associated mitophagy is recognized as a negative feedback response, which means mitophagy is considered to be protective by removing the over-accumulated ROS, however, some researchers believe that ROS-mediated overactive or insufficient mitophagy leads to cytotoxicity even cell death.

We found in the present study that Cr(VI) induced hepatotoxicity in a dose-dependent manner. Cr(VI) could also reduce mitochondrial mass, decrease the protein content of MRCCs and the copy number of mtDNA, and inhibit ATP production. These mitochondrial loss phenomena are attributed to Cr(VI)-induced excessive mitophagy rather than the inhibition of mitochondrial biogenesis. Interestingly, we found that Cr(VI) had no significant effect on mitochondrial biogenesis, which further confirmed the important and specific role of mitophagy in Cr(VI)-induced mitochondrial loss in hepatocytes. The inhibition of excessive mitophagy by ATG5 interference can reverse the loss of mitochondria, maintain the balance of energy metabolism, and alleviate the toxic effects induced by Cr(VI) exposure, indicating that excessive mitophagy is an important cause of Cr(VI)-induced hepatotoxicity. In addition,
mitochondrial loss is an important cause of mitochondrial-related diseases such as cardiovascular diseases, diabetes, and neurodegenerative diseases [22, 23]. For example, in diabetic mice, it was found that the number of mitochondrial DNA copies of islet cells was decreased, resulting in MMP loss, calcium homeostasis imbalance, glucose metabolism disorder, and insulin resistance. Some toxic substances such as 6-hydroxydopamine (6-OHDA) can inhibit the synthesis of MRCC I protein, cause the death of dopamine neurons in substantia nigra, and induce the symptoms of Parkinson's disease [24].

Mitophagy is the mechanism of mitochondrial degradation under various stress conditions, which has been observed in many diseases related to mitochondrial dysfunction. It has been reported that 6-OHDA can activate extracellular signal-regulated kinases 1/2 (ERK1/2) pathway in SH-SY5Y (human neuroblastoma cells), lead to excessive mitophagy, mitochondrial loss and even cell death [25], suggesting that mitophagy-mediated mitochondrial degradation can regulate the quantity and quality of mitochondria. As a quality control mechanism, mitophagy is responsible for eliminating damaged mitochondria [26], but the role of mitophagy in cell death is still controversial. Inhibition of autophagy makes hepatocytes more susceptible to apoptosis, probably because damaged mitochondria cannot be removed in time, resulting in energy supply disorders and cell death. However, the abnormal activation of mitophagy can remove or degrade excessive mitochondria, which makes cell physiological activity unsustainable and leads to fatal consequences. Whether caspase is activated or not, mitochondrial autophagic death has been recognized as another form of cell death in addition to apoptosis and necrosis [27]. Mitophagy is confirmed to control the quality and the number of mitochondria, while the excessive mitophagy promoted Cr(VI)-induced mitochondrial loss and cytotoxicity. Current data suggest that mitochondrial damage can trigger mitochondrial autophagic death. The occurrence of Cr(VI)-induced mitophagy can be confirmed by the formation of mitochondria-containing APs and mitochondria-containing ALs. Cr(VI) can damage mitochondria directly or indirectly and induce excessive mitochondrial autophagic clearance, thus we do not exclude that activation of mitophagy is a secondary response to Cr(VI)-induced mitochondrial damage. Conversely, the mitochondrial dysfunction can accelerate mitophagy activation, and excessive mitophagy
can further aggravate mitochondrial loss, thus forming a vicious cycle that will not terminate until cell death. This may be why inhibition of mitophagy is protective at the initial stage of Cr(VI) exposure, whereas at the later stage (48 h) the accumulation of damaged mitochondria accelerated cell death.

In summary, we confirmed in the present study that Cr(VI) exposure induced mitochondrial loss, energy metabolism disorders and cell death in L02 hepatocytes. In addition, Cr(VI) affected the autophagic flow of the hepatocytes by increasing APs formation, and the excessive mitophagy promoted Cr(VI)-induced mitochondrial loss and cytotoxicity. Based on the effect of Cr(VI) on mitophagy, choosing specific inhibitors of mitophagy can protect against Cr(VI)-induced hepatotoxicity and provide new clues to mitigate the health hazards caused by chromium.

**Author Contributions**

Yujing Zhang: methodology and writing-Original draft preparation; Huanfeng Bian: investigation and software; Yu Ma: data curation; Yuanyuan Xiao: writing-reviewing and editing; Fang Xiao: conceptualization and supervision.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

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