

Hexavalent chromium intoxication induces intrinsic and extrinsic apoptosis in human renal cells

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Abstract. Hexavalent chromium [Cr(VI)], is a well-known toxic form of the heavy metal chromium in the natural environment. Clinical evidence has indicated that exposure to Cr(VI) can cause severe renal damage. The production of reactive oxygen species (ROS) due to intracellular reduction of Cr(VI) is the main mechanism underlying the induction of cellular dysfunction and apoptosis. The present study aimed to investigate in detail the apoptotic pathways induced by Cr(VI)-exposure in a human immortalized proximal tubular epithelial cell line HK-2, in order to understand the mechanism involved therein. Exposure to 10 μ M potassium dichromate ($K_2Cr_2O_7$), a toxic compound of Cr(VI), significantly decreased cell viability after 24 and 48 h of incubation and induced intracellular ROS generation. The expression levels of markers that activate the apoptotic pathway including cleaved caspase-3 and poly (ADP-ribose) polymerase were significantly upregulated in $K_2Cr_2O_7$ -exposed HK-2 cells. In addition, the induction of intrinsic and extrinsic apoptotic markers was detected in $K_2Cr_2O_7$ -exposed HK-2 cells. In summary, the present study described for the first time the novel apoptotic mechanism of Cr(VI)-toxicity in human renal cells which may be beneficial in designing optimal clinical treatment for renal damage caused by acute Cr(VI) toxicity.

Introduction

Chromium is the sixth most abundant element in the earth's crust. Trivalent Cr(III) and hexavalent Cr(VI) chromium are the most stable oxidative states occurring in nature. Cr(VI) is more cytotoxic than Cr(III) due to its stronger oxidizing capacity and rapid absorption by the cells through non-specific anion carriers (1,2). Toxic Cr(VI) can be released into the environment through soil, sea water, and fresh water. As a result, individuals may be at a risk of exposure to Cr(VI) when consuming contaminated drinking water (3,4). The development of an optimal treatment scheme against acute or chronic Cr(VI) exposure remains a critical issue.

When Cr(VI) enters the cells, it can be rapidly reduced into Cr(III), resulting in the production of several reactive chromium intermediates and reactive oxygen species (ROS), all of which are responsible for altering normal cellular function and promoting apoptosis (5,6). Potassium dichromate ($K_2Cr_2O_7$) is a toxic compound of Cr(VI) that has been demonstrated to induce nephrotoxicity in humans and animals (7). In a clinical setting, acute and chronic Cr(VI) exposure can cause severe damage to proximal renal tubular cells and result in significant renal function deterioration, thereby requiring hemodialysis (8-11). Cr(VI) toxicity causes acute tubular necrosis, whereby Cr(VI) directly damages the tubular epithelium (12). Less frequently, Cr(VI) also causes interstitial renal injury (13,14). Furthermore, Cr exposure can lead to hepatorenal syndrome, severe coagulopathy, and intravascular hemolysis that may indirectly contribute to the aggravation of renal dysfunction (13).

Cr(VI) induces free radical production by Fenton-type or Haber-Weiss reaction or by reacting directly with cellular molecules, triggering multiple possible apoptotic signaling pathways in various cell types (15,16). Several studies have suggested that Cr(VI) toxicity induced cell apoptosis mainly via intrinsic mitochondrial pathways in several types of cells, including human tumor cell lines, anterior pituitary cells, hepatocytic cells, and also in a rat model (5,17-19). Although ROS is also linked to the extrinsic apoptotic pathway (20), it is not well understood whether the extrinsic apoptotic pathway is induced in renal cells post-Cr(VI) exposure. The aim of the present study was to investigate the activation of the extrinsic

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Abbreviations: Cr(VI), hexavalent chromium; ROS, reactive oxygen species; Cr(III), trivalent chromium; PARP, poly (ADP-ribose) polymerase; AIF, apoptosis-inducing factor

Key words: cell death, extrinsic apoptosis, intrinsic apoptosis, hexavalent chromium, human proximal tubular epithelial cell

apoptotic pathway in Cr(VI)-exposed HK-2 human immortalized proximal tubular epithelial cell line HK-2.

Materials and methods

HK-2 cell culture. HK-2 cells (ATCC CRL-2190), an immortalized proximal tubular epithelial cell line derived from normal adult human kidneys, were purchased from the American Type Culture Collection. Culture conditions have been described in our previous study (21).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay for cell viability. To determine the toxicity of chromium on kidney cells, HK-2 cells (1×10^4 cells) were seeded in each well of a 96-well culture plate (Falcon; BD Biosciences) in triplicates. The cells were exposed to potassium dichromate ($K_2Cr_2O_7$; Sigma-Aldrich; Merck KGaA), which was used as the source of chromium (VI), at concentrations of 0.1, 0.3, 1, 10, 30, and 100 μM for 24, 48, and 72 h. Cell viability was directly examined by an Eclipse Ti-U inverted microscope (Nikon Corporation) at a magnification of x400 and indirectly assayed by an MTT assay (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. The absorbance at 570 nm was determined using a microplate reader (Multiskan EX; LabSystems).

Oxidative stress assay. HK-2 cells (1×10^6) were incubated in 10-cm dishes with 5 μM 2'-7'-dichlorofluorescein diacetate (Sigma-Aldrich; Merck KGaA) at 37°C for 30 min. After centrifugation (1,000 x g) and washing in phosphate-buffered saline, the HK-2 cells were exposed to 10 μM $K_2Cr_2O_7$ each in triplicates. After 30 min of incubation, the fluorescence intensity, which is correlated to the concentration of hydroxyl radicals, was detected by Partec CyFlow (Sysmex Partec GmbH). The data were analyzed by FCS Express 4 Flow Cytometry (De Novo Software). All procedures were performed in the dark on ice.

Western blotting. Protein lysates were extracted using radio-immunoprecipitation lysis buffer (Amresco, LLC) containing 1% proteinase inhibitor on ice for 20 min. Proteins were quantified using the protein assay kit (based on Bradford method, Bio-Rad Laboratories, Inc.) and 30 μg protein per lane was loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis at 100 V for 1 h. The separated proteins were transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences; GE Healthcare) and blocked with 3% bovine serum albumin in Tris-buffered saline containing Tween-20 for 1 h. The membranes were incubated overnight at 4°C with commercial primary monoclonal antibodies against poly (ADP-ribose) polymerase (PARP; catalogue number 9542S, 1:1,000; Cell Signaling Technology, Inc.), pro-caspase-3 (catalogue number 06-735, 1:1,000; EMD Millipore), cleaved caspase-3 (catalogue number C8487, 1:1,000; Sigma-Aldrich; Merck KGaA), Bcl-2 (catalogue number 658702, 1:1,000; BioLegend, Inc.), Bax (catalogue number 5023, 1:1,000; Cell Signaling Technology, Inc.), cytochrome *c* (catalogue number 05-479, 1:500; EMD Millipore), Fas ligand (FasL; catalogue number 68405, 1:500), apoptosis-inducing factor (AIF; catalogue number 4642,

1:1,000), pro-caspase-8 (catalogue number 9746S, 1:1,000) and cleaved caspase-8 (catalogue number 9496S, 1:1,000; all from Cell Signaling Technology, Inc.). Then, the membranes were incubated with goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibodies (catalogue number 405306, 1:5,000) or anti-rabbit IgG HRP-conjugated antibodies (catalogue number 410406, 1:10,000; both from BioLegend, Inc.) at room temperature for 1 h. β -actin (catalogue number MAB1501, 1:1,000; Chemicon; EMD Millipore) was used as an endogenous control and detected by the same secondary antibodies as aforementioned. Target proteins were visualized using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc.) and HyBlot CL film (Denville Scientific, Inc.). The intensities of the bands were quantified with ImageJ software 1.52a (NIH).

Statistical analysis. All results were expressed as the mean \pm standard deviation (SD) of at least three independent experiments. Data were analyzed by analysis of variance (ANOVA) using SPSS20 software (IBM Corp.). Scheffe's test was used for post hoc analysis to compare all pairs of groups of the ANOVA test. Results were considered statistically significant at a value of $P < 0.05$.

Results

Cr(VI) exposure decreases cell viability and alters the morphology of HK-2 cells. To evaluate the toxic effect of Cr(VI) on renal tubular cells, a normal human proximal tubule epithelial cell line HK-2 was treated with different concentrations of $K_2Cr_2O_7$, which produced Cr(VI) in aqueous solution. Various concentrations of $K_2Cr_2O_7$ were added into culture medium and the cell viability was evaluated at 24 and 48 h post- $K_2Cr_2O_7$ intoxication. The viability of 10- μM $K_2Cr_2O_7$ -treated cells was approximately 50-60 and 20-30%, at 24 and 48 h, respectively (Fig. 1A). To evaluate the toxic effect of $K_2Cr_2O_7$ for 24-72 h, similar viability was obtained and is presented in Fig. 1B, compared to the control cells. The morphology of 10- μM $K_2Cr_2O_7$ -treated cells was altered. Significant cell shrinkage was observed when compared to the control cells (Fig. 1C). These results indicated that the morphology and viability of HK-2 cells were significantly affected upon exposure to 10 μM $K_2Cr_2O_7$ for 24-72 h.

Cr(VI) exposure increases intracellular ROS production in HK-2 cells. Previous studies have suggested that the production of ROS during the reduction of Cr(VI) to Cr(III) promoted apoptosis (5,6). Thus, the intracellular level of ROS was assessed in HK-2 cells exposed to 10 and 100 μM of $K_2Cr_2O_7$ for 30 min (Fig. 2A). The results revealed that the intracellular ROS level in 10 and 100 μM $K_2Cr_2O_7$ -exposed HK-2 cells was greater than that in control cells. Quantification of data also revealed similar results (Fig. 2B). A high level of ROS may be a factor that triggers cell death in $K_2Cr_2O_7$ -exposed HK-2 cells.

Cr(VI) exposure increases apoptosis in HK-2 cells. The results revealed that cells treated with 10 μM $K_2Cr_2O_7$ for 48 h exhibited a viability of ~20-30%. Since a significant reduction in cell viability from 24 to 48 h suggested that apoptosis-regulating

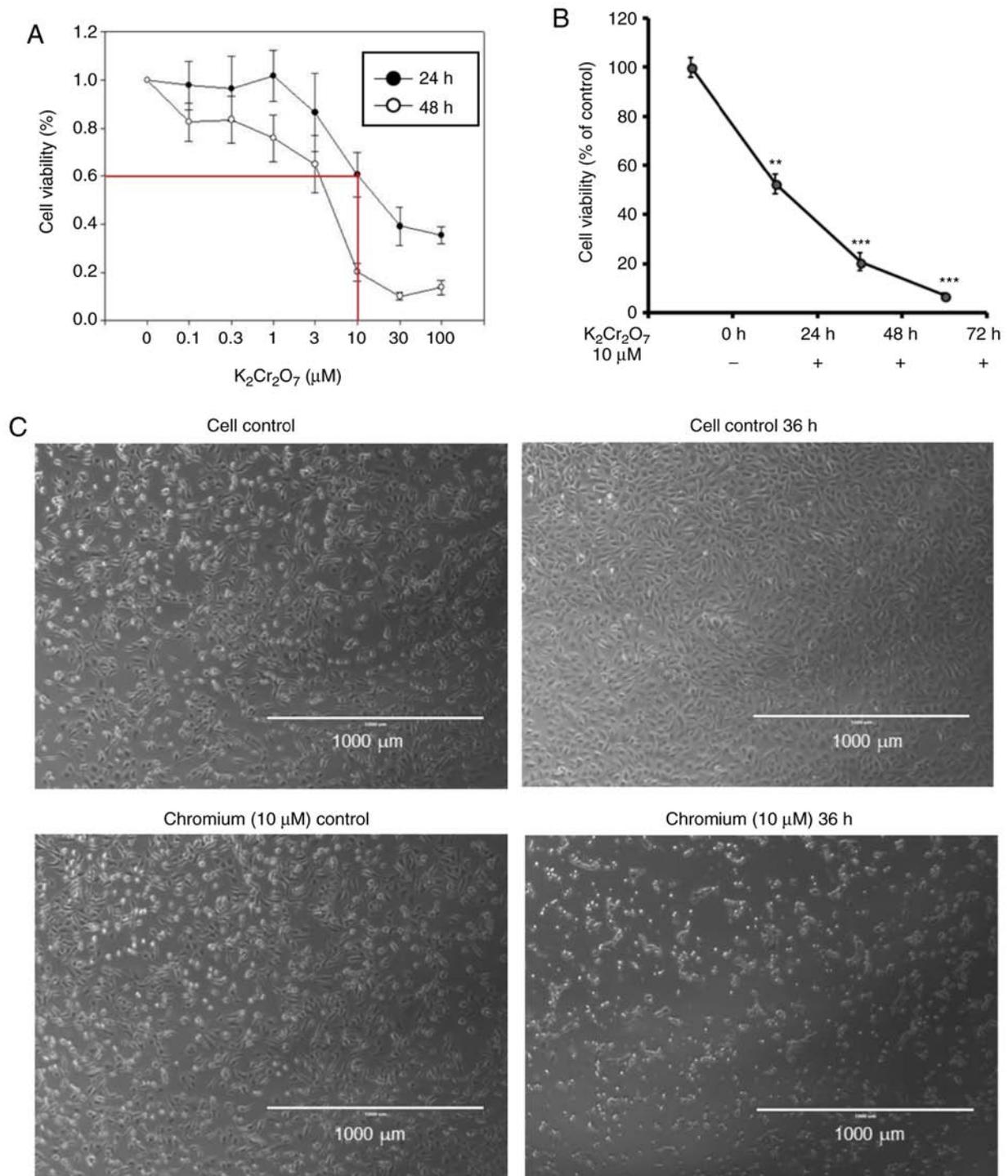


Figure 1. Toxic effect of $K_2Cr_2O_7$ in HK-2 cells. HK-2 cells were treated with various concentrations of $K_2Cr_2O_7$ and control HK-2 cells were treated with H_2O . (A) Cell viability at 24 and 48 h after exposure to various concentrations of $K_2Cr_2O_7$. (B) Cell viability at 0, 24, 48, and 72 h after exposure to 10 μM $K_2Cr_2O_7$. (C) Morphology of HK-2 cells after 36 h of exposure to 10 μM $K_2Cr_2O_7$. The scale bar indicates 1,000 μm . Data are presented as the mean \pm SD. ** $P < 0.01$ and *** $P < 0.001$ compared with 0 h, respectively. $K_2Cr_2O_7$, potassium dichromate.

proteins were induced, the protein lysates were collected during a 24 to 48-h time frame. The protein lysates of HK-2 cells exposed to $K_2Cr_2O_7$ were harvested at 24, 36, 40, and 45 h (Fig. 3A) and the quantitative results are presented in Fig. 3B-E. The activation of caspase-3 and cleavage of PARP indicated activation of the apoptotic pathway (22). After 24 h of $K_2Cr_2O_7$ exposure, the expression levels of cleaved PARP and cleaved caspase-3 were significantly upregulated. In addition, the expression of Bcl-2,

which prevents the release of mitochondrial apoptogenic factors such as cytochrome *c* and AIF (23), was significantly suppressed in $K_2Cr_2O_7$ -exposed HK-2 cells, indicating that 10 μM $K_2Cr_2O_7$ exposure induced apoptotic pathways at 24 h post- $K_2Cr_2O_7$ treatment. At other time-points, similar expression patterns were observed. However, there was no significant difference between the control and 10- μM $K_2Cr_2O_7$ -exposed cells at 36, 40 and 45 h due to high standard deviation.

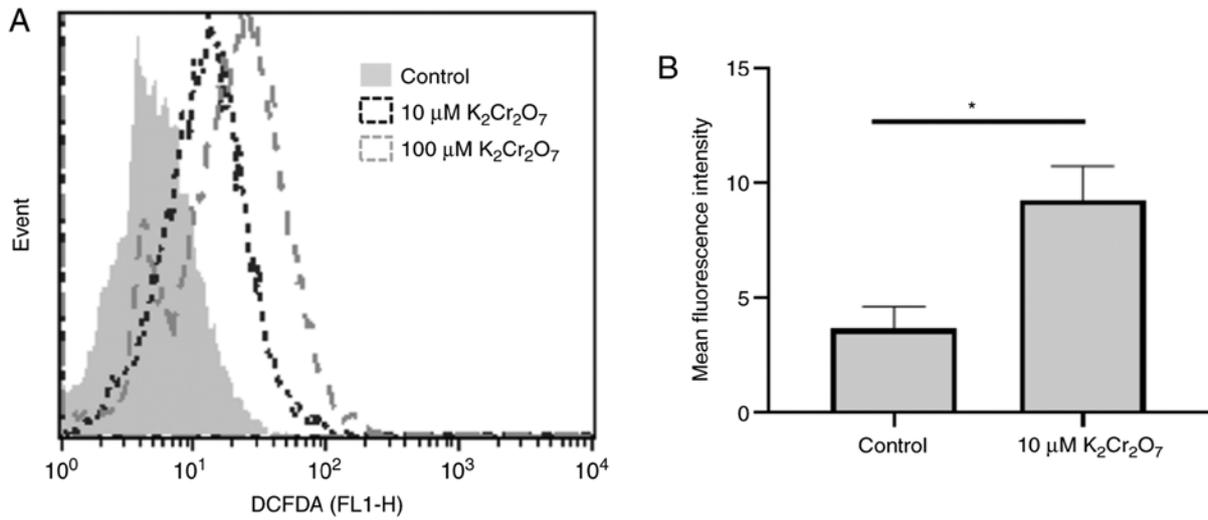


Figure 2. Intracellular ROS detection in K₂Cr₂O₇-treated HK-2 cells. (A) Detection of ROS levels by flow cytometry after 10 and 100 μM K₂Cr₂O₇ treatment for 30 min. (B) Quantification of ROS levels (n=3). Data are presented as the mean ± SD. *P<0.05, 10 μM K₂Cr₂O₇ exposure vs. the control. ROS, reactive oxygen species; K₂Cr₂O₇, potassium dichromate.

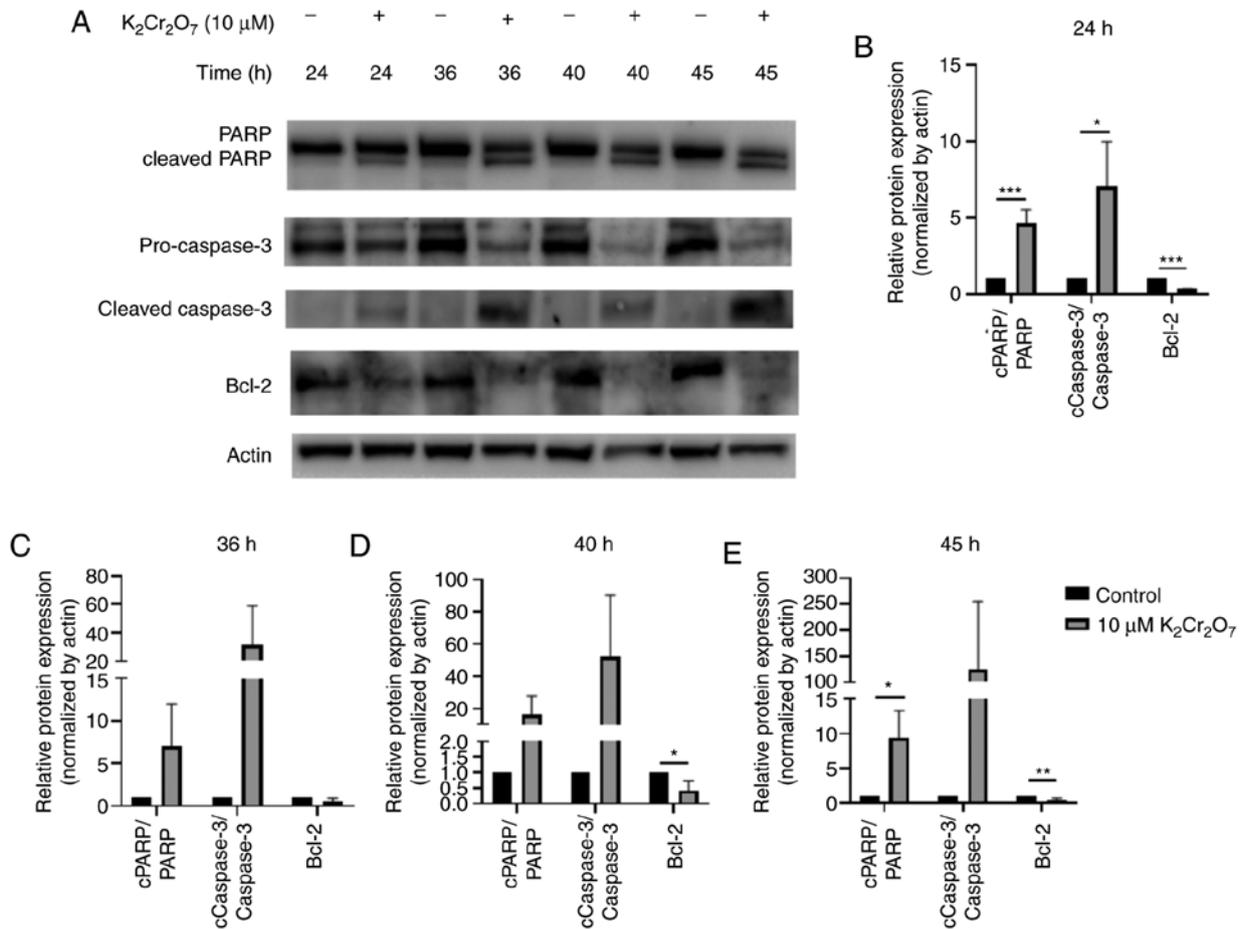


Figure 3. Evaluation of apoptotic markers. HK-2 cells were exposed to 10 μM K₂Cr₂O₇ and protein lysates were collected at different time-points (24, 36, 40, 45 h after K₂Cr₂O₇ treatment). (A) The expression of PARP, cleaved PARP, pro-caspase-3, cleaved caspase-3, and Bcl-2 in HK-2 cells. The relative protein expression levels at (B) 24 (C) 36 (D) 40 and (E) 45 h are presented. *P<0.05, **P<0.01 and ***P<0.001. 10 μM K₂Cr₂O₇ exposure vs. the control at various time-points. K₂Cr₂O₇, potassium dichromate; PARP, poly (ADP-ribose) polymerase; AIF, apoptosis-inducing factor.

Cr(VI) exposure increases not only the intrinsic, but also the extrinsic and caspase-independent apoptosis in HK-2 cells. Previous studies have revealed that Cr(VI) toxicity induced

cell apoptosis mainly via intrinsic mitochondrial pathways (5). Our results showed that the expression levels of molecules involved in the intrinsic pathway (BAX, cytochrome c),

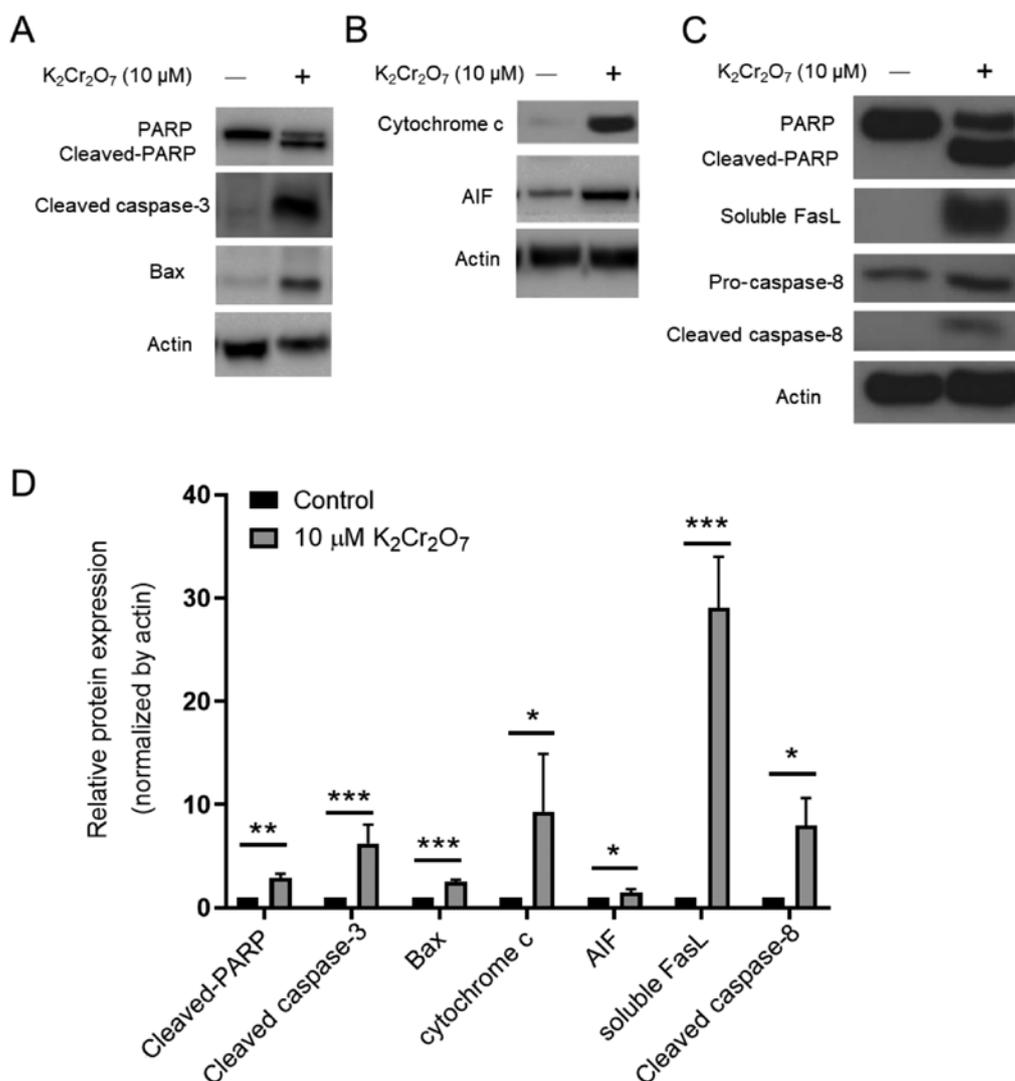


Figure 4. Evaluation of apoptotic pathways. (A) The expression levels of PARP, cleaved PARP, cleaved caspase-3, and Bax are presented. (B) The expression levels of AIF and cytochrome *c* are presented. (C) The expression levels of soluble FasL, pro-caspase-8, and cleaved caspase-8 were assessed. (D) Quantification of the western blot assay. The data represent the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, 10 μ M K₂Cr₂O₇ exposure vs. the control. PARP, poly (ADP-ribose) polymerase; AIF, apoptosis-inducing factor; FasL, Fas ligand; K₂Cr₂O₇, potassium dichromate.

caspase-independent pathway (AIF), and extrinsic pathway (soluble FasL and cleaved caspase-8) were significantly induced after 45 h of incubation in the presence of 10 μ M K₂Cr₂O₇ (Fig. 4). These findings indicated that these pathways contributed to Cr(VI)-mediated human renal cell death. The summary of this study is outlined in Fig. 5.

Discussion

Exposure to Cr(VI) affects various physiological events, and Cr(VI)-induced apoptosis has been reported by previous studies. In human diploid and normal fibroblasts and in HeLa human cervical cancer cells, reduced form of Cr(VI) caused DNA strand breaks (24-26). In U937 cells, exposure to 20 μ M Cr(VI) for 24 h induced intrinsic but not extrinsic apoptosis (27). Exposure to 30 μ M Cr(VI) induced both p53-dependent and independent intrinsic mitochondrial apoptosis in HCT116 human colon carcinoma cells (28). Activation of ribosomal protein L3 (rpL3) plays a role in chemotherapeutic drug-induced p53-independent apoptosis in colon and lung cancer cells (29,30). However, the

role of rpL3 in Cr(VI)-induced apoptotic processes is unclear. Another study revealed that Bcl-2 and p21 were downregulated after HCT-116 cells were exposed to 30 μ M Cr(VI) (5). In a rat model, a single subcutaneous injection of K₂Cr₂O₇ at 15 mg/kg induced the intrinsic mitochondrial pathway in kidneys (19). Clinically, Cr(VI) exposure often causes acute renal failure that may result in permanent hemodialysis or even death, and renal injury has mainly been detected in renal proximal tubular epithelial cells (31). Therefore, investigating the mechanism of Cr(VI)-induced apoptosis in renal cells is an important issue. To develop an optimal strategy to treat acute Cr(VI) intoxication, the apoptosis mechanism in human kidney cells under a curable dose was evaluated. According to a clinical study, chromium at a concentration of 10 mg/l (approximate 34 μ M) or greater in human blood is considered to be lethal for human life (32). A case study indicated that a patient with chromium blood concentration of 3.4 mg/l fortunately survived after intermittent hemodialysis treatment (33). Compared to cells exposed to 10 μ M of Cr(VI), low cell viability was observed when cells were exposed to 30 and 100 μ M for 24 and 48 h (Fig. 1A).

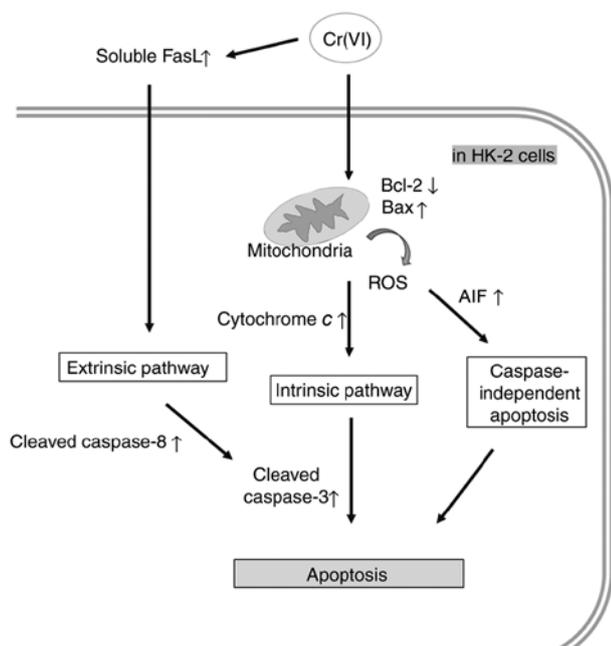


Figure 5. Schematic of the proposed hypothesis. Cr(VI) toxicity induced ROS and subsequently triggered intrinsic and extrinsic apoptotic pathways in human epithelial renal proximal tubule cells. Dashed lines indicate the unknown regulatory mechanism of sFasL post-Cr(VI) exposure. Cr(VI), hexavalent chromium; ROS, reactive oxygen species; sFasL, soluble FasL.

Conversely, cell viability was relatively high when cells were exposed to low concentrations of Cr(VI) ($<10 \mu\text{M}$). Therefore, the present results also indicated that higher concentrations were lethal and lower doses may not induce apoptosis. Cr(VI) toxicity at $10 \mu\text{M}$ (2.95 mg/l) was selected to induce apoptosis in the HK-2 human proximal tubular epithelial cell line.

The intrinsic pathway is regulated by pro-apoptotic and anti-apoptotic proteins such as Bax and Bcl-xL, respectively (22). These proteins alter the fate of cytochrome *c* release and activation of the caspase cascade, which regulate the permeability of the outer mitochondrial membrane (22). Increased Bax and decreased Bcl-2 protein expression indicated that the intrinsic mitochondrial apoptotic pathway was induced in HK-2 cells (Figs. 3 and 4). AIF is a pro-apoptotic protein released from the mitochondria, and its nuclear translocation caused DNA fragmentation and was involved in caspase-independent apoptosis (34,35). Extrinsic apoptotic pathways initiate apoptosis via the interaction between ligands and transmembrane receptors that belong to the tumor necrosis factor (TNF) receptor gene superfamily (22). Induction of extrinsic apoptosis was observed in mouse glomerular podocytes when they were exposed to toxic metals such as arsenite, cadmium, and mercury (36). Increased expression of soluble FasL and cleaved caspase-8 was detected after $10 \mu\text{M}$ Cr(VI) toxicity. Soluble FasL is well known as a degradation product of the membrane form of FasL by matrix metalloproteinases (37,38). Previous studies have documented that chromium had the potential to enhance the function of matrix metalloproteinases (39,40), which may explain the increased ratio between soluble and membrane FasL in the present results. In addition, the prevalence of the soluble form in association with the increased activation of caspase-8 (cleaved

caspase-8) indicated the possible active role of soluble FasL in chromium-induced HK-2 cell apoptosis. Therefore, the present results indicated that Cr(VI) toxicity for 48 h triggered the extrinsic apoptotic pathway. To the best of our knowledge, this study is the first to observe the induction of extrinsic apoptotic markers in Cr(VI)-exposed human renal cells. However, the mechanism of Cr(VI)-induced soluble FasL release is still unknown. The crosstalk between tumor necrosis factors and ROS may be a potential explanation (41), but further studies are required to confirm this hypothesis.

The limitation of the present study is that the conclusions were supported by one technique in one cell line. For confirmation, more experiments are required in future studies. For example, cell death can be also detected via BrdU staining assay and Annexin V staining assay at each time-point. Inhibition of intrinsic and extrinsic apoptosis-dependent pathways via specific inhibitors on caspase-8 and caspase-9 may further confirm the most critical pathways at specific time-points. To further investigate the role of Cr(VI)-induced ROS production, treatments with antioxidants or ROS scavengers are strategies for future experiments. In addition, performing animal experiments may strengthen our conclusion.

Collectively, not only intrinsic but also extrinsic apoptotic pathway-related apoptotic markers were activated in an immortalized proximal tubular epithelial cell line after exposure to $10 \mu\text{M}$ activated Cr(VI). The experimental dosage of Cr(VI) was similar to the Cr(VI) dosage that causes acute renal dysfunction. It is anticipated that the present study may be beneficial in designing optimal treatments for acute Cr(VI) toxicity in the future.

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Availability of data and materials

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

Authors' contributions

YHW, JCL and IJY designed the study. PLW and FWC performed the experiments. YHW, TYW, TJL, MCY, YLS, YHL, and IJY analyzed the data and interpreted the results. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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