Original Paper

Involvement of Calcium, Reactive Oxygen Species, and ATP in Hexavalent Chromium-Induced Damage in Red Blood Cells

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Key Words

Hexavalent chromium (Cr⁶⁺) • RBCs • Eryptosis

Abstract

Aim: The aim of this study was to investigate the mechanisms of Cr⁶⁺-induced red blood cells (RBCs) damage. **Methods:** The effect of Cr⁶⁺ exposure on RBCs was evaluated by hemolytic rate and blood gas assays. After exposure to 20 μM Cr⁶⁺, the percentage of phosphatidylserine (PS)-exposing cells, intracellular Ca²⁺, reactive oxygen species (ROS), and ATP levels were evaluated, and cell morphology was observed. RBCs were exposed to Cr⁶⁺ in different Ringer solutions to investigate the role of changes of Ca²⁺, ROS, and ATP levels in eryptosis and morphology. **Results:** The Cr⁶⁺-induced damage of RBCs was dose-dependent. After a 6 h incubation with Cr⁶⁺, RBCs exhibited significant Ca²⁺ influx, ROS increase, ATP depletion, and PS exposure, but displayed no obvious change in morphology at this time point. After 24 h Cr⁶⁺ exposure, the RBCs decreased in size, appeared to be spike-like, and had decreased forward scatter. Inhibition of Ca²⁺ influx attenuated PS-exposure caused by 6 h Cr⁶⁺ exposure, but did not prevent 24 h Cr⁶⁺ exposure-induced morphological change in RBCs. Inhibition of rapid ATP consumption using adenine significantly ameliorated Cr⁶⁺-caused PS-exposure at 12 h, 24 h and 48 h, and prevented 24 h Cr⁶⁺ incubation-induced morphological change in RBCs. **Conclusion:** Cr⁶⁺ can lead to eryptosis. Ca²⁺ influx, increased ROS levels, and rapid ATP consumption are closely related to Cr⁶⁺-induced RBCs damage. Ca²⁺ influx plays an extremely important role in Cr⁶⁺-mediated toxicity.

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Introduction

Chromium is prevalent in nature and is mainly distributed in rocks, soil, air, water, and living organisms. Chromium exists primarily in two oxidation states: trivalent chromium (Cr^{3+}) and hexavalent chromium (Cr^{6+}). Cr^{6+} compounds are the most common heavy metal pollutants that lead to occupational and environmental health hazards [1, 2]. Cr^{6+} can enter the human body in a variety of ways. Accumulation of Cr^{6+} can cause dermatitis and ulcers, resulting in damage to the liver, kidneys, heart, and reproductive organs, and it can even cause cancer [3-7]. Due to the genotoxicity and mutagenicity of chromium, the United States Environmental Protection Agency and the International Agency for Research on Cancer have recognized Cr^{6+} compounds as human carcinogens [8, 9].

In eukaryotic cells, Cr^{6+} crosses the cell membrane through $\text{SO}_4^{2-}/\text{HPO}_4^{2-}$ channels and enters into cells in the form of chromate [10]. Reduction of Cr^{6+} to Cr^{3+} produces free radicals, superoxide anions, and hydroxyl free radicals, which may cause direct or indirect damage to cells [11, 12], including apoptosis, oxidative stress, and dysfunctions in Ca^{2+} mobilization, cell cycle regulation, and energy metabolism [13-16].

Suicidal death of erythrocytes (eryptosis) is a process similar to apoptosis in nucleated cells [17]. Studies have shown that Ca^{2+} influx, ATP depletion, and oxidative stress are the main triggers of eryptosis [18-21]. Studies have found that Cr^{6+} can be taken up via a general anion channel protein (band 3 protein) in red blood cells [22]. Lupescu and colleagues confirmed that Ca^{2+} influx and ATP depletion participate in Cr^{6+} -induced eryptosis, but the detailed mechanisms of this process require further research [23]. This study aimed to investigate if additional factors are involved in Cr^{6+} -induced eryptosis and to evaluate the roles of these factors in the eryptosis process.

Materials and Methods

RBCs preparation

This study was approved by the Medical Ethics Review Committee of the Second Hospital Affiliated with the Third Military Medical University. Written informed consents were obtained from all human subjects involved in this study. Fresh (stored no more than 24 h) and leukocyte-free RBCs were obtained from healthy donors provided by the Chongqing Blood Center. The RBCs hematocrit was adjusted to 0.4% using Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM glucose, and 1 mM CaCl_2 ; pH 7.4) before incubation with hexavalent chromium (Cr^{6+} ; potassium dichromate, Aladdin, USA) at 37°C for 6, 12, 24, or 48 h. Ca^{2+} -free Ringer solution was made using 1 mM glycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (ethylene-glycol-tetraacetic acid, EGTA) as a substitute for 1 mM CaCl_2 . Adenine- and NAC (N-acetylcysteine) -Ringer solutions were made by adding 1.04 mM $\text{C}_5\text{H}_5\text{N}_5$ (Sangon, China) and 2 mM N-acetylcysteine (Sigma, USA), respectively.

Hemolytic rate measurement

RBCs were incubated with Cr^{6+} at gradient concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μM at 37°C for 48 h. Deionized water and Ringer solution were used as positive and negative controls, respectively. The incubation mixture was centrifuged at 10,016 g for 3 min, and the supernatant was collected for spectrophotometry. The absorbance (A) analysis wavelength was 570 nm with a reference at 655 nm. All experiments were conducted in triplicate in one donor's RBCs and were repeated at least three times in three donors' RBCs. The hemolytic rate was calculated with the following equation: $[(A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{sample}})] \times 100$ [24].

Blood gas analysis

RBCs were incubated with 20 μM Cr^{6+} at 37°C for 48 h. The blood gas profile, including pH, pO_2 , pCO_2 , HCO_3^- , K^+ , Na^+ , Ca^{2+} , Mg^{2+} , and Cl^- , was analyzed using the Critical Care Xpress Blood Gas Analyzer (Nova, USA).

Percentage of phosphatidylserine (PS)-exposing cells and morphology of RBCs

The PS was detected by Annexin-V-FLUOS and the percentage of PS-exposing RBCs was determined by measuring Annexin-V-FLUOS fluorescence-positive cells. RBCs were incubated with 20 μM Cr^{6+} in the following conditions: Ringer solution, Ca^{2+} -free Ringer solution, adenine-Ringer solution, and NAC-Ringer solution in a humidified incubator with 5% CO_2 at 37°C for 6, 12, 24, or 48 h. After incubation, RBCs were washed three times with PBS and resuspended in incubation buffer (HEPES buffer) and Annexin-V-FLUOS solution (Roche, Germany) at 20°C for 15 min under protection from light. The forward scatter (FSC) and Annexin-V-FLUOS fluorescence intensity were then analyzed in a FACS Calibur (BD, USA). Images were obtained using confocal microscopy (Carl Zeiss, 510 meta, Germany) with transmitted light and laser excitation. The excitation and emission wavelengths of flow cytometry and confocal microscopy were 488 and 530 nm, respectively. The percentage of PS-exposing RBCs was analyzed using Flowjo (Treestar, USA).

Measurement of Ca^{2+} , ATP, and ROS

The Ca^{2+} concentration in RBCs was measured using Fluo-3/AM (Invitrogen, USA) and flow cytometry (FACS Calibur, BD, USA). After two washes in Ringer solution, RBCs were incubated in Ringer solution containing 5 μM Fluo-3/AM and 5 mM CaCl_2 at 37°C for 30 min, washed twice in Ringer solution containing 5 mM CaCl_2 , and resuspended in Ringer solution. Fluo-3/AM-loaded RBCs were then incubated at 37°C for 30 min to allow complete de-esterification of intracellular Fluo-3/AM. The intracellular Ca^{2+} concentration was measured by determining Fluo-3 fluorescence intensity on a FACS Calibur (488 nm excitation and 530 nm emission) and the geometric means of fluorescence intensity were analyzed by Flowjo (Treestar, USA).

The ATP concentration was measured using an ATP Assay Kit (Beyotime, China). RBCs were washed twice in Ringer solution and added to the lysis buffer. Cell lysate was centrifuged at 12000 g for 10 min and supernatant was collected for ATP analysis. ATP samples were diluted at the following concentrations to make a standard curve of ATP: 3.90625, 7.8125, 15.625, 31.25, 62.5, 125, 250, and 500 μM . For ATP analysis, 100 μl ATP testing solution was added to each well of an enzyme-coated plate and after 5 min 100 μl supernatant was added to each well. The relative luciferase activity (RLU) was measured by Varioskan Flash Multimode Reader (Thermo Fisher, USA) and ATP concentration was calculated according to the standard curve.

ROS level was analyzed using the ROS Fluorescent Probe-DCFH (Beyotime, China) and a Varioskan Flash Multimode Reader (Thermo Fisher, USA). RBCs were washed in Ringer solution and incubated in 10 μM 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Beyotime, China) in Ringer solution at 37°C for 30 min. After three washes in Ringer solution, the fluorescence intensity was measured on a FACS Calibur (488 nm excitation and 530 nm emission) and geometric means of fluorescence intensity were analyzed by Flowjo (Treestar, USA).

Statistics

All experiments were conducted in triplicate and were repeated at least three times.

Mean values from at least three experiments were plotted and expressed as means $\pm$ SEM. Statistical analysis was performed using SPSS software v.18.0 (SPSS Inc., USA). A p-value less than 0.05 was considered as significant.

Results *Cr^{6+} dose-dependent hemolysis*

According to United States Environmental Protection Agency, the total chromium concentration (including Cr^{3+} and Cr^{6+}) in drinking water cannot exceed 100 $\mu\text{g/L}$ (approximately 1.9 μM), as Cr^{6+} can cause dramatic RBCs membrane injury, eryptosis, and even hemolysis [25]. In this study, to evaluate Cr^{6+} toxicity in human red blood cells, the lowest concentration we tested was 1.25 μM , which is still within the safety range. For the hemolytic rate test, RBCs were incubated with Cr^{6+} for 48 h at the following concentrations: 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μM . All samples showed different degrees of hemolysis, and the hemolytic rate displayed a Cr^{6+} dose-dependent pattern (Fig. 1). Incubation with 40 μM Cr^{6+} led to a hemolytic rate of greater than 5%. Statistical analysis revealed that the

Fig. 1. Dose-dependent effect of Cr^{6+} exposure on hemolysis in RBCs. Hemolytic activity of Cr^{6+} at different concentrations ranging from 1.25 to 160 μM for 48 h. Data represent the arithmetic means $\pm$ SEM ($n = 9$) of the percentage of hemolysis from three independent experiments. Deionized water and Ringer solution were used as positive and negative controls, respectively.

Fig. 2. Effects of a 24 h exposure of 20 μM Cr^{6+} on PS-exposure and morphological change in RBCs. (a) Percentage of Annexin-V-FLUOS-positive cells in Ringer solution in the absence or presence of Cr^{6+} . Data represent the arithmetic means $\pm$ SEM ($n = 9$) of the percentage of Annexin-V-FLUOS-positive cells from three independent experiments. Blue and red bars indicate the absence (control) and presence of 20 μM Cr^{6+} , respectively. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences compared to the absence of Cr^{6+} . (b) Bright field images for cell morphology and fluorescence images for Annexin-V-FLUOS-positive cells. b1 and b2 indicate the absence and presence of 20 μM Cr^{6+} , respectively. Scale bar = 10 μm .

hemolytic rate was significantly higher in RBCs incubated in Ringer solution containing 20 μM Cr^{6+} than in RBCs incubated in Ringer solution without Cr^{6+} ($4.37 \pm 0.24\%$ vs. $0.78 \pm 0.12\%$; $p < 0.05$). Therefore, we selected 20 μM Cr^{6+} as the dose for the following experiments. After 48 h incubation with 20 μM Cr^{6+} , most of the measured blood gas parameters did not change significantly and only the Na^+ and K^+ content changed slightly, suggesting that Cr^{6+} has little influence on acid-base metabolism and O_2 and CO_2 binding capacities.

Fig. 3. Ca^{2+} influx and eryptosis induced by Cr^{6+} . (a) Intracellular Ca^{2+} concentration after 6, 12, 24, and 48 h exposure to $20 \mu\text{M}$ Cr^{6+} . Blue and red bars indicate the absence (control) and presence of $20 \mu\text{M}$ Cr^{6+} , respectively. (b) Cell morphology and Fluo-3 fluorescence images for treatments without (b1) and with $20 \mu\text{M}$ Cr^{6+} (b2) after 6 h. (c) Intracellular Ca^{2+} concentration after different exposure times to $20 \mu\text{M}$ Cr^{6+} in Ringer and Ca^{2+} -free Ringer solutions. Blue, red, green, and purple bars represent the control, control plus Ca^{2+} -free, Cr^{6+} , and Cr^{6+} plus Ca^{2+} -free, respectively. (d) Percentage of Annexin-V-positive cells in normal Ringer and Ca^{2+} -free Ringer solutions with Cr^{6+} exposure. Data represent the arithmetic means $\pm$ SEM ($n = 9$) of the percentage of Annexin-V-FLUOS-positive cells (d) in RBCs from three independent experiments. (e) Cell morphology and Annexin-V-FLUOS fluorescence images of the cells exposed to Ringer solution or Ca^{2+} -free Ringer solution with or without $20 \mu\text{M}$ Cr^{6+} after 24 h. Scale bar = $10 \mu\text{m}$. * $p < 0.05$ and ** $p < 0.01$.

Cr⁶⁺-induced eryptosis

PS exposure is an important feature of eryptosis. To evaluate the effect of Cr^{6+} exposure on PS-exposure, RBCs were incubated with $20 \mu\text{M}$ Cr^{6+} for 6, 12, 24, or 48 h and were then subjected to flow cytometry analysis. A 6 h Cr^{6+} -exposure significantly increased the percentage of Annexin-V-positive cells compared to no Cr^{6+} exposure ($7.42 \pm 0.16\%$ vs. $5.83 \pm 0.16\%$; $p < 0.01$; Fig. 2a), and the percentage of PS-exposing RBCs was increased in an exposure time-dependent manner. Exposure to $20 \mu\text{M}$ Cr^{6+} for 48 h resulted in $42.20 \pm 1.88\%$ of PS-exposing RBCs, which was significantly higher than the percentage when exposed to only Ringer solution ($21.38 \pm 2.26\%$; $p < 0.01$). Images of Annexin-V-FLUOS-labeled RBCs were obtained immediately after a 24 h Cr^{6+} incubation using confocal microscopy. Compared to the control cells (Fig. 2b1), RBCs exposed to 24 h Cr^{6+} displayed apparent morphological changes, including dramatic collapses and spike-shaped cell membranes, and increased Annexin-V-FLUOS fluorescence (Fig. 2b2).

Ca²⁺ influx and Cr⁶⁺-induced eryptosis

Ca^{2+} influx has been observed in eryptosis; it is caused by many physical and chemical factors. The intracellular Ca^{2+} concentration was determined using the Ca^{2+} indicator, Fluo-3/AM, and flow cytometry. Compared to the control, incubation with $20 \mu\text{M}$ Cr^{6+} for 6, 12, 24 and 48 h significantly increased intracellular Ca^{2+} level in RBCs by 1.16-, 1.08-, 1.14- and 1.18-fold, respectively (Fig. 3a). Although 6 h Cr^{6+} exposure increased Ca^{2+} influx, fluorescence

Fig. 4. ATP depletion and eryptosis induced by Cr⁶⁺. (a) Intracellular ATP concentrations after 6, 12, 24, and 48 h of exposure to 20 μ M Cr⁶⁺. Blue and red bars indicate the absence (control) and presence of 20 μ M Cr⁶⁺, respectively. Effects of adenine-Ringer solution on intracellular ATP concentration (b), and PS-exposing rate (c). (d) Cell morphology and Annexin-V-FLUOS fluorescence images. Blue, red, green, and purple bars represent the control, control plus adenine, Cr⁶⁺, and Cr⁶⁺ plus adenine, respectively. Data represent the arithmetic means $\pm$ SEM (n = 9) of the ATP concentration (a, b) or the percentage of Annexin-V-FLUOS-positive cells (c) in RBCs from three independent experiments. Scale bar = 10 μ m. *p<0.05 and **p<0.01.

imaging revealed that Fluo-3-labeled RBCs displayed normal cell morphology (Fig. 3b1, b2).

We then blocked Ca²⁺ influx by exposing RBCs to Ca²⁺-free Ringer solution, and we observed PS-exposure and cell morphological changes after exposing RBCs to 20 μ M Cr⁶⁺ at different time points. Detection of a Fluo-3-fluorescence signal showed that incubation in Ca²⁺-free Ringer solution prevented time-dependent Ca²⁺ influx in RBCs (blue and red in Fig. 3c). In the presence of 20 μ M Cr⁶⁺, incubation in Ca²⁺-free Ringer solution inhibited Cr⁶⁺-induced Ca²⁺ influx at all tested time-points (purple in Fig. 3c). We further examined the effect of Ca²⁺ influx on PS exposure on RBCs. In the absence of Cr⁶⁺, RBCs incubated in Ca²⁺-free Ringer solution for 24 and 48 h showed significantly less PS-exposing cells compared to those incubated in Ringer solution (blue and red in Fig. 3d). In the presence of Cr⁶⁺, compared to RBCs incubated in Ca²⁺-containing Ringer solution RBCs incubated in Ca²⁺-free Ringer solution also showed significantly lower percentage of PS-exposing cells at the time points of 6 h, 24 h and 48 h (green and purple in Fig. 3d). These suggest that inhibition of Ca²⁺ influx reduced Cr⁶⁺-caused toxicity on PS exposure on RBCs. Although inhibition of Ca²⁺ influx reduced PS-exposing cells in the presence of 20 μ M Cr⁶⁺, apoptotic RBCs were still significantly higher than those in the control group in Ringer solution containing Ca²⁺ (purple and blue in Fig. 3d), suggesting that inhibition of Ca²⁺ influx did not completely block Cr⁶⁺-

Fig. 5. ROS and eryptosis induced by Cr^{6+} . (a) Relative ROS concentrations after 6, 12, 24, and 48 h of exposure to $20 \mu\text{M}$ Cr^{6+} . Blue and red bars indicate the absence (control) and presence of $20 \mu\text{M}$ Cr^{6+} , respectively. The effects of NAC-Ringer solution on ROS concentration (b) and the percentage of Annexin-V-positive cells (c). (d) Cell morphology and Annexin-V-FLUOS fluorescence images (d). Blue, red, green, and purple bars represent the control, control plus NAC, Cr^{6+} , and Cr^{6+} plus NAC, respectively. Data represent the arithmetic means $\pm$ SEM ($n = 9$) of the DCFH fluorescence (a.u.) (a, b) or the percentage of Annexin-V-FLUOS-positive cells (c) in RBCs from three independent experiments. Scale bar = $10 \mu\text{m}$. ** $p < 0.01$.

caused toxicity. Imaging results showed that after 24 h Cr^{6+} -exposure, compared to RBCs in Ringer solution (Fig. 3e3), although RBCs in Ca^{2+} -free Ringer solution displayed less Annexin-V-FLUOS fluorescence-positive cells they still exhibited a collapsed and spike-like morphology (Fig. 3e4), suggesting that inhibition Ca^{2+} influx did not completely prevent Cr^{6+} -induced morphological change in RBCs.

ATP depletion and Cr^{6+} -induced eryptosis

ATP is important for maintaining normal RBCs morphology. Fast depletion of ATP is the major cause for RBCs collapse and spike-like shape. The ATP assay results showed that incubation with $20 \mu\text{M}$ Cr^{6+} for 6, 12, 24 and 48 h led to decreases of intracellular ATP levels to 56.73%, 38.81%, 18.99% and 17.91% of the control group, respectively (Fig. 4a). The ATP depletion rate was rapidly increased throughout the incubation time. At 24 h, $20 \mu\text{M}$ Cr^{6+} -exposure resulted in a significant decrease in ATP level below 25% of basal level, which is a critical point for RBCs rejuvenation [20]. Adenine is an important component of RBCs preservation and can be used to produce ATP in RBCs. To investigate the effect of ATP depletion on Cr^{6+} -induced eryptosis, adenine was added to Ringer solution. ATP

Fig. 6. RBCs volume in Cr^{6+} -induced eryptosis. Histogram showing forward scatter of RBCs following exposure to 20 μM Cr^{6+} in Ringer solution (Cr^{6+}), Ca^{2+} -free Ringer solution (Cr^{6+} and Ca^{2+} -free), adenine-Ringer solution (Cr^{6+} and adenine), NAC-Ringer solution (Cr^{6+} and NAC), and Ringer solution (control).

concentration and the percentage of PS-exposing cells were evaluated in the presence or absence of Cr^{6+} . In the absence of Cr^{6+} , application of adenine significantly increased ATP concentrations in RBCs at 12 and 24 h (red in Fig. 4b). In the presence of Cr^{6+} , compared to RBCs without adenine supply RBCs in adenine-Ringer solution showed significantly higher ATP concentrations at 6, 12, and 24 h by 1.18-, 1.78-, and 1.51-fold, respectively (green and purple in Fig. 4b), but their ATP levels were still lower than those in the control group (blue in Fig. 4b). This suggests that application of adenine partially restored Cr^{6+} -induced rapid ATP depletion. In the presence of Cr^{6+} , we found significantly less PS-exposing cells with adenine supply at 12, 24 and 48 h (purple versus green in Fig. 4c), although the percentage of PS-exposing cells were still significantly higher than those in the control group (blue in Fig. 4c). Consistent with PS exposure result, fluorescence images also showed that compared to the control group (Fig. 4 d1 and d2) 24 h Cr^{6+} exposure resulted in a biconcave disk structure and collapse in RBCs (Fig. 4 d3) and adenine supply prevented 24 h Cr^{6+} incubation-induced morphological change in RBCs (Fig. 4d4). All these suggest that adenine supply can reduce rapid ATP depletion in RBCs but it only partially reverse Cr^{6+} -caused PS-exposure and morphological changes in RBCs.

ROS- and Cr^{6+} -induced eryptosis

Cr^{6+} that entered into the RBCs was ultimately reduced to Cr^{3+} , resulting in the production of ROS, a critical by-product. The ROS assay showed that a 6 h incubation with 20 μM Cr^{6+} significantly increased ROS level (10.70 ± 0.09 a.u. vs. 8.14 ± 0.05 a.u.; $p < 0.01$; Fig. 5a). The effect of Cr^{6+} exposure on ROS levels was mitigated over time. Although longer incubation times increased ROS levels, the increase was significantly lower compared to the Cr^{6+} -free treatment (Fig. 5a). This result suggested that Cr^{6+} -induced oxidative stress in RBCs mainly occurred in the early phase of exposure.

NAC is a common antioxidant and can ameliorate oxidative stress induced by heavy metals and pathological changes in RBCs [26, 27]. To a certain extent, the NAC-Ringer solution attenuated the Cr^{6+} -induced increase in ROS levels. After a 48 h Cr^{6+} exposure, cells incubated in the NAC-Ringer solution still showed significantly lower ROS levels compared to RBCs incubated in the Ringer solution (9.80 ± 0.19 a.u. vs. 14.40 ± 0.07 a.u.; $p < 0.01$; Fig. 5b). No significant difference in the percentage of PS-exposing cells was detected between these two groups ($45.31 \pm 0.83\%$ vs. $40.45 \pm 1.37\%$; $p = 0.311$; Fig. 5c). After a 24 h Cr^{6+} exposure, a large amount of Annexin-V-FLUOS-positive RBCs exhibited obvious shrinking and a spike-like appearance (Fig. 5d).

RBCs volume in Cr^{6+} -induced eryptosis

To evaluate the effects of Ca^{2+} influx, ATP depletion, and oxidative stress on Cr^{6+} -induced RBCs volume reduction, RBCs were incubated in Ca^{2+} -free Ringer solution, adenine-Ringer solution, and NAC-Ringer solution with or without Cr^{6+} for 48 h. Lupescu and colleagues

reported that the forward scatter of RBCs exposed to 20 μM Cr^{6+} for 48 h was decreased [23]. Blocking the Ca^{2+} influx alleviated the cell volume reduction induced by Cr^{6+} , and additional adenine maintained the cell volume. However, NAC had no effect on the volume reduction induced by Cr^{6+} exposure (Fig. 6).

Discussion

Cr^{6+} has been identified as a human carcinogen. The chances of exposure to Cr^{6+} in everyday life are gradually increasing. Industrial production, medical materials, and environmental pollution, along with materials that we are exposed to in daily life, such as joint replacements [28], porcelain teeth [29], stainless steel cookware [30], or even groundwater pollution [31], all increase the risk of Cr^{6+} exposure. Although we have long realized that RBCs have a special affinity for Cr^{6+} [32], studies focusing on Cr^{6+} -toxicity in RBCs are scarce.

In this study, we found that a 48 h exposure of erythrocytes to 20 μM Cr^{6+} resulted in significant hemolysis and that 40 μM Cr^{6+} induced up to a 5% hemolytic rate. Moreover, the Cr^{6+} -induced hemolysis was dose-dependent. Additional studies on Cr^{6+} -induced eryptosis showed that the eryptosis characterized by PS-positive cells reached 42.2%, though 20 μM Cr^{6+} exposure for 48 h caused less than 5% hemolysis. Ca^{2+} influx, rapid ATP depletion, and increased ROS levels all contributed to Cr^{6+} -induced eryptosis. However, only inhibition of rapid ATP depletion using an adenine-containing solution ameliorated Cr^{6+} -induced PS exposure to cell membranes and morphological changes in RBCs. Inhibition of Ca^{2+} influx using a Ca^{2+} -free solution decreased eryptosis, but it had no effect on rescuing the cell morphological change. The NAC antioxidant did not reduce eryptosis or rescue cell morphology. All of these results suggested that Ca^{2+} influx, rapid ATP depletion, and increased ROS could play different roles in Cr^{6+} -induced eryptosis.

Ca^{2+} influx has long been considered the most critical factor in eryptosis [33, 34]. A significant intracellular Ca^{2+} concentration increase is observed in eryptosis induced by almost all physicochemical factors and endogenous substances, including drugs [19, 35], metal ions [23], and diseases [36]. Increased Ca^{2+} concentration affects PS exposure through the activation of phospholipid scramblase [37]. Increased Ca^{2+} also influences RBCs morphology by affecting the skeleton flexibility and stability via the calcium-calmodulin complex (Ca-CaM) [38], the intracellular ion balance, and cell osmolality [39]. Under some circumstances, blocking Ca^{2+} influx can inhibit eryptosis in RBCs [40]. Thus far, no direct evidence has been reported that increased intracellular Ca^{2+} levels are vital to eryptosis. However, a study has reported the heterogeneity between the elevation of intracellular Ca^{2+} concentrations and increased eryptosis [41] where RBCs eryptosis occurs without the involvement of Ca^{2+} [20]. In this study, we observed that Ca^{2+} influx occurred within 6 h after Cr^{6+} exposure. As mature RBCs have no capacity to store Ca^{2+} , using Ca^{2+} -free Ringer solution completely blocked Cr^{6+} -induced Ca^{2+} influx, thereby resulting in a significant decrease in Cr^{6+} -induced eryptosis but no change in RBCs morphology and volume. Although Ca^{2+} influx plays a critical role in Cr^{6+} -induced eryptosis, this result suggested that other factors could also be involved in the process.

It has long been recognized that ATP is critical for the maintenance of RBCs biconcave disk morphology, excellent plasticity, and functional plasma membrane Ca^{2+} pumps (PMCA) [38, 42, 43]. Rapid ATP depletion is caused by excessive activation of the Ca^{2+} pump and has been considered a result of Ca^{2+} influx [38, 44]. A recent study reported that ATP depletion affects phosphorylation levels of the 4.1R and spectrin proteins, which are key proteins in the RBCs cytoskeletal network [45]. Depletion of the ATP level to below 25% that of normal RBCs eventually leads to cell membrane skeleton fracture and irreversible eryptosis of spike-shaped RBCs [20]. In our study, Cr^{6+} exposure rapidly depleted intracellular ATP, and a 12 h exposure of Cr^{6+} reduced the ATP level to 21.2% of the normal ATP level of RBCs. Additional adenine attenuated ATP depletion, reduced the percentage of PS-exposing cells, and maintained normal RBCs morphology and volume, thereby suggesting that the

intracellular ATP level is important in Cr^{6+} -induced eryptosis. According to previous reports, increased ROS may be a major mechanism mediating Cr^{6+} -induced injury in RBCs and other karyocytes [14, 46, 47] and increased ROS levels are closely related to eryptosis [48]. We also found that Cr^{6+} exposure significantly increased RBCs ROS levels and PS-exposing cells. However, reduced ROS levels via additional NAC in the Ringer solution showed no effect on rescuing eryptosis and morphological changes in RBCs, thus suggesting that the effect of increased ROS in eryptosis is not preventable.

In conclusion, although 20 μM Cr^{6+} exposure caused a less than 5% hemolytic rate, it resulted in dramatic increases in Ca^{2+} influx, ATP depletion, and ROS levels, leading to RBCs membrane shrinkage and PS exposure. Inhibition of Ca^{2+} influx and ATP depletion can ameliorate eryptosis in RBCs to a certain extent. Moreover, reduced ATP depletion can maintain RBCs morphology in response to Cr^{6+} exposure, suggesting that ATP depletion may not be just a by-pass result of increased Ca^{2+} influx during the process of Cr^{6+} -induced eryptosis. This result was consistent with the report that ATP is important for maintaining membrane dynamic equilibrium and cytoskeleton in RBCs [43, 45, 49]. The ROS toxicity effect on RBCs after Cr^{6+} exposure was not reversed by additional antioxidants.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

- 1 Zhang XH, Zhang X, Wang XC, Jin LF, Yang ZP, Jiang CX, Chen Q, Ren XB, Cao JZ, Wang Q, Zhu YM: Chronic occupational exposure to hexavalent chromium causes DNA damage in electroplating workers. *BMC Public Health* 2011;11:224.
- 2 Wise SS, Holmes AL, Wise JP Sr: Hexavalent chromium-induced DNA damage and repair mechanisms. *Rev Environ Health* 2008;23:39-57.
- 3 Nickens KP, Patierno SR, Ceryak S: Chromium genotoxicity: A double-edged sword. *Chem Biol Interact* 2010;188:276-288.
- 4 Zhitkovich A: Chromium in drinking water: sources, metabolism, and cancer risks. *Chem Res Toxicol* 2011;24:1617-1629.
- 5 Stout MD, Herbert RA, Kissling GE, Collins BJ, Travlos GS, Witt KL, Melnick RL, Abdo KM, Malarkey DE, Hooth MJ: Hexavalent chromium is carcinogenic to F344/N rats and B6C3F1 mice after chronic oral exposure. *Environ Health Perspect* 2009;117:716-722.
- 6 Beaumont JJ, Sedman RM, Reynolds SD, Sherman CD, Li LH, Howd RA, Sandy MS, Zeise L, Alexeeff GV: Cancer mortality in a Chinese population exposed to hexavalent chromium in drinking water. *Epidemiology* 2008;19:12-23.
- 7 Wise SS, Wise JP Sr: Chromium and genomic stability. *Mutat Res* 2012;733:78-82.
- 8 U.S. Environmental Protection Agency: Integrated Risk Information System on Chromium (VI). U.S. Environmental Protection Agency, 1999.
- 9 International Agency for Research on Cancer: Chromium, nickel and welding. *IARC Monogr Eval Carcinog Risks Hum* 1990;49:1-648.
- 10 Ding M, Shi X: Molecular mechanisms of Cr(VI) -induced carcinogenesis. *Mol Cell Biochem* 2002;234-235:293-300.

- 11 Salnikow K, Zhitkovich A: Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol* 2008;21:28-44.
- 12 Sedman RM, Beaumont J, McDonald TA, Reynolds S, Krowech G, Howd R: Review of the evidence regarding the carcinogenicity of hexavalent chromium in drinking water. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2006;24:155-182.
- 13 Ye J, Shi X: Gene expression profile in response to chromium-induced cell stress in A549 cells. *Mol Cell Biochem* 2001;222:189-197.
- 14 Zeng M, Xiao F, Zhong X, Jin F, Guan L, Wang A, Liu X, Zhong C: Reactive oxygen species play a central role in hexavalent chromium-induced apoptosis in Hep3B cells without the functional roles of p53 and caspase-3. *Cell Physiol Biochem* 2013;32:279-290.
- 15 Bartholomäus R, Irwin JA, Shi L, Smith SM, Levina A, Lay PA: Isolation, characterization, and nuclease activity of biologically relevant chromium(V) complexes with monosaccharides and model diols. Likely intermediates in chromium-induced cancers. *Inorg Chem* 2013;52:4282-4292.
- 16 Xiao F, Feng X, Zeng M, Guan L, Hu Q, Zhong C: Hexavalent chromium induces energy metabolism disturbance and p53-dependent cell cycle arrest via reactive oxygen species in L-02 hepatocytes. *Mol Cell Biochem* 2012;371:65-76.
- 17 Lang F, Lang KS, Lang PA, Huber SM, Wieder T: Mechanisms and significance of eryptosis. *Antioxid Redox Signal* 2006;8:1183-1192.
- 18 Lupescu A, Jilani K, Zbidah M, Lang F: Patulin-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:291-299.
- 19 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. *Toxins (Basel)* 2013;5:1230-1243.
- 20 Pompeo G, Girasole M, Cricenti A, Boumis G, Bellelli A, Amiconi S: Erythrocyte death in vitro induced by starvation in the absence of Ca(2+). *Biochim Biophys Acta* 2010;1798:1047-1055.
- 21 Soudani N, Ben Amara I, Troudi A, Hakim A, Bouaziz H, Ayadi Makni F, Zeghal KM, Zeghal N: Oxidative damage induced by chromium (VI) in rat erythrocytes: protective effect of selenium. *Physiol Biochem* 2011;67:577-588.
- 22 Ottenwaelder H, Wiegand HJ, Bolt HM: Uptake of 51Cr(VI) by human erythrocytes: evidence for a carrier-mediated transport mechanism. *Sci Total Environ* 1988;71:561-566.
- 23 Lupescu A, Jilani K, Zelenak C, Zbidah M, Qadri SM, Lang F: Hexavalent chromium-induced erythrocyte membrane phospholipid asymmetry. *Biometals* 2012;25:309-318.
- 24 Lin YS, Haynes CL: Impacts of mesoporous silica nanoparticle size, pore ordering, and pore integrity on hemolytic activity. *J Am Chem Soc* 2010;132:4834-4842.
- 25 Ahmad MK, Syma S, Mahmood R: Cr(VI) induces lipid peroxidation, protein oxidation and alters the activities of antioxidant enzymes in human erythrocytes. *Biol Trace Elem Res* 2011;144:426-435.
- 26 Kasperczyk S, Dobrakowski M, Kasperczyk A, Ostalowska A, Birkner E: The administration of N-acetylcysteine reduces oxidative stress and regulates glutathione metabolism in the blood cells of workers exposed to lead. *Clin Toxicol (Phila)* 2013;51:480-486.
- 27 Nur E, Brandjes DP, Teerlink T, Otten HM, Oude Elferink RP, Muskiet F, Evers LM, ten Cate H, Biemond BJ, Duits AJ, Schnog JJ; CURAMA study group: N-acetylcysteine reduces oxidative stress in sickle cell patients. *Ann Hematol* 2012;91:1097-1105.
- 28 Hasegawa M, Yoshida K, Wakabayashi H, Sudo A: Cobalt and chromium ion release after large-diameter metal-on-metal total hip arthroplasty. *J Arthroplasty* 2012;27:990-996.
- 29 Chen B, Xia G, Cao XM, Wang J, Xu BY, Huang P, Chen Y, Jiang QW: Urinary levels of nickel and chromium associated with dental restoration by nickel-chromium based alloys. *Int J Oral Sci* 2013;5:44-48.
- 30 Kamerud KL, Hobbie KA, Anderson KA: Stainless steel leaches nickel and chromium into foods during cooking. *J Agric Food Chem* 2013;61:9495-9501.
- 31 Sharma P, Bihari V, Agarwal SK, Verma V, Kesavachandran CN, Pangtey BS, Mathur N, Singh KP, Srivastava M, Goel SK: Groundwater contaminated with hexavalent chromium [Cr (VI)]: a health survey and clinical examination of community inhabitants (Kanpur, India). *PLoS One* 2012;7:e47877.
- 32 Wiegand HJ, Ottenwälder H, Bolt HM: Fast uptake kinetics in vitro of 51Cr (VI) by red blood cells of man and rat. *Arch Toxicol* 1985;57:31-34.
- 33 Lang E, Qadri SM, Lang F: Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol* 2012;44:1236-1243.

- 34 Lang F, Qadri SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif* 2012;33:125-130.
- 35 Lupescu A, Bissinger R, Jilani K, Lang F: Triggering of suicidal erythrocyte death by celecoxib. *Toxins (Basel)* 2013;5:1543-1554.
- 36 Mahmud H, Ruifrok WP, Westenbrink BD, Cannon MV, Vreeswijk-Baudoin I, van Gilst WH, Silljé HH, de Boer RA: Suicidal erythrocyte death, eryptosis, as a novel mechanism in heart failure-associated anaemia. *Cardiovasc Res* 2013;98:37-46.
- 37 Wolfs JL, Comfurius P, Bekers O, Zwaal RF, Balasubramanian K, Schroit AJ, Lindhout T, Bevers EM: Direct inhibition of phospholipid scrambling activity in erythrocytes by potassium ions. *Cell Mol Life Sci* 2009;66:314-323.
- 38 Bogdanova A, Makhro A, Wang J, Lipp P, Kaestner L: Calcium in red blood cells-a perilous balance. *Int J Mol Sci* 2013;14:9848-9872.
- 39 Zbidah M, Lupescu A, Shaik N, Lang F: Gossypol-induced suicidal erythrocyte death. *Toxicology* 2012;302:101-105.
- 40 Shaik N, Zbidah M, Lang F: Inhibition of Ca(2+) entry and suicidal erythrocyte death by naringin. *Cell Physiol Biochem* 2012;30:678-686.
- 41 Wang J, Wagner-Britz L, Bogdanova A, Ruppenthal S, Wiesen K, Kaiser E, Tian Q, Krause E, Bernhardt I, Lipp P, Philipp SE, Kaestner L: Morphologically homogeneous red blood cells present a heterogeneous response to hormonal stimulation. *PLoS One* 2013;8:e67697.
- 42 Park Y, Best CA, Badizadegan K, Dasari RR, Feld MS, Kuriabova T, Henle ML, Levine AJ, Popescu G: Measurement of red blood cell mechanics during morphological changes. *Proc Natl Acad Sci U S A* 2010;107:6731-6736.
- 43 Betz T, Lenz M, Joanny JF, Sykes C: ATP-dependent mechanics of red blood cells. *Proc Natl Acad Sci USA* 2009;106:15320-15325.
- 44 Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, Bernhardt I: Regulation of phosphatidylserine exposure in red blood cells. *Cell Physiol Biochem* 2011;28:847-856.
- 45 Picas L, Rico F, Deforet M, Scheuring S: Structural and mechanical heterogeneity of the erythrocyte membrane reveals hallmarks of membrane stability. *ACS Nano* 2013;7:1054-1063.
- 46 De Mattia G, Bravi MC, Laurenti O, De Luca O, Palmeri A, Sabatucci A, Mendico G, Ghiselli A: Impairment of cell and plasma redox state in subjects professionally exposed to chromium. *Am J Ind Med* 2004;46:120-125.
- 47 Guo L, Xiao Y, Wang Y: Hexavalent chromium-induced alteration of proteomic landscape in human skin fibroblast cells. *J Proteome Res* 2013;12:3511-3518.
- 48 Lang F, Abed M, Lang E, Föller M: Oxidative stress and suicidal erythrocyte death. *Antioxid Redox Signal* 2014;21:138-153.
- 49 Park Y, Best CA, Auth T, Gov NS, Safran SA, Popescu G, Suresh S, Feld MS: Metabolic remodeling of the human red blood cell membrane. *Proc Natl Acad Sci USA* 2010;107:1289-1294.