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Effects of chromic chloride on chick embryo fibroblast viability

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A B S T R A C T

The objective of this study is to evaluate the effects of chromic chloride ($CrCl_3$) on chick embryo fibroblast (CEF) viability. The cells were incubated with $CrCl_3$ (0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μM), and the viability was determined using MTT assay, morphological detection and flow cytometry. The results show that lower concentrations of $CrCl_3$ (0.02, 0.1, and 0.5 μM) did not damage CEF viability. At 0.1 μM, $CrCl_3$ can increase CEF viability ($P < 0.05$). However, at higher concentrations of $CrCl_3$ (2.5, 12.5, and 62.5 μM), the number of apoptotic and necrotic cells ($P < 0.01$) and intracellular reactive oxygen species ($P < 0.01$) increased. In addition, decreased mitochondrial membrane potential ($P < 0.01$) and enhanced intracellular calcium levels ($P < 0.01$) were observed after the exposure. Moreover, apoptotic morphological changes induced by these processes in CEF were confirmed using Hoechst 33258 staining. Cell death induced by higher concentrations of $CrCl_3$ was caused by an apoptotic and a necrotic mechanism, whereas the main mechanism of oxidative stress and induced mitochondrial dysfunction was apoptotic death. The induced apoptotic death in CEF is concentration- and time-dependent.

1. Introduction

Chromium (Cr) is ubiquitous in the environment and is naturally found in soils, rocks, and living organisms [1]. Chromium exists in different oxidation states from −2 to +6, of which Cr(III) and Cr(VI) are the two primary valence states that are widely used in industries [2]. Aside from causing allergic dermatitis and apoptotic cell death, Cr(VI) is also toxic, carcinogenic [3], and teratogenic to animals and humans [3–6]. By contrast, Cr(III) is a micronutrient that is important in biological activities involved in potentiating lipids and insulin and in carbohydrate metabolism [7–10]. Type II diabetes, cardiovascular

diseases, and nervous system disorders have been associated with Cr(III) deficiency [8,11]. Thus, patients with glucose, insulin, lipid, and related abnormalities have been affiliated with insufficient Cr in their diets, and the treatment for these diseases involves the addition of Cr in their diet [8].

Cr(VI) enters the cell via general anion channels and is subsequently reduced to Cr(III) and other intermediates by a series of enzymatically catalyzed reactions. On the other hand, Cr(III) cannot enter the cell [12–14]. However, the DNA-damaging effects of Cr(III) have been reported both in cellular and cell-free systems [15]. Cr(III) negatively affects enzyme activity, intracellular ATP levels, and oxygen consumption [16]. The carcinogenic mechanisms of Cr(III) might be related to its ability to generate hydroxyl radicals (−OH) through the Fenton reaction [17]. Evidence have shown the trans-generational carcinogenicity of Cr(III) in

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the descendant of male mice that were intraperitoneally exposed to chromic chloride (CrCl$_3$) [18], which may be correlated to alterations in hormonal signaling [19] and/or gene expression [20].

The objective of this study is to evaluate the effects of chromic chloride on chick embryo fibroblast (CEF) viability. The cytotoxic potential of specific Cr(III) complexes was determined at the cellular level in terms of DNA damage, alterations in cellular morphology, and changes in intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and intracellular free Ca^{2+}. The concentrations of CrCl$_3$ at which cell proliferation is promoted and at which cell death occurs were determined. By studying these complexes, we expect to gain insights on the mechanism of metal-mediated cellular damage and the suitability of adding CrCl$_3$ in pharmaceutical dietary supplements. This study also provides a theoretical basis for further research.

2. Materials and methods

2.1. Chemicals

All chemicals were of the highest-grade purity available. CrCl$_3$ was purchased from Aladdin Reagent Database Inc. DMEM and trypsin were purchased from Sigma–Aldrich, USA. Newborn calf serum (NCF) was purchased from Sijiqing Biological Engineering Material (Hangzhou, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate (DCFH-DA), Hoechst 33258 staining, rhodamine 123 (Rh 123), Fluo-3/AM, and Annexin V/PI detection apoptotic kits were obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Preparation of CEF cell culture monolayers

The CEF were prepared from 9- to 11-day-old chicken embryos (Hy-Line Brown LTD, Taiwan, and China). After removing the head, limbs, and viscera, the embryos were cut into pieces with sizes between 1 and 2 mm and washed in PBS. The CEF were prepared by three rounds of trypsinisation (0.25%) for 1 min at 37 °C [21]. After filtration, the CEF were cultured in DMEM with 5% NCF and antibiotics at 37 °C with 5% CO$_2$.

2.3. Chemical treatment

CrCl$_3$ was dissolved in the DMEM solution and was added to the CEF solution to produce final concentrations of 0, 0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μM and incubated at 24, 48, and 72 h. Cell viability was tested using the MTT assay.

2.4. Assessment of cell survival

Cell viability in the culture medium was determined by the MTT assay [22]. The cells were seeded at a density of 2×10^5 mL^{-1}, and only 100 μL of the DMEM medium containing 2% NCF was added to the 96-well plates. The CEF were then incubated with CrCl$_3$ at 0, 0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μM for 24 h, 48 h, and 72 h at 37 °C. At predetermined time points, 15 μL of the MTT solution was added into the 96 well plates and incubated at 37 °C for 4 h. The medium was then removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan [23,24]. The 96-well culture plate was agitated for 10 min on a shaker. The plates were spectrophotometrically evaluated at 570 nm, and the OD value was obtained. The assays were performed three times each.

2.5. Analysis of nuclear morphology

Apoptotic morphological changes in the nuclear chromatin were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (bisbenzimide) [25]. The CEF cells were seeded into 6-well plates at a cell density of 4×10^5 per well. After incubation with various concentrations of CrCl$_3$ (0, 0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μM) for 48 h, the control and treated cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, the cells were washed three times with PBS and incubated with 5 μg/mL Hoechst 33258 staining solution for 15 min. After three washes, the cells were viewed using an inverted fluorescence microscope (TE-2000-S, NIKON, Japan) at an excitation wavelength of 352 nm.

2.6. Flow cytometry with Annexin V/PI staining

About 2×10^5 cells/mL were seeded into 6-well plates, which were then incubated for about 5 h at 37 °C. Subsequently, the cells were incubated with various concentrations of CrCl$_3$ (0, 0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μM) for 48 h. After treatment, the cells were harvested and washed with PBS. The cells from each sample were suspended in 195 μL of 1× Annexin V-FITC binding buffer and 5 μL of Annexin V-FITC, and then incubated at room temperature for 10 min. Each sample was then centrifuged at $1000 \times g$ for 5 min, resuspended in 190 μL of the binding buffer, and 10 μL of propidium iodide (PI) working solution was added. The samples were then analyzed via flow cytometric methods (FCM) [26]. The population was analyzed using quadrant statistics. The cells in the lower left quadrant represented living cells, those in the lower right quadrant represented necrotic cells, those in the upper left quadrant represented early apoptotic cells, and those in the upper right quadrant represented late apoptotic cells.

2.7. Measurement of Intracellular ROS

Intracellular ROS was determined using a fluorescent DCFH-DA probe [27,28]. The cells were then treated in a similar manner as that in the previous section. The cells were washed with PBS and incubated with DCFH-DA at 37 °C for 30 min in the dark. The DCFH-DA was oxidized to the fluorescent DCF product by the ROS in the cells. The DCF fluorescence distribution of 10,000 cells was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by using a FACS Calibur flow cytometer (Becton Dickinson, USA).

Table 1
Effects of CrCl$_3$ on the survival of CEF at different time points.

Groups	OD values		
	24 h	48 h	72 h
A	0.436 ± 0.0130	0.461 ± 0.00547	0.422 ± 0.00234
B	0.456 ± 0.188	0.477 ± 0.00872	0.440 ± 0.00560
C	0.467 ± 0.0167[#]	0.490 ± 0.00445[#]	0.446 ± 0.00817[#]
D	0.423 ± 0.0140	0.437 ± 0.0369	0.411 ± 0.00432
E	0.406 ± 0.00489[#]	0.377 ± 0.0321[##]	0.317 ± 0.0299[##]
F	0.365 ± 0.0278[##]	0.349 ± 0.0317[##]	0.313 ± 0.0295[##]
G	0.344 ± 0.0304[##]	0.340 ± 0.0161[##]	0.309 ± 0.0199[##]

Among the groups: A control, B 0.02 μM CrCl$_3$, C 0.1 μM CrCl$_3$, D 0.5 μM CrCl$_3$, E 2.5 μM CrCl$_3$, F 12.5 μM CrCl$_3$, and G 62.5 μM CrCl$_3$. The values for each CrCl$_3$ concentration tested represent the average as mean± SD (n = 6). Comparing the exposed groups (B, C, D, E, F, and G) with the control.

[#] $P < 0.05$.
[##] $P < 0.01$ using one-way ANOVA.

2.8. Changes of mitochondrial membrane potential (MMP)

The changes in MMP were estimated using the fluorescent cationic dye Rh123 [29], which can specifically bind to mitochondria and has been used in numerous investigations to estimate MMP [30] with several modifications. The cells were treated in a similar manner as that in the previous section. Then treated cells were harvested, washed, and incubated with Rh123 (5 μg/mL) in PBS at 37 °C for 30 min in the dark. The cells were then washed twice with PBS, and the fluorescence was immediately measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by using a FACS Calibur flow cytometer (Becton Dickinson, USA).

2.9. Intracellular free Ca^{2+} measurements

The cells were treated in a similar manner as that in the previous section. The samples were loaded with the Ca^{2+} indicator Fluo-3/AM (1 μM; Beyotime, Jiangsu, China) for 30 min in the dark at 37 °C. The cells were then washed twice with PBS. The fluorescence intensity distribution of 10,000 cells was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by using a FACS Calibur flow cytometer (Becton Dickinson, USA). The fluorescence intensity distribution represents intracellular [Ca^{2+}]i.

2.10. Statistical analysis

Data are expressed as mean ± SD from at least three independent experiments that were performed in duplicates. Statistical significance between groups was determined based on one-way analysis of variance. The SPSS 17.0 software was used for statistical analyses. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of chromic chloride on CEF survival

Table 1 shows the viability of cells treated with different concentrations of CrCl$_3$, which was monitored by the MTT assay. Lower concentrations of CrCl$_3$ at 0.02 and 0.5 μM did not change CEF viability at any time point. At 0.1 μM, the effect was more apparent compared with the control sample ($P < 0.05$) at three time points. The difference at higher concentrations of 2.5, 12.5, and 62.5 μM were all significant compared with the control group at three time points, and the cytotoxic effects of the chosen CrCl$_3$ concentrations on CEF were time- and dose-dependent.

3.2. Effects of CrCl$_3$ on morphologic changes

The effects of various concentrations of CrCl$_3$ were also detected through morphological observation (Fig. 1). In Fig. 1a–c, majority of the cells were unaltered, and normal cell morphology with normal nuclear size and integrity was observed. However, when the CEF were exposed to higher concentrations of CrCl$_3$ (2.5 μM to 62.5 μM), cell body shrinkage and highly condensed nuclei were observed (Fig. 1d–g). Hoechst 33258 was used to assess the change in DNA and nuclear structures (Fig. 1A–G). Almost no apoptotic nuclei were found in the cells of the control group. At lower concentrations of Cr(III) (0.02 and 0.1 μM) (Fig. 1A–C), the nuclei were large and regular, whereas at higher concentrations of Cr(III) (2.5–62.5 μM), most of the nuclei were condensed, bright, and had a half-moon form, which are characteristics of apoptotic dead cells (Fig. 1D–G). In short, the nuclei of apoptotic cells were all significantly damaged compared with those of intact cells.

3.3. Effects of CrCl$_3$ on apoptosis induction

Cell apoptosis was investigated via flow cytometry by using an Annexin V-FITC apoptosis detection kit (Beyotime, Jiangshu, China). The cytometric analysis results reveal that at lower concentrations of CrCl$_3$ (0.02 and 0.1 μM), CEF apoptosis was inhibited, whereas at higher concentrations of CrCl$_3$ (2.5, 12.5, and 62.5 μM), CEF apoptosis was observed (Fig. 2). As shown in Table 2, the percentage of live cells exposed to lower concentrations of CrCl$_3$ (0.02 and 0.1 μM) significantly increased from 60.86% to 63.55% ($P < 0.05$). Similarly, significant differences were observed in the percentages of apoptotic and necrotic cells at a CrCl$_3$ concentration of 0.1 μM. However, when the cells were exposed to higher concentrations of CrCl$_3$ (0.5, 2.5, 12.5, and 62.5 μM) for 48 h, the percentage of apoptotic cells
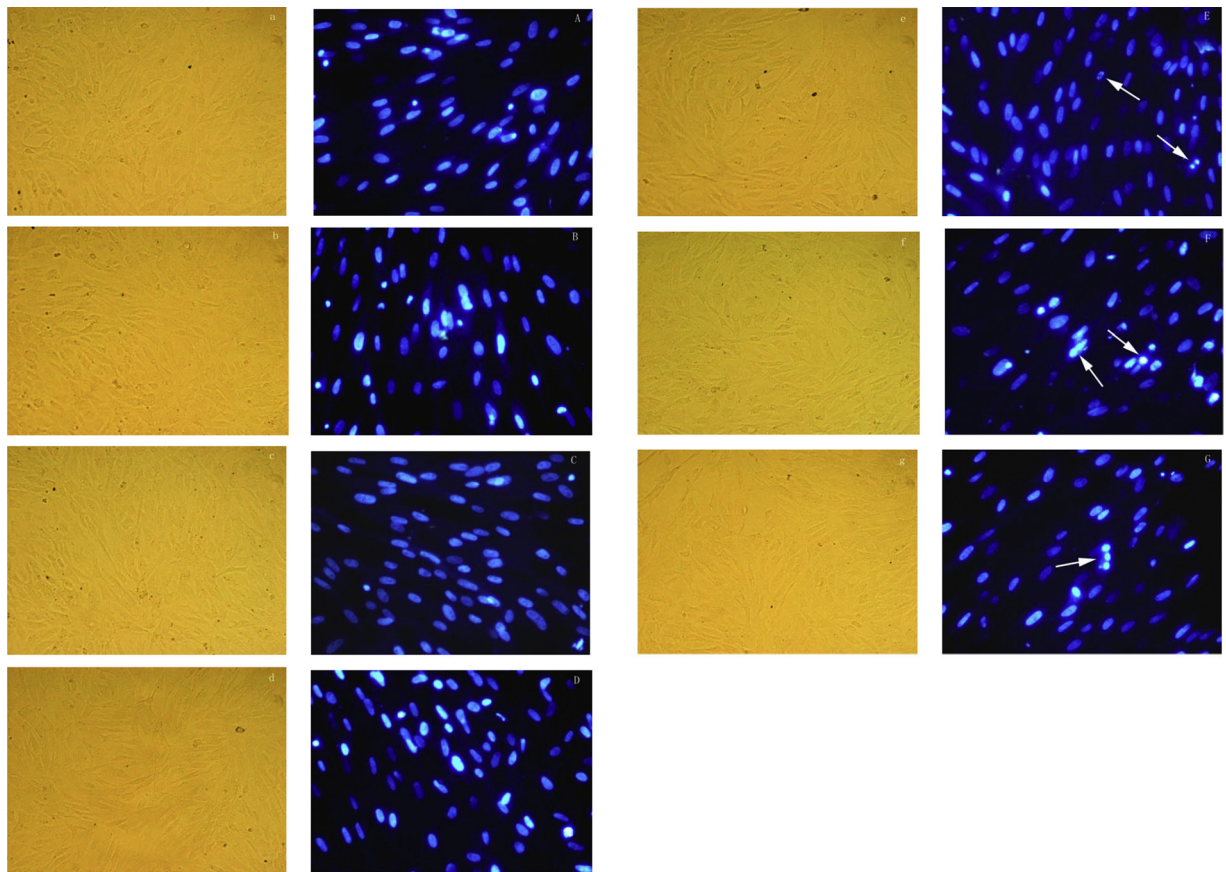
Fig. 1. Effects of Cr(III) on the morphologic changes. Control (a, A), 0.02 μM CrCl$_3$ (b, B), 0.1 μM CrCl$_3$ (c, C), 0.5 μM CrCl$_3$ (d, D), 2.5 μM CrCl$_3$ (e, E), 12.5 μM CrCl$_3$ (f, F), 62.5 μM CrCl$_3$ (g, G). Cellular morphology was observed with light microscope (a–g). In addition, nuclear morphology was observed by fluorescence microscope after stained with bisbenzimide (A–G). The changes of nuclei fragmentation with condensed chromatin exposed in 2.5 μM–62.5 μM CrCl$_3$ are evident. a–g, A–G = original magnification × 200.

significantly increased from 34.89% to 46.99% ($P < 0.01$) and dose-dependent cytotoxic effects were observed.

3.4. Effects of CrCl$_3$ on intracellular ROS levels in CEF

A fluorescent DCFH-DA probe was used to test the generation of intracellular ROS. The fluorescence of the

Table 2
Percent of live cells, necrotic cells and apoptotic cells in chicken embryo fibroblast treated with Cr(III) ($n = 4$).

Groups	Percent of live cells	Percent of apoptotic cells	Percent of necrotic cells
A	60.86 ± 0.44	34.89 ± 0.17	4.86 ± 0.53
B	61.26 ± 0.40[#]	34.41 ± 0.62	4.33 ± 0.35
C	63.55 ± 0.65[##]	30.83 ± 0.89[##]	5.63 ± 0.63[#]
D	58.79 ± 0.80[##]	36.69 ± 0.52[##]	4.53 ± 0.50
E	52.31 ± 0.65[##]	42.19 ± 0.72[##]	5.51 ± 0.21[#]
F	47.21 ± 0.15[##]	47.62 ± 0.35[##]	5.17 ± 0.30
G	44.55 ± 0.31[##]	46.99 ± 0.37[##]	8.46 ± 0.38[##]

A control, B 0.02 μM CrCl$_3$, C 0.1 μM CrCl$_3$, D 0.5 μM CrCl$_3$, E 2.5 μM CrCl$_3$, F 12.5 μM CrCl$_3$, and G 62.5 μM CrCl$_3$.

[#] $P < 0.05$.
[##] $P < 0.01$ using one-way ANOVA.

cells was examined on FCM at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. As shown in Fig. 3, the ROS level in the cells decreased when incubated at lower concentrations of CrCl$_3$ (0.02 and 0.1 μM) for 48 h. At a CrCl$_3$ concentration of 0.1 μM, the level of ROS was significantly lower ($P < 0.01$) than that of the control group. On the other hand, the level of ROS increased when the cells were exposed to higher concentrations of CrCl$_3$ (0.5, 2.5, 12.5, and 62.5 μM) for 48 h, and the increase occurred in a dose-dependent manner. The mean fluorescence intensity was significantly higher than that of the control group (287.75 ± 1.78, $P < 0.01$).

3.5. Changes in MMP in CEF

As shown in Fig. 4, the CEF treated with lower concentrations of CrCl$_3$ (0.02, 0.1, and 0.5 μM) for 48 h showed a significant increase in MMP except at 0.5 μM ($P < 0.01$). On the other hand, the MMP significantly decreased after the cells were treated with higher concentrations of CrCl$_3$ (2.5, 12.5, and 62.5 μM) for 48 h. The decrease occurred in a concentration-dependent manner ($P < 0.01$).
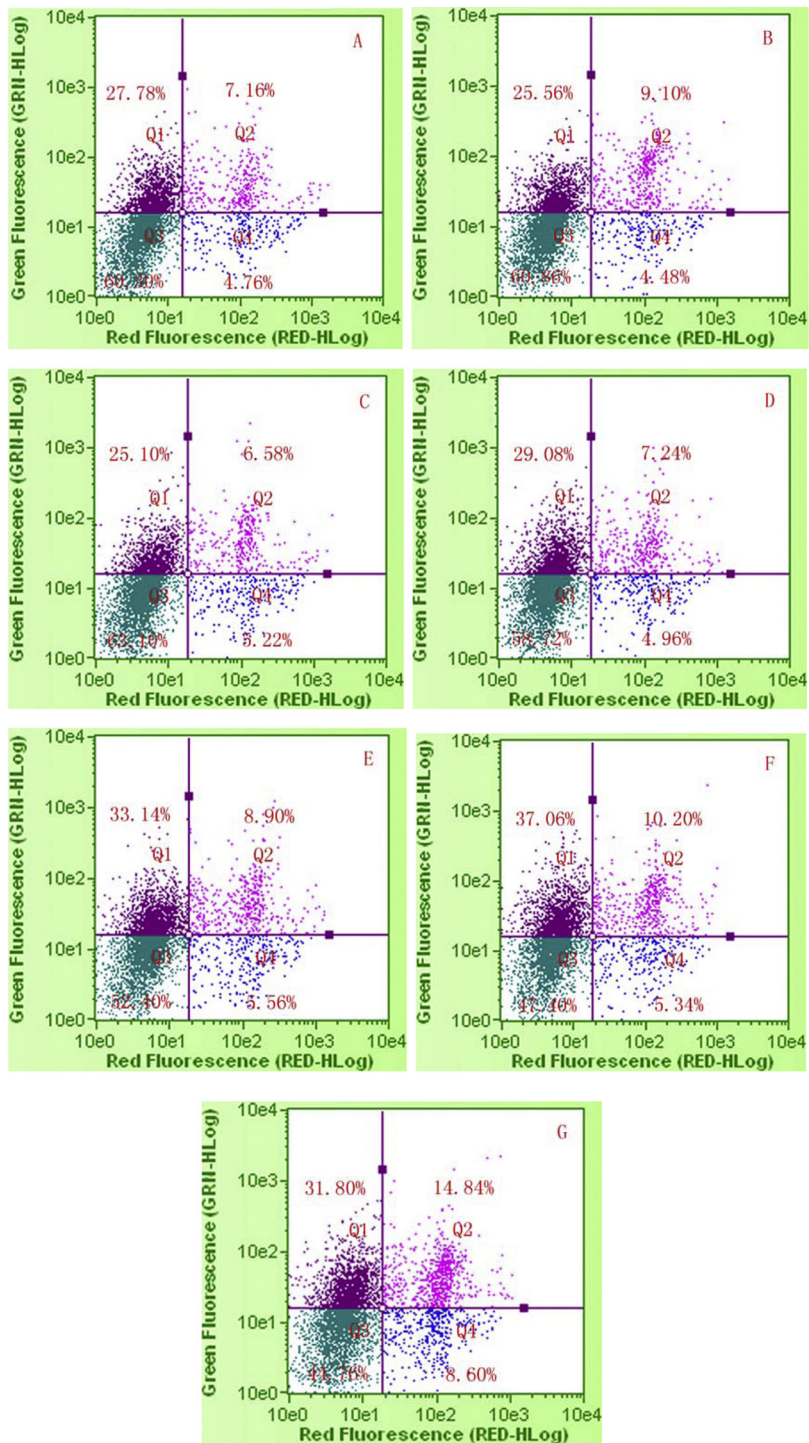
Fig. 2. Effects of Cr(III) on distribution of apoptotic cells. A control, B 0.02 μM CrCl₃, C 0.1 μM CrCl₃, D 0.5 μM CrCl₃, E 2.5 μM CrCl₃, F 12.5 μM CrCl₃, and G 62.5 μM CrCl₃. Q1: Early/primary apoptotic cells (Annexin V⁺/PI⁻); Q2: Late/secondary apoptotic cells (Annexin V⁺/PI⁺); Q3: Live cells (Annexin V⁻/PI⁻); Q4: Necrotic cells (Annexin V⁻/PI⁺).
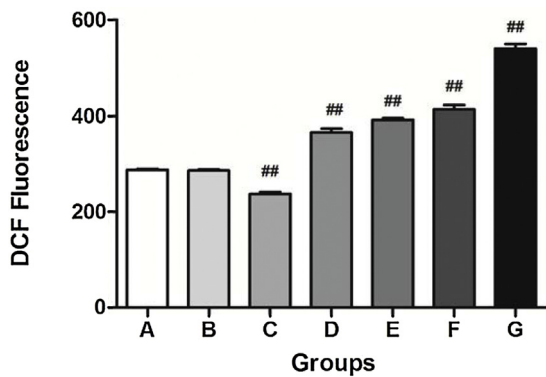
Fig. 3. Effects of Cr(III) on intracellular ROS level in CEF. The intracellular ROS as mean ± SD ($n = 4$). Cells were treated with: A control, B 0.02 μM CrCl$_3$, C 0.1 μM CrCl$_3$, D 0.5 μM CrCl$_3$, E 2.5 μM CrCl$_3$, F 12.5 μM CrCl$_3$, and G 62.5 μM CrCl$_3$ for 48 h. ([#]$P < 0.05$ vs. control and [##]$P < 0.01$ vs. control).

3.6. Effects of CrCl$_3$ on Intracellular [Ca^{2+}]i

As shown in Fig. 5, after treatment with different concentrations of CrCl$_3$ for 48 h, exposure to lower concentrations of CrCl$_3$ (0.1 μM) resulted in a marked decrease in intracellular [Ca^{2+}]i ($P < 0.01$). On the other hand, at higher concentrations of CrCl$_3$ (2.5, 12.5, and 62.5 μM) intracellular [Ca^{2+}]i was significantly enhanced ($P < 0.01$).

4. Discussion

Regarding the effect of chromium, researchers mainly studied lymphocytes [31,32], bacterial cells [33], and dermal fibroblasts [34]. However, in these animal studies, the effects of chromium on animal cells are rarely reported. Thus, this study aims to determine the effect of CrCl$_3$ on CEF viability at specific CrCl$_3$ concentrations. The selected dose range of CrCl$_3$ in the present study was 0.02–62.5 μM, which is within the acceptable limit for human consumption, specifically for pills that contain Cr(III) picolinate [35].

The cellular viability test results, in which 2×10^5 cells were incubated with different concentrations of CrCl$_3$ for
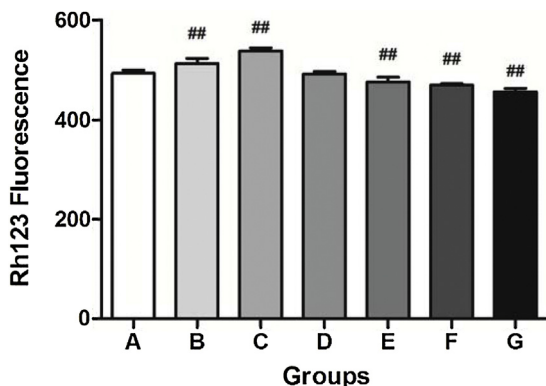

Fig. 4. The changes of MMP in CEF. The statistical interpretation of data expressed as mean ± SD ($n = 4$). Cells were treated with: A control, B 0.02 μM CrCl$_3$, C 0.1 μM CrCl$_3$, D 0.5 μM CrCl$_3$, E 2.5 μM CrCl$_3$, F 12.5 μM CrCl$_3$, and G 62.5 μM CrCl$_3$ for 48 h. ([#]$P < 0.05$ vs. control and [##]$P < 0.01$ vs. control).
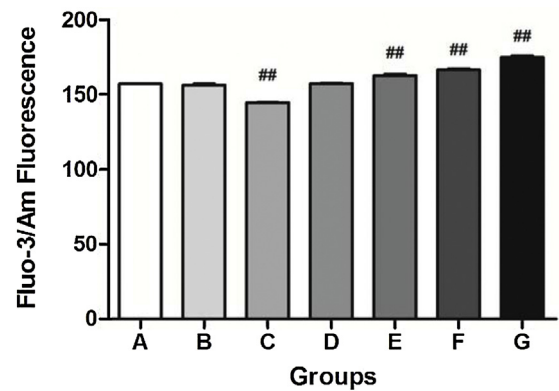

Fig. 5. Effects of Cr(III) on Intracellular [Ca^{2+}]i. The statistical interpretation of data expressed as mean ± SD ($n = 4$). Cells were treated with: A control, B 0.02 μM CrCl$_3$, C 0.1 μM CrCl$_3$, D 0.5 μM CrCl$_3$, E 2.5 μM CrCl$_3$, F 12.5 μM CrCl$_3$, and G 62.5 μM CrCl$_3$ for 48 h ([#]$P < 0.05$ vs. control and [##]$P < 0.01$ vs. control).

24, 48, and 72 h, show that at lower concentrations of CrCl$_3$ (0.02, 0.1, and 0.5 μM), cell toxicity was not observed at any time. At a CrCl$_3$ concentration of 0.1 μM, cell proliferation was significantly promoted ($P < 0.05$). However, at higher concentrations of CrCl$_3$ (2.5, 12.5, and 62.5 μM), cell toxicity was observed at three time periods ($P < 0.01$). The result also shows that at incubation with higher concentrations of CrCl$_3$ induced cell death, which is caused by both incubation time and metal concentration. The further experiments were performed to investigate if different concentrations of CrCl$_3$ lead to cell proliferation or necrosis. As shown in Table 2, cell death induced by higher concentrations of CrCl$_3$ is mediated by two mechanisms, namely, apoptotic and necrotic. The amount of apoptotic cells induced by higher concentrations of CrCl$_3$ significantly increased. Moreover, the number of necrotic cells and the cellular morphology confirm that the necrotic mechanism is responsible for cell death. By comparison, cell death is caused by the apoptotic mechanism at higher concentrations of CrCl$_3$.

Cellular morphology was assessed through Hoechst 33258 staining. Apoptotic morphological changes such as DNA fragmentation were also observed compared with intact cells. This result is consistent with that obtained from flow cytometric analysis.

In this study, the CEF incubated at higher concentration of CrCl$_3$ generated a significant amount of ROS (Fig. 3). This result further proves that oxidative stress has a key function in cell toxicity induced by CrCl$_3$, and the reverse can be said at lower concentrations of CrCl$_3$. Intracellular ROS is mainly produced in the mitochondria, which seriously damages the mitochondria [36]. Studies have demonstrated that intracellular ROS can regulate the initiation of apoptotic signaling [37,38]. In addition, excessive amounts of ROS may directly induce cell damage by oxidizing and inactivating proteins and lipids and by changing the normal cellular signaling pathways [39–41]. In the present study, the changes in the mitochondria could affect the intracellular ROS levels in CEF because CrCl$_3$ changes the MMP, which is consistent with the intracellular ROS levels. The increasing amount of ROS generated from the

intermembrane space can lead to mitochondrial dysfunction and contributes to cell death [42]. Studies have clearly shown that ROS is a associated with the decrease in MMP [43]. These results show that MMP has an important function in apoptosis induced by $CrCl_3$.

In addition, intracellular $[Ca^{2+}]i$ had an abnormal manifestation when the CEF was treated at higher concentrations of $CrCl_3$ (Fig. 5). These results also confirm that another important mechanism of cell apoptosis is caused by abnormal Ca^{2+} homeostasis when exposed to $CrCl_3$. Removing Ca^{2+} from the cytosol across the plasma membrane requires sufficient ATP [44,45]. Furthermore, normal mitochondria generates ATP if MMP breakdown results in a decrease in ATP levels [46]. Moreover, oxidative damage easily affects the mitochondria. Therefore, treatments with higher concentrations of $CrCl_3$ result in mitochondrial dysfunction, ROS overproduction. These results also lead to intracellular calcium overload.

5. Conclusions

The experimental results show that $CrCl_3$ concentrations below 0.5 μM do not have harmful effects on the CEF, whereas higher concentrations of $CrCl_3$ (2.5, 12.5, and 62.5 μM) affects the CEF mainly through oxidation damage, which leads to cell apoptosis, and occurs in a dose- and time-dependent manner. In conclusion, chromic chloride affected the viability of chick embryo fibroblast.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

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