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Hexavalent Chromium Induced Oxidative Stress and Toxicity on isolated human lymphocytes

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Abstract:

Introduction: The most toxic form of chromium (Cr) in the environment is the oxyanion chromate (Cr (VI)). In this form it is soluble and is transported into the cells. Chromate structurally resemble phosphate and sulfate, and can be transported into cells by the anion carrier.

Methods and Results: In this study, toxicity effects of Cr (VI) on isolated human lymphocytes was studied using accelerated cytotoxicity mechanisms screening (ACMS) technique. Human lymphocytes were isolated from blood of healthy persons using Ficoll-paque PLUS standard method. The trypan blue dye was used to cytotoxicity assay. The mechanistic parameters including reactive oxygen species (ROS), lysosomal membrane destabilization, mitochondrial membrane potential (MMP) collapse, lipid peroxidation, GSH and GSSG levels were assessed after 1, 2 and 3 hrs in potassium dichromate treated lymphocytes. The results indicate that toxicity of Cr (VI) was concentration dependent in human lymphocytes. Cr (VI) significantly ($p<0.05$) induced ROS production, MMP reduction, lysosomal membrane destabilization and lipid peroxidation in human lymphocytes. There was also a decrease in intracellular GSH and raise in extracellular GSSG levels in Cr (VI) treated lymphocytes.

Conclusion: Our findings revealed that ROS formation with subsequent cellular damages is the molecular mechanism for Cr (VI) induced human blood lymphocyte toxicity.

Keywords: Cytotoxicity; Chromium (VI); Human lymphocyte

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1. Introduction

Chromium (Cr) is one of well-known and the most important heavy metal that has important oxidant states of III and VI [1].Compared to Cr (III), the cross of Cr (VI) from cell membranes is easier due to ion carriers. Therefore, Cr (VI) is more toxic and is more attractive to researchers [2-4].Cr (VI) has many industrial applications due to its unique characteristics [4-6]. It has been shown that Cr (VI) have high mobility in the environment, which can induce toxic and irreversible effects in living organisms [6]. Today, pollution caused by Cr (VI) is a global concern for human health and the ecosystem [7].

Research has shown that Cr (VI) can accumulate in different tissues, which can lead to increased free radicals and oxidative stress [8].In recent years, toxicological studies on chromium toxicity have been of interest to researchers. It has been shown that *in vitro* studies can play an important role in understanding the mechanism of cytotoxicity and oxidative stress of compounds, including chromium[9]. Oxidative stress and excessive generation of reactive oxygen species (ROS) is one of the most important mechanisms by which Cr (VI) causes toxicity. In recent years, this mechanism has been considered by most researchers [1, 8, 10, 11].

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Oxidative stress is a process in which the accumulation and generation of ROS or oxidative capacity increases in comparison with the antioxidant capacity [12-15].It has been shown that excessive ROS generation damages macromolecules and also induces cell death. It has been shown that over-excessive of ROS generation cause damage to bio-macromolecules (DNA, lipid, and protein), and also the induction of cell death [5, 8, 16, 17]. Mitochondria is one of the most important sources of ROS generation. Studies using isolated mitochondria have shown that complexes I and III of the mitochondrial respiratory chain (MRC) play a crucial role in ROS generation [18, 19].Furthermore, mitochondria as a source of energy have been shown to play an important role in the physiological processes of the cell [20, 21].In addition, These organelle are the primary target of xenobiotics due to their sensitive structure and complex functions [20].
Accordingly, the aims of the present in vitro study were to determine the cytotoxic effect of Cr (VI) on human lymphocytes; including the effect of Cr (VI) on oxidative stress (through ROS, LPO, GSH and GSSG levels assay), mitochondrial damage (MMP collapse assay); and the probable effect of the metal ion on lysosomal membrane stability.

2. Materials & Methods

2.1. Ethics Statement

The present study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences as ID IR.SBMU.PHARMACY.REC.1397.212. After became aware of our study donors are asked to fill out the approval form.

2.2. Human Lymphocytes Isolation

In this study, lymphocyte specimens were isolated from 20 healthy donors aged between 18 and 30 years old. One of the exclusion criteria was the absence of disease at the time of sample collection. The standard Ficoll technique (Ficoll Paque Plus) using centrifugation (2500g for 20 min at 25 °C) is used to isolate lymphocytes. Then, isolated lymphocytes were suspended in erythrocyte lysis buffer. In the following, the cells were centrifuged (1500g for 10 min at 20 °C) after PBS addition. The supernatant was eliminated, and the cells were washed twice with RPMI with l-glutamine and 10% fetal bovine serum (FBS) at 2000*g* for 7 min. The cells were re-suspended in RPMI medium with L glutamine and 10 % FBS and counted using trypan blue exclusion dye. The viability and density of the humane lymphocytes was over 95%, and 10×10^6 cells/ml, respectively [22].

2.3. Lymphocyte exposure

The concentrations of the chromium used in the experiments were in the range from 0.05-2 mM. The humane lymphocytes were incubated with chromium at concentrations of 0.05-2 mM for 6 hour to evaluate cell viability. Furthermore, the humane lymphocytes were incubated with chromium at concentrations of 0.2, 0.4 and 0.8 mM for 1, 2 and 3 hour to evaluate the reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) collapse, lysosomal membrane damage, and GSH and GSSG levels. The incubation was done at 37 °C in 5% CO_2 atmosphere in the total darkness.

2.4. Lymphocytes Viability Assay

The trypan blue (0.4% w/v) exclusion test was used to the evaluation of cell viability of human lymphocyte. Lymphocytes were plated onto 96 well plate (1×104 cells/ml) and incubated with different concentration of chromium (0.05-2 mM) for 6 hour. After the incubation time (6 hour), the cell viability of humane lymphocytes was assayed with trypan Blue solution (0.4% w/v).

2.5. Lymphocytes Reactive Oxygen Species (ROS) generation

The 2′, 7′-dichlorodihydrofluoresceindiacetate (DCFH-DA) probe used to measure the production of ROS. Briefly, human lymphocytes were incubated with different concentrations of chromium (0.2, 0.4 and 0.8) for 1, 2 and 3 hours. After each incubation time, human lymphocytes were washed with PBS. Then, DCFH-DA at the concentration of 10 μM was used to measure intracellular ROS generation. Buyhylatedhydroxy toluene (BHT) was used as an antioxidant. Finally, the fluorescence intensity (DCF) was measured using the Shimadzu RF-5000 U fluorescence spectrophotometer (λ Ex = 495 nm, and λ Em= 530 nm) [23].

2.6. Lymphocytes Mitochondrial Membrane Potential (MMP) collapse

The rhodamine123 (Rh 123) probe used to measure the collapse of MMP. Briefly, human lymphocytes were incubated with different concentrations of chromium (0.2, 0.4 and 0.8) for 1, 2 and 3 hours. After each incubation time, human lymphocytes were washed with PBS. Then, Rh 123at the concentration of 10 μM was used to measure MMP collapse. Cyclosporine A was used as a mitochondrial permeability transition (MPT) pores blocker. Finally, the fluorescence intensity (Rh 123) was measured using the Shimadzu RF-5000 U fluorescence spectrophotometer (λ Ex = 470 nm, and λ Em= 540 nm) [23, 24].

2.7. Lysosomal membrane damage assay

The redistribution of acridine orange probe used to measure the damage (integrity) of the lysosomal membrane. Briefly, human lymphocytes were incubated with different concentrations of chromium (0.2, 0.4 and 0.8) for 1, 2 and 3 hours. After each incubation time,

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human lymphocytes were washed with PBS. Then, acridine orange at the concentration of 5 µM was used to measure lysosomal membrane damage. Chloroquine at concentration was used as a lysosomotropic agent. Finally, the fluorescence intensity (acridine orange) was measured using the Shimadzu RF-5000 U fluorescence spectrophotometer (λ Ex = 495 nm, and λ Em= 530 nm)[22, 23].

2.8. Lymphocytes Lipid Peroxidation (LPO) level

The Malondialdehyde (MDA) level was measured to assess lipid peroxidation (LPO).The level of MDA formed in each of the samples was evaluated through measuring the absorbance of the supernatant at 532 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria). MDA content was expressed as µg/mg protein[25].

2.9. Lymphocytes glutathione (GSH) and oxidized glutathione (GSSG) level

Briefly, after passing of 1, 2 and 3 hours, the 0.5 ml of TCA 10% was added to the cell, and then centrifuged at 11,000 RPM for 2 min, for GSH and GSSG assay 0.5 ml of supernatant were diluted by the addition of 4.5 ml phosphate-EDTA buffer. 100 µl of diluted supernatant was added to 2.8 ml phosphate-EDTA buffer and 100 µl of the OPT solution. After incubation for 15 min at room temperature, each sample was measured for GSH and GSSG level in quartz cuvettes using the Shimadzu RF-5000 U fluorescence spectrophotometer (λ Ex = 350 nm, and λ Em= 420 nm)[22, 26].

2.10. Statistical analysis results

Data were analyzed using one and two way analysis of variance followed by post hoc Tukey and Bonferroni test with GraphPad Prism 5 (Graphpad Software, La Jolla, CA). The value of P <0.05 was considered as statistically significant.

3. Results

3.1. Cr (VI) decreased cell viability

Figure 1 shows the effects of chromium on the viability of human lymphocytes. The results have shown that chromium (0.05-2 mM) significantly decrease the viability of human lymphocytes in a concentration-dependent manner. According to the results, the concentration of 0.4mM as determined as IC_{50} concentration at 6 hour following the exposure of human lymphocytes to chromium (0.05-2 mM) (Figure 1).Subsequently, in the current main study we used concentrations of 0.2 mM (1/2 IC_{50}), 0.4 mM (IC_{50}) and 0.8 mM (1.5 IC_{50}) for further experiments.

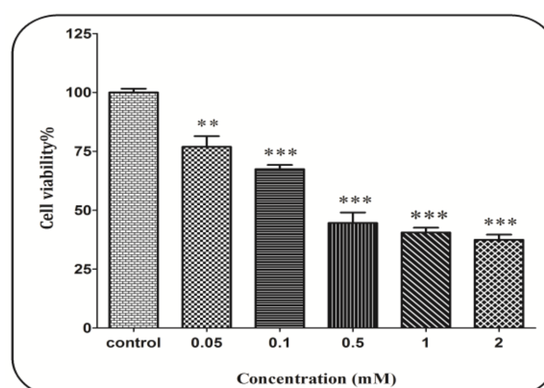

Figure 1. The effects of chromium on the viability of human lymphocytes. Viability determined using trypan blue exclusion dye after incubation of human lymphocytes with different concentration of chromium (0.05-2 mM). Chromium decreased the viability of human lymphocyte. Data are presented as mean ± SD. The one-way ANOVA test was carried out.$^{**}p<0.001$ and $^{***}p<0.001$ vs. control.

3.2. Cr (VI) increased reactive oxygen species (ROS) generation

As shown in Figure 2, chromium (0.2, 0.4 and 0.8 mM) has been able to increase the generation of ROS in human lymphocytes in a concentration and time-dependent manner. Also, buyhylatedhydroxy toluene (BHT) at the concentration of 50 µM is used as an antioxidant. When the humane lymphocytes were simultaneously treated with chromium (0.8 mM), and BHT (50 µM) the % fluorescence intensity was lower than that of chromium (0.8 mM) treated group. It means that BHT has been able to decrease the effects of chromium on increasing ROS production.

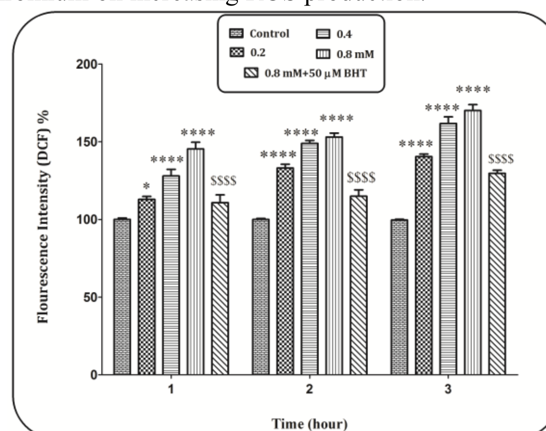

Figure 2. The effects of chromium on the ROS generation. ROS generation evaluated using DCFH-DA dye after incubation of human lymphocytes with different concentration of chromium (0.2, 0.4 and 0.8 mM). Chromium increased the ROS generation in human lymphocyte. Data are presented as mean ± SD. The two-way ANOVA test was carried out.$^{*}p<0.05$ and $^{****}p<0.0001$ vs. control, and $^{\$\$\$\$}p<0.0001$ vs. 0.8 mM.

3.3. Cr (VI) increased mitochondrial membrane potential (MMP) collapse

Results showed that chromium at a concentration 0.2 mM in 3 hour after incubation, and also at a

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concentrations 0.4and 0.8 mMin 1, 2 and 3 hour after incubation has been able to increase the collapse of MMP in human lymphocytes (Figure 3).Also, Cyclosporine A at the concentration of 5 µM is used as amitochondrial permeability transition (MPT) pores blocker. When the humane lymphocytes were simultaneously treated with chromium (0.8 mM), and Cyclosporine A (5 µM) the % fluorescence intensity was lower than that of chromium (0.8 mM) treated group (Figure 3).It means that BHT has been able to decrease the effects of chromium on increasing MMP collapse.

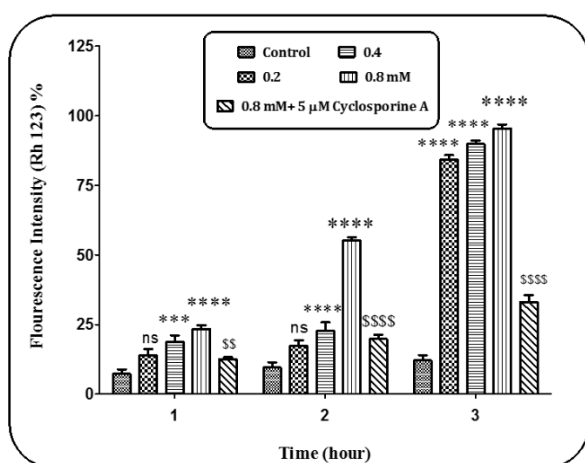

Figure 3. The effects of chromium on the MMP collapse. MMP collapse evaluated using Rh123 dye after incubation of human lymphocytes with different concentration of chromium (0.2, 0.4 and 0.8 mM). Chromium increased the MMP collapse in human lymphocyte. Data are presented as mean ± SD. The two-way ANOVA test was carried out.$^{***}p$<0.001 and $^{****}p$<0.0001 vs. control, $^{\$\$}p$<0.01 and $^{\$\$\$\$}p$<0.0001 vs. 0.8 mM.

3.4. Cr (VI) increased lysosomal membrane damage

Figure 4 displayslysosomal membrane damage of chromium (0.2, 0.4 and 0.8 mM) on human lymphocytes following 1, 2 and 3 h of incubation. Results showed that at all applied concentrations increased lysosomal membrane leakage after 1, 2 and 3 hour incubation. Also, chromium (0.8 mM) induced leakage of lysosomal membrane, decreased by pretreatment of human lymphocytes with chloroquine (100µM) as a lysosomotropic agent (Figure 4).

3.5. Cr (VI) increased Lipid Peroxidation (LPO) level

As shown in Figure5, chromium (0.2, 0.4 and 0.8 mM) has been able to increase the level of LPO in human lymphocytes in a concentration and time-dependent manner. Furthermore, chromium (0.8 mM)-induced LPO was inhibited by ROS scavenger (BHT; 50 µM) and MPT pore sealing agent (Cs.A; 5 µM). In addition to, the results showed that chromium (0.8 mM)-induced LPO was inhibited by GSH (2µM).

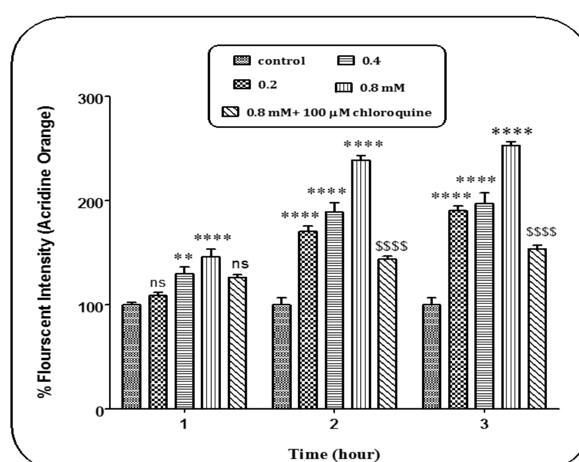

Figure 4. The effects of chromium on the lysosomal membrane damage. Lysosomal membrane damage evaluated using acridine orange dye after incubation of human lymphocytes with different concentration of chromium (0.2, 0.4 and 0.8 mM). Chromium increased the lysosomal membrane damage in human lymphocyte. Data are presented as mean ± SD. The two-way ANOVA test was carried out.$^{**}p$<0.01 and $^{****}p$<0.0001 vs. control, and $^{\$\$\$\$}p$<0.0001 vs. 0.8 mM).

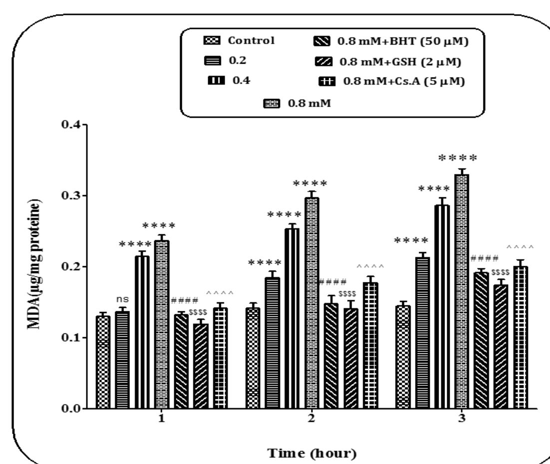

Figure 5. The effects of chromium on the lipid peroxidation (LPO) level. LPO level evaluated after incubation of human lymphocytes with different concentration of chromium (0.2, 0.4 and 0.8 mM). Chromium increased the LPO level in human lymphocyte. Data are presented as mean ± SD. The two-way ANOVA test was carried out.$^{****}p$<0.0001 vs. control. $^{\#\#\#\#}p$<0.0001 vs. 0.8 mM, $^{\$\$\$\$}p$<0.0001 vs. 0.8 mM and $^{\wedge\wedge\wedge\wedge}p$<0.0001 vs. 0.8 mM).

3.6. Cr (VI) decreased glutathione (GSH) level and increased oxidized glutathione (GSSG level

GSH is one of the main antioxidant systems in the cells. Furthermore, an imbalance between the generation of ROS, GSH and GSSG in cells can be associated with induction of oxidative stress. Our results showed that the exposure of human lymphocytes to chromium (0.2, 0.4 and 0.8 mM) at 1, 2, and 3 hours decreased the GSH level (Figure 6A) and increased the GSSG level (Figure 6B).

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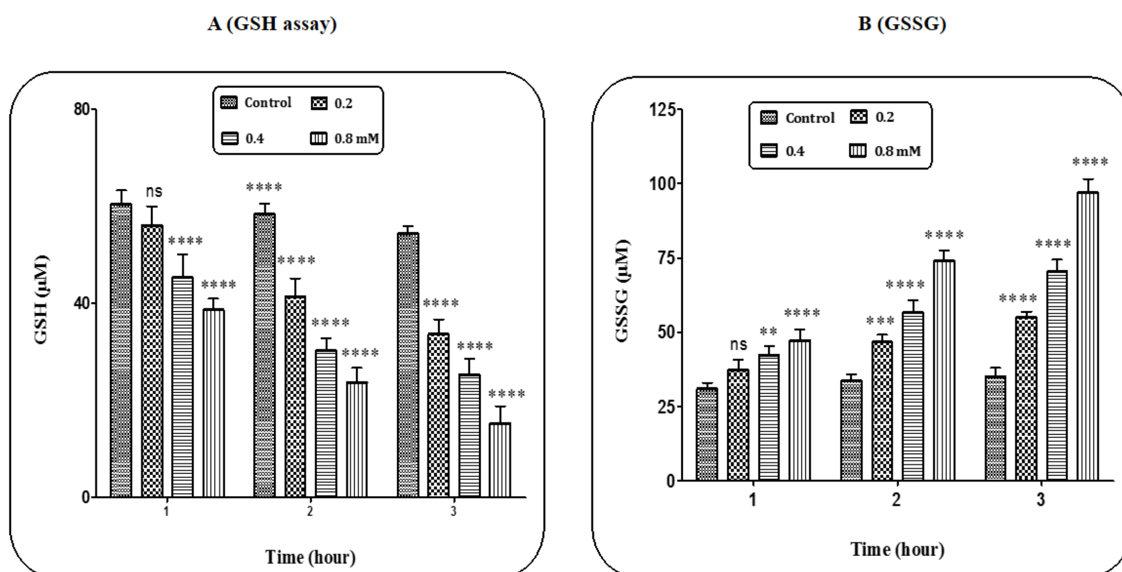

Figure 6. The effects of chromium on the GSH (A) and GSSG (B) levels. LPO level evaluated after incubation of human lymphocytes with different concentration of chromium (0.2, 0.4 and 0.8 mM). Chromium decreased the GSH level and increased the GSSG level in human lymphocyte. Data are presented as mean ± SD. The two-way ANOVA test was carried out. $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$ vs. control.

4. Discussion

Generally, Cr (VI) is more toxic than Cr (III). Cr (VI) displays toxicity due to reduction from Cr (VI) to Cr (III). In this reduction, the ROS is generated. It has been shown that the permeability of the cell membrane of Cr (III) is less than that of Cr (VI), and Cr (VI) passes via the cell membrane through an ion channel. Accordingly, the potential cytotoxic activity of Cr (VI) is higher than for Cr (III) [27].This research was designed to evaluate the *in vitro* cytotoxic effects of Cr (VI) on human lymphocytes. Therefore, we have further examined the effects of Cr (VI) on oxidative stress, mitochondrial and lysosomal membrane damage, and GSH and GSSG level in human lymphocytes. At first, human lymphocytes were treated with Cr (VI) and then oxidative stress, mitochondrial and lysosomal membrane damage, and GSH and GSSG level in human lymphocytes were evaluated. The results displayed that Cr (VI) showed a dose-dependent cytotoxic effects in human lymphocytes, and decreased cell viability. This result is agreement with the previous study, which showed that Cr (VI) decrease the HepG2 cells viability [9].

It has been well documented that Cr (VI) easily enter the cell through an ion channel (especially sulfate-anion channel) [4, 28].In the cell, it will eventually be converted to Cr (III) product. During this process, ROS such as hydrogen peroxide (H_2O_2) is generated [28].In this study, the fluorescence spectrophotometer assay displayed that 0.2, 0.4 and 0.8 mM Cr(VI) for 1, 2, and 3 hr exposure increased the H_2O_2levels. Furthermore, BHT shows antioxidant activity through inhibiting ROS generation. In mammalians cells, ROS play an important role in damage to cell membranes, cell death and other processes [28]. This result is agreement with the previous study, which showed that Cr (VI) could increase the ROS generation [7, 8, 29].Research has shown that mitochondrial respiratory chain (MRC) is one of the most main sources of ROS generation in different cells. In animal studies, it has been shown that electron leakage from MRC complexes (such as complex I) has led to generation of ROS[6].

The MMP of human lymphocytes was evaluated using Rh 123 assay and determined using fluorescence spectrophotometer. Based on the investigation on status of mitochondria in human lymphocytes exposed to Cr (VI), this study showed that Cr (VI) induced significant decline of MMP. Furthermore, Cs.A and inhibit MMP collapse due to Cr (VI). The decline in MMP leads to the matrix condensation and release of pro-apoptotic proteins such as cytochrome c to the inter-membrane space. Finally, the release of pro-apoptotic proteins induces cell death signaling cascade. It has also been shown that collapse in the MMP is one of the early events in apoptosis signaling [30].

In our research, the results showed that Cr (VI) increases LPO. MDA is measured as LPO indicator. Furthermore, BHT, Cs.A and GSH inhibit LPO induced by Cr (VI). These findings are in agreement with the previous study, which showed that Cr (VI) could increase the LPO [7]. It has been shown that increased ROS generation is responsible for LPO[16]. GSH is required at normal levels to reduce ROS generation (such as H_2O_2). An increase in ROS generation may result in GSH depletion [8].Therefore, the GSH depletion is another indicator of the cellular oxidative stress, and it may be accompanied by a GSSG increase [22].The results showed that when humane lymphocytes were incubated with Cr (VI), the

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GSH depletion occurs as a consequence of the ROS generation and the LPO.
The lysosomal membrane damage in human lymphocytes was evaluated using acridine orange assay. The incubation of human lymphocytes with Cr (VI) for 1, 2, and 3 hours resulted in damage to the lysosomal membrane. In the current research, the Cr (VI) induced acridine orange release was inhibited by the chloroquine, suggesting the involvement of intra-lysosomal Fenton's type reactions in Cr (VI) induced lysosomal membrane damage in human lymphocytes. Cr (VI), through oxidative stress and with increased ROS generation and damage to the mitochondrial membrane, can lead to cytochrome c release (pro-apoptotic proteins) from mitochondria and induction of cell death.

5. Conclusion

Cr (VI) is known as a lung carcinogen, and also has the ability to damage various tissues, including the liver and kidneys. In addition, Cr (VI) can enter the cell through the cell membrane and induce oxidative stress in human blood mononuclear cells including lymphocytes and monocytes. Considering the fact that these blood cells are most important initiators and coordinators of immune responses in mammalians, we can therefore conclude that Cr (VI) can potentially induce immune-toxicity in humans. Therefore, the precise knowledge of its toxicity mechanism can help to prevent these complications.

Acknowledgements

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Conflict of interest

The authors declare that there is no conflict of interest.

Ethics

The present study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences as ID IR.SBMU.PHARMACY.REC.1397.212. After became aware of our study donors are asked to fill out the approval form.

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