Time-order effects of vitamin C on hexavalent chromium-induced mitochondrial damage and DNA-protein crosslinks in cultured rat peripheral blood lymphocytes

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Abstract. Hexavalent chromium [Cr(VI)] and its compounds have extensive applications in many industries and are widely known to cause occupational diseases as well as carcinogenic effects in humans. Mitochondrial damage, which is important in Cr(VI)-induced cytotoxicity, may be characterized by the opening status of the permeability transition pore, the maintenance of the mitochondrial membrane potential and the level of malondialdehyde. The formation of DNA-protein crosslinks (DPCs) in target tissues appears to be the direct and primary genotoxic effect of Cr(VI) exposure, and the lymphocytic DPCs may be viewed as a biomarker of internal Cr(VI) accumulation. It is well known that vitamin C (vit C) is an important biological reducing agent in humans and animals, which is capable of reducing Cr(VI). Regardless of the evidence from cell culture and *in vivo* experiments of the protective effect of the antioxidant, vit C, following exposure to Cr(VI), no studies have been conducted to date to demonstrate the time-order effects of vit C on Cr(VI)-induced mitochondrial damage and DPC formation. In the present study, by using peripheral blood lymphocytes from Sprague-Dawley rats, we demonstrated that vit C pre- and co-treatment have a protective effect against Cr(VI)-induced loss of cell viability and mitochondrial damage, while only vit C co-treatment has a protective effect against the Cr(VI)-induced increase in DPCs. The mechanistic investigation revealed that cellular reactive oxygen species levels are correlated with Cr(VI)-induced mitochondrial damage, and that p53 expression is correlated with the Cr(VI)-induced increase in DPCs. We concluded that vit C exerts different time-order effects on Cr(VI)-induced mitochondrial damage and DPC formation, and that biomarkers, including DPC and p53, may be used in the assessment of the development of Cr(VI)-induced cancer. These findings facilitate more detailed follow-up of the Cr(VI)-exposure populations for secondary prevention.

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Abbreviations: PTP, permeability transition pore; MMP, mitochondrial membrane potential; DPC, DNA-protein crosslink; ROS, reactive oxygen species; PBLs, peripheral blood lymphocytes; MTT,3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyltetrazoliumbromide; ETC, electron transport chain; MRCC, mitochondrial respiratory chain complex

Key words: hexavalent chromium, vitamin C, time-order effect, DNA-protein crosslinks, peripheral blood lymphocytes

Introduction

Chromium (Cr) exists in the workplace mainly in two valence states: hexavalent Cr [Cr(VI)] and trivalent Cr [Cr(III)] (1). Cr(VI) has extensive applications in diverse industries, including artistic paints, electroplating and stainless steel welding; while Cr(III) is often used as a micronutrient or a dietary supplement (2). Cr(III) is known to be less toxic than Cr(VI), due to its inability to pass through transporters residing within the cell membrane (3). Cr(VI) and its compounds are widely known to cause contact dermatitis and nasal perforation, as well as carcinogenic effects in humans and animals (4). A large number of workers are exposed to Cr due to its widespread use. Occupational exposure to Cr(VI) mainly occurs via long-term chronic inhalation, and it is estimated that >700,000 workers are potentially exposed to high levels of Cr(VI) in China (5). Although attempts have been made to explore the toxicity of heavy metals in recent years, Cr(VI) has received little attention in comparison to Zn, Cu, Pb and Cd.

In addition to the production of ATP, mitochondria are involved in numerous other cellular functions, including metabolic regulation signaling and cell growth, differentiation and death (6). The mechanisms by which mitochondrial damage affects cell function are well documented. Numerous studies have demonstrated that Cr(VI) is capable of inducing a variety of types of DNA damage, including single-strand breaks, alkali-labile sites and DNA-protein crosslinks (DPCs) in various cells in cell culture and *in vivo* (7). The formation of DPCs in target tissues appears to be the direct and primary

genotoxic effect of Cr(VI) exposure. Welders (8), chrome platers (9) and leather tanners (10) have demonstrated increased levels of DPCs in peripheral blood lymphocytes (PBLs). A previous study supported the hypothesis that there is a strong correlation between lymphocytic DPCs and Cr levels in red blood cells (11), thus the lymphocytic DPCs may be viewed as a biomarker of internal Cr(VI) accumulation.

There is also evidence to suggest that Cr(VI) and its compounds are genotoxic and may induce gene mutations (12,13). Cr-DPCs are stable and ternary DNA adducts, which constitute a significant class of Cr-related genetic lesions, are able to block the normal processes of replication and transcription (14,15). Additionally, it has been demonstrated that oxidative stress, which occurs when reactive oxygen species (ROS) reach abnormally high levels, is able to affect the formation of ≤50% of the DPCs (16). ROS, including superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$) and non-radical molecules, such as hydrogen peroxide (H_2O_2), are known to initiate peroxidative cell damage (17). However, the relative contribution of ROS to Cr(VI)-induced DPCs is unclear and is the subject of debate.

Vitamin C (vit C), also known as ascorbic acid, is a water-soluble vitamin that has been demonstrated to be a key antioxidant, which is capable of reducing metal-induced lipid peroxidation and oxidative stress in animals (18). It is well known that vit C is an important biological reducing agent in humans and animals, which is capable of reducing Cr(VI) (19). Therefore, supplementation of vitamins from external sources has become necessary to prevent Cr(VI)-induced toxicity, including DNA damage. A previous *in vivo* study demonstrated that vit C significantly decreased the cytotoxicity and mutagenicity induced by Cr(VI) in rats and guinea pigs (20). However, there is also evidence to suggest that vit C may aggravate Cr(VI)-induced DNA damage by increasing Cr-DNA binding and DNA strand breaks *in vitro* (21). Therefore, this raised the possibility that vit C may have different time-order effects when antagonizing Cr(VI)-induced cytotoxicity and DNA damage. Regardless of the evidence from cell culture and *in vivo* experiments with regard to the protective effect of the antioxidant, vit C, following exposure to Cr(VI), no studies have been conducted to date to demonstrate the time-order effects of vit C on Cr(VI)-induced mitochondrial damage and DPC formation. Additionally, although there have been a large amount of studies concerned with understanding the molecular mechanisms associated with Cr(VI) exposure, the relative contributions of mitochondrial dysfunction and DNA damage to Cr(VI) toxicity are an area that as yet remains relatively unexplored. In the present study, we aimed to investigate the time-order effects of vit C on Cr(VI)-induced mitochondrial damage and DPCs in cultured rat PBLs. We also conducted a mechanistic study to demonstrate the role of ROS and p53 in Cr(VI)-induced mitochondrial damage and DPC formation.

Materials and methods

Animals. Male Sprague-Dawley (SD) 8-week-old rats were obtained from the animal center of Central South University (Changsha, Hunan, China). The rats were clinically normal, without infection or inflammation, and were housed in clean cages with free access to water and food. The animals were maintained in a 12-h light/dark cycle at a temperature of 22±2˚C and a humidity of 55±5%. The study was conducted with the approval of the Animal Care and Use Committee of Central South University.

Materials. Vit C, 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33258, Coomassie Brilliant Blue (G-250) and rhodamine 123 (Rh123) were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 culture medium, fetal bovine serum (FBS) and trypsin-EDTA (0.25%) were obtained from Gibco-BRL (Gaithersburg, MD, USA). Potassium dichromate ($K_2Cr_2O_7$) was obtained from Changsha Chemical Reagents Company (Changsha, Hunan, China) and was used as the standard reagent. All other chemicals and solvents were of analytical grade, HPLC grade or the optimum pharmaceutical grade.

Preparation of rat PBLs. The preparation of rat PBLs was performed as previously described with slight modifications (22,23). A peripheral blood sample (20 ml) was drawn from SD rats and collected in a heparinized tube. As the lymphocytes mainly comprised of mononuclear cell fractions, we immediately (within 2 h) isolated the PBLs by density gradient centrifugation (1,200 x g, 20 min) using Gradiaol L (Aqua-Medic, Kobylnica, Poland). The lymphocytes were then collected, and washed twice with 0.9% NaCl solution and twice with transport solution (20 mM HEPES, 150 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM pyruvate, pH 7.4). An adequate volume of transport solution was added to the isolated PBLs to obtain the density of 10^6 cells/ml.

Evaluation of cell viability. An MTT assay was performed to evaluate the cell viability as previously described; however, with slight modifications (24). The PBLs were seeded in each well of a 96-well plate with 100 μl medium containing 10^4 cells. $K_2Cr_2O_7$, vit C and the different combinations of the two chemicals of indicated final concentrations were added to each well, respectively. Control cells and medium controls without cells received dimethylsulfoxide (DMSO) only. Following incubation at 37˚C for the indicated time period, the PBLs were treated with 5 μl 5 mg/ml MTT for an additional 4 h at 37˚C, and then lysed in phosphate-buffered saline (PBS, pH 7.4) containing 20% sodium dodecyl sulfate (SDS) and 50% N,N-dimethylformamide (pH 4.5). The absorbance was read at 570 nm on a Versamax multiwell enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Measurement of DPC formation. The assay for measuring DPC formation was based on the binding of SDS to proteins and its lack of binding to DNA, according to the K-SDS assay developed by Zhitkovich and Costa (25). Protein-bound DNA is easily separated from free DNA, as protein-linked DNA precipitates with SDS, while free DNA remains in the supernatant when the cation is altered from Na to K. This method provided a direct measurement of DPC formation by analyzing the quantity of DNA in the SDS pellet. Following chemical exposure, the PBLs were washed and then resuspended in 100 μl ice-cold PBS. The suspensions were lysed with 500 μl

lysis buffer [1% SDS, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5] at -70˚C overnight. The frozen tubes were thawed at 65˚C in a water bath for 10 min, and then the DNA was sheared by passing 10 times through a syringe with a 21-gauge needle (foam must be avoided). Then, 100 μl precipitation solution (2.5 M KCl, 10 mM Tris-HCl, pH 7.4) was added and vortexed for 10 sec. The tubes were incubated at 65˚C for 10 min in a water bath, cooled on ice and then centrifuged at 6,000 x g for 5 min. Subsequently, the pellet was resuspended by adding 1 ml scavenging buffer solution (0.1 M KCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 7.4) and heating for 5 min at 65˚C. The heating step was then repeated and the pellet was washed three times. The pellet was resuspended in 500 μl scavenging buffer solution, heated to 65˚C and then digested with 500 μl 0.4 mg/ml protease K at 50˚C for 3 h. Subsequently, the pellet was incubated with 1 ml freshly prepared fluorescent dye (Hoechst 33258) to the final concentration of 200 mg/ml, for 30 min in the dark. A fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 450 nm was used to assay the DPC coefficient. The DPC coefficient was calculated as the ratio of the percentage of DNA involved in DPCs to the percentage of total DNA (DNA involved in DPCs and the unbound fraction of DNA).

Measurement of malondialdehyde (MDA) content. The PBLs were lysed using the Mammalian Cell Lysis kit, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). The total protein content of the PBLs was isolated and the protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay kit (Sigma-Aldrich). The measurement of MDA content was performed using the MDA Content Detection Assay kit (Jiancheng Institute of Biotechnology, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Measurement of the open permeability transition pore (PTP) rate. The PBLs were collected following chemical exposure and were then processed for mitochondrial isolation as previously described (26). The cell pellets were resuspended with solution A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, pH 7.5). The PBLs were homogenized by syringe homogenization and then centrifuged twice at 750 x g for 10 min at 4˚C. The supernatants were collected and centrifuged at 10,000 x g for 15 min at 4˚C, to obtain the mitochondrial pellets. Mitochondrial protein concentrations were estimated using Coomassie Brilliant Blue (G-250) according to the method developed by Bradford (27). The opening of the PTP was determined using a fluorescence spectrophotometer, with a wavelength of 520 nm for the excitation and emission, at 25˚C.

Measurement of the mitochondrial membrane potential (MMP). According to the method previously described by Emaus *et al* (28), with certain modifications, the MMP was monitored by the changes in the fluorescence of a specific fluorescent cationic dye, Rh123, the uptake of which by mitochondria is strongly dependent on the transmembrane potential. The absorbance was recorded by a fluorescence spectrophotometer at an excitation wavelength of 495 nm and an emission wavelength of 535 nm, at 25˚C. For each test, Rh123 (final concentration, 30 μM) was added to the mitochondrial suspension.

Measurement of ROS production. The mitochondrial ROS production was determined spectrofluorimetrically, by detecting the fluorescence intensity of 2',7'-dichlorofluorescein (DCF), the oxidized product of the membrane permeable fluoroprobe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes, Carlsbad, CA, USA). Briefly, following chemical exposure, $2x10^6$ PBLs were collected and incubated with 10 μM CM-H_2DCFDA solution for 1 h at 37˚C, in the dark. Fluorescence was measured with a fluorescence spectrometer with an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The cellular ROS levels were considered to be directly proportional to fluorescence intensity.

Western blot analysis. Western blot analysis was performed using the WesternBreeze Chemiluminescent Immunodetection kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Sample proteins (40 μg) were separated by electrophoresis on 10% SDS polyacrylamide gels (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies overnight at 4˚C, blocked with 4% non-fat milk and then incubated for 1 h with secondary antibodies at room temperature. The membranes were developed with the detection system and exposed to films. The primary antibody for p53 (PAb 240) (ab26) was purchased from Abcam (Cambridge, MA, USA) and β-actin was purchased from Cell Signaling Technology, Inc. (no. 4967; Danvers, MA, USA).

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA), in the Statistical Package for the Social Sciences (SPSS) v15.0 software (SPSS, Inc., Chicago, IL, USA), to assess the significance of the differences between groups. The results are expressed as mean ± standard deviation and were calculated from quantitative data obtained from three replicate experiments. $P<0.05$ was considered to indicate a statistically significant result.

Results

Time-order effect of vit C on Cr(VI)-induced inhibition of PBL viability. To investigate whether Cr(VI) affected the viability of PBLs, the response of cells to different doses (25, 50, 100, 200 and 400 μM) of Cr(VI) was tested using the MTT assay. As shown in Fig. 1A, Cr(VI) decreased the viability of PBLs in a dose-response manner. A concentration of 100 μM Cr(VI) caused ~30% inhibition of cell viability, and this Cr(VI) concentration was used for the following experiment. We then sought to determine whether different time-order treatments of vit C with Cr(VI) altered the inhibition of cell viability in the PBLs. In the vit C alone treatment group, >95% of the cells were alive, indicating that the concentrations of 100 and 200 μM vit C were not the cytotoxic concentrations in PBLs following treatment for 6 h. Both vit C pre- and

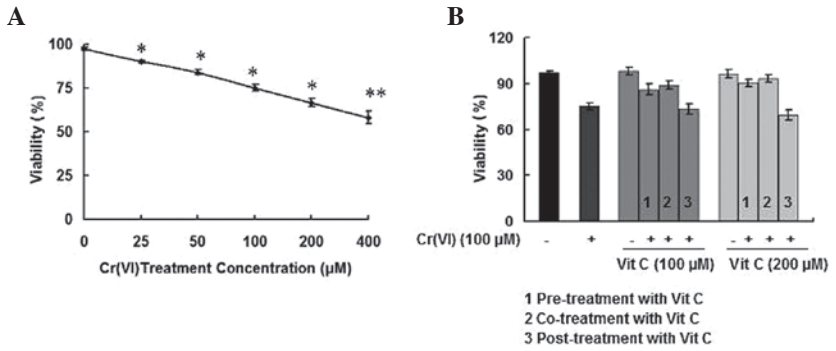

Figure 1. Time-order effect of vit C on Cr(VI)-induced inhibition of PBL viability. (A) The PBLs were incubated with different doses (25, 50, 100, 200 and 400 μM) of Cr(VI) for 6 h and were then analyzed by the MTT assay to detect cell viability. *P<0.05 and **P<0.01 compared with the control group. (B) The concentration of Cr(VI) that caused ~30% inhibition of cell viability (100 μM) was used for this experiment. Different time-order effects of vit C [pre-treatment with vit C for 2 h plus Cr(VI); co-treatment with Cr(VI) and vit C; and Cr(VI) treatment for 2 h plus vit C post-treatment] on Cr(VI)-induced inhibition of cell viability were assayed by the MTT method. Two concentrations of vit C (100 and 200 μM) were employed. Data are expressed as the mean ± standard deviation of three independent experiments. vit C, vitamin C; Cr(VI), hexavalent chromium; PBL, peripheral blood lymphocyte; MTT, 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide.

co-treatment protected PBLs from the deleterious effects of Cr(VI) toxicity, and resulted in higher viability compared with treatment with Cr(VI) alone (P<0.05), particularly in the 200-μM vit C groups. While co-treatment with vit C and Cr(VI) demonstrated the optimal protective effect on cell viability, vit C post-treatment did not rescue the decreased cell viability compared with treatment with Cr(VI) alone (P>0.05) (Fig. 1B). The results indicated that when PBLs were exposed to Cr(VI) together with different time orders of vit C treatments, vit C pre- and co-treatment exerted a protective effect against Cr(VI)-induced inhibition of cell viability.

Time-order effect of vit C on Cr(VI)-induced DPC formation in PBLs. Previous studies on Cr(VI)-induced toxicity suggested the potential utility of DPC lesions as biomarkers of exposure to Cr(VI) and its compounds (29). Treatment of PBLs with various concentrations of Cr(VI) (0, 25, 50, 100, 200 and 400 μM) for 6 h resulted in a significant increase in DPCs, in a dose-dependent manner (Fig. 2A). The DPC coefficients of the treatment groups receiving vit C alone (100 and 200 μM) were not significantly different compared with that of the control group (P>0.05). However, compared with the treatment groups receiving vit C alone, more DPC formation occurred in the Cr(VI) treatment groups that were pre-treated with vit C (P<0.05). Additionally, while there was no significant difference in DPC formation between the Cr(VI) treatment groups that received vit C post-treatment and the treatment group that received Cr(VI) alone (P>0.05), the combination treatment of Cr(VI) with vit C demonstrated a protective effect against DPC occurrence (P<0.05), particularly when the treatment concentration of vit C was 200 μM (Fig. 2B). The results indicated that when PBLs were exposed to Cr(VI) together with different time orders of vit C treatments, only vit C co-treatment exerted a protective effect against the Cr(VI)-induced increase in DPC formation.

Time-order effect of vit C on Cr(VI)-induced mitochondrial damage. In addition to the opening status of the PTP and the maintenance of the MMP, mitochondrial damage may also be evaluated by the content of MDA (30). As shown in Fig. 3, Cr(VI) treatment resulted in a significant increase in the MDA content and the open PTP rate; however, a decrease in MMP (signified by an increase in the light density of Rh123; P<0.05). The results indicated that when PBLs were exposed to Cr(VI) together with different time orders of vit C treatments, while the Cr(VI) treatment groups that received vit C post-treatment did not rescue mitochondrial damage, vit C pre-treatment and co-treatment demonstrated a protective effect against Cr(VI)-induced mitochondrial damage, particularly when the treatment concentration of vit C was 200 μM.

Cellular ROS levels correlate with Cr(VI)-induced mitochondrial damage. As ROS play a central role in Cr(VI)-induced cytotoxicity in different types of cells, we measured the ROS levels in PBLs exposed to various concentrations of Cr(VI) (25, 50, 100, 200 and 400 μM). By utilizing the oxidant-sensitive fluorogenic probe, CM-H_2DCFDA, we identified that Cr(VI) stimulation induced significantly higher values of DCF fluorescence in a dose-dependent manner compared with the control group (P<0.05), suggesting a greater quantity of cellular ROS were generated in the Cr(VI) treatment groups (Fig. 4A, upper panel). When detected under the fluorescence microscope, we observed that Cr(VI) (100 μM) induced a significantly higher level of fluorescence signals compared with the control group (Fig. 4A, bottom panel). We then determined the ROS levels in the groups of different time-order treatments of vit C with Cr(VI) in PBLs. As shown in Fig. 4B, the cells treated with 100 μM Cr(VI) and vit C exhibited up to a ~4-fold increase in ROS levels when compared with the control. The ROS levels of the treatment groups receiving vit C alone (100 and 200 μM) were not significantly different (P>0.05). Compared with the treatment groups receiving Cr(VI) alone, while the Cr(VI) plus vit C post-treatment group demonstrated no significant change in ROS levels (P>0.05), the Cr(VI) groups receiving vit C pre- and co-treatment demonstrated significantly lower ROS levels (P<0.05). As the changes in the ROS levels in the groups of different time-order treatments of vit C with Cr(VI) are similar to the changes in MDA content, open PTP rate, and MMP (Fig. 3), we concluded that cellular ROS levels were correlated with Cr(VI)-induced mitochondrial damage.

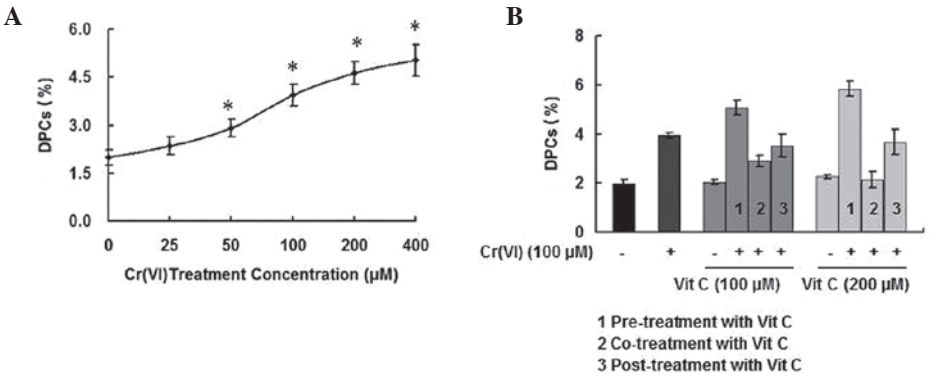

Figure 2. Time-order effect of vit C on Cr(VI)-induced increase in DPC formation in PBLs. (A) The PBLs were exposed to different doses (25, 50, 100, 200 and 400 μM) of Cr(VI) for 6 h, and were then processed for the detection of DPC formation using the K-SDS assay as described in Materials and methods. *P<0.05 and **P<0.01 compared with the control group. (B) Different time-order effects of vit C [pre-treatment with vit C for 2 h plus Cr(VI); co-treatment with Cr(VI) and vit C; and Cr(VI) treatment for 2 h plus vit C post-treatment] on Cr(VI)-induced increase in DPC formation were examined. Two concentrations of vit C (100 and 200 μM) were employed. Data are expressed as the mean ± standard deviation of three independent experiments. vit C, vitamin C; Cr(VI), hexavalent chromium; PBL, peripheral blood lymphocyte; DPC, DNA-protein crosslink; SDS, sodium dodecy sulfate.

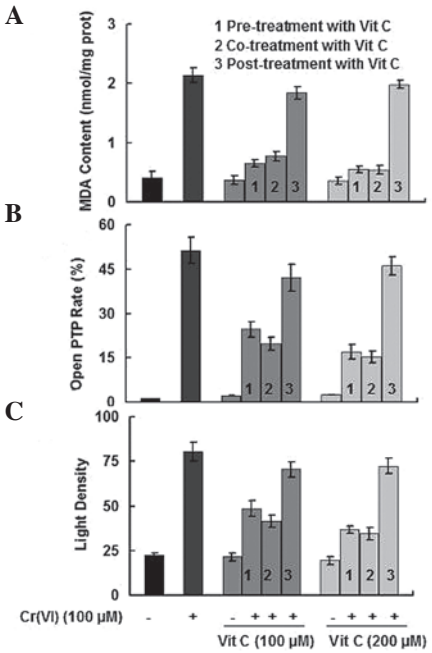

Figure 3. Time-order effect of vit C on Cr(VI)-induced mitochondrial damage in PBLs. Different time-order effects of vit C [pre-treatment with vit C for 2 h plus Cr(VI); co-treatment with Cr(VI) and vit C; and Cr(VI) treatment for 2 h plus vit C post-treatment] on Cr(VI)-induced mitochondrial damage were detected. Two concentrations of vit C (100 and 200 μM) were employed. (A) MDA content was determined using the MDA Content Detection Assay kit according to the manufacturer's instructions. (B) The PBLs were collected following chemical exposure and were then processed for mitochondrial isolation. The PTP open rate was examined using a fluorescence spectrophotometer. (C) The MMP was monitored by the fluorescence changes in a specific fluorescent cationic dye, Rh123. Data are expressed as the mean ± standard deviation of three independent experiments. vit C, vitamin C; Cr(VI), hexavalent chromium; PBL, peripheral blood lymphocyte; MDA, malondialdehyde; PTP, permeability transition pore; MMP, mitochondrial membrane potential; Rh123, rhodamine 123.

p53 expression correlates with the Cr(VI)-induced increase in DPC formation. A previous study suggested a possible correlation between p53 expression and DPC formation following formaldehyde exposure (31). Thus, we detected p53 expression levels using western blot analysis. Cr(VI) induced higher p53 expression levels. The p53 expression levels of the treatment groups receiving vit C alone (100 and 200 μM) were slightly decreased when compared with that of the control group. Compared with the treatment group receiving Cr(VI) alone, the p53 levels in the vit C pre-treatment plus Cr(VI) groups were significantly increased. Additionally, while the Cr(VI) plus vit C post-treatment groups revealed no significant change in p53 levels compared with the treatment groups receiving vit C alone, the Cr(VI) and vit C co-treatment groups demonstrated a significant decrease in p53 levels (Fig. 5). The changes in p53 expression in the groups of different time-order treatments of vit C with Cr(VI) corresponded with the changes in the DPC coefficient (Fig. 2B); thus, we concluded that p53 expression was correlated with the Cr(VI)-induced increase of DPC.

Discussion

Numerous studies have demonstrated that Cr(VI) is genotoxic and mutagenic to mammalian cells. Cr(VI) is transported into cells through the sulfate anion transport system (32), and is immediately reduced by the redox system to its stable form, Cr(III), once inside cells (33). It has been suggested that the ROS that are generated during the cellular reduction process may initiate the carcinogenic process by altering DNA structures (16). Various chemical and physical agents, which are known or suspected carcinogens, such as Cr(VI), are able to induce DPC formation. One of the most commonly occurring types of DNA damage following Cr(VI) exposure is DPC formation (29). DPCs are large helix-distorting lesions that interfere with DNA replication, transcription, repair and recombination, as well as with chromatin remodeling (34). By blocking the binding and progression of protein complexes, DPC formation results in severe cytotoxic and mutagenic outcomes (35,36). DPC levels are used as biomarkers for detecting Cr(VI)-associated genetic damage in the exposed individuals, as they are not significantly affected by age, gender and body weight (37). Experimental studies have suggested that Cr(VI) causes DPC formation either by Cr(III)-associated cross-linking reactions or by oxidative mechanisms (38,39).

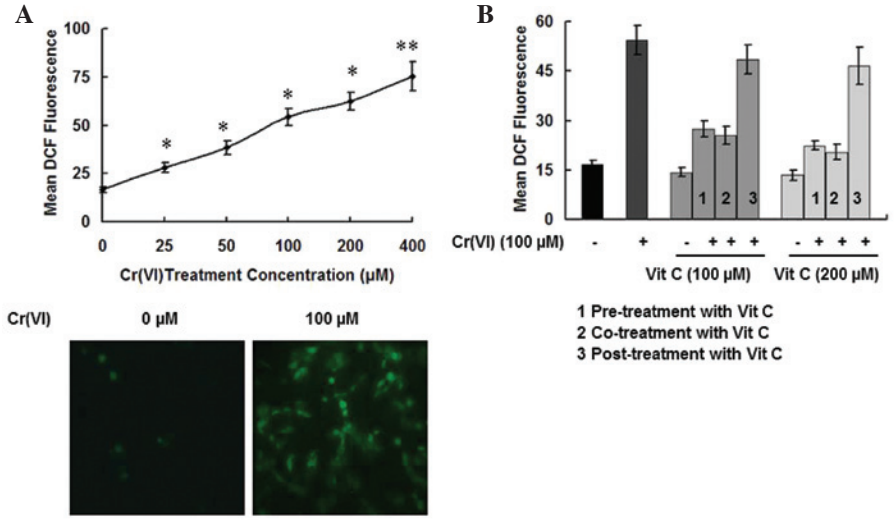

Figure 4. Time-order effect of vit C on Cr(VI)-induced ROS accumulation in PBLs. (A) Cr(VI) induces ROS accumulation. We measured the ROS levels in PBLs exposed to various concentrations of Cr(VI) (25, 50, 100, 200 and 400 μM) by utilizing the oxidant-sensitive fluorogenic probe CM-H_2DCFDA. The level of ROS production, which was considered to be directly proportional to fluorescence intensity, was quantitated by flow cytometry (upper panel) and was detected under a fluorescence microscope (bottom panel). *P<0.05 and **P<0.01 compared with the control group. (B) Different time-order effects of vit C [pre-treatment with vit C for 2 h plus Cr(VI); co-treatment with Cr(VI) and vit C; and Cr(VI) treatment for 2 h plus vit C post-treatment] on Cr(VI)-induced ROS accumulation were detected. Two concentrations of vit C (100 and 200 μM) were employed. Data are expressed as the mean ± standard deviation of three independent experiments. vit C, vitamin C; Cr(VI), hexavalent chromium; PBL, peripheral blood lymphocyte; ROS, reactive oxygen species; CM-H_2DCFDA, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate.

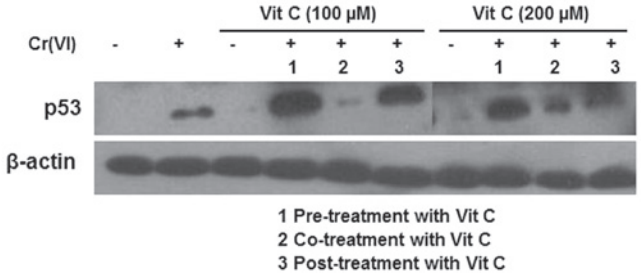

Figure 5. Time-order effect of vit C on Cr(VI)-induced p53 expression in PBLs. Different time-order effects of vit C [pre-treatment with vit C for 2 h plus Cr(VI); co-treatment with Cr(VI) and vit C; and Cr(VI) treatment for 2 h plus vit C post-treatment] on Cr(VI)-induced p53 expression were detected. Two concentrations of vit C (100 and 200 μM) were employed. The PBLs were cultured for a further 18 h following chemical exposure. Whole cell lysates were processed for western blot analysis to examine the expression levels of p53. Representative images of at least three independent experiments are shown. vit C, vitamin C; Cr(VI), hexavalent chromium; PBL, peripheral blood lymphocyte.

However, to what extent the DPCs are directly responsible for the formation of mutations and cancer remains unclear.

Mitochondria are well known for their functions of supplying cellular energy and signaling, as well as regulating the cell cycle and cell differentiation. Mitochondrial damage may lead to severe consequences, including cell death (40,41), and it is important in Cr(VI)-induced cytotoxicity (42). The PTP is an inner membrane megachannel, the opening of which results in mitochondrial swelling, depolarization and rupture of the outer membrane (43). The decrease in MMP leads to matrix condensation and the exposure of cytochrome c to the intermembrane space, which is involved in mediating mitochondrial dysfunction (44). The MDA content may be viewed as a biomarker of mitochondrial oxidative stress, as MDA may cause cellular toxic stress and form covalent protein adducts to interrupt the normal function of mitochondria (45). In the present study, we observed that Cr(VI) induced mitochondrial damage, which was characterized by the increased MDA content and open PTP rate, as well as the decreased MMP. In addition, we identified that ROS levels were correlated with mitochondrial damage.

ROS, including $O2^{\bullet-}$ and H_2O_2, are the products of normal cellular functioning; however, excessive ROS levels may also cause deleterious effects. Following Cr(VI) exposure, ROS were observed to be generated not only from the reduction process mediated by the redox system; however, also from the electrons leaked from the electron transport chain (ETC), when mitochondrial respiratory chain complexes (MRCC) were inhibited by Cr(VI) (46,47). ROS levels are correlated with mitochondrial damage, as mitochondrial dysfunction, including oxidative stress, results in ROS accumulation. This accumulation may aggravate mitochondrial damage by attacking mitochondrial DNA, deactivating specific enzymes and inhibiting the function of the ETC. By determining the time-order effect of the small molecule antioxidant, vit C, on Cr(VI)-induced mitochondrial damage in PBLs, we concluded that vit C pre-treatment and co-treatment had a protective effect against Cr(VI)-induced mitochondrial damage and loss of cell viability. This was due to the fact that vit C inhibited cellular ROS by suppressing ROS production and scavenging excessive accumulated ROS. It has been demonstrated that ROS accumulation occurred within only a few minutes following Cr(VI) exposure, thus the 2-h-postponed treatment of vit C was too late to clear the ROS accumulation and to rescue the ROS-induced damage. Therefore, this explains why, in our study, the Cr(VI) plus vit C post-treatment groups did not exhibit any protective effects against mitochondrial damage and loss of cell viability.

The tumor suppressor, p53, was identified in 1979, and it is well known that p53 activation is involved in growth arrest

and apoptosis. It was demonstrated that high p53 serum levels were identifiable several years prior to the cancer was able to be clinically detected (48). In a previous study by Hanaoka *et al*, high levels of serum pantropic p53 proteins were detected in workers that had a long history of Cr(VI) exposure, and thus were presumed to be at high risk for lung cancer (49). In the present study, we concluded that p53 expression was associated with the Cr(VI)-induced increase in DPC formation. We observed that when PBLs were exposed to Cr(VI) together with different time-orders of vit C treatments, only vit C co-treatment demonstrated a protective effect against the Cr(VI)-induced increase in DPCs. Cr(VI) may be reduced outside the cell by vit C to Cr(III), which cannot cross cell membranes due to its tendency to form insoluble hydrated complexes; this explains why the lowest number of DPCs occurred in the vit C co-treatment groups. The reason for the increased formation of DPCs in the vit C pre-treatment plus Cr(VI) group is that vit C pre-treatment enhanced the capacity of the cell to reduce Cr(VI), and the accumulation of Cr(III) resulted in the formation of Cr(III)-DNA binding adducts. This is consistent with a previous study conducted by Mattagajasingh *et al* (16). The Cr(VI) plus vit C post-treatment groups revealed no significant changes in DPC generation compared with the Cr(VI) alone group, as the postponed treatment with vit C was too late to halt the toxicity of Cr(VI).

Cr(III) is proposed to give rise to DPCs by directly mediating the cross linking between cellular DNA and proteins (50). A previous study provided evidence for a three-step cross-linking mechanism, which included reduction of Cr(VI) to Cr(III), Cr(III)-DNA binding and the generation of protein-Cr(III)-DNA crosslinks (51). Shaham *et al* demonstrated a possible causal correlation between p53 activation and DPC formation following the examination of the PBLs of 186 workers exposed to formaldehyde (31). The authors also suggested that p53 activation and DPC formation may represent steps in formaldehyde carcinogenesis. The mechanism whereby p53 activation correlates with DPC formation following Cr(VI) exposure in PBLs remains unclear. Previous studies have demonstrated that ROS are able to increase DPC levels by the initial oxidative lesions on DNA, as well as by the reactive products of lipid peroxidation, including MDA (52). In the present study, we did not observe a correlation between ROS levels and the formation of DPCs. Cr(VI) and its compound-induced gene mutations have been demonstrated in a variety of cell systems (53,54), and it is suggested that the induction of DPC formation is associated with chromosomal effects; however not with the occurrence of gene mutations (55). Thus, we inferred that Cr(VI)-induced DPCs were not the cause of gene mutations involved in carcinogenesis, and that the mutations of tumor-related genes following exposure to Cr(VI) are not a direct result of DPCs.

We concluded that vit C has different time-order effects on Cr(VI)-induced mitochondrial damage and DPC formation. Although further investigation is required, it is clear that the biomarkers, including DPC and p53, may be used in the assessment of the development of Cr(VI)-induced cancers. Therefore, this facilitates a closer follow-up of populations exposed to Cr(VI), for secondary prevention.

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