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Transcriptomics Evaluation of Hexavalent Chromium Toxicity in Human Dermal Fibroblasts

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Data deposition: The microarray data has been deposited in the Gene Expression Omnibus (GEO) Database, www.ncbi.nlm.nih.gov/geo (accession number GSE16349)

Disclaimer: "The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health."

Abstract

Significant exposure to hexavalent chromium, a metal with broad toxicity potential in humans, has been reported. In order to understand the mechanisms of dermal toxicity induced by hexavalent chromium, global gene expression profiling of human dermal fibroblasts exposed to potassium dichromate was performed. Microarray analysis of the gene expression profile in the fibroblasts treated with potassium dichromate identified significant differential expression of approximately 1,200 transcripts compared with the control cells. Functional categorization of the differentially expressed genes identified the enrichment of genes involved in several cellular processes, including apoptosis and oxidative stress, in the fibroblasts exposed to hexavalent chromium. Induction of apoptosis and oxidative stress in the dermal fibroblasts in response to their exposure to chromium was independently confirmed by additional experiments. The potassium dichromate-induced cytotoxicity, apoptosis, and oxidative stress were significantly blocked by the addition of ferrous sulfate, an agent known for its ability to reduce chromium to the insoluble and therefore impermeable trivalent form, to the cell culture medium. Taken together, our data provide insights into the potential mechanisms underlying the dermal toxicity of hexavalent chromium and provide experimental support for the proposed protective role of ferrous sulfate in hexavalent chromium-induced toxicity.

Keywords: Hexavalent chromium; Dermal toxicity; Gene expression; Oxidative stress; Apoptosis

Introduction

Chromium, inspite of being recognized as an essential trace element, is known to cause toxicity under conditions of excessive human exposure. Chromium has been detected as a contaminant in the environment, in various food items, in water, and in certain industrial and consumer products. Similarly, large quantities of chromium are used in various industries resulting in significant occupational exposure among workers. The potential of chromium, especially its hexavalent form, to result in toxicity has been recognized for a long period of time. Treating cells cultured *in vitro* with hexavalent chromium resulted in cytotoxicity [1], genotoxicity [2], apoptosis [3], and cell transformation [4]. Similarly, administration of hexavalent chromium to experimental animals results in toxicity of the lungs [5], kidney [6], skin [7], and reproductive system [8]. Epidemiological evidence is also available to demonstrate the toxicity of hexavalent chromium in humans [9]. Chromium has been classified as a toxic and carcinogenic metal in humans [10].

Occupational exposure to hexavalent chromium resulting in toxicity and illnesses is a major health concern among workers in the U.S. and elsewhere. Even though the principal route of human exposure to chromium is through inhalation, and lung has been identified as the primary target organ for its toxicity [11], significant human exposure to chromium also takes place through the skin [7]. In addition, skin is a major target organ for chromium toxicity [7,11]. For example, the widespread incidence of dermatitis noticed among construction workers is believed to be due to chromium present as a contaminant in cement [7]. The incidence of allergic contact dermatitis observed among construction workers who are occupationally exposed to cement is higher than that of the general population [12], and a good correlation has been observed between the chromium content of cement and the incidence of contact dermatitis among construction workers in several countries [13]. Inspite of such compelling evidence suggesting a definite role for hexavalent chromium in dermal toxicity, the underlying mechanism(s) responsible for its toxicity on skin are not fully understood.

Because of its ability to reduce the water soluble and highly toxic hexavalent chromium [Cr(VI)] to the insoluble and less toxic trivalent chromium [Cr(III)] [14], it is expected that the addition of ferrous sulfate ($FeSO_4$) to products containing Cr(VI), for example cement, may be protective against its toxicity. Support to this concept is derived from the observation that in many European countries the incidence of allergic contact dermatitis (ACD) among construction workers has diminished considerably since the addition of $FeSO_4$ to cement has been practiced [9]. However, a definite role for $FeSO_4$ in protecting against Cr(VI)-induced ACD has been disputed. Accordingly, better industrial hygiene practices and the more frequent use of improved personal protective devices have been attributed to be responsible

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for the decline in chromium-induced ACD noticed among cement workers. In addition, experimental evidence, derived either from *in vitro* cell culture or *in vivo* animal models, supporting a definite role for FeSO4 in Cr(VI)-induced ACD or any other type of toxicity is lacking. For example, no statistically significant difference in chromium content was noticed between skin samples exposed to cement with or without $FeSO_4$ [15] questioning the proposed protective role of $FeSO_4$ in chromium toxicity. The lack of experimental evidence supporting a definite protective role for $FeSO_4$ in chromium toxicity may be one of the reasons for not adding $FeSO_4$ to cement in many countries including the US.

Advances in high throughput gene expression profiling, such as microarray analysis, enable a comprehensive understanding of the effects of toxic chemicals in biological systems. In addition, gene expression profiling may provide mechanistic insights that may subsequently be employed to develop biomarkers to detect chemical toxicity as well as strategies to intervene chemical toxicity. Presently, the potential mechanism(s) of dermal toxicity induced by hexavalent chromium was investigated by analyzing the global gene expression profile in human dermal fibroblasts exposed to potassium dichromate. Our results demonstrated that exposure of human dermal fibroblasts to potassium dichromate resulted in toxicity, and this was associated with significant differential expression of a large number of genes including those involved in oxidative stress and apoptosis, as well as many other cellular processes. Furthermore, we have obtained data demonstrating the potential of $FeSO_4$ to prevent/reduce the potassium dichromate-induced cytotoxicity, oxidative stress, and apoptosis in human dermal fibroblasts. The present study findings may have implications in developing strategies to prevent/reduce human dermal illnesses caused by exposure to hexavalent chromium.

Materials and Methods

Cell culture and cytotoxicity studies

Culturing of mycoplasma-free human dermal fibroblasts (Catalogue number CRL 2076, ATCC, Manassas, VA) in Iscove's modified Dulbecco's medium and determination of cytotoxicity induced by hexavalent potassium dichromate [$K_2Cr_2O_7$, abbreviated Cr(VI), Sigma Chemical Company, St Louis, MO] were done exactly as described in our previous publication [1].

Gene expression studies

Microarray analysis of global gene expression profile: Exponentially growing dermal fibroblasts ($3x10^5$ cells) were cultured in T25 cell culture flasks. When the cells were approximately 70% confluent, Cr(VI) was added to the medium at a final concentration of 5μM (24-hour LC50 value; see Results section for details), and the cells were further cultured for 16-hours. Total RNA, free of contaminating DNA and proteins, was isolated from the cell, using the RNeasy Kit (Qiagen, Inc., Valencia, CA). The RNA was quantitated by UV-spectrophotometry, and the integrity was determined using a Bioanalyzer (Agilent Technology, Palo Alto, CA). Samples with an RNA Integrity Number (RIN) $\geq$8.0 were used in the gene expression studies.

The global gene expression profile of the control and Cr(VI) treated fibroblasts was determined using HumanRef-8 V2 Sentrix BeadChip array (Illumina, Inc, San Diego, CA). All microarray experiments were performed to comply with Minimal Information About a Microarray Experiment (MIAME) protocols. Biotin-labeled cRNA was generated from 375 ng RNA samples each by employing the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc, Austin, TX). Chip hybridizations, washing, Cy3-streptavidin staining, and scanning of the chips were performed on the BeadStation 500 platform (Illumina, Inc, San Diego, CA) following protocols provided by the manufacturer.

Microarray data analysis: Metrics files from the bead scanner were checked to ensure that all samples fluoresced at comparable levels before samples were loaded into the Beadstudio (Framework version 3.0.19.0) Gene Expression module v.3.0.14. Housekeeping, hybridization control, stringency and negative control genes were checked for proper chip detection. BeadArray expression data were then exported with mean fluorescent intensity across like beads and bead variance estimates into flat files for subsequent analysis.

Illumina BeadArray expression data were analyzed in Bioconductor using the 'lumi' and 'limma' packages. Bioconductor is a project for the analysis and comprehension of genomic data and operates in R, a statistical computing environment. The 'lumi' Bioconductor package was specifically developed to process Illumina microarrays and covers data input, quality control, variance stabilization, normalization, and gene annotation [16]. Normalized data were then analyzed using the 'limma' package in R. In short, limma fits a linear model for each gene, generates group means of expression, calculates p-values and log fold-changes which are converted to standard fold changes. The raw p values were corrected for false discovery rate (FDR) using the Benjamini and Hochberg procedure [17]. Only genes with FDR $\leq$0.05 and a fold change $\geq$1.8 compared with the controls were considered as significantly differentially expressed and used as input for subsequent bioinformatic analysis using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). IPA software is designed to map the biological relationship of the uploaded genes and classify them into categories according to published literature in the database. Hierarchical clustering of all the genes detected following hybridization of the chips was done by Euclidean distance metric/uncentered Pearson correlation method (http://www.cs.umd.edu/hcil/multi-cluster/).

Real-time PCR confirmation of microarray data: A total of twenty nine genes that were differentially expressed in response to Cr(VI) exposure in the fibroblasts were randomly selected for quantitative real-time PCR (QRT-PCR) analysis to confirm the microarray data. Nucleotide sequences of the primers employed to PCR amplify the selected genes are presented in Supplemental Table 1. The PCR amplification, detection of the PCR-amplified gene products, and their quantitations were performed with the 7500HT Fast Real Time PCR machine and SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). The expression levels of the genes were normalized to that of the housekeeping gene – beta-2-microglobulin (B-2-M), and the fold changes in expression compared with the controls were calculated using the formula 2^(-(ΔCt_target – ΔCt_B-2-M).

Apoptosis determination : Human dermal fibroblasts ($2x10^5$ cells/well) were cultured on sterile cover slips which were placed in 6 well cell culture plates and treated with Cr(VI) (5μM final concentration) for 16 hrs. After the exposure period, cells were washed in PBS, fixed with acetone, and stained with terminal transferase-mediated dUTP nick end-labeling (TUNEL) reagent (Promega Corporation, Madison, WI) and DAPI for detection of apoptotic and total nuclei, respectively. Apoptotic cells were detected under an Olympus AX70 fluorescence microscope using a standard fluorescein filter. The number of apoptotic nuclei (TUNEL-positive) was expressed as a percentage of total nuclei (DAPI-positive) per field. Relevant positive (cells pretreated with 6U DNase/ml) and negative (without TdT) controls were also included in the experiment.

Detection of oxidants in Cr(VI) exposed fibroblasts: The dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate,

acetyl ester (CM-H2DCFDA) purchased from Invitrogen Corporation (Carlsbad, CA) was used to determine the production of oxidizing species in the dermal fibroblasts exposed to Cr(VI). The yield of the fluorescent 2',7'-dichlorofluorescein (DCF), the oxidation product of DCFH, is considered as a reflection of the overall production of Cr(VI) reductive intermediates [18,19] and reactive oxygen species such as hydrogen peroxide (H_2O_2) [20] and hydroxyl radical ($OH^{\cdot}$) [21]. Cells were plated onto circular glass cover slips in 24-well plates and treated with 5 μM Cr(VI) for 1.5-hours. Staining of the control and treated cells with the dye and detection of the stained cells by confocal microscopy was done exactly as described in our previous study [22]. Images were recorded in pseudocolor, where low intensity sites appear blue and increasingly high intensity areas are displayed as green, yellow, red, or white with a pixel intensity of 0-255.

Role of $FeSO_4$ in Cr(VI) toxicity: In an effort to obtain experimental data to support the proposed protective role of FeSO4 against Cr(VI) toxicity, studies were conducted to determine cytotoxicity, apoptosis and oxidative stress in the dermal fibroblasts exposed to Cr(VI) and FeSO4. Exponentially growing dermal fibroblasts were treated with Cr(VI) in the presence or absence of FeSO4. Cr(VI) and FeSO4 were used at final concentrations of 5 μM and 40 μM, respectively. At the end of the exposure period, cytotoxicity (MTT assay), apoptosis (TUNEL assay), and oxidative stress (confocal microscopy) were determined as described in respective sections above.

Gene Symbol	Control	Cr (VI) treated	Fold change
HB2M	21.315 ± 0.117	21.842 ± 0.224	-
IL8	36.665 ± 0.321	29.026 ± 0.130*	+292.684
HMOX1	27.397 ± 0.136	25.4 ± .093*	+5.862
GDF15	26.673 ± 0.212	21.153 ± 0.215*	+67.378
FOS	33.544 ± 0.229	28.757 ± 0.103*	+40.544
RGS4	34.232 ± 0.202	30.691 ± 0.206*	+17.087
AXUD1	31.087 ± 0.084	28.287 ± 0.075*	+11.078
H3F3B	30.331 ± 0.189	28.063 ± 0.085*	+7.073
ANGPTL4	35.015 ± 0.241	32.2 ± 0.310*	+10.335
TNFRSF10D	27.235 ± 0.131	24.544 ± 0.234*	+9.485
GADD45A	26.291 ± 0.105	24.092 ± 0.096*	+6.746
GAS	26.127 ± 0.131	33.384 ± 0.124*	-104.17
DDIT4	28.138 ± 0.172	30.887 ± 0.247*	-4.576
KIAA1199	24.767 ± 0.239	29.408 ± 0.355*	-16.995
CXCL12	25.924 ± 0.141	30.841 ± 0.135*	-20.566
DKK1	22.619 ± 0.158	26.384 ± 0.132*	-9.259
HMGCR	30.413 ± 0.230	33.262 ± 0.114*	-4.905
KRT19	25.010 ± 0.129	29.197 ± 0.222*	-12.399
DHRS3	22.931 ± 0.318	26.164 ± 0.267*	-6.402
PTK2	27.950 ± 0.268	30.862 ± 0.406*	-5.128
TNS3	25.680 ± 0.169	30.639 ± 0.371*	-21.162
IRXL1	27.155 ± 0.198	31.224 ± 0.170*	-11.43
LMCD1	27.572 ± 0.201	31.893 ± 0.143*	-13.612
HOXA10	26.051 ± 0.220	28.967 ± 0.171*	-5.141
IFIT1	29.75 ± 0.425	35.132 ± 0.253*	-28.392
PPP1R3C	27.317 ± 0.298	29.058 ± 0.190*	-2.277
INSIG1	26.285 ± 0.293	30.156 ± 0.207*	-9.963
HMGCS1	26.772 ± 0.359	31.674 ± 0.167*	-20.353
SERPINB1	27.864 ± 0.135	31.006 ± 0.074*	-6.012
PRKD1	31.694 ±0.161	35.777 ± 0.230*	-11.317

Based on the microarray data, 29 genes were selected for real time PCR analysis to confirm their differential expression in the cells treated with Cr(VI). '+' and '-' represent overexpression and underexpression, respectively, of the genes in the Cr(VI) treated cells compared with the control cells. Data presented is the threshold cycle (CT) derived from real time PCR analysis and are mean ± S.E. of four independent experiments. The fold change in expression of the individual genes in the Cr(VI) treated cells compared with the control was calculated as described in the text. Statistical analysis of the data was done by Student's t test and a p value ≤0.05 was considered as statistically significant (*).

Table 1: Real Time PCR Confirmation of Differential Gene Expression in Human Dermal Fibroblasts Exposed to 5 μM Cr(VI).

Category	p-value*	Number of genes#
Cell Death	4.74E-09 - 1.82E-01	206
Cellular Growth and Proliferation	1.38E-06 - 1.82E-01	221
Cell Cycle	2.46E-03 - 1.82E-01	90
DNA Replication, Recombination, and Repair	4.28E-03 - 1.82E-01	55
Cellular Development	5.94E-03 - 1.82E-01	144
Gene Expression	9.22E-03 - 1.82E-01	142
Cell-To-Cell Signaling and Interaction	2.71E-02 - 1.82E-01	39
Cell Morphology	3.26E-02 - 1.82E-01	95
Cellular Movement	3.26E-02 - 1.82E-01	63
Cellular Assembly and Organization	5.56E-02 - 1.82E-01	24
Carbohydrate Metabolism	8.21E-02 - 1.76E-01	21
Lipid Metabolism	8.21E-02 - 1.68E-01	32
Molecular Transport	8.21E-02 - 1.76E-01	33
Nuclic Acid Metabolism	8.21E-02 - 1.82E-01	4
Small Molecule Biochemistry	8.21E-02 - 1.82E-01	48
Antigen Presentation	9.25E-02 - 1.76E-01	29
Cellular Function and Maintenance	9.47E-02 - 1.76E-01	21
Cell Signaling	1.14E-01 - 1.76E-01	44
Amino Acid Metabolism	1.35E-01 - 1.76E-01	4
Vitamin and Mineral Metabolism	1.35E-01 - 1.35E-01	3
Protein Synthesis	1.82E-01 - 1.82E-01	27

*The 'p-value' was calculated using the right-tailed Fisher Exact Test and corrected for false discovery rate by the Benjamini-Hochberg method. Only those categories with a p value ≤0.05 are considered significant and therefore are presented.
#The 'number of genes' represents the number of differentially expressed genes belonging to the specific IPA category in the Cr(VI) treated cells.

Table 2: IPA Classification of the Differentially Expressed Genes in the Cr(VI) treated Dermal Fibroblasts into Cellular and Molecular Functions Categories.

Results

Cr(VI) cytotoxicity in human dermal fibroblasts

Exposure of dermal fibroblasts to Cr(VI) resulted in concentration-dependent cytotoxicity and cell death and a 24-hour LC50 value of 5 μM was calculated from the concentration-response curve. These results were essentially similar to those of our previous publication [1] and are, therefore, not presented here. Exposure of fibroblasts to 5 μM Cr(VI) for 16-hours (the time interval employed in the case of gene expression experiment) resulted in <30% cell death (data not presented).

Differential gene expression profile in fibroblasts treated with Cr(VI)

Out of the 22185 transcripts that are represented on the Illumina Human Ref 8 microarray, the expressions of 10917 transcripts were detected in the Cr(VI) treated cells. Out of the 10917 transcripts that were detected on the microarray, 1153 genes were significantly differentially expressed ($p \leq 0.05$ adjusted for FDR by Benjamini and Hochberg correction and ≥1.8-fold change in expression) in the Cr(VI)-treated cells compared with the control (Supplemental Table 2). The 1153 differentially expressed genes consisted of 468 and 685 genes whose expressions were up- and down-regulated, respectively.

Results of the QRT-PCR analysis further confirmed the microarray data – expressions of all 29 genes selected for the PCR analysis were found significantly different in the Cr(VI)-treated cells compared with the control cells (Table 1).

The functional annotation of the differentially expressed genes by IPA identified several cellular functions, pathways, and networks that were significantly enriched in the fibroblasts due to their exposure to Cr(VI) (Table 2). More than 200 genes that were differentially expressed in the Cr(VI)-treated cells were found to be related to cell death. A further categorization of the differentially expressed genes involved in cell death function identified the differential expression of 170 genes that were involved in apoptosis (Table 3). Other major functional groups of the differentially expressed genes were cell-to-cell signaling and interaction (39 genes), cellular assembly and organization (24 genes), cellular growth and proliferation (221 genes), cellular development (144 genes), cell morphology (95 genes), cellular movement (63 genes), cell cycle (90 genes), and cellular function and maintenance (21 genes) (Table 2). IPA classification of the differentially expressed genes into diseases and disorders categories identified the differential expression of 302 and 30 genes involved in cancer and dermatological diseases and conditions, respectively (Table 4). In addition, several genes involved in diseases of other organs/tissues and systems were also found differentially expressed in the Cr(VI)-treated dermal fibroblasts.

Category	p-value*	Number of Genes#
Apoptosis	4.74E-09 - 1.82E-01	170
Cell Death	1.53E-08 - 1.81E-01	188
Survival	4.71E-02 - 1.35E-01	61
Cell Viability	3.07E-02 - 1.82E-01	26
Anoikis	8.50E-02 - 1.82E-01	10

*The 'p-value' was calculated using the right-tailed Fisher Exact Test and corrected for false discovery rate by the Benjamini-Hochberg method. Only those categories with a p value ≤0.05 are considered significant and therefore are presented.
#The 'number of genes' represents the number of differentially expressed genes belonging to the specific IPA category in the Cr(VI) treated cells.

Table 3: IPA Classification of the Differentially Expressed Genes in the Cr(VI) treated Dermal Fibroblasts into Categories Related to Cell Death Function.

Category	p-value*	Number of Genes#
Cancer	4.74E-09 - 1.82E-01	302
Gastrointestinal Disease	1.28E-05 - 1.82E-01	95
Reproductive System Disease	4.73E-05 - 1.82E-01	163
Genetic Disorder	2.46E-02 - 1.46E-01	96
Infectious Disease	1.23E-02 - 1.82E-01	6
Inflammatory Disease	1.23E-02 - 1.76E-01	52
Neurological Disease	1.23E-02 - 1.82E-01	46
Skeletal and Muscular Disorders	1.53E-02 - 1.76E-01	78
Dermatological Diseases and Conditions	1.88E-02 - 1.82E-01	30
Connective Tissue Disorders	3.06E-02 - 1.82E-01	84
Endocrine System Disorders	3.26E-02 - 1.82E-01	32
Inflammatory Response	3.26E-02 - 1.76E-01	29
Organismal Injury and Abnormalities	3.84E-02 - 1.35E-01	9
Hematological Disease	3.88E-02 - 1.81E-01	95
Renal and Urological Disease	4.71E-02 - 1.82E-01	26
Respiratory Disease	4.81E-02 - 1.46E-01	51
Immunological Disease	6.32E-02 - 1.82E-01	49
Hepatic System Disease	1.12E-01 - 1.12E-01	4
Developmental Disorder	1.20E-01 - 1.76E-01	10
Cardiovascular Disease	1.20E-01 - 1.82E-01	37
Metabolic Disease	1.50E-01 - 1.82E-01	12
Antimicrobial Response	1.76E-01 - 1.76E-01	3

*The 'p-value' was calculated using the right-tailed Fisher Exact Test and corrected for false discovery rate by the Benjamini-Hochberg method. Only those categories with a p value ≤0.05 are considered significant and therefore are presented.
#The 'number of genes' represents the number of differentially expressed genes belonging to the specific IPA category in the Cr(VI) treated cells.

Table 4: IPA Classification of the Differentially Expressed Genes in the Cr(VI) treated Dermal Fibroblasts into Diseases and Disorders Categories.

Category	p-value*	Number of Genes#
Cholesterol Biosynthesis	2.14E-04	7
Oxidative stress response mediated by NRF2	1.50E-02	20
VDR/RXR activation	1.50E-02	11
p53 Signaling	3.38E-02	11
Hormone Receptor Regulated Cholesterol Metabolism	3.90E-02	3
Oxidative Stress	4.49E-01	5

*The 'p-value' was calculated using the right-tailed Fisher Exact Test and corrected for false discovery rate by the Benjamini-Hochberg method. Only those categories with a p value ≤0.05 are considered significant and therefore are presented.
#The 'number of genes' represents the number of differentially expressed genes belonging to the specific IPA category in the Cr(VI) treated cells.

Table 5: IPA Classification of the Differentially Expressed Genes in the Cr(VI) treated Dermal Fibroblasts into Toxicology Categories.

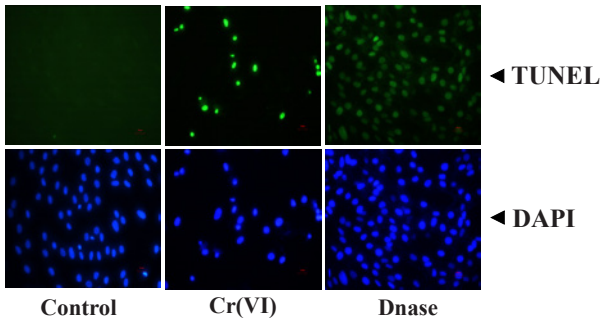


Figure 1: Cr(VI)-induced apoptosis in human dermal fibroblasts. Human dermal fibroblasts were treated with 5μM Cr(VI) for 16 and 24-hours and the cells were stained with TUNEL reagent and DAPI to detect apoptotic and total nuclei, respectively. Cells treated with DNase were used as positive control for apoptosis. The experiment was repeated four times, and the results of a representative 24-hour experiment are presented. TUNEL positive apoptotic nuclei are presented in the upper row, and the same cells stained with DAPI showing apoptotic and non-apoptotic nuclei are presented in the lower row.

Oxidative stress was the most prominent toxicological category enriched by Cr(VI) as identified by the IPA analysis of the differentially expressed genes (Table 5). Out of the 25 oxidative stress related genes that were differentially expressed in the Cr(VI)-treated cells, 20 genes belonged to the IPA category of oxidative stress response mediated by Nrf2.

Cr(VI)-induced apoptosis in dermal fibroblasts

Exposure of the human dermal fibroblasts to 5 μM Cr(VI) resulted in induction of apoptosis as evidenced from the number of TUNEL-positive apoptotic nuclei in the treated cells compared with the control (Figure 1).

Cr(VI)-induced oxidative stress in dermal fibroblasts

Exposure of dermal fibroblasts to Cr(VI) resulted in oxidative stress as evidenced from the results of the confocal microscopy analysis (Figure 2). Compared with the control cells, the fibroblasts treated with Cr(VI) exhibited a significantly elevated cellular level of oxidants capable of inducing oxidative stress .

Inhibition of Cr(VI)-induced cytotoxicity, apoptosis, and oxidative stress by $FeSO_4$

Addition of $FeSO_4$ to the cell culture medium significantly blocked Cr(VI)- induced cytotoxicity (Figure 3A), apoptosis (Figure 3B), and reductive metabolism of Cr(VI) resulting in the generation of reactive

oxidant metabolites and toxic reactive oxygen species capable of inducing oxidative stress (Figure 2).

Discussion

Recent developments in transcriptomics have facilitated a better understanding of the potential for chemicals to cause toxicity as well as the molecular mechanisms underlying their toxicity. In the present study, exposure of human dermal fibroblasts to Cr(VI) resulted in toxicity as well as significant differential expression of approximately 1,200 genes as demonstrated by the microarray data. Functional analysis of the differentially expressed genes facilitated a better understanding of the mechanisms potentially underlying the dermal toxicity of Cr(VI) through identification of multiple cellular processes/functions/pathways/networks that are affected as a consequence of exposure to Cr(VI).

The present study is a follow-up of one of our previous publications[1] in which we employed a microarray that contained 263 genes, whose expression are indicative of stress and toxicity, to determine the mechanisms underlying Cr(VI)-induced dermal toxicity. The microarray employed in the present study contained more than 22,000 genes involved in a variety of cellular functions and, therefore, is expected to provide a broader understanding of the dermal toxicity potential of Cr(VI) as well as further insight into the possible mechanisms underlying the toxicity.

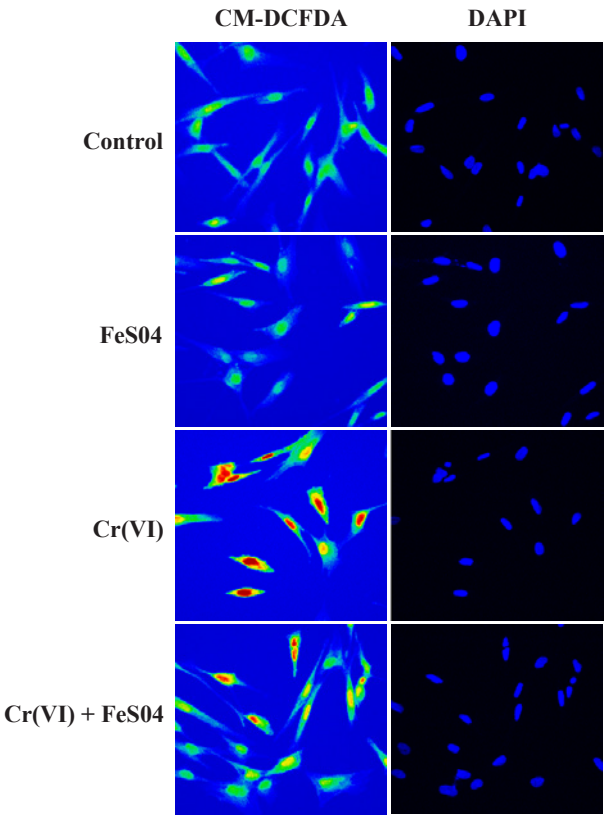
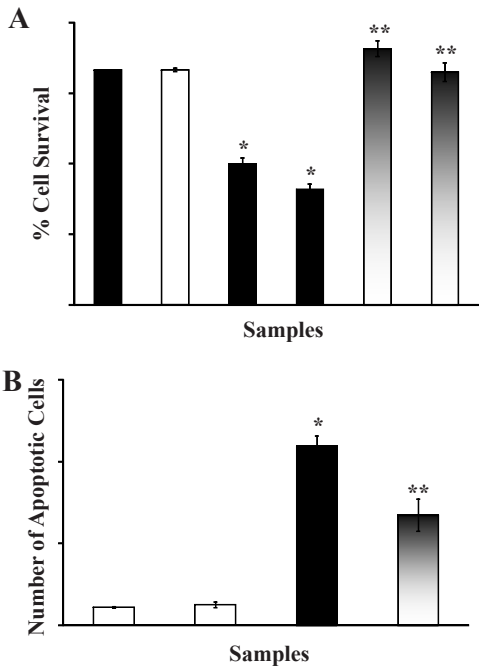


Figure 2: Detection of reactive oxygen species (ROS) in human dermal fibroblasts exposed to Cr(VI) with or without $FeSO_4$. Exponentially growing human dermal fibroblasts were exposed to 5 μM Cr(VI) with or without 40 μM $FeSO_4$ as described in the Materials and methods section. The cells were stained with the fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), capable of detecting Cr(VI) reductive metabolites and reactive oxygen species and the nuclear dye, DAPI. The stained cells were detected by confocal microscopy and a representative field is presented. The various colors in the photographs represent the cellular level of ROS in decreasing order as follows: red > yellow > green > blue.



*Statistically significant ($p \leq 0.05$) compared with the untreated control cells.
**Statistically significant ($p \leq 0.05$) compared with the corresponding Cr(VI) treated cells.

Figure 3: $FeSO_4$-mediated blocking of cytotoxicity (A) and apoptosis (B) in human dermal fibroblasts treated with Cr(VI). Fibroblasts were treated with Cr(VI) and $FeSO_4$ at the indicated concentrations and cytotoxicity and apoptosis were determined by MTT assay and TUNEL assay, respectively, as described in the text. The experiment was repeated four times and the data is presented as mean ± S.E. For TUNEL assay, four independent microscopic fields, each consisting of 30-50 cells, were counted per experiment and the data is used. Statistical analysis was done by Tukey's Honest Significant Difference (HSD) procedure .

Exposure of dermal fibroblasts to Cr(VI) resulted in toxicity and this was associated with significant differential expression of approximately 1,200 genes in the treated cells compared with the controls. The cytotoxicity data obtained in the present investigation is in agreement with our previous report [1] and further supports the dermal toxicity potential of Cr(VI) [7]. Many of the genes that were differentially expressed in our previous study [1] were also found differentially expressed in the current study. However, quantitative differences in the expression of those genes were noticed between the two studies. This may be attributed to the differences in the microarray platforms employed and/or the duration of Cr(VI) exposure (16-hours in the present study vs 2, 6, and 24-hours in the previous study). Bioinformatic analysis of the differentially expressed genes in the Cr(VI) treated dermal fibroblasts suggested the potential of Cr(VI) to cause cancer, inflammatory, immunological, endocrinological, metabolic, and genetic diseases in the skin. In addition, classification of the differentially expressed genes in the Cr(VI) exposed cells into various biological categories provided insight regarding the potential mechanisms underlying the Cr(VI)-induced dermal toxicity. As presented in Table 2, a large number of genes involved in vital molecular and cellular functions such as development, growth, differentiation, proliferation, signaling, metabolism, movement, transport, cell cycle, etc., were found differentially expressed in the Cr(VI)-treated cells compared to the untreated control cells. It is possible that exposure

of dermal fibroblasts to a toxic concentration of Cr(VI) resulted in the abnormal expression of several genes thereby interfering with the biological and molecular functions mediated by these genes so as to result in cytotoxicity and cell death. Alternatively, some of the gene expression changes observed may represent adaptive responses of the cells to combat Cr(VI) toxicity or effects secondary to the toxicity of the chemical. Many of the cellular functions which were interrupted by Cr(VI) exposure in the dermal fibroblasts were also affected in human peripheral blood mononuclear cells [23] and human pulmonary epithelial cell line A549 [24] exposed to Cr(VI) suggesting the possible involvement of common mechanisms in the toxicity of Cr(VI) in cell lines derived from different target organs/tissues.

Reactive oxygen species (ROS) responsible for the induction of oxidative stress play a central role in the toxicity of transition metals including Cr(VI). Upon entering the cells, Cr(VI) is reduced both enzymatically [25,26] and non-enzymatically [14] to intermediates of lower valences; this is often accompanied by the generation of ROS resulting in oxidative stress and toxicity. Unless efficiently detoxified and immediately removed, the ROS generated can interact with intracellular targets to result in toxicity [14]. The microarray findings, in conjunction with those of the confocal microscopy study, support the existence of pro-oxidant conditions in the Cr(VI) treated dermal fibroblasts capable of resulting in toxicity. Superoxide dismutase (SOD), responsible for the dismutation of superoxide anion to result in the generation of H_2O_2, was found significantly overexpressed in the Cr(VI)-treated cells. The H_2O_2 thus generated, if not detoxified immediately, may result in its cellular accumulation resulting in toxicity [27]. Catalase plays a major role in detoxifying H_2O_2 [28], and in the Cr(VI)-treated cells the expression of catalase gene was significantly down-regulated compared with the control cells. The overexpression of SOD along with the down-regulation of catalase gene expression should have facilitated increased generation and accumulation of H_2O_2 in the Cr(VI) treated cells. The H_2O_2 accumulated in the cells can interact with cellular targets to result in toxicity. The confocal microscopy results demonstrating the elevated cellular level of oxidants such as the reductive intermediates of Cr(VI) and reactive oxygen species in the Cr(VI) treated cells further supported the microarray findings. Several stress response genes including HO-1 [29], members of the heat shock proteins [30], metallothionein [31], growth differentiation factor 15 [32], etc, were found significantly overexpressed in the Cr(VI) treated fibroblasts, further supporting the induction of cellular stress in response to Cr(VI) exposure .

Cells, in general, defend against oxidative stress and the resulting toxicity induced by ROS by activating the cellular defense machinery collectively referred to as oxidative stress response. The transcription factor - nuclear factor-E2 related factor (Nrf2), is a key member of the cellular antioxidant response system and facilitates the detoxification of ROS by transcriptional activation of one or several phase II detoxifying genes and stress-inducible genes [33]. The activation of the Nrf2 signaling pathway in response to exposure of mouse hepa1c1c7 cells to Cr(VI) has been recently reported [34] as a protective mechanism against chromium-induced apoptosis. Similarly, a definite role for the Nrf2-mediated antioxidant response system in protecting hepatocytes against chromium-induced stress and toxicity has been implicated based on the results of studies conducted in rats [35]. The reported protective role of Nrf2 against oxidative stress is presumably due to the antioxidant response element (ARE)-mediated induction of expression of phase II detoxifying genes and stress-inducible genes [33]. Expression of several genes encoding cytoprotective detoxification enzymes is coordinately regulated by Nrf2- a redox sensitive transcription factor, through its interaction with the antioxidant responsive element (ARE) present in the 5' flanking region of these genes [36]. The activation of genes involved in the oxidative stress response mediated by Nrf2 (Table 5) in the fibroblasts in response to Cr(VI) exposure, therefore, can be considered as an adaptive response of the cells to detoxify ROS to result in protection against Cr(VI) toxicity.

Apoptosis is a physiological adaptation whereby cells undergo programmed death in response to unrepairable DNA damage induced by toxic chemicals. The structural and functional damage to DNA observed in cells exposed to Cr(VI) is attributed both to the reactive chromium intermediates and the ROS generated during the intracellular reduction of Cr(VI) [37]. It is well established that Cr(VI) induces generation of ROS and concomitant DNA damage leading to apoptosis in cells [38]. The Gadd45a gene, whose expression is considered as a marker for cellular response to DNA damage [39], was found significantly overexpressed in the fibroblasts treated with Cr(VI). Gadd45a interacts with proliferating cell nuclear antigen (PCNA), a nuclear protein that plays a central role in DNA damage repair [40]. The significant overexpression of both Gadd45a and PCNA in the Cr(VI) treated cells may, therefore, be considered an adaptive response of the cells to repair the DNA damage induced by Cr(VI) metabolites and/or the ROS generated. Functional categorization of the differentially expressed genes in the Cr(VI) treated cells identified the differential expression of several genes involved in apoptosis (Table 3) potentially resulting in the induction of apoptosis as a likely response of the fibroblasts to Cr(VI) exposure. Pro-apoptotic genes such as phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) [41], B-cell CLL/lymphoma 6 (BCL6) [42], tumor protein p53 inducible nuclear protein 1 (TP53INP1) [43], tumor necrosis factor receptor superfamily, member 10 A and B (TNFRS10A and B) [44], BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) [45], death effector domain containing 2 (DEDD2) [46], etc, were overexpressed while antiapoptotic genes such as cyclin dependent kinase 9 (CDK9) [47], protein kinase C alpha (PKCA) [48], fas apoptotic inhibitory molecule (FAIM) [49], kruppel-like factor 2 (KLF2) [50], etc, were repressed in the Cr(VI) exposed cells facilitating apoptosis induction. The results of TUNEL assay further confirmed the microarray findings and demonstrated the induction of apoptosis as a cellular response of the fibroblasts to Cr(VI) toxicity. Several apoptotic response genes, including those involved in the p53 signaling pathway such as OKL38 [51], transcription factor E2F1 [52], and Gadd45a [53], were overexpressed in the Cr(VI)-treated fibroblasts suggesting the possible involvement of mechanisms dependent on p53, an important stress-responsive and apoptotic regulatory gene [54], in Cr(VI)-induced apoptosis and cytotoxicity in the cells.

Because of the widespread presence of chromium as a contaminant in various consumer products and in cement and the ability of chromium to cause dermal toxicity, especially ACD, some investigators have suggested restricting the amount of Cr(VI) present in consumer products, such as detergents, to <5 ppm [55]. Addition of reducing agents, for example $FeSO_4$, to facilitate the reduction of water-soluble and toxic Cr(VI) to insoluble and, therefore, less permeable trivalent chromium has been suggested as a feasible approach to prevent or at least to reduce the toxicity of Cr(VI) that is present as a contaminant in various industrial and consumer products. For example, a significant reduction in the incidence of ACD has been observed among construction workers in certain European countries where the addition of $FeSO_4$ to cement has been mandated [56]. However, in spite of the limited epidemiological evidence demonstrating the beneficial effect of $FeSO_4$ to prevent the toxicity and illnesses resulting from exposure to Cr(VI), the addition of $FeSO_4$ to products that contain Cr(VI) is not universally accepted or practiced in several countries, including the US. One of the reasons for this might be the lack of adequate experimental data to support the protective role of $FeSO_4$ in the Cr(VI)-induced

toxicity. Currently, by employing human dermal fibroblasts as an *in vitro* experimental model for human skin, we have obtained data to demonstrate the potential protective role of $FeSO_4$ in the apoptosis, oxidative stress, differential gene expression, and cytotoxicity induced by hexavalent chromium. The present findings warrant additional animal and epidemiological studies to confirm the protective role of $FeSO_4$ in Cr(VI)-induced dermal toxicity for potential application in cases of human exposure to Cr(VI) and the resulting toxicity and illnesses.

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