

## Exposure to low levels of hexavalent chromium: target doses and comparative effects on two human pulmonary cell lines

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**Abstract.** Intracellular reduction of hexavalent chromium [Cr(VI)] is associated with the production of reactive oxygen species (ROS) and subsequent oxidative damage to different intracellular molecules like DNA, proteins and lipids is believed to contribute to the process of carcinogenesis. Aim of this study was to develop a model to establish a relationship between intracellular and macromolecule-bound chromium and some biomarkers of oxidative stress in two *in vitro* cell lines. Human lung adenocarcinoma (A549) and human bronchial epithelial (BEAS2B) cells were exposed for 3, 8 and 24 hours to relatively low doses (0.5 – 1 – 2  $\mu$ M) of Cr(VI), i.e., to concentrations similar to what measured and reported by some authors in unexposed subjects and chromate workers. The results show that the differential cytotoxicity of Cr(VI) on the A549 and BEAS2B cell lines may be related both to their different polymorphism of Glutathione S-transferases genes and probably to their unlike permeability to Cr(VI). The glutathione decrease and the induction of HO-1 observed only in BEAS2B cells after Cr(VI) exposure strengthen the idea that glutathione S-transferases activity may accelerate the reduction of Cr(VI) to Cr(III) with the concomitant induction of oxidative stress. In conclusion, the determination of intracellular Cr in cellular models can be considered an important step in comparing *in vitro* and *in vivo* models on the basis of target doses and a promising approach to study the effects of pneumotoxic compounds. ([www.actabiomedica.it](http://www.actabiomedica.it))

**Key words:** Chromium, oxidative stress, haeme oxygenase, glutathione-S-transferase, BEAS2B cell line

### Introduction

Cr(VI)-containing compounds are extensively used in many plating, welding and pigment production plants. Epidemiological and experimental evidence indicates that occupational exposure to Cr(VI) compounds is associated with lung cancer (1-4).

Therefore, Cr(VI) is included in the class I of carcinogens (5). The respiratory tract is considered the primary target organ for Cr(VI) compounds, being the inhalation the main route of entry into human body (1, 2).

Cr(III), which is also an essential nutrient involved in the metabolism of glucose, insulin and blood

lipids (6), is less toxic than Cr(VI) (5,7). Under physiological conditions, Cr(VI) forms soluble oxyanions (e.g. chromate) that can cross cell membranes through anionic channels or non-specific anionic transporters (8,9), whereas Cr(III) does not usually cross the cell membrane. However, once inside cells, Cr(VI) undergoes rapid reduction by various cell components, such as ascorbate, glutathione (GSH) and cysteine (10-14). The main reduction products, which include Cr(III) and the unstable tetravalent [Cr(IV)] and pentavalent forms [Cr(V)], are primarily responsible for Cr(VI) toxicity (15, 16). In addition, intracellular reduction of Cr(VI) is associated with the production of reactive oxygen species (ROS) (17-19).

The intracellular reduction is complex and involves multiple pathways including metabolism by polymorphic Glutathione S-transferases. This family of enzymes is a defence system against the damaging effects of oxidative stress and catalyzes the nucleophilic addition of glutathione to electrophilic acceptors including toxic products. De Flora (20) has proposed that these enzymes could play a rate-limiting role in the reduction of Cr(VI) to Cr(III). Among GSTs, both GSTM1 and GSTP1 polymorphisms have been associated to lung diseases, including lung cancer (21). Homozygous gene deletion of *GSTM1* leads to the absence of the respective enzyme in about 50% of Caucasians. The *GSTP1* gene contains two polymorphic sites, respectively at exon 5 (Ile105>Val105) and exon 6 (Ala114>Val114), giving rise to four different alleles: *GSTP1\*A* (Ile105-Ala114), *GSTP1\*B* (Val105-Ala114), *GSTP1\*C* (Val105-Val114), *GSTP1\*D* (Ile105-Val114). Compared with the wild type protein encoded by *GSTP1\*A* allele, variant proteins show *in vitro* either a reduced half-life or a different catalytic efficiency toward different substrates (22, 23)

Cr(III) forms stable adducts with nucleophile sites of DNA and proteins, thus generating DNA cross-links with other macromolecules (cysteine-Cr(III)-DNA or glutathione-Cr(III)-DNA) considered to be the main modifications after *in vitro* exposure to Cr(VI) (16, 24, 25). As a result, alterations as gene mutations, sister chromatid changes, and chromosomal aberrations have been reported in literature, as well as effects on RNA synthesis, DNA replication, and DNA repair systems (16). Also Cr(V), the insta-

ble form, seems able to induce oxidative stress (26-29) through direct bond on DNA (16, 30, 31).

Some *in vitro* studies have shown that Cr(VI) exposure affects the transcriptional induction of cell cycle inhibition and apoptosis-inducing genes by means of multiple cell cycle dysregulation mechanisms that seem to be cell-type specific (16). This complicates any direct comparison of *in vitro* and *in vivo* results (particularly in the case of Cr(VI)-induced pulmonary neoplasms), and the exact molecular mechanisms of Cr toxicity in complex biological systems are still unclear.

To evaluate and perform a direct comparison between some *in vivo* situations using *in vitro* models, we have exposed two different pulmonary cell lines to Cr VI concentrations similar to what measured and reported by some authors in unexposed subjects and chromate workers (32-35).

We have selected low doses of Cr(VI) (0.5, 1 and 2  $\mu$ M) and we have analysed well-known biomarkers of oxidative stress, including TBARS concentrations (Thiobarbituric Acid Reactive Substances), heme oxygenase-1 (HO-1) expression and glutathione (GSH) depletion (36) in two *in vitro* pulmonary cell lines: human lung adenocarcinoma (A549) and human bronchial epithelial (BEAS2B). Moreover, these pulmonary cells differ each other for their genetic pattern on GST enzymes thus allowing a better comprehension on the exact role of intracellular transferases activities. This approach can allow a correct extrapolation of *in vitro* sub-toxic target Cr concentrations that may be useful for relating *in vitro* models to existing *in vivo* data.

## Material and methods

### Cell cultures

The A549 human lung adenocarcinoma and BEAS2B immortalised human bronchial epithelial cells were purchased from the American Type Culture Collection (Rockville, MD). The former were maintained in RPMI medium supplemented with 10% inactivated fetal bovine serum and penicillin/streptomycin, while the latter were cultured in LHC-9/RPMI medium and used for experiments from passage

50 to passage 60. Sub-confluent cells were exposed to 0.5, 1 and 2  $\mu\text{M}$  concentrations of Cr(VI) for measurement of selected markers of oxidative stress.

#### *Cell viability*

Cell viability was evaluated using the MTT assay (37). Briefly, two hours before the end of incubation, 10  $\mu\text{l}$  of a 5 mg/ml MTT solution were added to each well. The mitochondrial dhydrogenases of viable cells cleave the tetrazolium ring, thus yielding purple formazan crystals that are insoluble in aqueous media. At the end of the incubation, the crystals were dissolved by adding 100  $\mu\text{l}$ /well of solubilisation solution (10% Triton X-100 and 0.1 N HCl in 125 ml of anhydrous isopropanol), and the absorbance of the resulting purple solution was measured at a wavelength of 570 nm using a multiwell spectrophotometer (Thermo Lab-system Multiskan Ascent). The samples were cultured in eight replicates at each tested concentration, and the experiments were performed in duplicate. Untreated controls and blanks were incubated in the same plates under the same conditions.

#### *DNA isolation and characterisation of genetic polymorphisms*

Genomic DNA was isolated from about  $5 \times 10^6$  cells using a commercial kit (PUREGENE; Gentra Systems; Minneapolis, MN, USA). The genetic polymorphisms were characterised according to previously described methods, either using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocols in the case of GSTP1 (38, 39) or by multiplex PCR protocol, using  $\beta$ -globin gene as an internal control, for GSTM1 and GSTT1 (40). The BEAS 2B showed the following genotypes: *GSTM1 pos*, *GSTT1 null*, *GSTP1 BB*, whereas A549 resulted *GSTM1 null*, *GSTT1 null* and *GSTP1 BC*.

#### *Intracellular Cr levels*

The intracellular concentration of Cr was determined (in the supernatant phase) by distinguishing total Cr and the non-diffusible fraction bound to intracellular macromolecules (bound Cr). Briefly, the

cell pellets were collected, freeze/thawed three times, and centrifuged (1200 g for 5 min), and the supernatant was divided into two aliquots. The first aliquot was immediately analysed by means of electrothermal atomic absorption with Zeeman's effect background correction (ETAAS), using a 220 Z atomic absorption spectrometer (VARIAN, Palo Alto, CA) in order to estimate the total intracellular level of Cr. The second aliquot was filtered three times (30 minutes at 5000 g) in Amicon Centrifugal Filter Devices (Millipore Corporation, Bedford, MA), using a cut-off filter of 5000 Da after further volume re-equilibrations with water. The expected fraction of only diffusible Cr in the upper part of the filter was less than 1/50 (2%), and the bound Cr was measured by means of ETAAS as total Cr. A standard of 10  $\mu\text{g/l}$  of Cr(VI) prepared in the supernatant of unexposed cells diluting a certificate standard of 1 g/l of Cr(VI) (Fluka, Milan, Italy) was placed every 10 samples in order to assess the stability of the signal. The standard curve [0-30  $\mu\text{g/l}$ ] was directly prepared in the supernatant of unexposed cells in order to reduce matrix effect on Cr ETAAS detection. The limit of detection (LOD) was 0.05  $\mu\text{g/l}$ .

#### *Cell GSH content*

The GSH content of the epithelial cells was assessed in cell lysates prepared after washing the cells with ice-cold PBS (41). Briefly, washed cells were lysed by adding ice-cold lysis buffer (0.6% [w/v] sulfosalicylic acid, 0.1% [v/v] Triton X-100, 5 mM EDTA in 0.1 M potassium phosphate buffer, pH 7.5) and incubating for 10 min on ice. The lysates were harvested, and cell pellets obtained after centrifugation were disrupted using a Teflon pestle followed by vortexing. This solution was cleared by centrifugation, and GSH content of supernatant was assessed using Tietze's method (42). The GSH content was calculated using a standard curve, and expressed as nmol/mg protein. The protein content of the lysates was determined using the bicinchonic acid (BCA) method (Pierce Chemical Co, Rockford, IL).

#### *TBARS assay*

Cell lipid peroxidation products were identified by measuring thiobarbituric acid reactive substances

(TBARS), adapted for cells as previously described by Vettori et al. (36). Briefly, the cells were collected, freeze/thawed three times and, after centrifugation at 3000 g for 5 min, an aliquot of supernatant was mixed with an equal volume of 0.2 M orthophosphoric acid and a 1/20 volume of thiobarbituric acid in 0.1 M NaOH. After 45 min at 95°C, the TBARS were extracted using *n*-butanol/NaCl. After further centrifugation (3000g for 10 min), the upper solution was collected and its TBARS concentration measured using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Cary, NC) (excitation 515, emission 545). Malondialdehyde was used as the standard for the calibration curve (range 0-10 µM). TBARS values were expressed as percentage of control.

#### RNA isolation

Total RNA was extracted by means of the method of Chomczynski and Sacchi (43) using a commercially available reagent (Trizol; Gibco BRL, Gaithersburg, MD), and digested with Dnase I (DNA-free kit; Ambion Inc., Austin, TX) in order to remove any genomic DNA contamination. The RNA was checked for integrity and purity by means of native 1% agarose gel electrophoresis in a denaturing Tris-borate-EDTA buffer, pH 8.3, containing 6.5% Ficoll, 0.005% bromphenol blue and 3.5 M urea (RNA Ladder; New England Biolabs Inc., Beverly, MA). Total RNA was quantified using the RiboGreen probe (Molecular Probes, Eugene, OR) and a Cary Eclipse fluorescence spectrophotometer equipped with a microplate reader (Varian Inc, Cary, NC).

#### Gene expression analysis

First-strand cDNA was synthesised using 2.5 µM of Random Decamers (Ambion Inc., Austin, TX), 400 ng of total RNA and 200 U of SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and the subsequently diluted 1:2 in nuclease-free water (Ambion Inc., Austin, TX) for use in downstream applications.

RT-PCR reactions containing 2 µl of template cDNA, 300 nM of forward and reverse primer, and 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad, Hercules,

CA) in a final volume of 25 µl, were set up in duplicate and run on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each assay also included four serial dilutions of a test cDNA used to build a standard curve, and a negative control. The amplification protocol consisted of 3 min at 95°C to activate the Hot-Start i*Taq* DNA polymerase, 40 cycles at 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and a final melting step with a gradual increase in temperature from 50°C to 94°C in order to verify the absence of non-specific products. Specific oligonucleotide primers spanning the exon-exon junctions were designed for the HO-1 gene (GeneBank Accession No. NM\_002133) using Primer3 software (Forward: 5'-TCCGATGGGTCTTACTC-3'; Reverse: 5'-TAAGGAAGCCAGCCAAGAGA-3'), which is freely accessible online (<http://frodo.wi.mit.edu>).

Previously published primer sequences were chosen to amplify the following internal control genes: hydroxymethylbilane synthase (*HMBS*), succinate dehydrogenase complex subunit A (*SDHA*), and hypoxanthine phosphoribosyl-transferase I (*HPRT*) (44).

HO-1 gene expression levels were normalised to the three most stable internal control (housekeeping) genes as determined using the *geNorm* application for Microsoft Excel, and then averaged over separate experiments, each repeated three times.

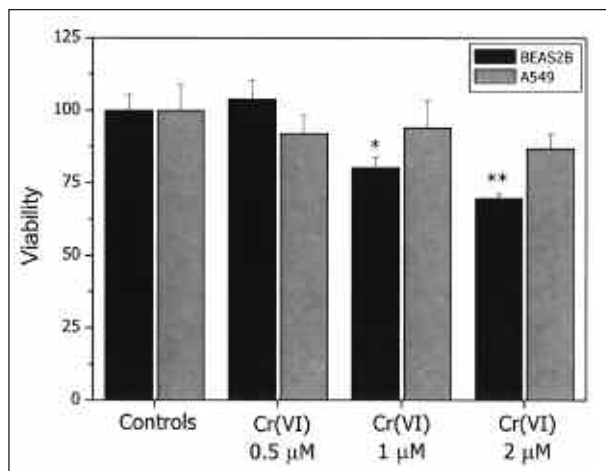
#### Statistical analysis

Each parameter was measured at least in triplicate, and data in all graphs and tables are expressed as mean values ± SD. The Student *t*-test was used for comparisons involving two samples; ANOVA followed by Tukey's *post hoc* test was applied if more than two samples were compared. A *p* value of 0.05 was considered significant. All of the the analyses were performed using SPSS 14.0 software (SPSS, USA).

## Results

#### Cell viability

Figure 1 shows the effect of chromium hexavalent on the viability of bronchial epithelial (BEAS2B)



**Figure 1.** Viability of BEAS2B and A549 cells after 24 hours' exposure to Cr(VI) 0.5, 1 and 2  $\mu\text{M}$ , respectively. One-way ANOVA followed by Dunnett's *post hoc* test: \*  $p < 0.05$  vs untreated control; \*\*  $p < 0.01$  vs untreated control

and adenocarcinoma (A549) cells. There was no significant change in the viability of A549 cells, whereas the viability of BEAS2B cells significantly decreased to about 80% after 24 hour exposure to 1  $\mu\text{M}$  of Cr(VI) and to about 65% after exposure to 2  $\mu\text{M}$ ; the concentration of 0.5  $\mu\text{M}$  had no effect.

#### Intracellular Cr(VI) levels

Figures 2A-C-E show the accumulation kinetics of Cr at different concentrations. After exposure to 0.5  $\mu\text{M}$  Cr(VI), BEAS2B cells tended to accumulate more Cr than A549 cells at all time points (Fig. 2A); moreover, there was a significant decrease in total Cr in A549 cells from 8h to 24h. The trend was similar after exposure to 1  $\mu\text{M}$  Cr(VI) (Fig. 2C), whereas the behaviour of the two cell types did not differ after treatment with 2  $\mu\text{M}$  (Fig. 2E). BEAS2B cells exposed to Cr(VI) 1 and 2  $\mu\text{M}$  showed similar accumulation kinetics.

Figures 2B-D-F show the fraction of Cr bound to intracellular macromolecules (MW >5 kDa) in the same samples. In general, the differences between A549 and BEAS2B cells were less evident (but sometimes significant) after 3 h and 8 h in comparison with total Cr, but the levels of bound Cr were significantly higher in the BEAS2B cells after 24 h exposure to 0.5 and 1  $\mu\text{M}$  Cr(VI).

The ratios of Cr bound to intracellular macromolecules and total Cr concentration are shown in Figs. 3A-C. At the lowest concentration (Fig. 3A), A549 cells tended to bind higher fractions of Cr than BEAS2B cells (Fig. 3A). At the Cr(VI) concentration of 1  $\mu\text{M}$ , the percentage of bound Cr in A549 cells was similar at all time points, whereas that in BEAS2B cells was increased over time (Fig. 3B). Finally, at Cr(VI) 2  $\mu\text{M}$ , the only significant difference was between 8 h and 24 h in the case of BEAS2B cells (Fig. 3C).

#### Lipid peroxidation

We measured TBARS levels in lysates of A549 and BEAS2B cells after exposure to 0.5, 1 and 2  $\mu\text{M}$  Cr(VI) for 3, 8 and 24 h (Tab. 1A-B). After exposure to 0.5  $\mu\text{M}$ , the A549 cells showed a statistically significant increase in TBARS only at 3 h; treatment with 1  $\mu\text{M}$  induced an increase after 8 and 24 hours; and the highest concentration (2  $\mu\text{M}$ ) induced a significant increase after 3 and 24 hours of exposure (Table 1A). The effect of Cr on TBARS behaviour in BEAS2B cells was completely different, particularly after prolonged exposure (24 h). Table 1B shows that the highest Cr(VI) concentrations (1 and 2  $\mu\text{M}$ ) increased TBARS levels only after 8 h; they then decreased up to 24 h, when the difference was significant after exposure to 2  $\mu\text{M}$ .

#### GSH levels

Cr exposure did not induce any significant alteration in GSH levels in A549 (Tab. 2A) while a weak decrease is observed in BEAS2B cells after 3h of exposure to all the Cr(VI) concentrations considered (Tab. 2B).

#### HO-1 RNA levels

Figure 4A shows HO-1 expression in A549 cells, which significantly decreased only after 3 h exposure to Cr(VI) 1  $\mu\text{M}$  ( $p < 0.05$  vs control) and significantly increased only after 24 h exposure to Cr(VI) 2  $\mu\text{M}$  ( $p < 0.01$  vs control). Like that of TBARS, the behaviour of HO-1 expression in BEAS2B cells was com-

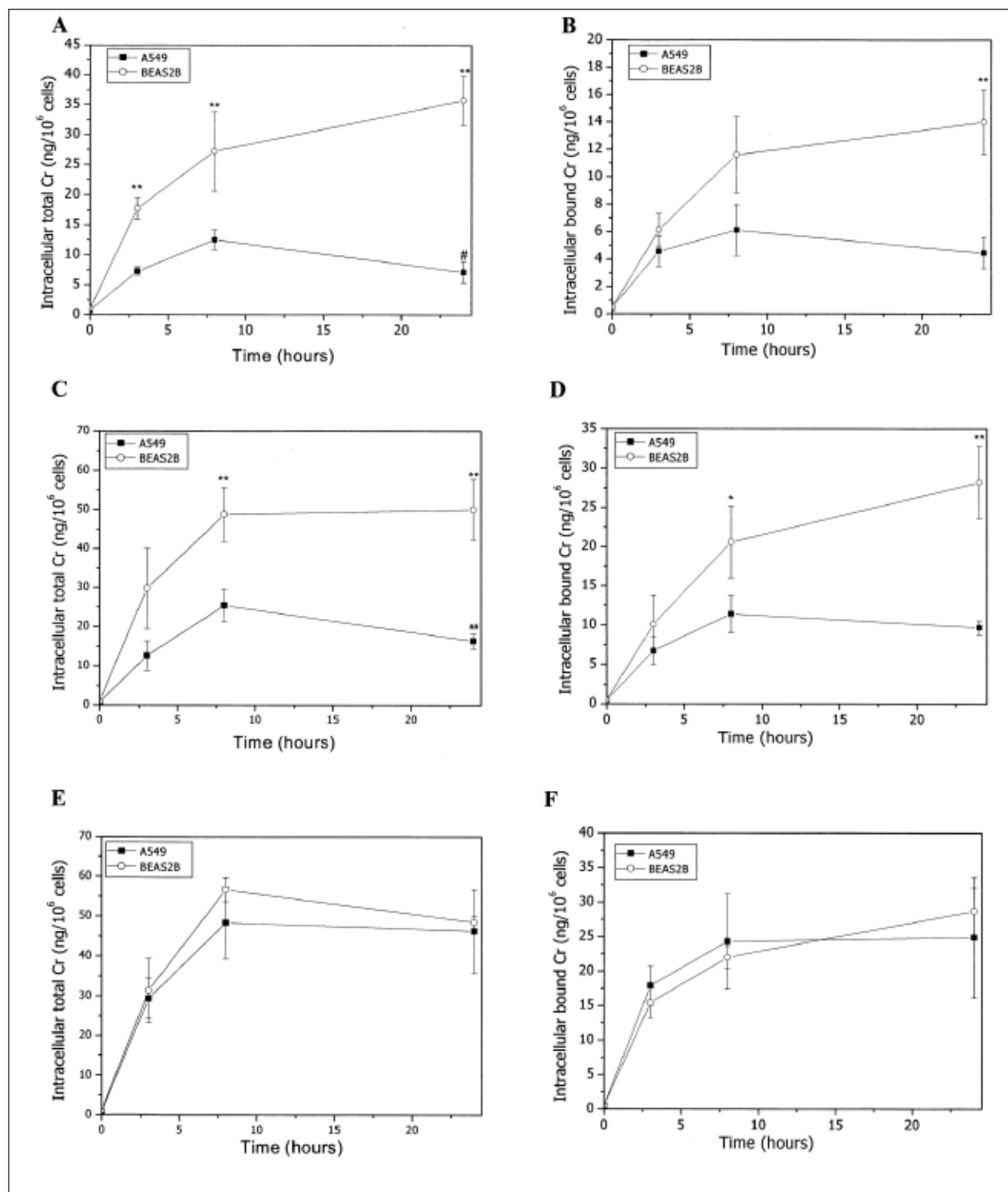
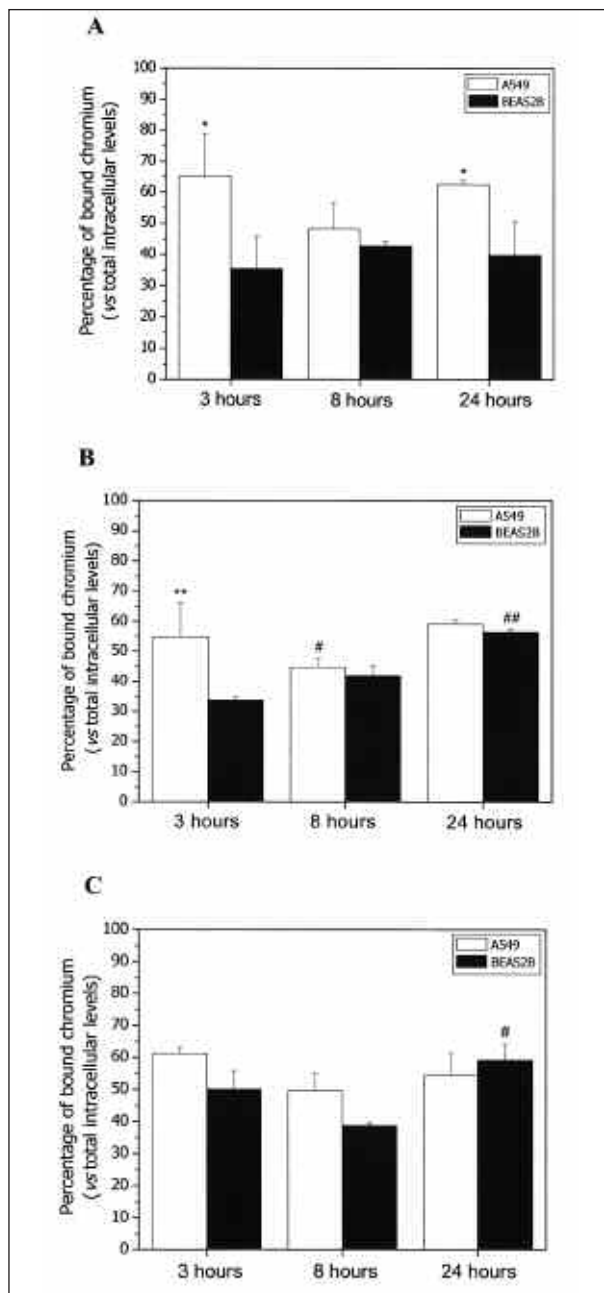
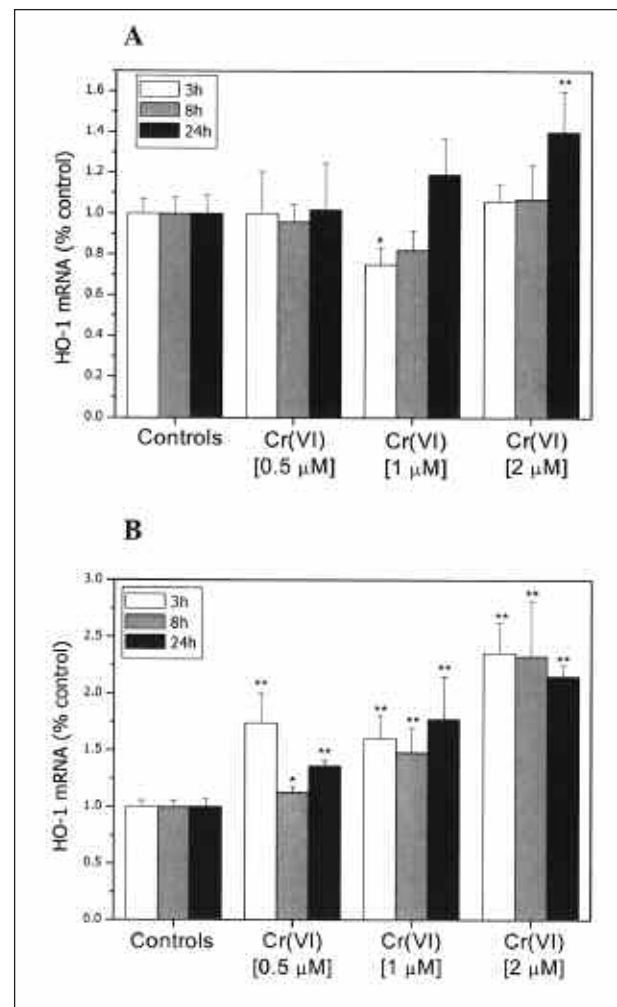


Figure 2. Intracellular concentrations of chromium (respectively A, C, E) and macromolecule-bound chromium (respectively B, D, F) in both cell lines after exposure for 3, 8 and 24 hours to Cr(VI) 0.5, 1, and 2  $\mu$ M. Student t-test at every time point to compare different cell lines, and ANOVA followed by Tukey's *post hoc* test to compare different time points in the same cell line. \* $p < 0.05$  and \*\* $p < 0.01$  vs A549; #  $p < 0.05$  vs 8 hours in the same cell line



**Figure 3** Percentage of macromolecule-bound chromium in relation to total intracellular levels in (A) BEAS2B and A549 cells after exposure to Cr(VI) 0.5  $\mu$ M for 3, 8 and 24 hours. \* $p$ <0.05 vs BEAS2B; (B) BEAS2B and A549 cells after exposure to Cr(VI) 1  $\mu$ M for 3, 8 and 24 hours. \*\* $p$ <0.05 vs BEAS2B; # $p$ <0.05, ## $p$ <0.01 vs 3 hours; (C) BEAS2B and A549 cells after exposure to Cr(VI) 2  $\mu$ M for 3, 8 and 24 hours. # $p$ <0.05 vs 8 hours. Student t-test at every time point was performed to compare different cell lines, and ANOVA followed by Tukey's *post hoc* test to compare different time points in the same cell line.



**Figure 4.** Heme oxygenase-1 expression in A549 cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu$ M of Cr(VI) (A) and Heme Oxygenase-1 expression in BEAS2B cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu$ M of Cr(VI) (B). One-way ANOVA followed by Dunnett's *post hoc* test was performed. \* $p$ <0.05 vs untreated control; \*\*  $p$ <0.01 vs untreated control

pletely different for BEAS2B cells: a significantly increased HO-1 expression was observed under all tested conditions (Fig. 4B).

## Discussion

The results of this study show that the exposure to relatively low concentrations (0.5, 1 and 2  $\mu$ M) of Cr(VI) on cell viability, lipid peroxidation and HO-1 expression in A549 and BEAS2B human pulmonary

**Table 1A.** TBARS levels in A549 cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu\text{M}$  of Cr(VI). One-way ANOVA followed by Dunnett's *post hoc* test

	TBARS levels (% of control)			
	Control	Cr(VI) 0.5 $\mu\text{M}$	Cr(VI) 1 $\mu\text{M}$	Cr(VI) 2 $\mu\text{M}$
3 h	100.0 $\pm$ 2.2	172.0 $\pm$ 28.0*	142.4 $\pm$ 23.9	191.6 $\pm$ 24.9*
8 h	100.0 $\pm$ 15.0	128.6 $\pm$ 17.8	154.5 $\pm$ 26.6*	134.6 $\pm$ 17.6
24 h	100.0 $\pm$ 32.2	94.8 $\pm$ 8.2	142.4 $\pm$ 21.0*	200.0 $\pm$ 15.5*

\*  $p < 0.05$  vs untreated control; \*\*  $p < 0.01$  vs untreated control**Table 1B.** TBARS levels in BEAS2B cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu\text{M}$  of Cr(VI). One-way ANOVA followed by Dunnett's *post hoc* test

	TBARS levels (% of control)			
	Control	Cr(VI) 0.5 $\mu\text{M}$	Cr(VI) 1 $\mu\text{M}$	Cr(VI) 2 $\mu\text{M}$
3 h	100.0 $\pm$ 36.3	122.2 $\pm$ 0.4	147.6 $\pm$ 5.7	150.0 $\pm$ 28.9
8 h	100 $\pm$ 16.6	129.0 $\pm$ 2.6	207.0 $\pm$ 6.2*	178.9 $\pm$ 11.0*
24 h	100 $\pm$ 2.4	66.6 $\pm$ 20.5	61.2 $\pm$ 24.3	40.4 $\pm$ 2.5*

\*  $p < 0.05$  vs untreated control; \*\*  $p < 0.01$  vs untreated control**Table 2A.** GSH levels in A549 cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu\text{M}$  of Cr(VI). One-way ANOVA followed by Dunnett's *post hoc* test

	GSH levels (% of control)			
	Control	Cr(VI) 0.5 $\mu\text{M}$	Cr(VI) 1 $\mu\text{M}$	Cr(VI) 2 $\mu\text{M}$
3 h	100 $\pm$ 2.3	102.2 $\pm$ 1.6	104.3 $\pm$ 0.6	101.4 $\pm$ 0.8
8 h	100 $\pm$ 3.8	81.3 $\pm$ 23.1	94.1 $\pm$ 1.1	85.0 $\pm$ 15.3
24 h	100 $\pm$ 2.1	101.8 $\pm$ 1.1	98.6 $\pm$ 4.9	104.2 $\pm$ 3.2

**Table 2B.** GSH levels in BEAS2B cells exposed for 3, 8 and 24 hours to 0.5, 1 and 2  $\mu\text{M}$  of Cr(VI). One-way ANOVA followed by Dunnett's *post hoc* test

	GSH levels (% of control)			
	Control	Cr(VI) 0.5 $\mu\text{M}$	Cr(VI) 1 $\mu\text{M}$	Cr(VI) 2 $\mu\text{M}$
3 h	100 $\pm$ 3.9	74.1 $\pm$ 36.8	86.8 $\pm$ 17.7	82.5 $\pm$ 15.6
8 h	100 $\pm$ 2.5	110.5 $\pm$ 11.1	106.8 $\pm$ 8.0	105.3 $\pm$ 5.9
24 h	100 $\pm$ 2.3	84.8 $\pm$ 22.9	103.2 $\pm$ 6.3	100.1 $\pm$ 2.3

cell lines is related to the intracellular levels of total and bound Cr. The different concentrations inside the cells and the unlike observed behaviour could be partially justified on the basis of different *GSTM1* and *GSTP1* polymorphisms that distinguish these cell lines.

The selected doses which were used in our experiments came from some extrapolation from *in vivo* data reported by Tsuneta et al. (35) or by Raithel et al.

(34). Given that  $10^6$  cells weigh about 1 mg, the maximum intracellular dose was 12–50  $\mu\text{g/g}$  for the A549 cells, and 30–55  $\mu\text{g/g}$  for the BEAS2B cells and, on the basis of the observed cytotoxic effects in BEAS2B, a dose of about 50  $\mu\text{g/g}$  could be considered the LOAEL (Lowest Observed Adverse Effect Level) for cytotoxicity in these *in vitro* models. In unexposed subjects, tissue Cr levels are less than 5  $\mu\text{g/g}$  (32, 33), but Tsuneta et al. (35) found that the mean Cr content

in the peripheral lung tissue in chromate workers with lung cancer was 36.7  $\mu\text{g/g}$  wet, and Raithel et al. (34) found concentrations of between 10 and 30  $\mu\text{g/g}$  wet in welders, especially flame sprayers. Thus, the concentrations we used in this study are comparable to the levels found in occupationally exposed subjects. This approach could be very useful to compare *in vitro* models and *in vivo* findings, and could provide further information concerning the kinetic mechanisms and the alterations related to Cr(VI) exposure.

As already reported and pointed out by Martin et al. (30), none of the three doses (0.5 – 1 – 2  $\mu\text{M}$ ) of Cr(VI) altered the viability of A549 cells. Only exposure to 10  $\mu\text{M}$  of Cr(VI) induced a decrease in the percentage of cell survival. Similarly, Liu et al. (18) observed an inhibition of cell proliferation only at Cr(VI) concentrations of at least 5  $\mu\text{M}$  and after 48 h exposure. A consistent trend has also been found in other pulmonary cell lines (45). On the contrary, BEAS2B cells seemed to be more sensitive, and treatment with 1 and 2  $\mu\text{M}$  Cr(VI) significantly decreased their viability to respectively 80% and 65% of control levels. This result is also in agreement with previously published data: Pascal and Tessier (46) reported a 60% reduction in viability after 48h exposure to Cr(VI) 2  $\mu\text{M}$ .

The difference in sensitivity of the two cell lines may be partially explained by differences in their kinetics of Cr accumulation. After exposure to 0.5 and 1.0  $\mu\text{M}$  Cr(VI), total intracellular Cr was twice as high in BEAS2B cells, and bound Cr levels were also higher. Moreover, at 2  $\mu\text{M}$  the effect on viability was particularly evident in the BEAS2B cells, but both cell lines showed similar kinetics of accumulation of total and bound Cr, suggesting that this aspect alone is not sufficient to explain the different cytotoxic effects of Cr (VI).

In order to better understand the different sensitivity of these two cell lines, their non homogeneous profile of GST classes relevant to lung cell metabolism seems to play a major role. In particular, A549 cells bear a GSTP1 isoform (BC) functionally different from that expressed by BEAS2B (GSTP1 BB) (22,23) and lack GSTM1 activity, that is present in the other cell line. Thus, theoretically, on the basis of genotype, the two cell lines should differ in their reducing capability. This seems to be confirmed by experimental re-

sults, as the BEAS2B line expresses a better reduction of Cr species from Cr(VI) to Cr(III). As further confirmation, the study of GSH contents shows an early drop in GSH content in BEAS2B, probably due to a consumption by enzyme catalyzed reactions, whereas in A549 cells the drop is delayed, probably consequent to non enzymatic consumption of the molecule. Therefore, different genetic patterns could be an important and complementary aspect to justify the different cellular sensibility after Cr(VI) exposure.

In this study we also assessed TBARS and HO-1 expression, as the target doses we used could be related to other more specific effects than cell viability. Many published studies have described and analysed the effects of Cr exposure on different *in vitro* cell lines (30, 47, 48), particularly its ability to generate oxidative stress (49-51).

We have considered the expression of the inducible isoenzyme heme oxygenase-1 (HO-1), as marker of oxidative stress, involved in cytoprotective mechanisms. The products of the catalyzed enzymatic reaction, bilirubin (BR) and (subsequently) biliverdin (BV) have antioxidant properties (52), confirmed by experimental *in vitro* studies (53-55). HO-1 expression was different in A549 and BEAS2B cells. Kweon et al. (56) have shown that A549 cells express high constitutive levels of HO-1, and therefore it is possible that our low Cr(VI) doses were unable to induce any additional expression except at the highest concentration (2  $\mu\text{M}$ ) after 24 hour exposure; however, the basal HO-1 levels in our A549 cells did not prevent moderate lipid peroxidation (see Tab. 1A).

In contrast, HO-1 expression in BEAS2B cells significantly increased under all tested conditions. Basal HO-1 expression is lower in BEAS2B than in A549 cells, but it has been shown that BEAS2B cells can considerably over-express HO-1 shortly after cell shock (57). This difference could be due to the different abilities of the two cell lines to reduce Cr(VI) according to GST polymorphism that would result in different activities relevant to Cr(VI) biotransformation.

Prolonged over-expression of HO-1 could also be related to the relatively low TBARS levels, which were below control levels after 24 h exposure to the highest Cr(VI) concentration of 2  $\mu\text{M}$ , in agreement with

other studies showing that TBARS levels are decreased by bilirubin (the levels of which are presumably increased by HO-1 induction) (52,58) thus confirming the antioxidant role of the isoenzyme.

In conclusion, the different kinetics and effects of low Cr(VI) doses in the A549 and BEAS2B cell lines may be related either to their different genetic profile modulating their ability to reduce Cr(VI) to Cr(III) or to their permeability to Cr(VI) through anion channels. However, differences in cytotoxicity were found even when the kinetic profiles of total and bound intracellular Cr were similar in the two cell lines, e.g., at 2  $\mu$ M Cr(VI), which suggests that the different pattern of GST enzymes rather than membrane permeability accounts for the differential sensitivity of these cell lines to Cr(VI). Intracellular Cr concentrations were consistent with Cr levels found in pulmonary tissue of exposed workers, thus indicating that comparing *in vitro* and *in vivo* models on the basis of target doses is a promising approach to study the effects of pneumotoxic compounds.

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