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The oxidation of glutathione by cobalt/tungsten carbide contributes to hard metal-induced oxidative stress

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Abstract
The occupational exposure to cobalt/tungsten carbide (Co/WC) dusts causes asthma and interstitial fibrosis. The International Agency for Research on Cancer (IARC) recently classified the mixture Co/WC as probably carcinogenic to humans (group 2A). The mechanism of action of Co/WC involves particle driven generation of Reactive Oxygen Species (ROS) with consequent oxidative damage. The present study evaluates the reactivity of Co/WC dust toward glutathione (GSH) and cysteine (Cys). Co/WC oxidized thiols through a mechanism involving the generation of sulphur-centred radicals. The results are consistent with the oxidation taking place at surface active sites, a part of which is accessible only to Cys S-H groups, but not to GSH ones. Such a reaction, with consequent irreversible depletion of antioxidant defenses of cells, will potentiate the oxidative stress caused by particle and cell generated ROS.

Keywords: *Glutathione, hard metals, free radicals, particle toxicity, Co/WC*

Abbreviations: *ROS, Reactive Oxygen Species; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline*-N-*oxide; PB, phosphate buffer.*

Introduction

'Hard metals' are industrial materials made up by sintering tungsten carbide (WC) particles (usually more than 80%) with small amounts of small particles of metallic cobalt (usually 5–10%) [1]. Depending upon their applications other minor components may be employed (e.g. Ti, Ta, Nb).

Hard metals are widely used as their unique mechanical properties (hardness, resistance) make them suitable for the manufacture of cutting tools, drilling and mining equipments [2].

Workers exposed to hard metal dusts may develop various lung diseases from asthma to interstitial fibrosis and lung cancer. While asthma may be caused by cobalt alone [3], lung fibrosis and cancer occur only when Co is associated with WC (in hard metal) or microdiamonds [4–6]. The IARC (International Agency for Research on Cancer) has classified hard metal dusts as 'probably carcinogenic to humans (Group 2A)' on the basis of sufficient evidence in experimental animals but limited evidence in humans for increased risk of lung cancer [7]. Cobalt alone was only classified as 'possibly carcinogenic to humans

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(Group 2B)' [7]. Several studies report that metallic cobalt acquires a higher genotoxicity when associated to WC or to other carbides [8–12].

The adverse effects caused by Co/WC mixtures may be explained by a specific interaction between cobalt and WC particles which generates a new entity with the potential to release a high amount of Reactive Oxygen Species (ROS) causing oxidative damage to cells and tissues [1,13,14]. The mechanism hypothesized for the generation of ROS involves the translocation of electrons from cobalt to the WC surface and the simultaneous reduction of oxygen dissolved in water to superoxide-like species [1,13,15], alongside with release of Co^{2+} ions into solution [16,17].

Inhaled particles interact primarily with the lung-lining layer made up by surfactants, proteins and rich in glutathione (the tripeptide γ-Glu-Cys-Gly, GSH) and ascorbic acid [18–20]. GSH acts as a ROS scavenger, thus constituting one of the first lines of defense against lung injury due to the over-production of ROS. Both ascorbic acid and GSH are able to scavenge superoxide and hydroxyl radicals [21–23]. GSH and cysteine residues in proteins also have an important role in redox regulation [24].

The antioxidant activity of GSH and ascorbic acid is due to their oxidation by radicals or metal ions to glutathione disulphide (GSSG) or other mixed disulphides (GSSR) and to dehydroascorbic acid, respectively.

GSH and ascorbic acid levels are strictly related, since GSH act as hydrogen donors for the enzyme ascorbate dehydrogenase, whose main function is to maintain high the level of ascorbic acid.

It was previously reported that the mixture Co/WC, by extracting hydrogen atoms following the homolytic cleavage of the C-H bond, generates carbon-centred radicals [1]. Considering that hydrogen extraction is the first step in the oxidation of thiols to disulphide, we investigated whether the mixture Co/WC would also react with thiol groups in GSH. The study was also carried out with cysteine (Cys), the aminoacid holding the SH group in GSH.

Antioxidant depletion may contribute to the toxic potential of hard metal dusts, since a depletion of such antioxidant defenses would increase the effects caused by ROS generated by inhaled particles, thus adding another piece of evidence to the hypothesis of an oxidative stress at the basis of the mechanisms of hard metal lung diseases.

Experimental

Materials

A finely divided pure metallic cobalt (Co, 99.8%) and tungsten carbide (WC, 99.5%) were purchased from Strem Chemicals (MA, USA). Co/WC mixture was prepared by simple mixing of the above powders (Co 6.0%, WC 94.0%) in an agate mortar in a proportion close to that of the industrial mixture as done in previous studies by some of us [1].

Reagents

5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), purchased from Alexis-biochemicals (CA, USA), was dissolved in distilled water, stored in the dark and used without any supplementary treatment. All other reagents were purchased from Sigma-Aldrich. For all experiments ultrapure MilliQ water was used.

Methods

Surface area measurement. The specific surface area (SSA) of Co/WC mixture (1.76 m^2/g) has been measured by means of the BET method based on N_2 adsorption at $-196^{\circ}C$ using a ASAP 2020 apparatus (Micromeritics, USA).

Cys and GSH consumption. The reaction of Cys and GSH with the Co/WC mixture was measured by incubating increasing amounts of powder (1–5 mg) with 20 ml of a 0.1 mM solution of Cys or GSH. The suspension was incubated at 25°C for 10 min and filtered through a filtering membrane (cellulose acetate, pores diameter 0.20 μm). The concentration of Cys and GSH was measured spectrophotometrically (Uvikon, Kontron Instruments, Inc., Everett, MA; $\lambda = 412$ nm) by using the Ellman's reagent [25]. The concentration of thiols in the supernatant were also measured after longer incubation times, but no further reduction of the concentration was observed after 10 min. In order to evaluate the amount of thiols consumed by the dust, the residual concentration of thiols after incubation with the dust was compared with a control thiol solution kept in the same conditions as the reacting mixture but with no dust. The experiments were performed with the simple components (WC and Co powders) in amounts corresponding to the respective content in the Co/WC mixture. All the experiments have been repeated three times.

Reaction of Cys and GSH with the superoxide radical. Superoxide radicals were produced with the xanthine/xanthine oxidase system by means of increasing concentration of xanthine (from 1.2 μM to 8.4 μM) in the presence of 3.5 mU/ml solution of xanthine oxidase. The reaction of the superoxide with a 0.05 mM solution of Cys and GSH was carried out directly in the cuvettes. The concentration of Cys and GSH was measured spectrophotometrically (Uvikon, Kontron Instruments, Inc., Everett, MA; $\lambda = 412$ nm) by using the Ellman's reagent [25]. In order to evaluate the amount of thiols consumed, the residual

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concentration of thiols after incubation with the dusts was subtracted from the control solution (no dust).

ESR spectroscopy. The release of radical species was monitored by Electron Paramagnetic Resonance (ESR) spectroscopy (Miniscope 100 X-band ESR spectrometer, Magnettech, Germany) by using the spin-trapping technique with the spin trapping agent 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO).

Generation of thiyl radicals. Seven milligrams of dust was suspended in 0.25 ml of 0.15 M DEPMPO solution in potassium phosphate buffer (PB) 1 M (pH 7.4). The reaction was started by adding to the suspension 0.25 ml of 1 M GSH or Cys solution in PB. The spectra were recorded on aliquots of 50 μl progressively withdrawn up to 1 h and filtered through a filtering membrane (cellulose acetate, pores diameter 0.20 μm).

Simulation of the ESR spectra. The simulations of the ESR experimental signals were performed using the software Winsim 2002 (National Institute of Environmental Health Science, National Institutes of Health, Bethesda, MD). The hyperfine splitting constants obtained from the optimization of the simulation were compared with those reported in the literature (NIESH STBD database).

Electrochemical study of GSH oxidation. The consumption of GSH as the result of the electrochemical reaction involving Co/WC was tested using an electrochemical cell as previously reported in more detail by some of us [26]. The cathode was a paste WC electrode, obtained by mixing the WC powder (5 g) at high pressure (7 t/cm^2). In order to overcome the poor mechanical properties of WC, 20 μl of n-dodecane as binder were added. The anode was a pure Co foil. Both the electrodes (geometrical surface of the electrodes: 7.55 cm^2) were supported on graphite and separated from each other by a felt separator supporting the electrolyte (1 m potassium phosphate buffer, pH 7.4) (Figure 3A). A 0.1 mm solution of GSH in 1 m potassium phosphate buffer (pH 7.4) was percolated through the felt separator and continuously collected at the bottom of the apparatus for up to 1 h. Every aliquot collected contains the solution percolated in 5 min. The concentration of the residual GSH was measured spectrophotometrically (Uvikon, Kontron Instruments, Inc., Everett, MA; $\lambda = 412$ nm) by using Ellman's reagent as previously described.

NMR measurements. The reaction between WC/Co and GSH or Cys has been performed in the same conditions described in paragraph 2.3.2 by using D_2O instead of H_2O. After the reaction, the suspensions were filtered through cellulose acetate filters (diameter 0.45 μm) and analysed as such. Cys ^{1}H NMR (600 MHz, D_2O): $\delta = 3.9$ (bs, 1H, CH), 3.00 (dd, $\mathcal{J} = 14.7$, 5.4 Hz, 1H, CH_2), 2.92 (dd, $\mathcal{J} = 14.7$, 3.9 Hz, 1H, CH_2), Cystine ^{1}H NMR (600 MHz, D_2O): $\delta = 4.0$ (dd, 4.0, 8.1 Hz, 1H, CH), 3.3 (dd, $\mathcal{J} = 4.0$, 15.0 Hz, 1H, CH_2), 3.1(dd, $\mathcal{J} = 8.1$, 15.0, Hz, 1H, CH_2).

Statistical analysis

Differences between data were assessed by using one-way Analysis of Variance (ANOVA) and Tukey's tests (software: SPSS 11.0 for Windows, SPSS Inc. Chicago, IL). Linear regression and correlation were calculated by using a commercial software (Origin 6.1, OriginLab Corporation).

Results

Reactivity of Co/WC toward thiols

The reactivity of the Co/WC mixture and of the single components with the thiol group is illustrated in Figure 1A (Cys) and B (GSH) which report the Cys and GSH concentration following a 10 min incubation in the respective solutions.

Co and WC alone exhibit a very low reactivity toward Cys and no reactivity toward GSH. Conversely, the mixture Co/WC dramatically decreases the concentration of Cys (Figure 1A) and at lesser extent of GSH (Figure 1B). With increment of the dust in suspension a dose effect relationship, with a linear dependence of thiol consumption vs amount of dust, is observed (Figure 2). The amount of Cys consumed per unit surface exposed of the particles, obtained from the linear regression of experimental data (0.089 mmol/m^2) is $\sim$5-fold what found for GSH (0.019 mmol/m^2).

As for the generation of ROS [1,13,15] a direct contact between Co and WC particles was required and we investigated whether this was also a requisite for the reaction with thiols. For this purpose an electrochemical cell (Figure 3A) was employed. If Co and WC electrodes are separated GSH is not consumed. Conversely, a significant consumption of GSH is observed (Figure 3B) after connecting the two electrodes by a copper connector wire. The electrochemical aspects of this experiment are reported in Francia et al. [26].

The oxidation of thiol groups to disulphide bridges generally occurs via a radical mechanism involving the generation of intermediate thiyl radicals. To determine if any sulphur-centred radicals were generated during the reaction, Co/WC dust was suspended in a buffered solution of GSH in the presence of the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO). The ESR signal registered on the suspension of Co/WC just after the

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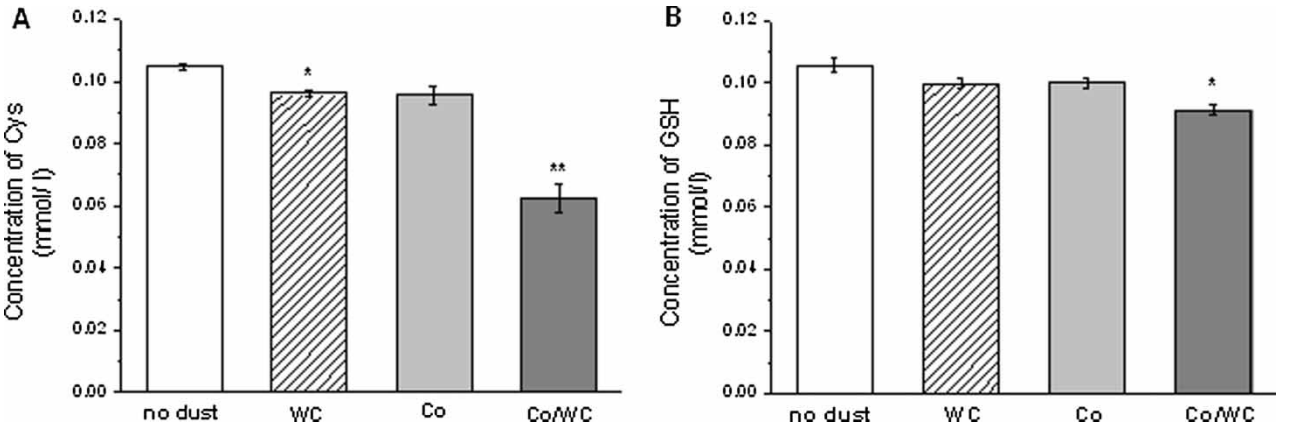

Figure 1. Reactivity of Co/WC mixture toward thiols. Residual concentration of (A) Cys and (B) GSH measured in the supernatant of a suspension of Co (0.3 mg), WC (5 mg) and the mixture Co/WC (5 mg) in a 0.1 mM solution of the thiols. The data are expressed as the mean values of three separate determinations ± SE. Vs ctrl: $*p < 0.01$, $**p < 0.001$.

addition of the reagents is shown in Figure 4, spectrum c. The eight lines signal recorded was simulated (spectrum d). The splitting constants $a_N = 13.6$ G, $a_H = 14.1$ G and $a_P = 43.9$ G obtained from the simulated spectrum are assigned to the DEPMPO/GS• adduct [27]. The signal was not detected with the two separate components of the dust (Co and WC) (spectra a, b). No signal was detected when the reaction was conducted in the presence of Cys: such results are not surprising since the adducts of nitrone spin-trap with Cys are known to exhibit a low half-life [28]

^{1}H NMR spectrometry was employed to identify the nature of the oxidized products of thiols. The spectrum recorded on the starting solution of Cys (Figure 5, spectrum a) exhibits a small amount of cystine, which may be identified since the signals of both CH_2 and CH hydrogens appears downfield shifted by respect Cys [29]. On the spectrum recorded after the reaction (Figure 5, spectrum b) no signal correspondent to Cys is observed, suggesting a full oxidation to cystine. The signal of an unidentified secondary product however is also present in the spectrum (doublet of doublet at 4.1 ppm and the partially overlapped signal under the methylene of cystine at 3.3 ppm). This secondary product could be assigned to cysteine sulphonic acid [30].

^{1}H NMR analysis was also performed on the product of oxidation of GSH. In this case, the amount of product recovered after reaction was under the limit of detection of this technique.

Reactivity of the superoxide radical in solution toward GSH and Cys

Increasing amount of superoxide radical was generated by the xanthine/xanthine oxidase system in the presence of GSH or Cys in the absence of any dust. The amount of thiols consumed, measured spectrophotometrically (Ellman's reagent), showed that

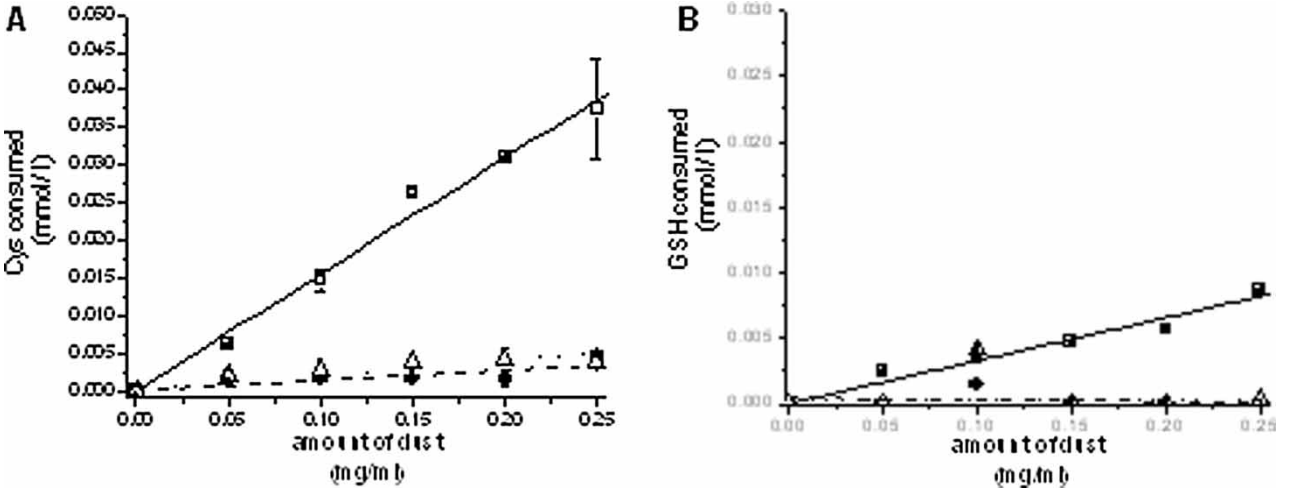

Figure 2. Correlation between the amount of Co/WC mixture and the amount of thiols consumed. Amount of (A) Cys and (B) GSH consumed by increasing amount of Co (●), WC (△) and Co/WC (□) dusts in a suspension of the sample in a 0.1 mM solution of the thiols. The data are expressed as the mean values of three separate determinations ± SE. The lines corresponds to the regression through the experimental data.

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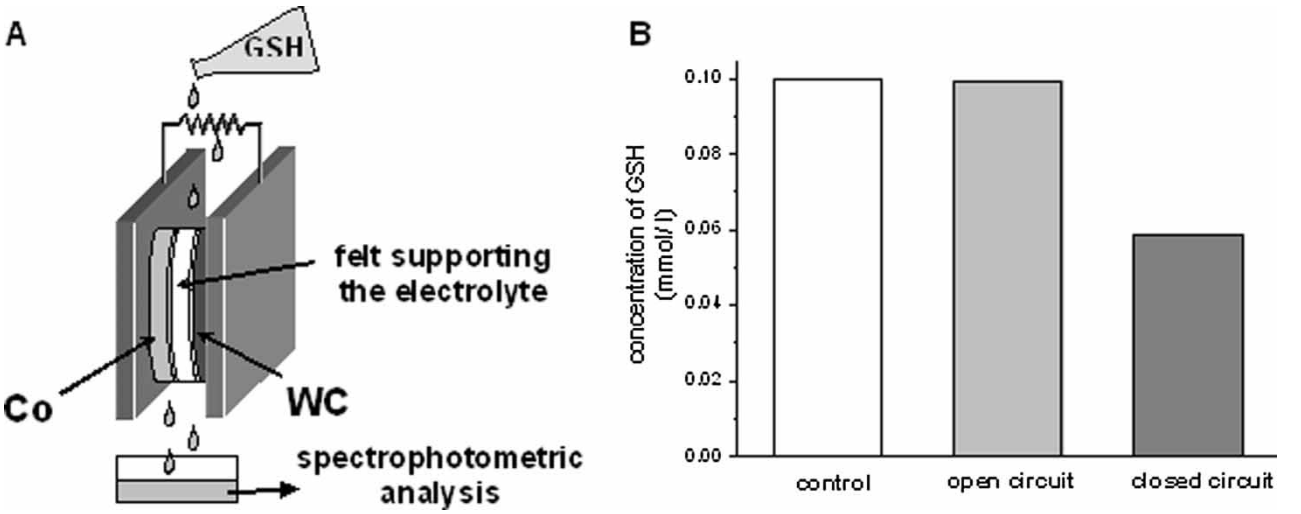

Figure 3. (A) Scheme of the electrochemical apparatus used to evidence the oxidation of GSH following the contact between Co and WC dusts. (B) Concentration of glutathione in the solution collected at the bottom of the felt supporting the electrolyte. Control: concentration of the droplets added to the felt; open circuit: regime concentration in the electrolyte after continuous adding of droplets; closed circuit: concentration 10 min after the circuit was closed.

superoxide reacts with both thiols to the same extent (Figure 6). No differences in the amount of GSH and Cys consumed were found.

Discussion

Depletion of GSH by Co/WC system

The antioxidant defenses of cells, including a range of enzymes, proteins and small molecules, may effectively keep under control an increased level of oxidant species if the levels of antioxidants are maintained sufficiently high. Conversely, if a depletion of such species occurs, any oxidative damage will be enhanced.

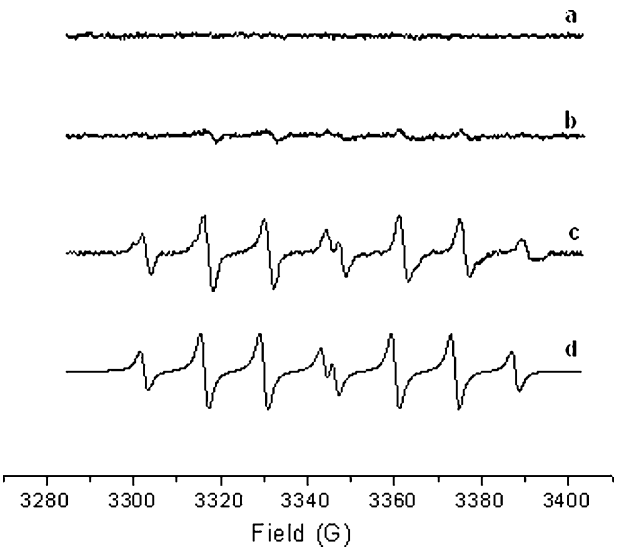

Figure 4. Identification of the glutathionyl radical. ESR spectra registered on a suspension of Co (spectrum a), WC (spectrum b) and Co/WC (spectrum c) in a buffered solution (pH 7.4, PB, 1 M) of GSH 0.5 M and DEPMPO 0.075 M. Spectrum d is the simulation for spectrum c. The eight lines signal characterized by the splitting constants $a_N = 13.6$ G, $a_H = 14.1$ G and $a_P = 43.9$ G in spectrum c is assigned to the DEPMPO/GS$^{\cdot}$ adduct. Instruments settings: receiver gain 9×10^2, microwave power 10 mW; modulation amplitude 1G; scan time 70 s, two scans.

Following exposure to toxic particulates, e.g. asbestos and artificial fibres, a depletion of GSH was reported [20,31–35]. Previous studies reported a direct reaction of GSH with the surface of quartz particles [36] and amosite asbestos [20], suggesting a marked depletion of GSH *in vivo* which would favour oxidative stress.

The antioxidant properties of GSH are based on its ability to act as a hydrogen donor, through the thiol group of Cys which may be converted to the correspondent thiyl radicals by reacting with other radical species. The sulphur-centred radicals formed easily dimerize to form disulphide bridges [37]:

$$\text{R-SH} + \text{R}^{\bullet} \rightarrow \text{R-S}^{\bullet} + \text{RH}$$

$$2\text{R-S}^{\bullet} \rightarrow \text{R-S-S-R}$$

Thiyl radicals may also react with Cys residues of proteins to form mixed disulphide, also involved in redox regulation [24], or react with oxygen to form products at a higher oxidation state [38].

The aim of the present paper is to evaluate if Co/WC mixture, which is the toxic entity in hard metals, would react similarly to quartz and asbestos [20,31,32,36], with the thiol group in Cys both as a simple molecule or as a GSH component, causing a depletion of GSH.

The concentration of GSH and Cys is significantly reduced in the presence of the Co/WC mixture, while the single components alone do not react or react to a much lesser extent with GSH (Figure 1B) and Cys (Figure 1A). The extent of the reduction of the thiols concentration correlates to the amount of dust and, consequently, with the surface exposed (Figure 2). If

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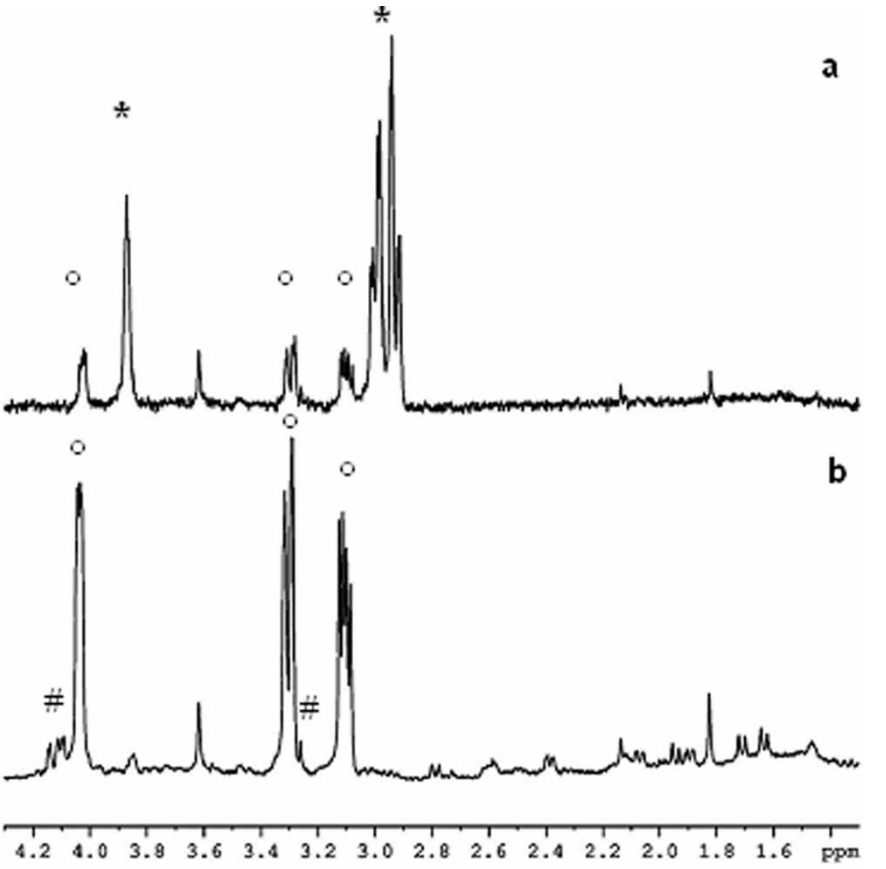

Figure 5. ^{1}H NMR spectra of the products of the reaction between Cys and Co/WC. (A) starting solution of Cys, (B) the solution after contact with Co/WC dust. *Cys; ○ cystine; # unidentified secondary product.

the amount of GSH or Cys consumed is reported on a unit of surface area basis, the specific reactivity of the dusts toward GSH and Cys is obtained. The reactivity of Co/WC mixture toward thiols is quite substantial, reaching values of 0.089 mmol/m^2 for Cys and 0.019 mmol/m^2 for GSH, i.e. Cys alone

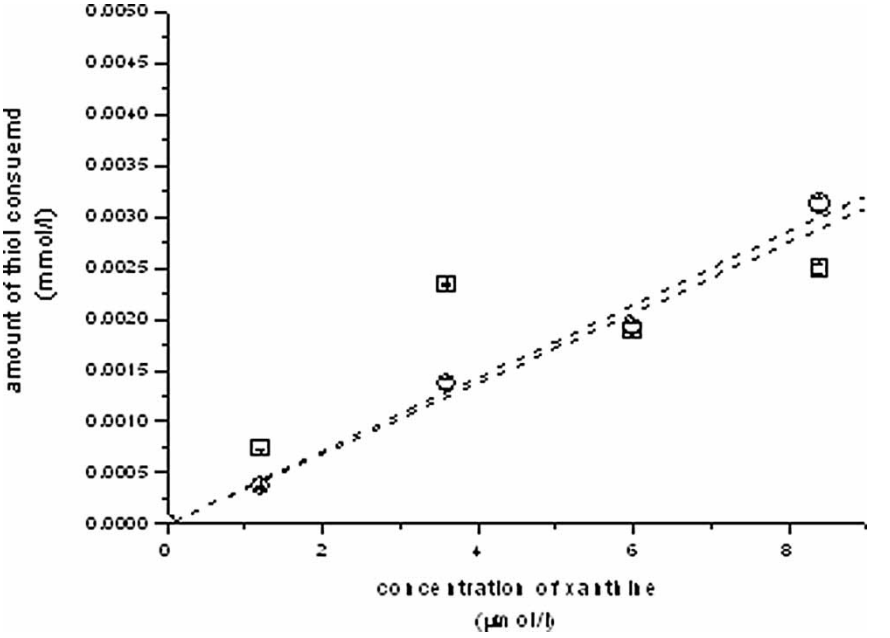

Figure 6. Consumption of GSH ○ and Cys □ by superoxide radical generated in solution by the xanthine/xanthine oxidase system. Data are expressed as the mean values of three separate determinations ± SE. The lines corresponds to the regression through the experimental data.

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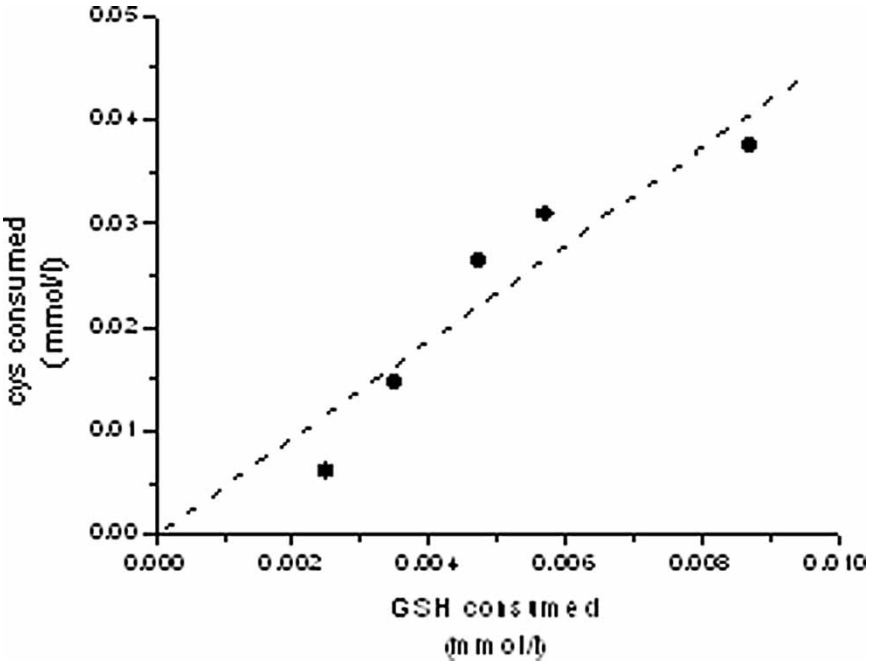

Figure 7. Amount of Cys vs amount of GSH consumed for each of the tested doses of material. The straight line is obtained from the regression through the experimental data ($p < 0.01$). The slope of the line (4.7) indicates the ratio between Cys and GSH consumption, i.e. among about five surface sites reacting with Cys, only one will react with GSH.

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appears to react with Co/WC to a larger extent than the cysteinyl fragment in the tripeptide GSH. Such consumption is the consequence of an oxidation of the thiols caused by Co/WC which occurs for GSH, but likely also for Cys, via the formation of a sulphur-centred radicals (Figure 4) [27]. The reaction appears to go even beyond the simple formation of disulphuric bridges, as supported by the production of a secondary product (Figure 5) [30] in the reaction of Co/WC with Cys.

Mechanism of oxidation of thiols

The extent of oxidation is directly related to the amount of dust in the suspension (Figure 2). The reaction will occur either at the surface of the particles or as a consequence of ROS released by the solid. The exposed surface obviously increases by increasing the amount of dust in suspension and the observed linear correlation (Figure 2) suggests a mechanism dependent from the amount of surface exposed.

The amount of Cys consumed is much higher than that of GSH. Reporting for all the tested doses the amount of Cys or GSH consumed, the experimental points lay confidently on a straight line passing through the origin (Figure 7). The slope indicates that among five surface reactive sites which would oxidize Cys, one only would be able to oxidize GSH.

Since no differences in the reactivity of Cys and GSH toward superoxide in solution were observed (Figure 6) the higher reactivity of Cys in respect to GSH suggests the occurrence of a direct reaction with reactive sites at the particle surface. In this case, in fact, a steric hindrance effect may account for the lower reactivity of the less accessible –SH group in GSH.

The nature of such reactive sites remain unknown, but the presence of superoxide or superoxide-like species may be hypothesized. Unfortunately, such radical species, when bound at the surface, may not be identified in the ESR spectroscopy of the solid because of the electrical conductivity of the particles mixture.

Co and WC alone did not consume GSH (Figure 1B) or consumed a minor amount of Cys (Figure 1A) compared to the Co/WC mixture. Therefore, similarly to what was observed for the homolytic cleavage of the C-H bond [1], the contact between Co and WC particles is necessary for the generation of active sites as confirmed by the electrochemical study (Figure 3).

Relevance of the results for hard-metal-induced lung diseases

The data presented herein reveal the existence of an alternative reaction which would further deplete the tissue fluids of GSH and Cys. The ability of Co/WC dusts to oxidize GSH and Cys may contribute to the overall effects caused by the inhalation of hard metal dusts, by inhibiting the antioxidant defences and enhancing the Co/WC or cell-derived oxidative stress. Since it is impossible to predict the amplitude of such a reaction *in vivo*, the involvement of the observed oxidation of thiols at the surface of Co/WC mixtures

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in the overall mechanism of toxicity is difficult to asses. It was previously reported that the exposure to Co^{2+} ions causes a depletion of thiols, but this depletion was not related to cell dysfunction [39,40]. The amount of GSH consumed by the Co/WC mixture in the conditions used in our experiments (0.02 mmol/L) are lower than the physiological concentration of GSH in lung tissues (0.5–10 mm [18]). The long-term persistence of the particles in the lung or inside cells, however, may progressively lead to a consistent depletion of GSH with remarkable loss in antioxidant defences. Furthermore, the formation of glutathionyl radicals, disulphides and, possibly, secondary oxygenated products during the reaction may also have some important effects on the regulation of the redox system of cells.

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