Molecular and Cellular Biochemistry **279:** 17–23, 2005.
DOI: 10.1007/s11010-005-8211-6
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Effect of stainless steel manual metal arc welding fume on free radical production, DNA damage, and apoptosis induction

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Abstract

Questions exist concerning the potential carcinogenic effects after welding fume exposure. Welding processes that use stainless steel (SS) materials can produce fumes that may contain metals (e.g., Cr, Ni) known to be carcinogenic to humans. The objective was to determine the effect of *in vitro* and *in vivo* welding fume treatment on free radical generation, DNA damage, cytotoxicity and apoptosis induction, all factors possibly involved with the pathogenesis of lung cancer. SS welding fume was collected during manual metal arc welding (MMA). Elemental analysis indicated that the MMA-SS sample was highly soluble in water, and a majority (87%) of the soluble metal was Cr. Using electron spin resonance (ESR), the SS welding fume had the ability to produce the biologically reactive hydroxyl radical ($^{\bullet}OH$), likely as a result of the reduction of Cr(VI) to Cr(V). *In vitro* treatment with the MMA-SS sample caused a concentration-dependent increase in DNA damage and lung macrophage death. In addition, a time-dependent increase in the number of apoptotic cells in lung tissue was observed after *in vivo* treatment with the welding fume. In summary, a soluble MMA-SS welding fume was found to generate reactive oxygen species and cause DNA damage, lung macrophage cytotoxicity and *in vivo* lung cell apoptosis. These responses have been shown to be involved in various toxicological and carcinogenic processes. The effects observed appear to be related to the soluble component of the MMA-SS sample that is predominately Cr. A more comprehensive *in vivo* animal study is ongoing in the laboratory that is continuing these experiments to try to elucidate the potential mechanisms that may be involved with welding fume-induced lung disease. (Mol Cell Biochem **279:** 17–23, 2005)

Key words: apoptosis, chromium, electron spin resonance, free radical, stainless steel, welding fume

Introduction

Welding processes produce gaseous and aerosol by-products that are composed of a complex mixture of metal oxides [1]. Welding fume is vapourised metal that reacts with air and forms particles that are of respirable size. The metal composition of the fume is mostly derived from the welding electrode or wire consumed during the process. Welding processes that use stainless steel (SS) materials can produce fumes that may contain metals known to be carcinogenic to humans. Two such metals include chromium (Cr) and nickel (Ni).

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It has been estimated that nearly 400,000 workers in the United States were employed full-time as welders, cutters, solderers and brazers during the year of 2002 [2]. However, it is believed that greater than two million workers worldwide perform welding as part of their work duties. The health effects of welding are oftentimes difficult to study because welders work in a variety of locations (e.g., well-ventilated indoor and outdoor setting vs. poorly ventilated confined spaces) and are exposed to a number of substances (e.g., asbestos, silica and solvents) that may be toxic to the respiratory system.

One serious health concern associated with the inhalation of welding fumes is the possible development of lung cancer. *In vitro* studies have indicated that SS welding fumes are both toxic and mutagenic to mammalian cells [3, 4]. The International Agency for Research on Cancer (IARC) has concluded that welding fumes were "possibly carcinogenic" to humans, despite the fact that the finding was based on limited evidence in humans and inadequate evidence in laboratory animals [5]. A number of epidemiology studies have observed a significant elevated risk for lung cancer among welders [6–9], whereas others have not [10–12]. Animals studies addressing this issue are mostly non-existent [as reviewed by 13, 14].

Many questions remain unanswered concerning the potential carcinogenic effects of welding fume inhalation. In addition, incomplete information exists regarding the causality and possible underlying molecular mechanisms associated with welding fume exposure and pulmonary disease. It was the goal of this study to determine the effect of *in vitro* and *in vivo* welding fume treatment on free radical generation, DNA damage, cytotoxicity and apoptosis induction, all factors possibly involved with the pathogenesis of lung cancer. SS welding fume was collected during manual metal arc welding (MMA), a commonly used process by the welding community.

Materials and methods

Reagents

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), iron sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione reductase (GSSG-R), sodium formate, and RPMI 1640 culture media. Sodium dichromate dihydrate ($Na_2Cr_2O_7 \cdot 2H_2O$) was purchased from Aldrich Chemical Co., Milwaukee, WI. Catalase was purchased from Boehringer-Mannheim, Indianapolis, IN. Phosphate-buffered saline (PBS) and DNA λ *Hind* III fragments were purchased from Gibco BRL, Gaithersburg, MD. The spin trap, DMPO, was purified by charcoal decolourisation and vacuum distillation to remove any possible electron spin resonance detectable impurities. PBS was treated with Chelex 100 (Bio-Rad Laboratories, Richmond, VA) to remove any possible transition metal ion contaminants.

Welding fume collection and characterization

The collection of the welding fume was previously described by Antonini *et al.* [15]. The welding fume sample was obtained from Lincoln Electric Co. (Cleveland, OH), courtesy of Kenneth Brown. Briefly, the fume was generated in a cubical open-front fume chamber (volume $= 1\ m^3$) by a skilled welder during manual metal arc welding using a stainless steel electrode (MMA-SS) and collected on 24 cm qualitative cellulose filter paper (Fisher Scientific, Pittsburgh, PA) during intermittent 2 min periods of welding. The amounts of seven different metals commonly found in welding fume (Cr, Cu, Fe, Mn, Ni, Ti and V) were measured using inductively coupled argon plasma atomic emission spectroscopy (ICP-AES) according to the method of NIOSH [16] and were previously reported [17]. See Table 1 for elemental composition of welding sample used in the study. The particle size of the fume was found to be of respirable size with a count mean diameter of $<2.0\ \mu m$ as previously reported [15].

In addition, the MMA-SS sample was divided into soluble and insoluble components. The particle sample was suspended in PBS and incubated for 24 h at 37 °C. After incubation, the sample was centrifuged at $12{,}000 \times g$ for 30 min. The supernatant of the sample (soluble fraction) was recovered and filtered with 0.22 μm filters (Millipore Corp., Bedford, MA). Analysis of the metal constituents of the soluble fractions was determined by ICP-AES (see Table 1).

Table 1. Elemental composition of MMA-SS welding fume[a]

Welding sample	Dry weight ($\mu g/mg$)	Weight (%)[b]
Total particle suspension		
Fe	33.4	41.1
Cr	23.2	28.5
Mn	13.6	16.7
Ni	2.06	2.53
Soluble fraction only		
Fe	0.2	0.46
Cr	38.2	87.2
Mn	5.1	11.6
Ni	0.3	0.68

[a]Data presented were modified from [17].
[b]Relative to the specific metals analysed.

Laboratory animals

Experiments that involved animals used adult male Sprague-Dawley rats (Hla: [SD] CVF; 200–250 g; Hilltop Laboratory Animals, Scottdale PA). The animals were free of endogenous viral pathogens, parasites, mycoplasmas, Helicobacter and CAR Bacillus. They were kept in ventilated cages and provided with HEPA-filtered air, autoclaved Prolab 3500 diet and tap water *ad libitum* under a controlled light cycle (12 h light/12 h dark) and temperature (22–24 °C) conditions. Facilities are AAALAC-accredited, specific pathogen-free and environmentally controlled. The rats were acclimated to the animal facility for at least 1 week after arrival. All procedures involving animals were performed under protocols approved by the NIOSH Institutional Animal Care and Use Committee.

Free radical production

Electron spin resonance (ESR) with spin trapping was used to examine free radical generation. Spin trapping involves the addition reaction of a short-lived radical with a paramagnetic compound (e.g., spin trap) to form a relatively long-lived free radical product, termed the spin adduct, which can be studied with conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radical trapped, and the pattern of hyperfine splittings of the spin adduct is generally characteristic of the original, short-lived, trapped radical. To determine the presence of hydroxyl radicals ($^{\bullet}OH$) and Cr(V), DMPO was used as a spin trap. Measurements were made with a Bruker EMX spectrometer and a flat cell assembly (Bruker Instruments Inc., Billeria, MA). Acquisit software (Bruker Instruments Inc.) was used for data collection and analysis. The Fenton reaction ($FeSO_4 + H_2O_2$) was used to generate $^{\bullet}OH$ radicals as a positive control for one system, and Cr(VI) ($Na_2Cr_2O_7$), NADPH and glutathione reductase (GSSG-R) were used in another one. Catalase and sodium formate also were used in the ESR studies to further define free radical generation. For final concentrations for reactants used for ESR studies, see Fig. 1.

In vitro *DNA strand breakage*

A DNA strand break assay was carried out according to methods described earlier [18]. Briefly, reactions were performed in 10 mM PBS (pH 7.4) in 1.5 ml polypropylene tubes at 37 °C. Each reaction mixture contained 10 μg DNA λ *Hind* III fragments in a total volume of 100 μl of buffer. To this solution, 2 μl of gel loading buffer (50 mM EDTA, 2.5% sodium dodecyl sulfate and 0.1% bromophenol blue) were added, and then electrophoresis was performed in 0.7% agarose at 1–2 V/cm in 40 mM tris acetate buffer containing 2 mM EDTA (pH 8.0). Gels were stained in ethidium bromide

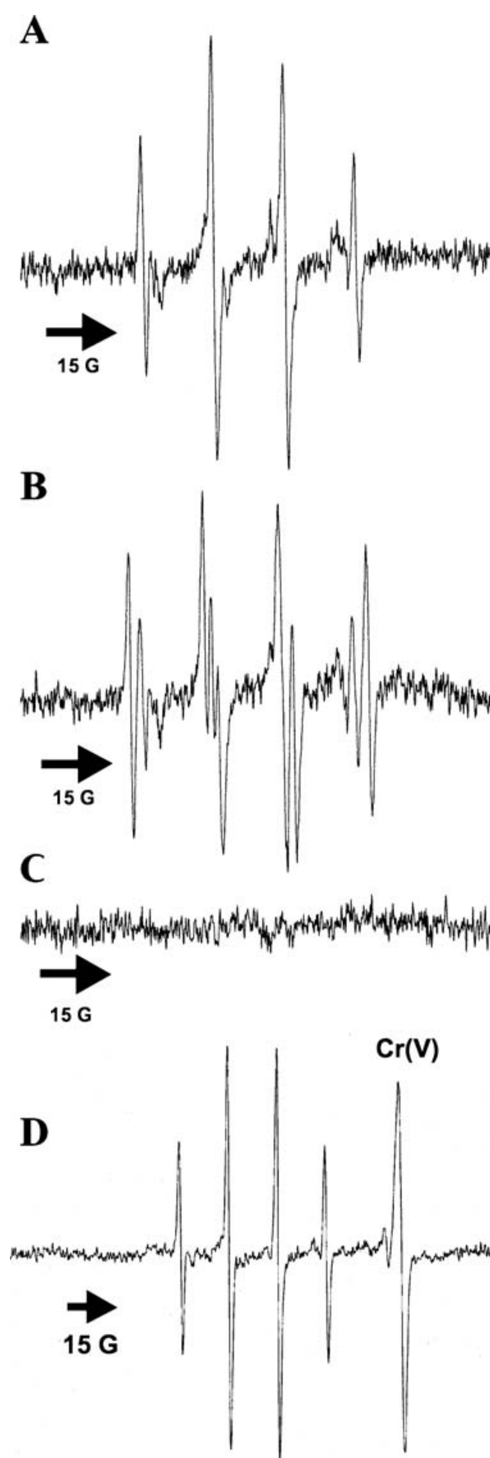

Fig. 1. Electron spin resonance spectra of MMA-SS welding fume (1.0 mg/ml) with DMPO (100 mM). (A) MMA-SS with H_2O_2 (10 mM); (B) MMA-SS with H_2O_2 (10 mM) and sodium formate (500 mM); (C) MMA-SS with H_2O_2 (10 mM) and catalase (2000 U/ml); (D) MMA-SS with NADPH (5 mM) and GSSG-R (0.5 mg/ml); Cr(V) peak has been indicated. All arrows represent 15 G and direction of scan.

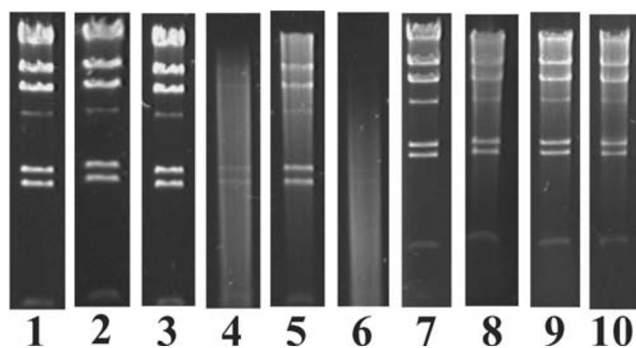

Fig. 2. *In vitro* DNA damage caused by MMA-SS welding fume. (Lane 1) untreated control DNA; (Lane 2) DNA + H_2O_2 (10 mM); (Lane 3) DNA + MMA-SS (1.0 mg/ml); (Lane 4) DNA + H_2O_2 (10 mM) + $FeSO_4$ (1 mM; positive control); (Lane 5) DNA + H_2O_2 (10 mM) + MMA-SS (0.1 mg/ml); (Lane 6) DNA + H_2O_2 (10 mM) + MMA-SS (1.0 mg/ml); (Lane 7) DNA + NADPH (0.1 mM) + GSSG-R (0.05 mg/ml); (Lane 8) DNA + NADPH (0.1 mM) + GSSG-R (0.05 mg/ml) + $Na_2Cr_2O_7$ (2 mM; Cr(VI) positive control); (Lane 9) DNA + NADPH (0.1 mM) + GSSG-R (0.05 mg/ml) + MMA-SS (0.1 mg/ml); (Lane 10) DNA + NADPH (0.1 mM) + GSSG-R (0.05 mg/ml) + MMA-SS (1.0 mg/ml).

(5 μg/ml) for 30 min and photographed under UV light using a Stratagene Eagle Eye II (Stratagene Inc., La Jolla, CA, USA). The Fenton reaction ($FeSO_4$ + H_2O_2) was used to generate $^\bullet$OH radicals as a positive control for one system, and Cr(VI), NADPH, and GSSG-R were used in another one. For final concentrations for reactants used for DNA damage studies, see Fig. 2.

In vitro *cell viability*

Bronchoalveolar lavage was performed on untreated male Sprague-Dawley rats to recover primary lung macrophages as previously described [15]. It was found that >98% of the recovered cells were macrophages. Viability of the recovered cells was assessed by trypan blue exclusion and found to be >95%. The lung macrophages were suspended in sterile RPMI 1640 culture medium (pH 7.4) enriched with 10% fetal bovine serum and suspended in a 1.5 ml microfuge tubes at a concentration of 3×10^5 cells/ml. The macrophages were treated with 6.25, 25 or 100 μg/ml of MMA-SS particles suspended in sterile RPMI and incubated for 24 h at 37 °C containing air and 5% CO_2 ($n = 6$ rats/particle group). Before cell treatment, all particles were sonicated for 1 min. The control group received an equal volume of sterile PBS. Cell viability was measured on aliquots from each microfuge tube by trypan blue exclusion.

In vivo *lung treatment and apoptosis detection*

Male Sprague–Dawley rats were intratracheally instilled with 1 mg/100 g body weight of the MMA-SS welding fume sample that had been suspended in sterile PBS according to the method of Reasor and Antonini [19]. Control animals were intratracheally treated with sterile PBS (vehicle control). At days 3, 6 and 10 after treatment, the animals were humanely euthanized by an intraperitoneal injection of sodium pentobarbital (>100 mg/kg body weight; Bulter Co., Columbus, OH), and their lungs were inflated with Tissue-Tek O.C.T. compound (Sakura Finetek, Inc., Torrance, CA) to capacity. Lung slices were placed in OCT in vinyl cryomolds, frozen in liquid nitrogen-cooled isopentane, and sections cut at 6 μm were placed on microscope slides for apoptosis the assay. Apoptosis of pulmonary cells was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using a TUNEL assay kit (Promega, Madison, WI). In this apoptotic detection system, the fragmented DNA of apoptotic cells is measured by enzymatically incorporating fluorescein-12-dUTP at the 3′-OH end of the DNA using Terminal Deoxynucleotidyl Transferase (TdT). The frozen lung sections were vapour-fixed with paraformaldehyde and treated with protease. For a positive control, a slide was prepared using a DNase 1 incubation for 30 min at room temperature to produce nicked DNA. For a negative control, the TdT enzyme was replaced by distilled water in the reaction mix. Propidium iodide was applied as a counter stain, after which the slides were rinsed in distilled water and cover slipped using anti-fade Gel Mount (Biomeda, Foster City, CA). The slides were examined using a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY) using a 40× objective.

Statistical analysis

Data in Fig. 3 are presented as means ± S.E.M. Comparisons between means in Fig. 3 were made using ANOVA followed by Tukey's *post hoc* test. Statistical significance was established when $p < 0.05$.

Results

ESR was used to assess the ability of MMA-SS welding fume to produce free radicals in a cell-free system. When the welding fume sample was reacted with H_2O_2 in the presence of DMPO, a characteristic DMPO-$^\bullet$OH spin adduct signal was produced, indicating the generation of $^\bullet$OH radicals (Fig. 1A). The spectrum produced consisted of a 1:2:2:1 quartet with splitting of $a_H = a_N = 14.9$ G. Based on these splitting constants, the 1:2:2:1 quartet was assigned to a DMPO-$^\bullet$OH adduct. With the addition of sodium formate to the system, the intensity of the DMPO-$^\bullet$OH spin adduct signal was decreased and resulted in the appearance of a new spin adduct signal with hyperfine splittings of $a_H = 15.8$ G

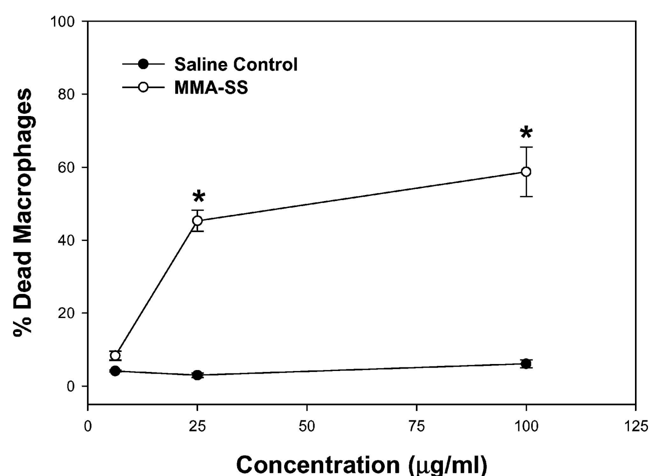

Fig. 3. Percentage of dead macrophages as measured by trypan blue exclusion after a 24 h incubation with MMA-SS welding fume or saline (vehicle control). Final concentrations of MMA-SS welding fumes were 6.25, 25 and 100 μg/ml. Values are means ± S.E.M. ($n = 6$); The asterisk (*) indicates values significantly greater than all saline control values and the MMA-SS value at the lowest concentration ($p < 0.05$).

and $a_N = 18.8$ G (Fig. 1B). These splittings are typical of those of a DMPO-$^{\bullet}COO^-$ adduct, also demonstrating that $^{\bullet}OH$ radicals were generated. After the addition of catalase, an enzyme that converts H_2O_2 to water and oxygen, $^{\bullet}OH$ radical generation was completely blocked (Fig. 1C). Although Cr(VI) cannot be directly measured with ESR, its reduction to Cr(V) with the generation of the $^{\bullet}OH$ radical can be monitored [20]. When reacting the welding fume sample with NADPH and GSSG-R in the presence of DMPO, a Cr(V) signal with an accompanying DMPO-$^{\bullet}OH$ signal was formed (Fig. 1D).

An *in vitro* DNA strand breakage assay was used to determine whether MMA-SS welding particles damaged DNA as a result of the generation of $^{\bullet}OH$ radicals (Fig. 2). The bands of the plasmid DNA are sharp and distinct when no DNA damage is present as was observed in lanes 1 (DNA only), 2 (DNA + H_2O_2), 3 (DNA + MMA-SS), and 7 (DNA + NADPH + GSSG-R). When DNA and H_2O_2 were added together with $FeSO_4$ (lane 4), the DNA was damaged as evidenced by a smearing of the DNA bands, likely resulting from $^{\bullet}OH$ radical formation via the Fenton reaction. When DNA and H_2O_2 were added together with 0.1 or 1.0 mg/ml MMA-SS welding sample (lanes 5 and 6, respectively), a concentration-dependent increase in DNA damage was observed, resulting from $^{\bullet}OH$ radical formation. The addition of DNA with NADPH and GSSG-R in the presence of $Na_2Cr_2O_7$ (Cr(VI) positive control, lane 8), 0.1 mg/ml of MMA-SS (lane 9), or 1.0 mg/ml of MMA-SS (lane 10) caused DNA damage, resulting in the smeared appearance of the bands due to the generation of $^{\bullet}OH$ from the reduction of Cr(VI) to Cr(V).

To determine the potential cytotoxic effect of MMA-SS welding fume on cell viability, primary rat lung macrophages were incubated for 24 h with different concentrations of the particle sample (Fig. 3). Treatment of the cells with the MMA-SS particles caused a concentration-dependent increase in macrophage death. At the lowest concentration (6.25 μg/ml), cell death was not significantly different when comparing the saline control group with the MMA-SS group. However, at the two higher particle concentrations (25 and 100 μg/ml), macrophage death was significantly elevated after treatment with the MMA-SS particles compared to saline.

To assess *in vivo* apoptosis induction in lung tissue after intratracheal instillation of MMA-SS welding particles, a TUNEL assay was performed (Fig. 4). Treatment with the welding sample caused a time-dependent increase in the number of green/yellow-stained cell nuclei (indicative of apoptosis) in the airspaces and lung parenchyma as depicted in representative lung tissue slices. The number of apoptotic cells was significantly increased in the MMA-SS group at days 6 and 10 compared to the saline control group.

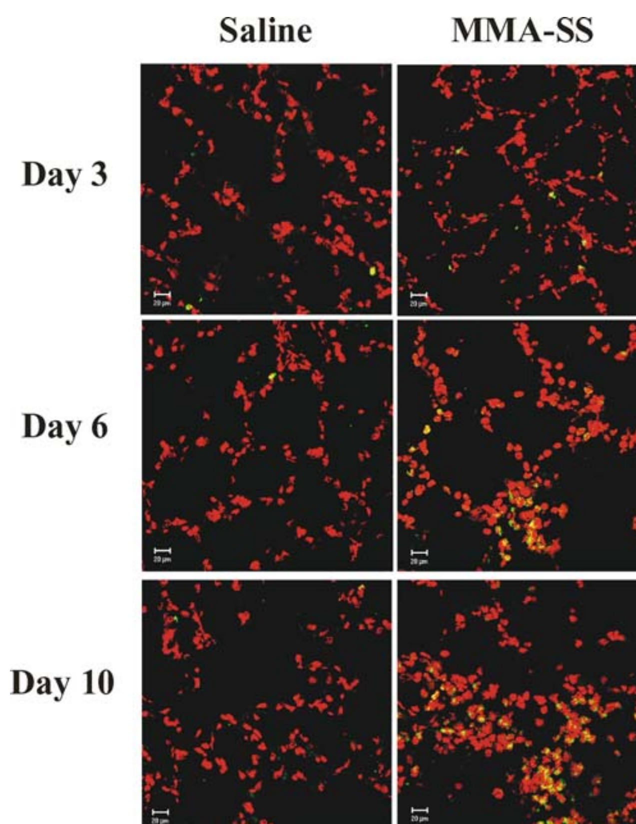

Fig. 4. Representative images of *in vivo* apoptosis induction at days 3, 6 and 10 after intratracheal treatment with MMA-SS (1 mg/100 g body weight) or the equivalent volume of sterile saline (vehicle control). TUNEL assay was performed on frozen lung sections. Green/yellow nuclei are stained positive by TUNEL, indicating apoptotic DNA nicking in the cell nucleus. All other cell nuclei stained red with propidium iodide.

Discussion

Welding fumes contain transition metals, such as Fe, Cr, and Ni, that are capable of generating free radicals. In previous animal and cell culture studies, we have observed that treatment with SS welding fumes may cause the production of free radicals by pulmonary cells [15, 21, 22]. SS welding fumes differ from other welding fumes (e.g., mild steel) in that Cr is present in significant amounts. It has been shown that SS welding particles contain Cr(VI) [23]. Previous analysis of the MMA-SS welding particle sample used in the current study indicated that the fume was quite soluble in water, and a majority (87%) of the soluble metal was Cr [15].

Soluble Cr(VI) has been shown to enter cells in a dose-dependent manner at low concentrations [24]. Once inside the cell, Cr(VI) is reduced to Cr(V) and Cr(IV). In addition, molecular oxygen is reduced to $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ radical during redox cycling. It was demonstrated in the current study that the highly soluble MMA-SS welding fume reacted with H_2O_2 to generate $^{\bullet}OH$ via a Fenton-type reaction. Also, in the presence of NADPH and GSSG-R, the MMA-SS sample generated both a Cr(V) intermediate and a $^{\bullet}OH$ radical. Previously, it was observed that mild steel and water-insoluble SS welding fumes did not generate free radicals compared to MMA-SS fumes when assessed by ESR under the same reaction conditions [17]. Thus, it appears the reactivity of the MMA-SS fume is likely due to the presence of Cr and resides within the soluble fraction of the sample that is predominately Cr.

It was hypothesized that free radical reactions mediated by Cr in soluble MMA-SS welding fumes may be involved in lung disease development. Reactive oxygen species have been reported to play a key role in Cr-induced DNA damage [25]. Cell cycle surveillance mechanisms set checkpoints for cell cycle progression. If the cell damage by Cr(VI) results in DNA breaks, cell cycle progression will be transiently delayed to allow the cell to repair the damaged DNA. If the DNA is damaged severely enough, the cell will undergo apoptosis. Apoptosis is a defence mechanism by which the body can control cell number in tissues and by which damaged cells can be eliminated. In diseases such as cancer, there may be an imbalance between the rate of cell division and that of elimination of damaged cells, influencing the anomalous accumulation of neoplastic cells [26, 27].

Several studies have shown that Cr(VI) induces DNA damage in a variety of *in vitro* test systems [24, 25, 28–30]. Using an *in vitro* DNA strand breakage assay, we observed that MMA-SS welding fume caused a concentration-dependent increase in DNA damage that was similar to what was observed when using a soluble Cr salt, likely as a result from $^{\bullet}OH$ radical formation. In addition, the MMA-SS sample used in the current study caused a concentration-dependent increase in lung macrophage death. Previously, we have shown that the soluble MMA-SS welding fume was more cytotoxic than mild steel and insoluble SS welding fumes [15], further implicating Cr within the soluble fraction as the potential agent of concern.

In addtion, it was our interest to evaluate the apoptotic response of the lung using an *in vivo* animal model. D'Agostini *et al.* [31] used the TUNEL method to assess apoptosis of cells from embedded lung tissue after intratracheal treatment with sodium dichromate. They observed a marked increase in the number of apoptotic cells in both the bronchial epithelium and lung parenchyma of Cr(VI)-treated rats. Using a similar method in the current study, we saw a time-dependent increase in the number of apoptotic cells in the lung airspaces and parenchyma of animals intratracheally instilled with the MMA-SS welding sample.

In summary, it has been demonstrated in the described study that soluble MMA-SS welding fume can induce the generation of reactive oxygen species that may result in DNA damage, lung macrophage cytotoxicity and *in vivo* lung cell apoptosis. These types of responses have been shown to be involved in various toxicological and carcinogenic processes. The effects observed appear to be related to the soluble component of the MMA-SS sample that is predominately Cr. A larger scale study is currently underway in the laboratory to continue these experiments to try to further examine these observations and attempt to elucidate the potential mechanisms that may be involved with welding fume-induced lung responses. Experiments are being performed that are comparing the *in vivo* potential of different welding fume samples with vastly different chemical composition to cause DNA damage and apoptosis. It is also important to evaluate the role that the insoluble fraction of the welding fume sample has on the pulmonary responses after exposure. Thus, comparisons are being made in ongoing studies between the insoluble and soluble fractions of the MMA-SS fume as well as with soluble Cr salts. In addition, tumour-susceptible animal models (e.g., A/J mice) are being used to assess the tumourigenicity of SS welding fumes.

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