Role of metal-induced reactive oxygen species generation in lung responses caused by residual oil fly ash

ANTHONY B LEWIS[†], MICHAEL D TAYLOR, JENNY R ROBERTS, STEPHEN S LEONARD, XIANGLIN SHI and JAMES M ANTONINI*

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA

[†]Department of Safety and Environmental Management, College of Engineering and Mineral Resources, Morgantown, WV 26506, USA

**Corresponding author (Fax, 304 285-5938; Email, jga6@cdc.gov)*

Inhalation of residual oil fly ash (ROFA) increases pulmonary morbidity in exposed workers. We examined the role of reactive oxygen species (ROS) in ROFA-induced lung injury. ROFA was collected from a precipitator at Boston Edison Co., Everett, MA, USA. ROFA (ROFA-total) was suspended in saline, incubated for 24 h at 37°C, centrifuged, and separated into its soluble (ROFA-sol.) and insoluble (ROFA-insol.) fractions. Sprague-Dawley rats were intratracheally instilled with saline or ROFA-total or ROFA-sol. or ROFA-insol. (1 mg/100 g body wt.). Lung tissue and bronchoalveolar lavage cells were harvested at 4, 24, and 72 h after instillation. Chemiluminescence (CL) of recovered cells was measured as an index of ROS production, and tissue-lipid-peroxidation was assessed to determine oxidative injury. Significant amounts of Al, Fe, and Ni were present in ROFA-sol., whereas ROFA-insol. contained Fe, V, and Al. Using electron spin resonance (ESR), significantly more hydroxyl radicals were measured in ROFA-sol. as compared to ROFA-insol. None of the ROFA samples had an effect on CL or lipid peroxidation at 4 h. Treatment with ROFA-total and ROFA-insol. caused significant increases in both CL (at 24 h) and lipid peroxidation (at 24 and 72 h) when compared to saline control value. ROFA-sol. significantly reduced CL production at 72 h after treatment and had no effect on lipid peroxidation at any time point. In summary, ROFA, particularly its soluble fraction, generated a metal-dependent hydroxyl radical as measured by a cell-free ESR assay. However, cellular oxidant production and tissue injury were observed mostly with the ROFA-total and ROFA-insol. particulate forms. ROS generated by ROFA-sol. as measured by ESR appear not to play a major role in the lung injury caused after ROFA exposure.

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1. Introduction

Epidemiology has demonstrated that inhalation of elevated levels of ambient air pollutants is associated with increased respiratory morbidity and mortality (Schwartz 1994). Residual oil fly ash (ROFA) is a particulate pollutant released into the environment due to combustion of fossil fuels. ROFA is a complex mixture composed of metals, sulphates, acids, and other unknown materials. It has been estimated that fly ash contributes more than $2{\cdot}5 \times 10^5$ tons to the ambient air particulate matter burden in the United States annually (Costa and Dreher 1997).

Keywords. Electron spin resonance; lung; reactive oxygen species; residual oil fly ash

Abbreviations used: AM, Alveolar macrophage; BAL, bronchoalveolar lavage; CL, Chemiluminescence; Def, deferoxamine; ESR, electron spin resonance; ROFA, residual oil fly ash; ROS, reactive oxygen species.

Occupational exposure to ROFA has been associated with adverse respiratory health effects in exposed workers (Hauser *et al* 1995). The bioavailability of soluble transition metals has been implicated as one of the mechanisms by which ROFA may damage the lungs (Dreher *et al* 1997; Kodavanti *et al* 1998). The generation of highly reactive oxygen species (ROS) also perhaps results in the pulmonary injury and inflammation associated with ROFA (Kadiiska *et al* 1997). ROS also play an important role in the pathogenesis of occupational lung disease (Vallyathan and Shi 1997). The objective of this study was to characterize the ability of ROFA to produce ROS with electron spin resonance (ESR) by analysing the soluble and insoluble fraction of a collected particulate sample from an operating power plant. In addition, an animal model was used to determine if ROS, generated by ROFA, can affect cellular oxidant production and cause lung injury and inflammation.

2. Materials and methods

2.1 *Animals*

Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA, USA) weighing ~ 250 g were used. They were fed on a conventional laboratory diet and given tap water *ad libitum*, housed in clean air, viral- and antigen-free room with restricted access, and allowed to acclimate in an AAALAC-approved animal facility for one week before use.

2.2 *ROFA*

ROFA was collected from a precipitator at Boston Edison Co., Mystic Power Plant #4, Everett, MA, USA. Particle size of the ROFA sample was characterized by scanning electron microscope (JSM-#5600 SEM, JEOL Ltd., Peabody, MA, USA). The ROFA particles were of respirable size with a count mean diameter of 2·2 μm. ROFA particles were suspended in sterile phosphate-buffered saline (PBS), pH 7·4, at a concentration of 6·67 mg/ml and sonicated for 1 min. The samples were further divided into their soluble and insoluble components as follows. The ROFA particle suspension (ROFA-total) in PBS was incubated for 24 h at 37°C in a shaking incubator. After incubation, the samples were centrifuged at 12,000 *g* for 30 min. The supernatant (ROFA-sol.) was recovered and filtered with a 0·22 μm filter, whereas the pellet (ROFA-insol.) was resuspended with initial volume of the PBS. pH of each of the ROFA samples (total, sol., and insol.) was measured, and the elemental composition was determined using inductively coupled argon plasma, atomic emission spectroscopy (NIOSH 1994).

2.3 *Free radical measurement*

ESR was used to detect short-lived free radical intermediates in the ROFA-total, ROFA-sol., and ROFA-insol. samples in a cell-free system as previously described by Leonard *et al* (2000). ESR measurements were made using a Bruker ESP 300E spectrometer and a flat cell assembly. For detection and characterization of the generated free radicals, 0·1 M 5,5-dimethyl-1-pyrroline-oxide (DMPO) was used as a spin trap. For all samples, the metal chelator deferoxamine (Def) and antioxidant enzyme catalase were added to the reaction. A SPEX 300 program (US EPR, Inc., Clarksville, MD, USA) was used for data acquisition and analysis.

2.4 *ROFA treatment*

Rats were lightly anesthetized by an intraperitoneal injection of 0·6 ml of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN, USA) and intratracheally instilled with 1·0 mg/100 g body wt. of ROFA dissolved in 300 μl of saline as previously described by Reasor and Antonini (2001). In addition, ROFA-sol. and ROFA-insol. samples were administered by intratracheal instillation using volumes equivalent to the ROFA-total instillate. Animals in the vehicle control group were intratracheally dosed with 300 μl of sterile saline. In one set of experiments, ROFA-total was incubated with 2·5 mM Def for 24 h before intratracheal instillation. As a control, one group received saline which was incubated with Def for 24 h.

2.5 *Bronchoalveolar lavage*

At 4, 24, and 72 h after instillation of the ROFA samples, bronchoalveolar lavage (BAL) was performed on the right lungs as previously described by Antonini *et al* (2000). Total cell numbers and differentials were determined with a Coulter Multisizer II (Coulter Electronics, Hialeah, FL, USA).

2.6 *Luminol-dependent chemiluminescence*

Luminol-dependent chemiluminescence (CL) was measured with an automated Berthold Autolumat LB 953 luminometer (Wallace, Inc., Gaithersburg, MD, USA) as described previously by Antonini *et al* (1994). Luminol was used as an amplifier to enhance detection of light, while 3 μM of phorbol myristate acetate (PMA; Sigma Chemical Company, St. Louis, MO, USA) was added immediately prior to the measurement of CL to activate the lung phagocytes. CL was measured for 15 min at 37°C, and the integral of counts per minute (cpm) vs time was calculated.

CL was calculated as the total counts of stimulated cells minus the total counts of the corresponding resting cells.

2.7 *Lipid peroxidation/lung injury*

Non-lavaged left lungs were collected from treated animals, weighed, suspended in water, and homogenized. The homogenate was centrifuged at 1,500 *g* for 10 min, and the supernatant was decanted and frozen at – 80°C for subsequent analysis. The lipid peroxidation products malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE), were measured using Bioxytech LPO-586^{TM} Colorimetric Assay for Lipid Peroxidation Markers (Oxis International, Inc., Portland, OR, USA).

Within the acellular first fraction of BAL fluid, albumin content was determined colorimetrically at 628 nm, based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma Chemical Company, St. Louis, MO, USA).

2.8 *Statistical analysis*

Results are expressed as means ± standard error (SE) of measurement. The significance of the interaction among the different treatment groups for the different parameters at each time point was assessed using an analysis of variance (ANOVA). The significance of difference between individual groups was analysed using the Tukey-Kramer post-hoc test. For all analyses, the criterion of significance was set at $P < 0{\cdot}05$.

3. Results

Elemental analysis of the ROFA samples is shown in table 1. Both the ROFA-total (pH 5·0) and ROFA-sol. (pH 4·1) samples were acidic, whereas the ROFA-insol. sample was neutral (pH 7·06). Significant amounts of Ni, Al, Ca, Fe, and Zn were present in ROFA-sol., whereas ROFA-insol. was mostly comprised of Fe, V, and Al. ESR was used to assess the oxidant potential of each of the samples in a cell-free system. A spectrum representative of a hydroxyl radical (1 : 2 : 2 : 1 quartet) was obtained when each of the samples was treated with H_2O_2 (figure 1). The response was much stronger for the ROFA-total and ROFA-sol. samples compared to ROFA-insol. Antioxidant catalase, as well as metal chelator, deferoxamine, significantly reduced generated hydroxyl radical signal for each of the ROFA sample.

Table 1. Element mass (μg/mg) and pH.

Elements	ROFA-total	ROFA-insoluble	ROFA-soluble
Fe	122	93·2	18·6
Al	60·9	32·0	23·3
V	46·0	41·6	0·58
Ni	38·4	5·50	27·9
Ca	30·6	3·58	22·6
Zn	5·34	0·56	4·34
pH	5·0	7·06	4·1

Note. Trace elements: Ba, Cd, Co, Cr, Cu, Mn, Pb.

The number of alveolar macrophages (AMs) and neutrophils (PMNs) recovered by BAL was determined (figure 2). No significant differences in AM numbers were observed among the groups at 4, 24, and 72 h after instillation of the ROFA samples (figure 2A). The number of PMNs recovered from the ROFA-total and ROFA-insol. groups was significantly increased at all time points when compared to saline group, with PMN numbers decreasing at 72 h (figure 2B). ROFA-sol. PMN-numbers were not different from control numbers at 4 h, but were significantly higher with time at both 24 and 72 h.

The cellular oxidant potential was measured by luminol-dependent CL (figure 3). No significant difference was observed among the groups in the measurement of PMA-stimulated CL 4 h after treatment (figure 3A). At 24 h, CL of the BAL cells from each of the ROFA groups was significantly elevated when compared with the saline control. Interestingly, at 72 h, CL for the ROFA-sol. group was significantly reduced as compared to saline control values. Pretreatment of the ROFA-total sample with Def before intratracheal instillation significantly reduced PMA-stimulated CL of recovered BAL cells 24 h after treatment (figure 3B).

For the assessment of lung injury, BAL albumin and lipid peroxidation were measured (figure 4). Albumin levels in the BAL were significantly elevated at 4 and 24 h for the ROFA-total group and at 24 h for the ROFA-insol. when compared to the saline control (figure 4A). Albumin levels for ROFA-sol. were not different from saline control values at any time point. By 72 h, no significant difference was observed in albumin levels among groups. No difference in lipid peroxidation was observed among the groups at 4 h (figure 4B). Treatment with ROFA-total and ROFA-insol. significantly increased lung lipid peroxidation at 24 and 72 h when compared to saline control. ROFA-sol. had no effect on lung lipid peroxidation at any time point.

4. Discussion

Pulmonary treatment of laboratory animals with ROFA is pneumotoxic (Dreher *et al* 1997; Kodavanti *et al* 1998). The observed lung damage was shown to be dose-dependent and transient. It was the goal of our current study to assess the role that ROS play in the lung disease associated with ROFA exposure. Using ESR, the ROFA

sample along with its soluble and insoluble fractions produced the highly reactive hydroxyl radical in presence of H_2O_2. The hydroxyl signal was greater in ROFA-sol. fraction compared to ROFA-insol. fraction. In addition, this response was significantly reduced after treatment with the metal chelator, Def, suggesting that the soluble metals associated with ROFA were likely responsible for the generated ROS. Kadiiska *et al* (1997) demonstrated that pulmonary administration of the soluble fraction of ROFA induced the *in vivo* generation of carbon-centered free radical adducts as assessed by ESR. Due to these ESR findings, it would be reasonable to hypothesize that the lung damage associated with ROFA is caused by the ROS generated from soluble metals.

It has been previously demonstrated that water-soluble transition metals are likely the causative agents of ROFA-induced acute injury (Dreher *et al* 1997). Transition metal content, bioavailability, and their interactions were shown to influence the lung responses to ROFA. However, in our current study, lung injury and inflammation

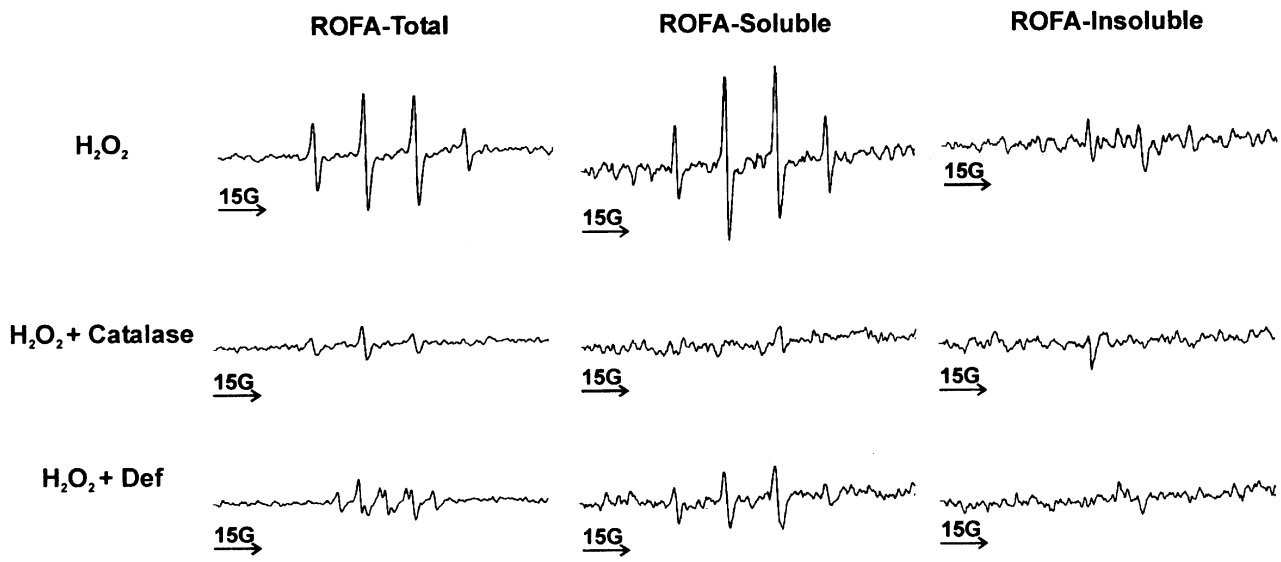

Figure 1. Electron spin resonance spectra of ROFA samples using spin trapping with DMPO in addition to H_2O_2; H_2O_2 + catalase; and H_2O_2 + Def.

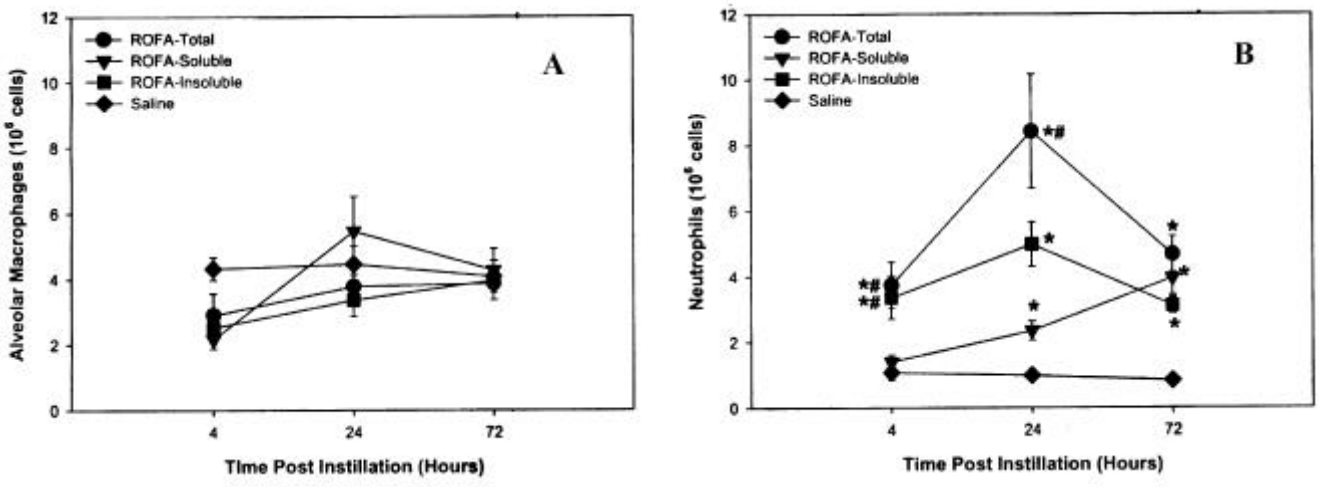

Figure 2. Number of alveolar macrophages **(A)** and neutrophils **(B)** recovered after treatment with ROFA-total, ROFA-sol., ROFA-insol., and saline. Values are means ± SE (n = 5–13 rats/group). *, Significantly greater than saline within a time point; #, significantly greater than ROFA-sol. group within a time point; $P < 0{\cdot}05$.

and lavage-cell-oxidant production were significantly elevated after exposure to the particulate forms of ROFA (ROFA-total and ROFA-insol.). The observed lung damage may be a result of the oxidants produced by the activation of lung AMs and PMNs after exposure to the particulate component of ROFA rather than the elevated presence of ROS associated with ROFA-sol. The soluble fraction of ROFA seemed to have little effect on lung injury at any of the time points examined in our investigation.

In agreement with our results, Imrich *et al* (2000) demonstrated that the bioactivity of ambient air particles for *in vitro* AM responses, such as activation, cytokine secretion, and phagocytosis, is governed almost entirely by the insoluble material present. However, other studies have demonstrated the important role in the response of lung epithelial cells to soluble metals of ROFA (Carter *et al* 1997; Dye *et al* 1997). Differences in lung responses observed by various laboratories are likely due to the heterogeneity in the physical and chemical composition of ROFA or ambient air particulate samples. Ambient air particle samples collected on different days (Imrich *et al* 2000) and ROFA collected from different locations within the same power plant (Kodavanti *et al* 1998) have

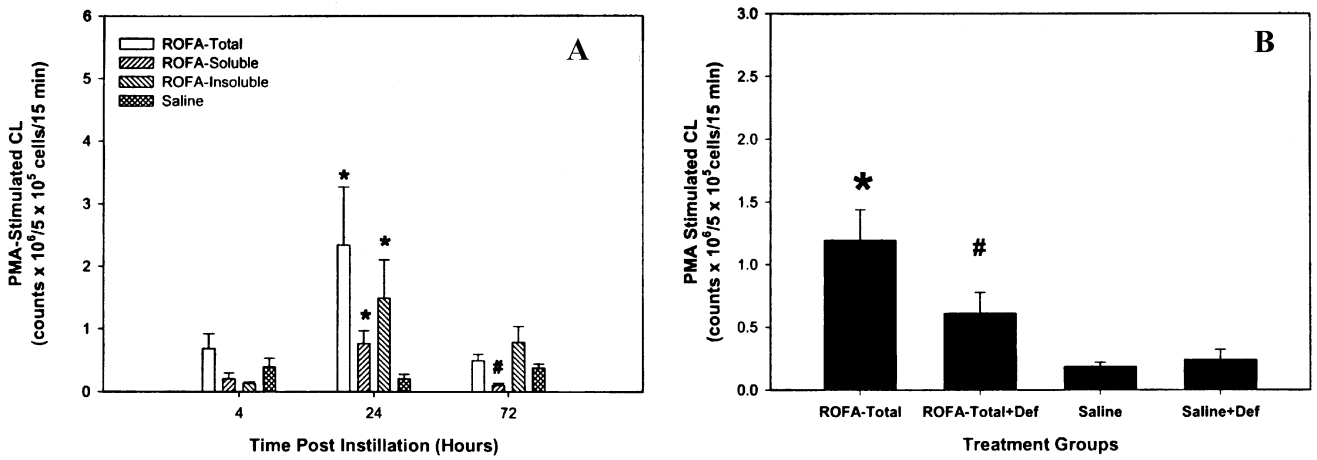

Figure 3. PMA-stimulated CL of lung lavage cells after treatment with ROFA-total, ROFA-sol., ROFA-insol., and saline over time **(A)** and at 24 h after intratracheal instillation of ROFA-total pretreated with 2·5 mM deferoxamine (**B**). Values are means ± SE (n = 5–13 rats/group). *, Significantly greater than saline; #, significantly less than other groups in **(A)** and significantly less than ROFA-total in **(B)**; $P < 0{\cdot}05$.

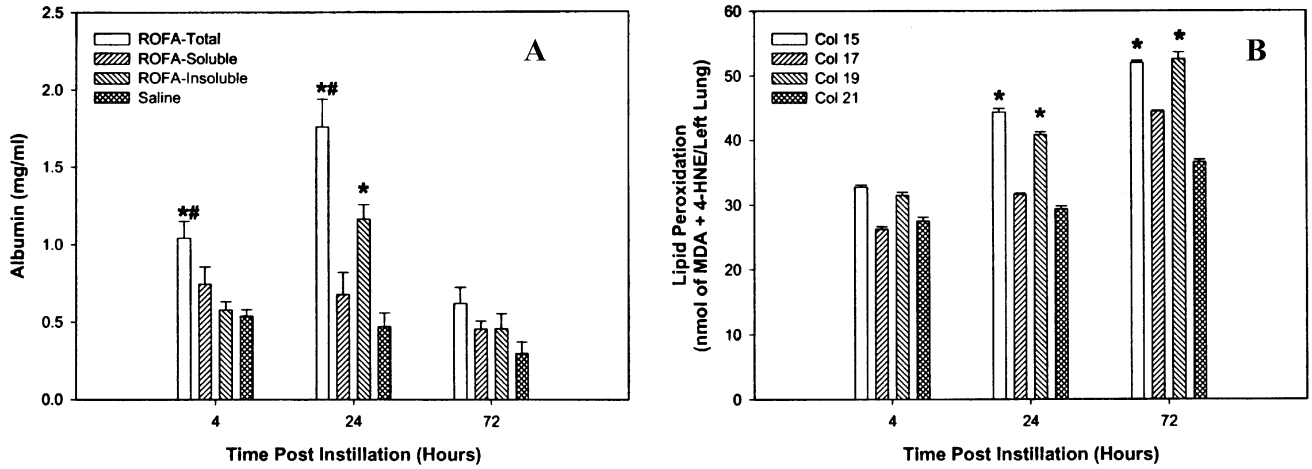

Figure 4. First lavage fraction fluid albumin content **(A)** and lung tissue lipid peroxidation products **(B)** after treatment with ROFA-total, ROFA-sol., ROFA-insol., and saline. Values are means ± SE (n = 5–13 rats/group). *, Significantly greater than saline within a time point; #, significantly greater than ROFA-insol. and ROFA-sol. groups within a time point; $P < 0{\cdot}05$.

been shown to demonstrate a wide range of biologic activity. Thus, the pneumotoxic profile of collected workplace samples and environmental particulate samples is likely to be influenced by the elemental composition of the particular sample.

In the assessment of ROFA samples with different chemical characteristics, Kodavanti *et al* (1998) indicated that soluble V appears to be associated with pulmonary inflammation and AM activation. The ROFA used in our current study contained little V in the soluble fraction, which may explain the observed lack of pulmonary effect after administration of ROFA-sol. Metal-metal interactions also may have an impact on the pulmonary injury associated with ROFA exposure. When Ni, V, and Fe were combined, lung pathology and cytokine production caused by the three metals together were less severe than by Ni alone (Kodavanti *et al* 1997). In addition, Dreher *et al* (1997) observed that when V and Ni were mixed together, lung damage caused by the mixture was less compared to the injury caused by Ni alone. Since our ROFA sample contained a significant amount of soluble Ni, the potential pneumotoxic responses of the other soluble metals present in the ROFA sample may be suppressed by the presence of Ni.

Interestingly in our study, the effects of ROFA-sol. appeared to be delayed compared to the more acute effects of ROFA-insol. PMN number increased over time, while CL was significantly depressed at 72 h after treatment with ROFA-sol. In our previous study we showed that ROFA-sol. pretreatment with a bacterial pathogen before pulmonary inoculation increased morbidity and mortality after infection (Roberts *et al* 2001). This further suggests the potential suppressive effects of lung defense responses due to the soluble Ni associated with ROFA.

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