

# Cardiopulmonary Effects of Nebulized Residual Oil Fly Ash in Anesthetized Pigs

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Abbreviated Title: Cardiopulmonary Effects of Nebulized ROFA

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**Abstract:** Exposure to ambient air pollution particles can be associated with pulmonary, hematological and cardiac effects in humans. In order to establish an animal model suitable to study the mechanisms underlying these effects, we exposed 14 intubated, anesthetized pigs to nebulized residual oil fly ash (ROFA, 2mg/kgBW) and monitored pulmonary, cardiac and hemodynamic endpoints. We found: (1) elevated nickel concentrations in the blood of treated animals showing the systemic distribution of soluble ROFA constituents; (2) increased pulmonary artery pressure; (3) pathological and histochemical changes in lung tissues that are consistent with an inflammatory lung injury; (4) electrocardiographic changes that demonstrate the shortening of cardiac depolarization times; and (5) altered heart rate dependent changes in cardiac repolarization. These data identify pathophysiological changes that may suggest potential mechanisms linking PM exposure to mortality.

**Key Words:** • particulate matter • air pollution • cardiovascular disease • sudden cardiac death • myocardial infarction • action potential

**Introduction:** Abundant epidemiological data associates fine particulate air pollution and adverse health effects.(Samet J, 2000) Of special interest is the observation that increased concentrations of fine particulate matter (PM) is associated with cardiovascular events,(Zanobetti A, 2000a) particularly among the elderly(Pope CI, 1992, Schwartz J, 1994) and those with concomitant cardiopulmonary disease.(Zanobetti A, 2000b) Despite the strength of those associations, a biological mechanism accounting for the link between respired PM and cardiovascular events has not been identified. Establishing such a mechanism is complicated by the complex composition of air pollutants and varied size and surface area of PM (Loomis D, 2000) and the statistical methods required for analysis of population based studies.(Gamble J, 1998, Gamble J, 1996, Schwartz J, 2000) There is general agreement, however, that air pollution contributes to cardiovascular risk and merits comprehensive scientific study.(Cifuentes L, 2001, Pope CI, 1995)

Experimental animal models serve an important role for the study of mechanisms of human disease. Models utilizing rodents(Costa DL, 1997, Godleski JJ, 2000, Oberdörster G, 2000) and dogs(Godleski JJ, 2000)are providing data regarding the effect of PM on cardiopulmonary physiology. However, the relevance of small mammalian models with co-morbid conditions to human disease is uncertain. Our short-term goal is to establish a porcine model for the study of the effects of PM on cardiovascular and hematological endpoints. To achieve this goal, we utilized nebulized residual oil fly ash (ROFA) whose cardiopulmonary toxicity is well characterized in small mammals.(Costa DL, 1994, Killingsworth C, 1997) As a first step, we sought to examine the effects of nebulized ROFA in intubated, anesthetized pigs on measures of pulmonary inflammation previously established in rodent models.

Concurrently, ROFA-induced effects on pulmonary and systemic arterial pressures and on cardiac repolarization were measured. Our long-term goal is to incorporate diabetes, coronary atherosclerosis, healed infarction and heart failure into the model as a means to identify mechanisms responsible for the increased cardiovascular risk attributed to PM exposure in those clinical subgroups.

Our studies showed that nebulized ROFA caused an increase in blood nickel concentration, thus confirming the absorption and systemic distribution of the soluble components of this agent. Further, these studies demonstrated that an inflammatory process in the lungs occurred as a result of ROFA exposure. The observed injury was associated with a moderate increase in pulmonary arterial pressures and the expression of interleukin-8 on the alveolar surface. Finally, our studies demonstrated shorter activation recovery intervals (ARI, a surrogate measure of cardiac repolarization (Haws CW, 1990)) following exposure to ROFA, thereby indicating reduced cardiac action potential duration and altered heart rate dependency.

## **Materials and Methods:**

**Animal Preparation:** Domestic swine of either sex (25-35kgBW, n=14) obtained from Walnut Hill Farms, NC, were pretreated with analgesia (Duragesic, 25 $\mu$ g/hr) 24hrs prior to surgery. These studies were performed in compliance with guidelines established by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, NIH publication No. 85-23, revised 1996). On the day of the study, animals were immobilized with ketamine HCl (500mg) and anesthetized with sodium pentothal (25mg/kgBW). Anesthesia was maintained using  $\alpha$ -chloralose (30mg/kg/hr, i.v.). The animals were intubated and ventilated with supplemental O<sub>2</sub> using a Harvard dual phase respirator having a 25% inspiration/75% expiration duty cycle. Body temperature was monitored and maintained at 38°C using a Gaymar T-pump water blanket. Arterial blood gases were monitored and adjustments maintained pO<sub>2</sub> >80mmHg, pCO<sub>2</sub> between 35 and 40mmHg and pH between 7.35 and 7.45. Arterial and venous femoral catheters were placed using standard techniques and served as a site for fluid infusions and blood sample withdrawals. A midsternal thoracotomy exposed the heart. The heart was suspended in a pericardial cradle and instrumented with potentiometric electrodes as outlined below. Those electrodes enabled the assessment of local cardiac electrical activity. After electrode stability was achieved, ROFA (2mg/kgBW) suspended in 5cc isotonic saline or 5cc isotonic saline alone was nebulized (1cc/min) using a DeVilbiss AeroSonic nebulizer connected in-line on the inspiration limb of the ventilator.

**Instrumentation:** A Swan-Ganz pulmonary artery catheter was advanced from the right internal jugular vein to the pulmonary artery for the measurement of right atrial (RAP) and pulmonary artery pressures

( $\Delta$ PA). Alternately, high fidelity Millar pressure transducers were installed and purse-string sutured directly into the right atrium and pulmonary artery for those assessments. Aortic pressure (AOP) was monitored at the level of the descending aorta distal to the arch using a fluid-filled arterial catheter in concert with a Millar pressure transducer. Unipolar electrograms were collected from the right and left ventricular free-walls using 0.007" stainless steel wire electrodes installed at various depths using a hypodermic needle assembly.(Johnson TA, 1991) These, in turn, were referenced to a large silver/silver chloride electrode positioned at the base of the aortic root. In all, sixteen electrodes were installed: 4 in the right heart and 4 in each of three levels in the left heart relative to the epicardial surface (*epicardial*, 1-4mm deep; *midmyocardial*, 5-8mm deep; *endocardial*, more than 8mm deep). Activation recovery intervals (ARIs, surrogate markers of action potential durations) were assessed from the unipolar electrograms by determining the elapsed time between the maximum negative slope of the activation complex and the maximum positive slope of the T-wave. (Haws CW, 1990) A standard Lead II ECG was installed for the measurement of heart rate. Hearts spontaneously beating >90 BPM were not paced during routine data collections. When the heart rate fell below 90 BPM the right atrium was paced with a Medtronic pacemaker. Prior to and at hourly intervals following exposure, hearts were paced at accelerated rates of 120, 150, 180 and, if tolerated, 240 BPM to assess the known rate-dependent effect of cycle length (the reciprocal of heart rate) on ARI.

**Data Collection and Analysis:** Electrogram data was amplified (50x) and filtered (0-500Hz @ -1dB) using high impedance instrumentation amplifiers.(Johnson TA, 1990) Data collection began before instillation of ROFA or saline alone. The Lead II ECG,  $\Delta$ PA, AOP, RAP and all electrogram data was digitized at 2KHz using a National Instruments ATMIO-64E3 ADC installed in a Pentium II PC. Data was collected at that rate for 2 seconds at 5-minute intervals after exposure and during hourly pacing studies over the course of the 3-6 hour observation period. Data collection and post-experimental processing was performed using *FlexiDAQ*<sup>TM</sup> software developed in our laboratory. These data were exported to Prism® spreadsheets for final data processing and statistical analysis using ANOVA and group or paired *t*-tests. Significance was established at  $p \leq 0.05$ .

**Lung and Blood Analysis:** Arterial blood (5cc) was drawn for ICP-MS Ni plasma analysis in ROFA (n=7) and saline (n=1) treated animals. Those blood samples were taken at t=0, 5, 30, 60, 120, 180, 240 and 300 minutes post-exposure. Post-experimentally, bronchoalveolar lavage (BAL) was performed on excised lungs from both groups (saline, n=5; ROFA, n=6). PMN (%) and BAL protein ( $\mu$ g/ml) were assessed to determine the extent of lung inflammation and injury. Further, sections from those lungs were inflation fixed in 4% paraformaldehyde at 25 cm H<sub>2</sub>O. After embedding in paraffin and staining (hematoxylin and eosin), tissue was microscopically examined for gross pathological changes. Immunohistochemistry for IL-8 was also

performed on fixed tissue. Sections were incubated with anti-human rabbit IL-8 antibody (San Diego, CA) and, sequentially, biotinylated secondary antibody, HRP-streptavidin complex and HRP substrate in order to assess IL-8 production. Slides were photographed with a Nikon Coolpix 990 digital camera attached to a Nikon E600 light microscope under differential interference contrast illumination.

**Cardiac Parameters:** Cardiac output is defined as the volume of blood ejected from the heart and is measured in liters/minute. Cardiac output was calculated using the Fick principle. (Grossman, 2000) The Fick method requires the measurement of the O<sub>2</sub> saturation in arterial and mixed venous blood, the hematocrit and the O<sub>2</sub> consumption. Arterial blood was obtained from the aorta, while simultaneously obtaining mixed venous blood from the pulmonary artery via the Swan-Ganz catheter. The O<sub>2</sub> saturation was measured with a clinical blood gas instrument. The O<sub>2</sub> consumption was assumed to be 125 ml/min during the entire study. Stroke volume is the volume of blood ejected from the heart during a single contraction and is measured in ml/beat. Stroke volume was calculated by dividing the cardiac output by the heart rate.

## Results:

### ROFA Exposure and Blood Ni Concentrations:

In animals exposed to nebulized ROFA, Ni concentrations in the blood increased as shown in Figure 1. Ni concentrations reached a peak 30 minutes after exposure and were 3-fold greater than baseline five hours later. It is noteworthy that the variability of the Ni concentrations was substantial. An increase in the Ni concentrations in the blood was not detected in the saline control.

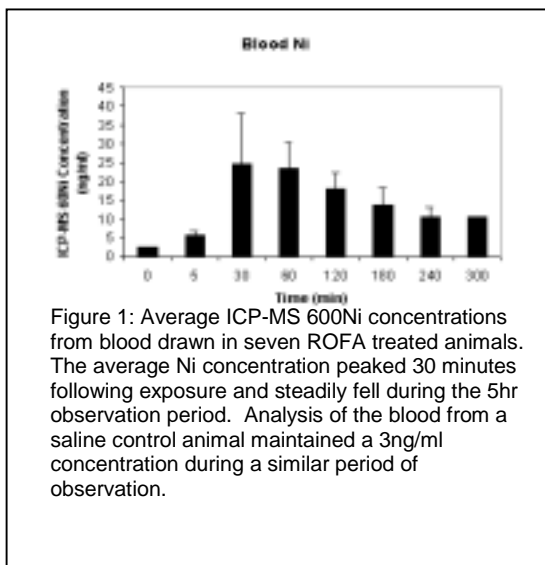
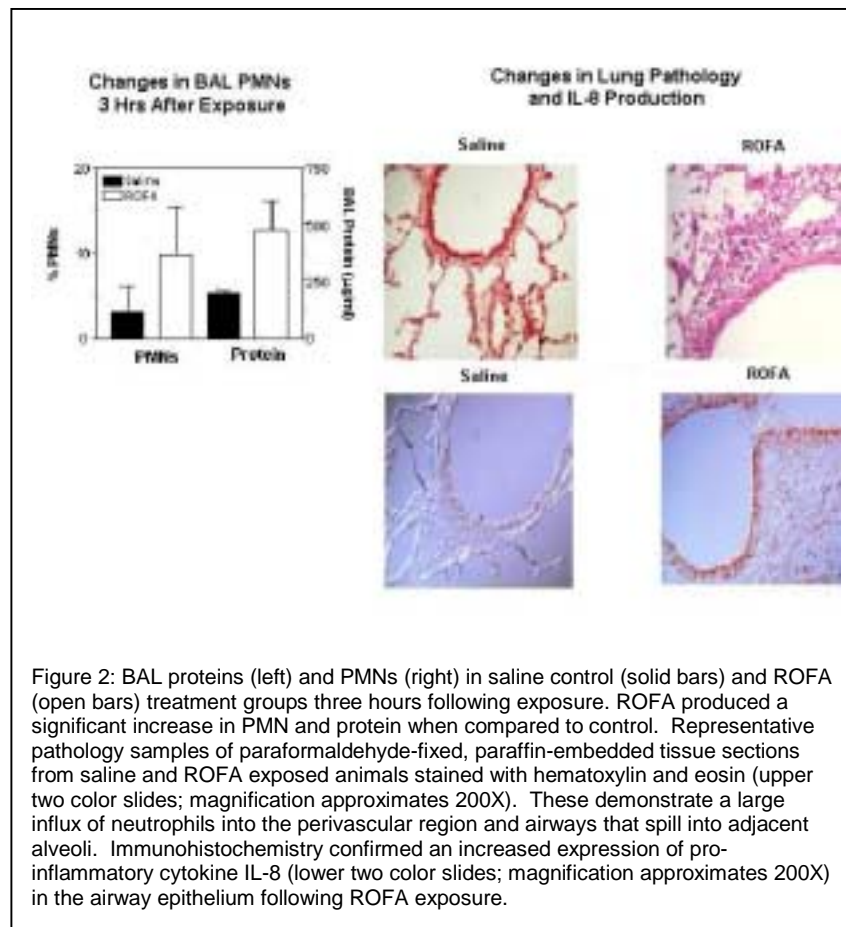


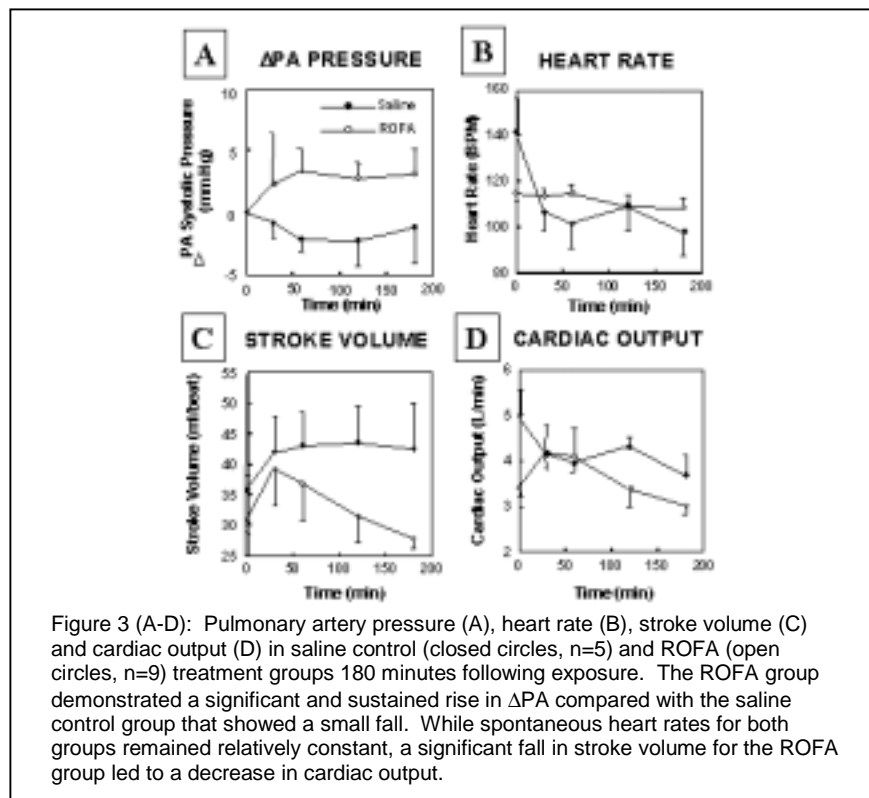
Figure 1: Average ICP-MS 600Ni concentrations from blood drawn in seven ROFA treated animals. The average Ni concentration peaked 30 minutes following exposure and steadily fell during the 5hr observation period. Analysis of the blood from a saline control animal maintained a 3ng/ml concentration during a similar period of observation.

**Pulmonary Effects:** Exposure to nebulized ROFA caused pulmonary inflammation and injury as shown in Figure 2. ROFA increased the PMNs and protein concentration in the BAL when compared to saline controls. PMNs increased 4-fold for the ROFA group and the BAL proteins more than doubled. These results were significant. Microscopic examination of lung tissue after ROFA exposure revealed an

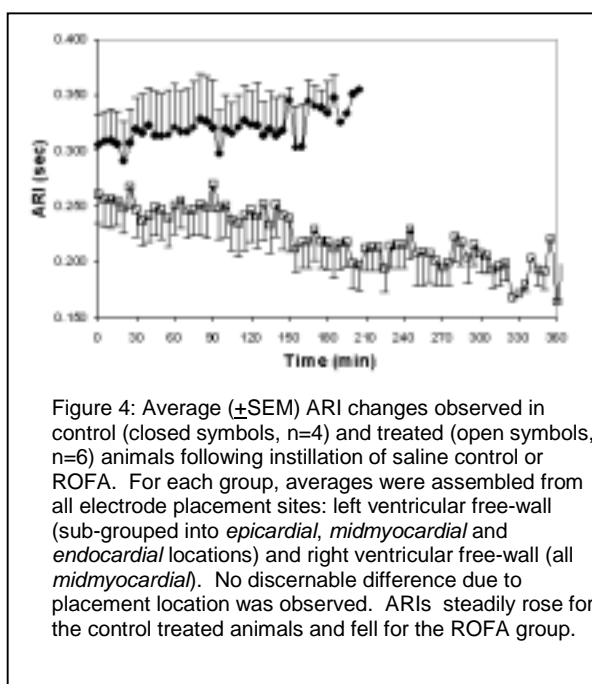


influx of neutrophils into the perivascular tissues and the airways (Figure 2). In addition, there was an increased expression of IL-8 in the airway after nebulization of the particles into the animal. Following exposure to ROFA, animals showed consistent hemodynamic responses in the pulmonary vasculature. ROFA generated a 2 to 3mmHg rise in the PA pressure, whereas the PA pressure fell 2 to 3mmHg in the saline control group ( $p < 0.05$  at 180min). These data are presented in Figure 3A.

**Cardiac Effects:** Figures 3B-D show changes in heart rate, stroke volume and cardiac output. Cardiac output fell for the ROFA group, largely due to a decrease in stroke volume ( $p < 0.05$ ) rather than a reduced heart rate ( $p = n.s.$ ). The cardiac electrical response to ROFA exposure can be seen in Figure 4 where ARI has been plotted as a function of exposure time for both saline control and ROFA groups. The summary data presented (mean  $\pm$  SEM) for control (solid symbols) and ROFA (open symbols) exposures include all electrodes within the right ventricle and those within different regions of the left ventricle (*epicardial*, *midmyocardial* and *endocardial*). No discernable differences were observed between the various regions of the heart in spite of the fact that saline exposure tended to lengthen ARI while ROFA shortened it in all



regions. Because it is known that heart rate modulates ARI, we evaluated the impact of ROFA by assessing that relationship using several heart rates at hourly intervals following exposure. Figure 5 shows that ROFA exposure produced a diminished slope for the relationship of ARI to cycle length (CL, the reciprocal of heart rate), thereby attenuating the known rate-dependence of ARI to heart rate.



## Discussion:

The purpose of this study was to determine the feasibility of using an in vivo porcine model to assess the effects of PM on blood, pulmonary and cardiac end-points. To achieve that goal, the effects of nebulized ROFA (2mg/kgBW) delivered via an endotracheal tube were compared to saline control in an open-chest porcine model. ROFA served as a surrogate for PM because it has an established pulmonary toxicity in small mammals (Dreher KL, 1997, Pritchard RJ, 1996) and humans (Ghio AJ, 2001, Woodin MA, 1998). Yet, its effects on cardiac function have not been established previously in a large animal model. Consistent with previous studies, nebulized ROFA induced an inflammatory response and injury in the lung as measured by increased PMNs, albumin and total protein in the BAL fluid. These inflammatory

responses were associated with the development of mild pulmonary hypertension and increases in  $\text{FIO}_2$  requirements. Cardiac effects include decreased cardiac output, stroke volume and activation recovery intervals and an attenuation of the slope of the relationship between the speeding of the heart rate with the shortening of activation recovery intervals.

**ROFA Exposure:** The chemical composition of the ROFA administered in this study was previously characterized and contains vanadium and nickel at elevated concentrations (Dreher KL, 1997). As such, blood concentrations of Ni provided a convenient method to assess the exposure of these animals to ROFA with the nebulization technique. As shown in Figure 1, the concentration of Ni in the blood increased following exposure, peaked at 30 minutes and decreased thereafter.

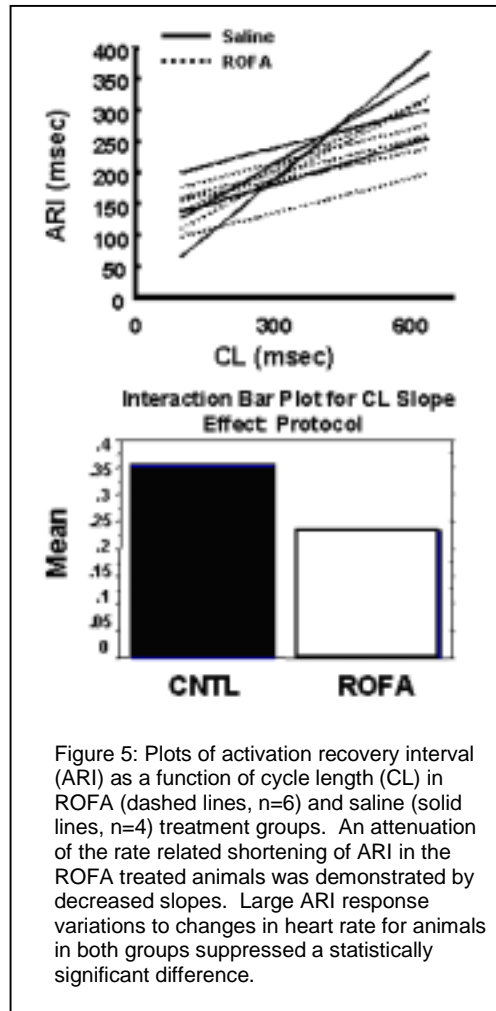


Figure 5: Plots of activation recovery interval (ARI) as a function of cycle length (CL) in ROFA (dashed lines, n=6) and saline (solid lines, n=4) treatment groups. An attenuation of the rate related shortening of ARI in the ROFA treated animals was demonstrated by decreased slopes. Large ARI response variations to changes in heart rate for animals in both groups suppressed a statistically significant difference.

Importantly, despite the administration of identical quantities of ROFA (normalized for body weight), the peak Ni concentrations varied considerably. Therefore, this result may suggest either that the nebulization technique did not provide consistent distribution to the lungs of the pigs or, alternately, that our findings represent individual responses of a genetically heterogeneous porcine population. Future investigations will be designed to study concentrated ambient PM collected on filters and, therefore, preliminary studies are warranted to insure uniform distribution of dry powers in the lung of the pig.

**Pulmonary Responses:** Exposure to nebulized ROFA caused acute pulmonary inflammation as evidenced by the appearance of PMNs and protein in the BAL fluid similar to that measured in small mammals.(Pritchard RJ, 1996) Immunohistochemical analysis of lung sections showed an increased expression of IL-8 on the surface of the alveoli consistent with findings in human cells.(Carter JD, 1997) The measured inflammatory response was associated with the appearance of mild pulmonary hypertension, an observation made previously in isolated, perfused rabbit lungs following intratracheal instillation of ROFA.(Huang Y-CT, 2001 (in press))

**Cardiac Responses:** Surprisingly, cardiac output and stroke volume decreased in the pigs exposed to ROFA. These changes in cardiac hemodynamics occurred without a change in systemic blood pressure, thereby implying an increase in peripheral vascular resistance. One can speculate that soluble components of ROFA, cytokines produced in the lungs or altered neural input to the peripheral vasculature contribute to an increase in peripheral resistance. Further studies are required to confirm these findings and establish the mechanism.

In a previous study, electrocardiographic ST segment changes occurred in dogs exposed to concentrated ambient PM.(Godleski JJ, 2000) Those changes may have been related to regional changes in cardiac repolarization. In these studies, we utilized a well-established method to measure the heterogeneity of cardiac repolarization, as determined by activation recovery intervals, in a large mammal heart. In the porcine model, ARIs (*i.e.*, the elapsed time in the unipolar electrogram between the maximum negative deflection of the activation complex to the maximum positive upstroke of the repolarization wave) correlates to the action potential duration.(Haws CW, 1990) As shown in Figure 4, ARIs tended to increase over time in the control pigs while ARIs tended to shorten after ROFA exposure. Cardiac loading conditions may also affect the repolarization of the heart. The decrease in the ARIs after ROFA exposure may be related to decreased ventricular volume loading suggested by the decreased stroke volume,(Zhu WX, 1997). However, direct effects on cardiac channels cannot be excluded. The progressive shortening of ARIs over time in those pigs exposed to ROFA is likely related to progressive decreases in local action potential durations. Hypoxia can also shorten action potential duration. However, arterial pO<sub>2</sub> was maintained >80 mmHg with supplemental oxygen in each of these studies. Thus, it is unlikely that hypoxia contributed to the measured

changes in ARIs. Such repolarization changes, when heterogeneous, facilitate arrhythmia formation. Although we confirmed the inherent heterogeneity of ARIs of ventricular myocardium, we did not find an increase of heterogeneity caused by ROFA.

## **Development of Clinically Relevant Porcine**

**Models:** Epidemiological studies show that the relative risk of PM is substantially higher for pulmonary effects when compared to cardiovascular effects.(Moolgavkar S, 2000) However, the prevalence of cardiovascular disease in the population exceeds COPD several fold. Consequently, the aggregate risk is substantially higher for cardiovascular events. Recently, data has appeared indicating that risk for cardiovascular events may be greatest for high-risk subgroups (Peters A, 2001, Peters A, 2000, Zanobetti A, 2000b), rather than for those with no history of clinically recognized heart disease or life-threatening comorbidities.(Levy D, 2001) These include individuals with a previous history of serious ventricular arrhythmia,(Peters A, 2000, Zanobetti A, 2000b) a predisposition to myocardial infarction(Peters A, 2001) and congestive heart failure.(Zanobetti A, 2000b)

Likewise, studies in rodents indicate that co-morbid states, such as pulmonary hypertension, increase the risk associated with PM exposure. Epidemiological data supports the notion that PM irritates the airways of the lungs and worsens pre-existing pulmonary and cardiac disease. As such, animal models were developed to study the effect of PM in animals with compromised cardiopulmonary function. Those included rats with monocrotaline-induced pulmonary hypertension,(Costa DL, 1994, Killingsworth C, 1997) cardiomyopathic hamsters (Gordon T, 2000) and dogs with myocardial ischemia.(Godleski JJ, 2000) For example, when monocrotaline-treated rats inhaled OFA, mortality and pulmonary inflammation increased more than in controls.(Costa DL, 1997, Killingsworth C, 1997) However, the increased mortality among the OFA-exposed, monocrotaline-treated rats remains unexplained.

**Summary:** ROFA produces cardiopulmonary effects in a large animal model. Further studies are needed to assess the effects of these cardiopulmonary changes on susceptibility to sudden cardiac death (SCD). Subsequent studies must extend these findings to other fine particles including concentrated ambient PM and PM containing man-made ultrafine particles with specific formulations. We speculate that cardiac deaths associated with exposure to PM can result directly from effects on cardiac electrophysiology, altered autonomic regulation and/or coronary thrombosis in subjects at high-risk for SCD. The development of large animal models to simulate high-risk human disease states, such as myocardial ischemia, coronary atherosclerosis, healed myocardial infarction and congestive heart failure with and without superimposed metabolic and neurological abnormalities (e.g. diabetes mellitus), are essential for a comprehensive understanding of the mechanisms of airborne PMs on cardiovascular health.

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## Legends:

Figure 1: Average ICP-MS 600Ni concentrations from blood drawn in seven ROFA treated animals. The average Ni concentration peaked 30 minutes following exposure and steadily fell during the 5hr observation period. Analysis of the blood from a saline control animal maintained a 3ng/ml concentration during a similar period of observation.

Figure 2: BAL proteins (left) and PMNs (right) in saline control (solid bars) and ROFA (open bars) treatment groups three hours following exposure. ROFA produced a significant increase in PMN and protein when compared to control. Representative pathology samples of paraformaldehyde-fixed, paraffin-embedded tissue sections from saline and ROFA exposed animals stained with hematoxylin and eosin (upper two color slides; magnification approximates 200X). These demonstrate a large influx of neutrophils into the perivascular region and airways that spill into adjacent alveoli. Immunohistochemistry confirmed an increased expression of pro-inflammatory cytokine IL-8 (lower two color slides; magnification approximates 200X) in the airway epithelium following ROFA exposure.

Figure 3 (A-D): Pulmonary artery pressure (A), heart rate (B), stroke volume (C) and cardiac output (D) in saline control (closed circles, n=5) and ROFA (open circles, n=9) treatment groups 180 minutes following exposure. The ROFA group demonstrated a significant and sustained rise in  $\Delta$ PA compared with the saline control group that showed a small fall. While spontaneous heart rates for both groups remained relatively constant, a significant fall in stroke volume for the ROFA group led to a decrease in cardiac output.

Figure 4: Average ( $\pm$ SEM) ARI changes observed in control (closed symbols, n=4) and treated (open symbols, n=6) animals following instillation of saline control or ROFA. For each group, averages were assembled from all electrode placement sites: left ventricular free-wall (sub-grouped into *epicardial*, *midmyocardial* and *endocardial* locations) and right ventricular free-wall (all *midmyocardial*). No discernable difference due to placement location was observed. ARIs steadily rose for the control treated animals and fell for the ROFA group.

Figure 5: Plots of activation recovery interval (ARI) as a function of cycle length (CL) in ROFA (dashed lines, n=6) and saline (solid lines, n=4) treatment groups. An attenuation of the rate related shortening of ARI in the ROFA treated animals was demonstrated by decreased slopes. Large ARI response variations to changes in heart rate for animals in both groups suppressed a statistically significant difference.