

Abstract

Matthew J. Campen. Cardiovascular and Thermoregulatory Toxicity of an Emission Source Particulate in Healthy and Compromised Rats. (Under the direction of Dr. W.P. Watkinson)

Recent epidemiological studies have demonstrated a positive association between concentrations of ambient particulate matter (PM) and cardiopulmonary morbidity and mortality; however, there are limited toxicological data to support these findings. In rodents, exposure to certain xenobiotics causes an acute hypothermic response, characterized by decreases in body core temperature (T_{CO}), heart rate (HR), and oxygen consumption. This study investigates the cardiovascular toxicity of PM and its potential relationship to the hypothermic response following intratracheal instillation of residual oil fly ash (ROFA), an emission source pollutant, in conscious, unrestrained male Sprague-Dawley rats. Four phases of this study were conducted to examine the effects of ROFA (0.0, 0.25, 1.0, or 2.5 mg/rat) in normal rats and in three models of compromised animals: 1) cold exposed rats, 2) rats with pulmonary inflammation, and 3) rats with pulmonary inflammation and hypertension. HR, T_{CO} , and electrocardiographic (ECG) data were monitored continuously in these rats via a radiotelemetric data acquisition system for 96 hours following instillation.

T_{CO} and HR decreased in a dose dependent manner in normal subjects following ROFA instillation. ECG data showed rate-related changes as well as increases in frequency and severity of arrhythmias, particularly atrioventricular nodal block and premature beats. Responses were exacerbated in all compromised animal models

compared to those seen in healthy animals. Lethality was observed only in animals with concurrent pulmonary hypertension and inflammation and was preceded by severe decreases in HR and T_{CO} .

These data demonstrate increased toxicity of emission source particulates in animals with cardiopulmonary disease and support epidemiological studies that report positive correlations between increased PM concentrations and excess mortality in cardiopulmonary-compromised patients.

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Quality Assurance

All data from this study are stored in Room M-209 of the Environmental Research Center of the U.S. Environmental Protection Agency, located in Research Triangle Park, NC 27711. All original heart rate and core temperature data are stored in C:\DQ4\DATA*. * on a Northgate Computer (U.S.EPA ID#666927). The figures in this report present data which has been clipped of artifactual data and averaged within dose groups. These processed data are filed on the NHEERL network drive N:\CAMP\MPDATA*. *. ECG data are stored both on magnetic tape and printed on chart recorder paper.

Introduction

Recent epidemiological studies (Schwartz, 1994; Dockery and Schwartz, 1995) have reported increased morbidity and mortality associated with concentrations of ambient particulate matter (PM) at or below levels considered to be safe by the U.S. Environmental Protection Agency (EPA). These effects appear to be more pronounced in human subpopulations with pulmonary or cardiovascular illness (Burnett *et al.*, 1995; Schwartz and Morris, 1995). However, controlled experimental evidence for PM cardiotoxicity in humans and animals is limited, especially for sensitive populations, leaving the question of causality in dispute. The present studies investigated the effects of residual oil fly ash (ROFA), a source-derived airborne pollutant, on the cardiopulmonary and thermoregulatory responses of healthy and compromised rodents. Three potential risk factors were studied to examine their role(s) in modulating the toxicity of ROFA: 1) cold ambient temperature (T_a), 2) ozone-induced pulmonary inflammation, and 3) monocrotaline-induced pulmonary inflammation and hypertension.

Previously this laboratory has reported on an induced hypothermic response in rodents exposed to a variety of xenobiotic agents (Gordon *et al.*, 1988; Watkinson and Gordon, 1993; Watkinson *et al.*, 1995). This response is characterized primarily by significant dose-related decreases in core body temperature (T_{co}) and heart rate (HR), as

well as other physiological parameters, all of which are subject to modulation by a number of experimental conditions. A moderate hypothermia, defined as a T_{CO} decrease of approximately $2^{\circ}C$, appears to be advantageous, while a more severe decrease in T_{CO} ($>3^{\circ}C$) appears to exacerbate toxicity and decrease survivability (Watkinson and Gordon, 1993). Importantly, changes in T_{CO} alter the normal physiology of the rodent and may affect the extrapolation of animal data to the human situation.

Historical perspective on air pollution

Air quality has been an environmental health issue since the ancient Greek and Roman times, when wood smoke polluted the more densely populated areas. While obvious examples of point-source toxic emissions exist, such as the methyl isocyanate disaster at Bhopal, India, non-specific source pollution is by far more common and of greater environmental and public health concern. This century has produced several dramatic illustrations of the extreme adverse health consequences of high air pollution levels originating from non-specific sources. In 1930, the Meuse Valley of Belgium suffered a severe toxic fog which lasted several days and increased the mortality rate 10-fold (Furkitt, 1931). For three days in 1948, a dense smog settled on the town of Donora, Pennsylvania, causing a similar change in mortality over a two week period (Shrenk *et al.*, 1949).

In an analysis of the Belgium fog mortality data, Furkitt (1931) estimated that such a disaster in London, where the exposed population was considerably higher, could potentially result in well over three thousand deaths. Indeed, two decades later, four

thousand excess deaths over a period of five days were attributed to the December 5, 1952 London fog episode (Logan, 1953), leaving little doubt as to the lethal potential of air pollution.

Historically, only such disastrous air pollution episodes were considered a major public health concern. However, it has recently been recognized that certain commonly encountered pollutants can have adverse consequences on human health. Under the Clean Air Act of 1970, the EPA established National Ambient Air Quality Standards (NAAQS) for six airborne pollutants which are reported human toxicants: PM, ozone (O₃), carbon monoxide (CO), sulfur dioxide (SO₂), lead (Pb), and nitrogen dioxide (NO₂). By law, these attainment levels are "pure risk" standards, established for the absolute protection of public health with particular regard to sensitive subgroups. As such, these standards do not consider either technical limitations or the economic feasibility of reducing pollution to acceptable levels. These standards are reevaluated at least every five years to incorporate new relevant information gained since the previous health risk assessment.

Origin and Distribution of PM

Ambient PM originates from various sources ranging from roadside dust to iron smelting furnaces, and is classified as coarse, fine, or ultrafine based on a trimodal distribution of geometric mean diameters: 4.9 μm, 0.21 μm, or 18 nm, respectively (Wilson *et al.*, 1977). Combustion processes produce smaller particles than do mechanical processes such as grinding and blasting. Most non-combustion produced particulates are non-

respirable and are therefore not considered to be of major health concern. The majority of combustion produced particulates are emitted from automobiles and coal- or oil-burning electrical plants.

While the strict California automobile emissions policy reflects a recognition of the pollution difficulties in that state caused by high levels of traffic, many developing nations have foregone the establishment of environmental policy to encourage rapid industrial growth, often producing daily PM averages over twice the U.S. allowable limit. It is common in Cairo, for example, to see levels of total suspended particulates (TSP) upwards of $400 \mu\text{g}/\text{m}^3$ (Costa and Amdur, 1996). In the U.S., most regions have been able to achieve the attainment requirements of the NAAQS, although many large cities, particularly industrial areas, which lie in valleys and/or are subject to weather patterns which stagnate the pollution dispersion (inversions) often surpass the limit. Among these areas are Los Angeles and Bakersfield, CA, Denver, CO, and many cities along the Ohio River Valley.

Previous measurement schemes to quantify ambient concentrations of PM include coefficient of haze, TSP, and British smoke, among others. Unlike the NAAQS PM measure, these schemes include large particulates which are less important from a respiratory than from an aesthetic standpoint.

For current regulatory purposes, PM concentration measurements exclude large particulates ($>10 \mu\text{m}$), as they are considered "non-respirable". The designations PM10 (particles $<10 \mu\text{m}$) and PM2.5 (particles $<2.5 \mu\text{m}$) are used to denote specific mass measurements. For PM10, the measure used under the current policy, the national

attainment requirement for 24 hour averaged maximum concentration is $150 \mu\text{g}/\text{m}^3$ and the yearly average limit is $50 \mu\text{g}/\text{m}^3$.

Contributions of Recent Epidemiology Studies

Many epidemiological studies have been conducted in regions of the U.S. and throughout the world to assess the adverse health consequences of airborne PM. Early research designs were not sophisticated enough to accurately quantify the level of risk associated with low levels of air pollution. However, recent studies utilizing time series analyses of large community populations have demonstrated a significant association between increased mortality and levels of air pollution previously considered innocuous. Several of these studies, detailed below, have reported stronger associations in certain susceptible subpopulations.

Epidemiological associations between PM and adverse health effects appear to be stronger in studies which limit their analyses of daily hospital admissions data to cardiovascular and/or respiratory patients. Hospital admission due to chronic obstructive pulmonary disease (COPD) seems to have the highest significant PM-associated risk. Burnett *et al.* (1995) found an excess of COPD hospital admissions of 4.8% (95% confidence interval: 2.1 to 6.9) related to an increase of ambient particulate sulfate of $13 \mu\text{g}/\text{m}^3$. Average excess hospital admissions for all respiratory disease was 3.5% (95% CI: 2.3 to 4.7) after statistical adjustment for ozone and temperature parameters. In addition, an association was found between ambient sulfate levels and cardiovascular admissions,

specifically coronary artery disease (excess of 2.3%; 95% CI: 0.7 to 3.8), cardiac dysrhythmia (1.3%; 95% CI: -2.0 to 4.6), and heart failure (3.0%; 95% CI: 0.6 to 5.3).

Schwartz and Dockery (1992) demonstrated excess mortality associated with a 100 $\mu\text{g}/\text{m}^3$ increase in TSP in an eight year study in Philadelphia. COPD mortality increased 19% (95% CI: 0 to 42%) and pneumonia increased 11% (95% CI: -3 to 27%) for the given rise in TSP. Excess mortality due to cardiovascular disease was also significantly correlated (10% increase; 95% CI: 6 to 14%).

In a study of PM10 health effects in Detroit, Schwartz and Morris (1995) found similar increased relative risk for coronary artery disease (Risk Ratio = 1.018; 95% CI: 1.005 to 1.032), heart failure (RR = 1.032; 95% CI: 1.012 to 1.052), and cardiac dysrhythmia (RR = 1.019; 95% CI: 0.996 to 1.044) hospital admissions in the elderly. An overwhelming number of studies in other cities and areas (e.g. Philadelphia, Ontario, Utah Valley) has also identified similar susceptible groups (Schwartz, 1994).

Confounding in Epidemiology Studies

While these studies report consistent and significant associations, both the biological plausibility of the PM associated health risk and the issue of residual confounding remain as outstanding questions for researchers to answer before PM can be considered a causative factor. Laboratory studies in animals and humans, described in greater detail below, have not been able to demonstrate significant adverse health effects at or below the current NAAQS attainment levels. The epidemiological studies have been further criticized for their

failure to conclusively separate PM₁₀ effects from those of other pollutants and for their inability to measure individual dose.

Particulate air pollution appears to correlate more specifically with daily mortality than do other pollutants such as SO₂, O₃, or CO in the experimental designs commonly used (Dockery *et al.*, 1993). However, using an alternative model, Moolgavkar *et al.* (1995) reevaluated Philadelphia mortality data originally investigated by Schwartz and Dockery (1992) and found that the observed effects between SO₂ and TSP could not be separated statistically. It has been argued that the apparent consistency of results across studies is due to a similarity of methods, and that residual confounding has not yet been accounted for sufficiently (Moolgavkar, 1994; Dockery, 1994). Dockery (1993) has emphasized the need for epidemiologists to find more creative, elegant designs to overcome the current limitations of the published studies, particularly in light of the EPA's refusal to infer a causal relationship between PM and adverse health effects based solely on epidemiology studies.

Particulate characteristics such as size, number, and composition can affect the magnitude and quality of associations observed, which may account for the variance across studies of reported risk levels (Thurston, 1996). Despite suggestive evidence from animal studies, there is a paucity of epidemiological research examining the relationship between increased health risk and specific PM constituents. Also, in many of the studies individual exposure is based on ambient levels monitored at single measurement stations; such limited measurements do not account for individual variability in outdoor exposure duration or physical activity. The percentage of indoor versus outdoor exposure is often greater than

70%, depending on the region, population, and season (Costa and Amdur, 1996).

Potential Changes in Current Policy

Current proposals to lower the PM₁₀ mass standard or change the measurement to a PM_{2.5} standard are being evaluated based partially on new findings suggesting that smaller, "fine" particulates (PM_{2.5}) are more closely associated with adverse human health consequences (Dockery and Schwartz, 1995). Coarse particulates (>3 microns) deposit in the upper airways and are eliminated relatively quickly via mucociliary clearance, while higher fractions of fine particulates deposit in the bronchoalveolar regions where clearance is much slower (Casarett, 1972). Dockery *et al.* (1993) demonstrated a stronger link between adverse health effects and fine PM than with any other measure of PM, O₃, or aerosol acidity. This and other similar studies reporting a stronger PM_{2.5} association have been questioned, however, based on the inability to correctly assess PM_{2.5} concentrations and individual human exposures.

Other proposals have suggested changing the 24 hour averaged PM₁₀ standard to a 1 hour average mass limit, based on brief excursions which surpass the 150 µg/m³ limit without driving the 24 hour average beyond attainment standards (RAMTRAC Corp, 1995). Many cities in the U.S. may exceed the 150 µg/m³ limit, especially during rush hour periods, but still remain in attainment for the 24 hour average. Whether these brief elevations contribute disproportionately to the epidemiologically observed morbidity and mortality remains unclear.

Particulate Matter Toxicology

As the myocardium is not in direct contact with ambient air, the strong association between PM levels and cardiac morbidity is unexpected. The assessment of adverse extrapulmonary effects of PM is particularly difficult with respect to the heart, as several pathways may be involved. Pulmonary deposition and retention of particulates in the lungs is well characterized, but only recently have researchers begun investigating clearance of particles by mucociliary action, macrophage phagocytosis, and uptake into the alveolar interstitium with subsequent distribution to extrapulmonary sites. The cardiovascular effects of PM could potentially be mediated by such translocation of particles or their constituents directly to cardiac tissue, although there is limited evidence to support such a theory. Alternatively, these effects may be caused by release of cytokines or other inflammatory mediators into the circulation by inflammatory or damaged cells, by hypoxia as a result of mismatched gas exchange across damaged or inflamed alveolar membranes, by neurohumoral action mediated by activation of pulmonary nerve fibers, or by some combination of these mechanisms. The plausibility of these pathways is discussed in greater detail below.

Deposition, Retention, Distribution and Clearance of PM

Dosage of particles is dependent on the exposure duration, size and concentration of the particles, as well as the frequency and depth of breathing. Individual health can also play a role, as evidenced by increased deposition of ultrafine PM in patients with obstructive lung

disease (Anderson *et al.*, 1990). A particle's regional deposition in the lung is influenced primarily by its aerodynamic diameter. Larger particles (>5 microns) having sufficient inertia to overcome the turbulent airflow of the upper airways normally deposit by impaction in the nares or bronchus. Environmentally relevant doses of these particles can be cleared to the esophagus by mucociliary action and are eventually removed by expectoration or swallowing. Particles of 1-5 μm deposit primarily by sedimentation in the airways and in the alveolar region where they can be phagocytized by alveolar macrophages which subsequently migrate to ciliated airways or enter the interstitium. Movement of non-phagocytized particles into the epithelial interstitium occurs at a rate which varies directly with dose and is inversely proportional to particle size (Ferin *et al.*, 1992; Oberdörster *et al.*, 1992).

Retention of PM is dependent on the initial deposition, and on the ability of the individual to clear the particle by the mechanisms mentioned above. Decreasing the effectiveness of clearance/defense mechanisms (mucociliary tract, phagocytes, etc.) will prolong the retention time and compound exposures. Under normal exposure conditions and given sufficient time, the lung can effectively clear particles via these mechanisms. However, this process is slowed considerably in the elderly and individuals with respiratory disease, e.g., chronic bronchitis, bronchial carcinoma, and asthma. Properly functioning respiratory defense mechanisms in animals can be overwhelmed by PM, but only with prolonged exposures several times greater than the current PM₁₀ NAAQS (Adalis *et al.*,

1978; Fairchild *et al.*, 1975).

Size of PM determines not only its regional deposition, but also its eventual fate within the lung. Phagocytosis of particles by macrophages is maximal when the particles are between 1.0 and 2.0 microns (Tabata and Ikada, 1987), although under conditions of low total lung burden, there is reasonably efficient macrophage uptake of smaller particles. In rats, ultrafine titanium dioxide particles rapidly enter the interstitium and come in contact with tissue macrophages, while larger particles (0.2 microns) are engulfed by alveolar macrophages and only enter the interstitium at or beyond 1 mg doses (Ferin and Feldstein, 1978). Oberdörster *et al.* (1992) confirmed this by demonstrating distribution differences between particles of 20 nm and particles of 250 nm. By re-instilling particle-containing alveolar macrophages into naive rats, they also demonstrated a protective role for phagocytosis. Oberdörster *et al.* (1992) also hypothesized that the higher the degree of interstitial deposition, the greater the chemotactic response, which would result in inflammatory thickening of the alveolar wall.

In addition to size, PM toxicity appears to be influenced by chemical composition and properties such as surface charge and solubility. Studies of various compounds have produced different results in terms of absorbance and distribution, as well as physiological effects. ROFA, the PM sample used in the present studies and described in further detail below, contains several soluble surface-complexed transition metals, all of which are observable in ambient derived PM samples, although often in smaller concentrations. These metals, particularly nickel (Ni), iron (Fe), and vanadium (V), appear to be important

mediators of ROFA toxicity as measured by airway hyperreactivity in rats (Dreher *et al.*, 1995). Ghio *et al.* (1992) demonstrated increases in lavagable protein and cellularity, as well as inflammatory changes in the cellular profile due to Fe loaded on silica. In a study of nickel compounds, Benson *et al.* (1986) found lower toxicity to the nearly insoluble Ni oxide as compared with highly soluble Ni chloride, Ni subsulfide, and Ni sulfate. Ni chloride has also been found to decrease ciliary beat frequency in hamsters, further impairing the pulmonary defense mechanisms (Adalis *et al.*, 1978). PM-complexed metals likely induce a Fenton-like reaction in the deep regions of the lung, leading to oxidant formation, cellular damage, and inflammatory cell recruitment (Costa *et al.*, 1996).

Both free and macrophage-associated particles gain access to the lymph system via interstitial uptake (Takahashi *et al.*, 1987). The elimination of such particles from the lymphatic system has not been thoroughly researched, and it is unclear whether or not PM or its constituents can be translocated to other organs. Reports examining blood and organ tissue for increases in transition metal concentration following concentrated ambient PM and fuel oil ash inhalation have been inconclusive (Godleski, 1996). However, nickel administered by *ip* injection to albino rats was preferentially sequestered into the myocardium at 7 and 14 days after exposure (Mathur *et al.*, 1978), and particles cleared from the lungs by mucociliary action are normally swallowed and enter the gastrointestinal tract. The transition metals which complex the surface of particles are often associated with contraction/conduction irregularities in studies examining isolated myocardial tissue (Evangelou and Kalfakakou, 1993), and, due to their solubility, may access extrapulmonary

sites through the lymphatics and/or circulation. The distribution kinetics of these metals suggest that while their direct translocation to cardiac tissue is plausible, it most likely occurs over a period of weeks and therefore could not account for the acute mortality changes associated with PM.

Animal and Human Studies of Pulmonary Effects

PM has been shown to cause lung function deficits in animals. Gross *et al.* (1992) instilled Fischer-344 rats with 10 mg or 40 mg silica and found dose-related decreases in inspiratory capacity and gas diffusing capacity over a period of one to six months. Similar results were reported by Wright *et al.* (1988) one month following instillation of 30 mg quartz in female Sprague-Dawley rats. Both studies reported increased amounts of nodular fibrosis over the course of several weeks to which these lung function changes were attributed.

PM and some of its isolated components have also been shown to alter immune defense function which may increase the infection rate and severity of pneumonia or acute bronchiolitis. Hatch *et al.* (1985) demonstrated significant excess mortality in influenza-infected mice exposed to various samples of PM. Demonstrations of decreased antibody production (Spiegelberg *et al.*, 1984) and virus-induced lung consolidation (Hahon *et al.*, 1985) in rodents caused by exposure to certain dusts (diesel exhaust and Ni oxide, respectively) suggest the immune response is only affected following several weeks of exposure.

There are a limited number of published studies on humans in a controlled environment. Yang and Yang (1994) demonstrated deficits in lung function tests and methacholine reactivity in asthmatic patients at a PM10 concentration of 202 $\mu\text{g}/\text{m}^3$. While the results demonstrated adverse effects at environmentally relevant exposure levels, they were confounded due to high levels of NO_2 , SO_2 , and CO contained within the air pollution mixture used in this study. Since all of these compounds are criteria pollutants and can affect the cardiopulmonary system, it is difficult to isolate the bronchoconstrictive effects of PM10.

Inhalation of radiolabelled particles (Technegas) for lung imaging procedures caused hypoxemia in 87% of subjects after only a few breaths (James *et al.*, 1992). These 50 nm particles are primarily carbon with surface complexed $^{99}\text{Tc}^{\text{m}}$. Hypoxemia was greater than 10% in over a third of the patients, which could lead to serious physiological consequences for patients with COPD or coronary/cerebral vessel disease.

Pathways for Potential Myocardial Insult

Pathways by which PM may affect the myocardium or signal conduction have been hypothesized, and some or all of the pathways posited may produce the observed effects. With no evidence of extrapulmonary distribution of PM or its constituents, it is likely that PM affects the heart by its action on the lungs. It is possible that the observed cardiovascular association with increased PM is merely the result of misdiagnosed pulmonary disease, especially end-stage COPD when cardiac abnormalities are common due

to hypoxemia (Bates, 1992). Alternatively, substantial PM-induced increases in endothelial permeability and resultant pulmonary edema could increase left atrial systolic pressure, which can, in turn, lead to slight but physiologically significant arrhythmias. The possibility of particle-induced (primarily ultrafines) chronic alveolar inflammation altering serum composition and predisposing individuals to clot formation and thrombii has been proposed (Seaton *et al.*, 1995) but is as yet unproven. Unpublished studies from this lab investigating changes in clotting time and fibrinolytic activity related to ROFA exposure were inconclusive (Gardner, personal communication). Other laboratories have reported inflammatory cytokine production and release following exposure to low levels ($9 \mu\text{g}/\text{m}^3$) of a Teflon combustion particle (median diameter 26 nm; Oberdörster, 1995). Circulating cytokines may contribute to the increased numbers of cardiac macrophages reported following exposure to concentrated PM (Godleski, 1996)

Neural pathways represent another possible mechanism by which PM could influence cardiac function. Studies of canine reflex response to ozone demonstrated the differential activity of non-myelinated afferent bronchial C-fibers during exposure (Coleridge, 1993). It is now believed that these reflex fibers act to decrease exposure of the lower airways to damaging oxidants by constricting the upper airways and reducing the tidal volume. PM possibly activates reflex receptors in the upper or lower airways which could alter cardiac responsiveness or function. This pathway is likely to be more significant in animal studies where dosages are often well in excess of environmentally relevant human exposures.

Methodological Justifications

The Electrocardiograph and Toxicology

Electrocardiography in the rat, as in humans, is an important diagnostic tool for determining cardiotoxicity. Many indices of heart function may be assessed through careful, systematic analysis of ECG rhythm and waveform parameters. The ECG pattern in healthy individuals (figure 1) reflects the depolarization and repolarization of cardiac tissue and is fairly constant in form. Cardiac arrhythmias by definition impede the consistent rhythmic depolarization essential for efficient cardiac output. Monitoring such alterations in the ECG waveform following exposure to toxic substances provides insight into both the magnitude and the underlying mechanism(s) of the cardiac insult.

Cardiac tissue of rats behaves similarly to that of humans, and the respective ECG waveforms, created by the summation of depolarizations of individual myocytes, are also remarkably similar. Despite the commonalities, there are several important differences in the rat ECG patterns, most notably, 1) increased HR, 2) sloping baseline of P-wave, 3) absent Q-wave, 4) no S-T segment, and 5) lack of isoelectric segment between T and P (figure 1). Some of these differences are attributable, to an extent, to the increased HR in rats.

Specific changes in individual components of the ECG may be associated with

specific changes in cardiac function. During hypoxia in humans, for example, the ST segment becomes depressed with the prolonged ventricular repolarization time caused by decreased ATP levels. QT dispersion, a measure of regional differences in myocardial repolarization due to hypoxemia, has been found to be predictive of sudden unexpected death in cardiac patients (Barr *et al.*, 1994).

Decreases in nerve conduction time produce varying degrees of atrioventricular (AV) node and bundle branch block and are characterized by prolonged P-R intervals and notched R waves, respectively. The occurrence of such specific arrhythmias may serve to implicate specific mechanistic or disease patterns. While occasional premature contractions or skipped beats are normal and generally innocuous, frequent episodes of multiple abnormal or missed beats may indicate more serious sinoatrial (SA) or AV node dysfunction.

The ECG signal can also be used to assist diagnoses of heart disease, and can be of prognostic value, as well. For example, a decrease in heart rate variability, which measures the sensitivity of the cardiac tissue to changes in adrenergic stimuli, is associated with mortality in patients who have suffered previous myocardial infarction. Increased activity of the vagus nerve acts to depress the rate of SA node firing and decrease contractility, while adrenergic substances increase rate and contractility via interaction with beta receptors on cardiac tissues. The SA node conducts impulses independently, but in the absence of stressors the vagus nerve maintains a tonic down regulation of this SA node rate. In healthy individuals, sensitivity to these varying stimuli is reflected by a high heart rate variability, while in patients with heart disease this responsiveness is decreased for reasons as yet

uncertain.

Known toxins (*e.g.*, zinc, nickel, anthracyclines) and endogenous substances (*e.g.* adrenergics, cholinergics) can alter cardiac conduction in a manner observable by ECG changes. Isolated guinea pig hearts perfused with 30 μM ZnCl_2 displayed marked arrhythmias from 10 minutes following the initial exposure until the end of the 65 minute observation period (Evangelou, 1993). These arrhythmias included complete and incomplete AV blockade, left BB blockade, and ventricular flutter. Also of interest was a sinus bradycardia which was elicited at lower doses of ZnCl_2 , as this response has been shown for several other toxins in intact animal studies.

Temperature, Hypothermia, and the Laboratory Rodent

Many previous studies have demonstrated a decrease in T_{CO} in the rodent following exposure to a variety of xenobiotic agents, including ozone, chlordimeform, and carbon monoxide (Gordon *et al.*, 1988; Watkinson and Gordon, 1993; Gautier and Bonora, 1994). Other physiological and metabolic functions such as HR, minute ventilation, and oxygen consumption also decline in a manner well correlated with T_{CO} , creating an overall metabolic depression referred to as the hypothermic response. It has been proposed that a moderate hypothermic response (characterized by an $\approx 2^\circ\text{C}$ decrease in T_{CO}) may be advantageous as demonstrated by a decreased toxic response and increased survival, while more severe hypothermia ($>3^\circ\text{C}$) may exacerbate the toxic effects and decrease survival (Watkinson and Gordon, 1993).

Rodents primarily dissipate heat through unfurred areas, such as the tail, ears, and feet, as well as through respiratory exchange. The tail skin temperature has been reported to increase as much as 9°C following toxicant exposure due to the dilation of the tail artery and subsequent shunting of blood flow to the tail. Rats typically display smaller decreases in T_{CO} than mice following the same dosage of toxin. This effect is presumably due to the increased tail surface area to body weight ratio in the mouse, as mass is an important determinant of heat capacity, while heat dissipation is proportional to the tail skin surface area. Since HR decreases along with T_{CO} , it has been suggested that this response is mediated by an increased vagal nerve tone, although the precise mechanism(s) from stimulus to response is uncertain.

The thermoregulatory response to xenobiotics is controlled behaviorally as well as physiologically. Rats will select cooler ambient temperatures (T_a) within a temperature gradient cage to optimize their heat dissipation following toxicant exposures, but when the T_a is maintained higher or lower than the preferred optimum, toxicity is increased and survival is compromised (Gordon, 1988; Watkinson and Gordon, 1993). Manipulation of the T_a also affects the magnitude of the decrease in T_{CO} , as well as that of the heart rate (Watkinson et al., 1992). Conversely, rats with fever caused by exposure to the pyrogen methamphetamine (MDMA, "Ecstasy") demonstrated increased survival in colder environments and in cages without bedding or other restrictions to heat dissipation (Watkinson, personal communication).

Hypoxemia caused by decreased available oxygen or CO exposure in rats also

induces a hypothermic response which is capable of being modulated by changes in the T_a (Gautier & Bonora, 1994). The advantage of reducing T_{CO} during hypoxia is well documented in clinical and experimental research of brain and cardiac tissue damage during oxygen reperfusion (Gambassi *et al.*, 1994). It has been suggested that hypothermia allows a reduction in the metabolism of oxygen, which, during periods of ischemia, delays uncoupling of oxidative phosphorylation pathways and subsequent production of oxygen radicals. Decreasing metabolism may also decrease the dose of inhalable toxins by lowering the minute ventilation.

Humans rarely exhibit hypothermia except in instances of severe illness or prolonged exposure to cold. Reports of acute accidental exposure to toxins generally find no change in human T_{CO} nor evidence of bradycardia, unless poisoning is severe. Importantly, the hypothermic response in rodents creates a physiologically different model for toxicological studies as the differences in T_{CO} , HR, and metabolism presumably alter the toxicokinetics and disposition of the studied xenobiotic. Since humans do not suffer hypothermia at environmentally relevant exposures, the extrapolation of animal data to reference doses for humans becomes problematic.

Residual Oil Fly Ash

The PM used in the present study, ROFA, is a source-derived pollutant extracted from the emission stack of an oil-burning power plant in Florida, with a mmad of 1.95 ± 0.18 microns and a geometric mean of 2.19 ± 0.22 . Chromatographic analysis of ROFA

constituents revealed concentrations of several transition metals, including iron, nickel, and vanadium that were several orders of magnitude higher than normal ambient levels. Table 1 shows the relative weights of all detectable constituents, along with the average make-up of ambient PM. Endotoxin concentration was insignificant, only accounting for 2.5 pg/mg.

ROFA is a particularly toxic component of ambient PM, possibly due to the high levels of soluble transition metals, several of which are reported to be arrhythmogenic agents. Gavett *et al.* (1996), using two samples of particulates, demonstrated a difference in toxicity driven primarily by increased levels of Zn²⁺ and Fe²⁺. Samples of ROFA which have been washed to remove excess soluble transition metals (V, Fe, Ni) elicited decreased toxic responses in Sprague-Dawley rats (Dreher, 1995).

Animal Models of Disease

While evidence of PM-associated morbidity and mortality is observed in the general population, a high degree of tolerance to PM toxicity exists in healthy individuals (Yang and Yang, 1994). The epidemiological studies which limit analyses to subjects with cardiopulmonary disease show stronger associations (Burnett, *et al.*, 1995; Schwartz *et al.*, 1995), suggesting that such disease may cause heightened susceptibility to PM. It is therefore appropriate to study animal models of disease that approximate the susceptible human subpopulations which appear to be driving the statistical associations seen epidemiologically, *i.e.*, cardiovascular and pulmonary disease patients. Thus, this study examined the toxic effects of PM in animal models chosen to simulate three known risk

factors: 1) cold-acclimated rats, 2) rats with pulmonary inflammation, and 3) rats with pulmonary inflammation and hypertension.

Cold Acclimation Model

Winter months are associated with higher mortality in humans caused by cardiovascular disorders. The nature of this excess mortality is unknown, although studies have demonstrated that many human cardiovascular disease risk factors, e.g. cholesterol, triglycerides, body mass, blood pressure, fibrinogen, and clotting factors, attain peak serum levels during January or February (Woodhouse et al., 1994). High density lipoprotein concentrations fall roughly 10% when daily air temperature decreases from 20°C to 0°C (Elwood et al., 1993). In most of these studies, however, daily activity is not controlled for and likely plays a significant role. In a study comparing heart disease risk factors in rural and urban men, Reddy et al. (1994) found higher levels of cholesterol, low density lipoprotein, and triglycerides, and lower levels of high density lipoproteins in males living in urbanized areas. The authors attributed part of this association to the higher levels of air pollution in urban areas, although significant differences were also seen in dietary behavior and no quantitative analysis of air quality, indoor or outdoor, was performed.

When exposed to colder ambient temperatures, blood pressure (BP) and HR in rats increase, which, under chronic conditions may lead to left ventricular hypertrophy (van Bergen *et al.*, 1992). Rats acutely exposed to cold T_a (5°C) increase HR immediately (≈ 100 bpm) and increase mean BP 10 mmHg within 60 minutes of exposure (Fregly *et al.*, 1989).

Cardiovascular response (BP and HR) to the adrenergic agonists isoproterenol and phenylephrine is less sensitive in cold-acclimated rats than in rats maintained in normal temperature, a characteristic which is similar to diseased hearts in humans with decreased heart rate variability.

Cold-acclimatization involves a number of behavioral and biochemical changes following initial exposure to lower temperatures. Shivering thermogenesis is the first mechanism of adaptation, peaking after roughly 24 hours of cold then gradually reducing to normal over several weeks (Gordon, 1993). Release of norepinephrine and epinephrine stimulates HR and peripheral vasoconstriction, as well as the release of glycogen and free fatty acids from the liver to begin non-shivering thermogenesis. In order for the rat to maintain thermogenesis and normal T_{CO_2} , its cardiovascular system must increase its output to match the increased oxygen metabolism. HR in rats housed at 10°C ambient temperature will rise 20% over rodents acclimated to 22°C (Watkinson *et al.*, 1995), with concomitant increases in oxygen consumption from 60 to 100% (Banet, 1988; Jansky, 1966).

Pulmonary Inflammation Model

Pulmonary inflammation is common to many lung diseases including asthma, bronchitis, and COPD. Differences in inflammatory cell profiles are important from a diagnostic standpoint, particularly early in the development of disease. Asthmatics have high levels of eosinophils and mast cells, while macrophages and plasma cells are more prominent in COPD and bronchitis (Jeffery, 1994). Acute exposure to O_3 produces a

cellular infiltrate similar to the latter two diseases without the progressive damage and fibrosis which are common in the chronic disease states.

O₃ is a tropospheric criteria pollutant which can attain levels over 0.5 ppm in highly polluted cities around the world. Acute human exposure to high concentrations of O₃ results in pulmonary function deficits, increased airway reactivity, epithelial cell damage, and enzyme inactivation (Jimba *et al.*, 1995; Pino *et al.*, 1992). A mouse study, using a similar protocol to that of the present study described below, found reduced cardiac protein synthesis at 24 hours post exposure, posing a possible complication in interpreting the data from this study if rat myocardium behaves similarly (Kelly and Birch, 1993). Another concern is that ozone has been found to enhance epithelial particle uptake in a dose related manner in cell cultures (Churg *et al.*, 1996).

In a study by Pino *et al.* (1991), O₃ was administered to rats for 6 hours at 1 ppm, resulting in increased lavagable protein and neutrophils and increased alveolar monocytes (the latter result was reported, but non-significant) at 18 hours following the cessation of exposure. The same protocol for ozone exposure was used in the present study prior to ROFA instillation as a model of lung inflammation.

Costa *et al.* (1996) proposed that inflammation of the lungs increases the constituents capable of undergoing Fenton-like reactions with PM surface-complexed metals to produce oxygen radicals. Kodavanti *et al.* (1996) administered ROFA to bleomycin-treated rats as a model of chronic airway fibrosis and showed increased response of BAL parameters during ongoing fibrosis, but not at peak inflammation.

Another notable effect of ozone in rodents is its ability to induce a hypothermic response, as previously described. The magnitude of this response is proportional to the ozone concentration, and duration and frequency of exposure, and can be modulated by ambient temperature (Watkinson *et al.*, 1997). This response rapidly resolves itself following the cessation of exposure. Sub-chronic studies have shown that this acute response to ozone, as well as the development of inflammation, diminishes over several days of treatment, presumably due to the increased expression of anti-oxidant enzymes.

Pulmonary Inflammation and Hypertension Model

Pulmonary disease, particularly COPD and bronchitis, are associated with excess hospital admissions during episodes of high PM pollution. Often associated with tobacco smoke, COPD is an inflammatory disease of the bronchi and bronchioles which is similar in many ways to asthma. Persistent cellular infiltrate, consisting primarily of macrophages and plasma cells, develops into mucous hypersecretion, smooth muscle hypertrophy, and fibrosis and stenotic narrowing of the bronchioli (Jeffery, 1992). COPD is associated with a progressive deterioration of functional capacity and can develop into irreversible emphysema (Jeffery, 1994). Loss of vasculature in moderate to severely affected patients, along with occasional atelectasis and increased heterogeneity of gas perfusion ratios (Marshall *et al.*, 1993), increases the susceptibility of these individuals to hypoxemic/ischemic situations.

Primary pulmonary hypertension is rare, idiopathic, and occurs most often in young

females. Secondary pulmonary hypertension, however, often occurs in COPD patients as a result of prolonged inflammation and fibrotic vessel obstruction. Since both forms of pulmonary hypertension result in increased work loads on the right ventricle, they can lead to cor pulmonale, or hypertrophy and sometimes failure of the right ventricle.

Monocrotaline (MCT; Aldrich Chemical Corp, Milwaukee, WI), a toxin extracted from the seeds of *Crotalaria spectabilis*, is selective for endothelial tissue and is used to induce pulmonary hypertension in laboratory animals. To form the putative toxin monocrotaline pyrrole (MCTP), MCT must be bioactivated by hepatic cytochrome P450 PCN-E. Due to alternative metabolic elimination pathways, less than one tenth of the total MCT becomes oxidized to the pyrrolic form (Yan and Huxtable, 1994). The half life of MCTP in the circulation is roughly five seconds due to its unstable electrophilic nature in blood (Bruner *et al.*, 1986), which, together with the three second blood circulation time in the average rat, suggests that the majority of MCTP becomes degraded by the time it completes its second pass (Roth and Reindel, 1991). It follows logically that the first capillary bed that MCTP encounters after its formation would be a primary target tissue, and, indeed, the pulmonary vasculature is the site of greatest insult in rats following MCT injection.

Hayashi *et al.* (1967) first reported the use of MCT for a rat model of cor pulmonale, or hypertrophy of the right ventricle due to increased pulmonary artery pressure (PAP). Later studies supported this model with measurements demonstrating elevated PAP in rats ten days following MCT administration (Reindel *et al.*, 1990). The pathogenesis of MCT-

induced pulmonary hypertension occurs over a period of two to three weeks, beyond which the dosed animals gradually succumb to the chronic cardiopulmonary damage. Toxicological effects are not seen *in vivo* until three days post injection, although *in vitro* studies of endothelium show inhibition of cell proliferation within the first 24 hours (Roth and Reindel, 1990). Congestive heart failure has been noted and occurs as early as three weeks following injection (Pelá *et al.*, 1990). Feeding experiments by Blaustein *et al.* (1965) demonstrated lipid-containing arterial lesions in rats eating MCT-containing chow. These atheromatous lesions were notable in the coronary arteries after several weeks of feeding at low doses (50 mg/kg of chow). Other extrapulmonary effects of MCT have been observed in the liver, kidney, and left ventricle (Guzowski and Salgado, 1987).

The precise mechanism of MCT toxicity remains a topic of continued research, although repair and regeneration abilities of the pulmonary endothelium seem to be greatly compromised by MCT. Elevations of growth factors TGF- β 1, β 2, and β 3 were observed in rat lung tissue from four days to three weeks following MCT administration, while concentrations of growth factors PDGF-B and endothelin declined over this time (Arcot *et al.*, 1993). Inhibition of endothelial cell proliferation is thought to be caused by MCTP-induced DNA cross-linking (Wagner *et al.*, 1993), which may also be central to the endothelial cell wall deterioration. Lipke *et al.* (1993) attributed the elevated PAP blood pressure to increased deposition of fibronectin and laminin which causes a narrowing of the capillary lumen in the lungs. Fibrinolytic activity in lungs has been shown to decrease following administration of MCTP, possibly due to altered expression of plasminogen

activator inhibitor by endothelial cells (Schultze and Roth, 1993).

Materials and Methods

General. These studies monitored T_{CO} , HR, and ECG of rats exposed to ROFA via intratracheal instillation. Data acquisition was facilitated by the use of surgically implanted radiotelemetry devices (Data Sciences International Inc., St Paul, MN). Toxic effects were examined and compared among four groups: 1) animals housed at normal ambient T_a (20-24°C), 2) animals housed at cold ambient T_a (10 - 14°C), 3) animals with pulmonary inflammation, and 4) animals with pulmonary inflammation and hypertension.

Animals. Male 60 day old Sprague-Dawley rats (Charles River Laboratory, Raleigh, NC) were used in all protocols. Rats were allowed one week following delivery for acclimation and recovery of normal circadian rhythm before the experiments began. Following surgery, rats were individually housed in plexiglas cages (28 × 17 × 12 cm) within a specially designed, climate controlled exposure chamber. T_a was maintained at 21-24°C unless otherwise noted for a specific protocol and the relative humidity ranged from 40-65%. A

twelve hour light-dark cycle was imposed. Food and water were available ad libitum throughout the experiment.

Experimental Preparation. Animals were anesthetized with pentobarbital sodium (Abbott Laboratories, North Chicago, IL; 50 mg/kg, i.p.) and incised at the midline. Using a 16g needle, the peritoneum was punctured on either side of the incision, roughly 1cm lateral, through which the ECG leads were passed from the peritoneal cavity to the subcutaneous region. These leads were then positioned such that the positive lead was caudal to the right clavicle and the negative lead placed just below the left forelimb (figure 2). The ends of these leads were secured subcutaneously with sutures. The body of the transmitter was placed unsutured within the peritoneal cavity and the peritoneal muscle wall incision was closed with suture. Cutaneous incisions were closed with either sutures or autoclips.

All procedures were performed using clean surgical technique, and all instruments were sterilized either in ethanol (100%) or by autoclaving. Warm water bottles were placed within the cages for 1-2 hours post-surgery to help animals regain normothermia and speed recovery from anesthesia. Animals were allowed a minimum of five days for recovery from surgery and the reestablishment of circadian rhythm.

Experimental Protocol. These studies were conducted in four phases (see below). Within each phase, animals were arbitrarily assigned to groups (n=4 per group) receiving one of four doses of ROFA (0, 0.25, 1.0, 2.5 mg) by intratracheal instillation, unless otherwise noted in the specific protocols. ROFA was suspended in saline such that all instillates were 0.3 cc in volume, and all doses were administered to the rats while under light halothane anesthesia. At the time of instillation, rats ranged in body weight from 320-410g.

Phase 1: ROFA exposure in healthy animals.

Twelve days post-surgery, animals were divided into four groups (n = 4 per group) and instilled with one of four doses of ROFA (0.0, 0.25, 1.0, or 2.5 mg). Animals were maintained at an T_b of 21-24°C and monitored telemetrically for 96 hours, at which time they were anesthetized (sodium pentobarbital, 60 mg/kg, ip) and euthanized via exsanguination and pneumothorax. Whole lung and heart tissue were dissected and treated as described below.

Phase 2: ROFA exposure in cold-acclimated animal.

With the exception of a lowered ambient temperature, the protocol for the second study was identical to that of the first. For this study, animals were allowed five days to recover from surgery after which chamber temperature was lowered to 10°C. Five additional days were allowed for the animals to acclimate to the cold temperature. ROFA

was then administered on the tenth day and the protocol proceeded as above.

Phase 3: ROFA exposure in animals with pre-existing pulmonary inflammation.

Following a 31 day recovery from surgery, rats were exposed to either ambient ozone (1 ppm×6 hours) or filtered air 18 hours prior to ROFA or saline instillation. Dosing groups were divided as follows: Air/Saline (n=3), Ozone/Saline (n=4), Air/ROFA (n=4), Ozone/ROFA (n=4). Data acquisition and serum collection were carried out as described below.

Phase 4: ROFA exposure in animals with MCT-induced pulmonary inflammation and hypertension.

MCT (60 mg/kg, ip) was administered to all rats in this phase at twelve days following surgery. To permit the development of pulmonary hypertension, twelve additional days were allowed before ROFA was administered. ROFA instillation occurred on the 24th day after surgery and, as above, animals were monitored for 96 hours. At the end of the monitoring period, surviving animals were anesthetized (pentobarbital, 50 mg/kg) for serum retrieval. Lungs and heart ventricles from all animals were dissected and weighed.

Telemetry Data Acquisition and Analysis. Ten second strip chart recordings (Astromed, West Warwick, RI) of the filtered and amplified ECG signals of all sixteen animals per phase were printed every five minutes for the first hour after ROFA instillation, every 10

minutes for the next six hours, and every thirty minutes for the remainder of the 96 hour monitoring period. T_{CO_2} , HR and activity data for all rats were collected by a computerized acquisition system (Dataquest IV, Data Sciences International Inc., St Paul, MN; figure 3) at ten minute intervals and stored on disk for the duration of the study. These files were then sorted and processed to eliminate obvious signal errors and artifacts. Thus, temperature values above 40°C and below 30°C and HR values greater than 500 bpm and lower than 200 bpm were removed from further analysis. Excluded values represented less than 5% of all acquired data points.

Tissue Sample Collection and Analysis. At the conclusion of the 96 hour monitoring period, individual samples of whole blood were withdrawn from the aorta of pentobarbital anesthetized rats and centrifuged (1500 rpm × 10 minutes) to separate serum, which was promptly transferred to sample tubes and frozen (-80°C). Serum was assayed for specific enzyme markers of cardiac damage (creatine kinase-mb, lactose dehydrogenase, and hydroxybutyl dehydrogenase), as well as more general markers of liver and renal dysfunction (sorbital dehydrogenase, alkaline phosphatase, bile acids, lactose dehydrogenase, alanine aminotransferase, and aspartate aminotransferase). All samples were assayed using commercially prepared kits, controls, and standards from Sigma Chemical Company (St. Louis, MO). All assays were modified for use on the Cobas Fara II centrifugal spectrophotometer (Hoffman-La Roche, Branchbury, NJ). For each rat, the heart was excised, trimmed of atrial and arterial tissue, washed twice with saline to remove excess

blood, and blotted dry. The right ventricle was dissected away from the left ventricle and septum and both sections were weighed and recorded as the ratio of the weights (right ventricle/left ventricle + septum). The lung and trachea were dissected at the base of the larynx, cleared of any residual external tissue, and weighed. All lungs were fixed for morphologic analysis by inflation with 4% paraformaldehyde (\approx 10 ml per lung).

Statistics: Sorted and processed HR and T_{CO} data were averaged within dosage groups and analyzed by Tukey's fixed effects ANOVA. All terminal endpoints (serum enzymes, organ weights, etc.) were averaged by group and compared via Student's paired t-test. Probability values of ≤ 0.01 were considered significant.

Results

Core Temperature

ROFA instillation elicited a transient dose-dependent decrease in T_{CO} in animals housed at 22°C. Animals administered saline exhibited a brief elevation in T_{CO} due to handling, but returned to preexposure levels within a few minutes. T_{CO} of high dose animals decreased 2.2°C (group mean) and returned to control values within six hours post-instillation (figure 4). Low and medium dose rats displayed less severe T_{CO} decreases (0.6°C and 1.5°C, respectively), with durations considerably less than those of the high dose animals. The T_{CO} response of animals housed at 10°C was similarly dose-related, but potentiated compared to that of the 22°C animals. The T_{CO} of the high dose rats housed at 10°C decreased 3.5°C, returning to control values approximately six hours following ROFA administration (figure 5). The T_{CO} of low and medium dose rats in the low temperature regimen decreased 0.5°C and 2.9°C, respectively. In both studies control animals displayed an increase in T_{CO} which was both brief (< one hour) and moderate (<0.5°C).

In both T_a regimens, the high dose rats exhibited a delayed hypothermia of up to 1.0°C. This delayed response was most pronounced over a range of approximately 24-36 hours following ROFA instillation in animals housed at 22°C and over a range of 12-72

hours post-instillation in animals housed at 10°C.

The T_{CO} of animals with ozone-induced pulmonary inflammation decreased an additional 1.8°C compared to that of healthy animals given the same dose (2.5 mg) of ROFA (figure 6). This hypothermia lasted approximately 18 hours and then returned to control values. A slight, but significant delayed response was observed in ROFA-administered animals.

Animals with MCT-induced pulmonary hypertension decreased T_{CO} in a dose-dependent manner immediately following ROFA administration (figure 7). T_{CO} of low dose rats decreased 1.0°C immediately following ROFA administration, but returned to pre-exposure levels within two hours. Significant deviations from control values (up to 0.5°C) were observed in this low dose group sporadically from 30–42 hours after instillation. The acute T_{CO} decrease was dose dependent (3.0°C and 3.5°C below control in medium and high, respectively), however, the high dose group returned briefly to control values while the medium dose group steadily decreased throughout the study. At 24 hours post-instillation, the T_{CO} of the high dose group also began a gradual decline which remained 1.0°C below that for the saline control group at the end of the study.

Heart Rate

In general, HR response patterns were highly correlated with T_{CO} responses in all groups. In all regimens, saline animals exhibited slight increases in HR for a few minutes immediately following handling. Average HR of medium dose rats housed at 22°C

decreased 75 bpm below control for one hour immediately following instillation, while the HR of high dose animals decreased 95 bpm below control for a four hour duration (figure 8). HR of low dose rats did not significantly differ from that of saline-treated animals. Rats housed at cold T_a demonstrated a two-fold greater decrease in their HR response, with medium dose rats decreasing HR 160 bpm, and high dose rats decreasing HR 175 bpm (figure 9). Low dose rats housed at 10°C also demonstrated a brief but significant decrease over controls (63 bpm), driven in part by the transient HR increases observed in control animals following handling.

High dose animals of both the 22°C and 10°C T_a studies also displayed a delayed bradycardic response. The 22°C T_a animals returned to control values roughly four hours post-instillation, but exhibited further decreases in HR beginning at 12 hours which were markedly lower than control from 24-36 hours following instillation. High dose animals in the 10°C T_a study never returned to control levels and demonstrated significant differences (up to 160 bpm) from control values for over 48 hours.

Ozone-induced inflammation potentiated the decrease in HR over air-exposed rats receiving the same dose of ROFA (124 bpm versus 95 bpm), however, the delayed response was weaker in the ozone pretreated rats (figure 10). While the nature of this diminished response is unclear, it could potentially be due to induction of antioxidant enzymes by O_3 . HR was markedly decreased following ozone exposure but returned to control values before ROFA instillations were conducted. HR in ozone treated rats displayed a rebound effect on day three, rising 0.5°C above control.

Animals with MCT-induced pulmonary hypertension demonstrated decreased HR in response to all doses of ROFA (55, 107, and 122 bpm in low, medium, and high dose, respectively; figure 11). Low dose rats returned quickly to control values with the exception of one lethality, but displayed significant decreases in HR from controls during dark periods when activity is normally elevated. High dose rats returned to control levels 24 hours following instillation, but thereafter demonstrated a progressive bradycardia for the remainder of the monitoring period. Medium dose rats responded similarly to the high dose rats, but did not return to control values and displayed a greater decrease in HR. It should be noted that this response is probably a statistical anomaly, driven in part by the low subject number. Disproportional lethalties (1, 3, and 2 in the low, medium and high dose groups, respectively) precluded analysis of both the high and medium groups with the statistical models used for the previous regimens.

Arrhythmias and Electrocardiogram Changes

In all studies, exposure to ROFA was associated with a dose-dependent increase in the frequency of cardiac arrhythmias. Several types of arrhythmias were commonly observed, ranging in severity from occasional innocuous premature contractions and R-R interval changes (figure 12) to serious conduction deficits such as prolonged AV blockade and bundle branch blocks (figure 13).

In rats housed at an T_a 22°C, the frequency of arrhythmias was increased in high and medium dose groups for the first 24 hours, but slowly subsided thereafter (table 2). The

medium dose animals of the cold T_a study behaved in a similar manner, but the frequency of arrhythmic events in the high dose animals did not diminish over the second 24 hour period (table 3). Ozone-induced pulmonary inflammation did not appear to have any marked effect on the frequency or severity of arrhythmic events when compared to untreated animals receiving ROFA (table 4).

MCT-treated animals displayed increases in the frequency and severity of arrhythmic events following all doses of ROFA (table 5), and the frequency of arrhythmias was elevated at the end of the 96 hour monitoring period for all three groups receiving ROFA. Depression of the S-T segment area was observed in several of the surviving animals. Lethalities occurred in all dose groups except control and were preceded by severe hypothermia and bradycardia, as well as distinct ECG abnormalities. These abnormalities were generally expressed in one of two ways: 1) depressed S-T segments which lasted over an hour accompanied by gradual myocardial failure (figure 14), or 2) sudden myocardial failure with associated conduction abnormalities (figure 15).

Gross Organ Pathology and Serum Analysis

In all studies, ROFA caused a dose-dependent increase in lung wet weight and produced observable inflammation, necrosis, and focal edema of the lung. RV/LVS was not affected by ROFA in any study, although MCT-treated rats displayed significant ($p < 0.001$) hypertrophy of the right ventricle ($RV/LVS = 0.3479 \pm 0.058$) compared to rats not receiving MCT (0.2630 ± 0.056). Euthanized MCT-treated rats had a significantly higher

RV/LVS ratio ($p < 0.01$) than rats that died during the study (0.3077 ± 0.034).

No significant alterations in serum enzymes were observed in response to ROFA exposure. MCT-treated animals had increases in liver biomarkers (lactose dehydrogenase, bile acids) and one cardiac marker, hydroxybutyl dehydrogenase (HBDH), over animals not receiving MCT, but these showed no association with ROFA dose. The single surviving medium dose MCT-treated animal had severely elevated levels of most hepatic and cardiac enzymes (table 8).

Discussion

The results of the present study support those of previous studies which observed significant dose-related decreases in T_{CO} and HR in rodents following exposure to xenobiotic agents. These results also support epidemiological studies which suggest that preexisting cardiopulmonary disease may be a risk factor for PM-related toxicity (Burnett *et al.*, 1995; Schwartz and Morris, 1995), and implicate both conductive and hypoxemic ECG mechanisms in cardiac -related lethalties

The hypothermic response elicited by ROFA appeared to exhibit a biphasic pattern and was exacerbated by changes in experimental conditions and animal models of cardiopulmonary disease. The acute phase of these parameter decreases potentially represents an inherent dose-dependent response to xenobiotic agents similar to that previously reported for ozone or chlordimeform exposure (Watkinson and Gordon, 1993), while the delayed phase may be related to subsequent inflammation or edema. The delayed phase of the observed response is perhaps unique to ROFA, as previously reported hypothermic responses to ozone were of an acute nature and were resolved following cessation of exposure (Watkinson *et al.*, 1994). The delayed hypothermia elicited by ROFA

coincides with the development of pulmonary inflammation and resultant edema; as respiratory exchange is a means of thermoregulation in the rat, such inflammation may increase heat dissipation and at least partially promote the observed delayed phase hypothermia. Several previous studies have reported increases in PMN, macrophages, and total cells in bronchoalveolar lavage fluid 24-72 hours following instillation of various xenobiotics (Hirano *et al.*, 1990; Bowden and Adamson, 1978). Specifically, Bowden and Adamson (1978) demonstrated a delayed increase of lavagable and interstitial inflammatory cells following instillation of carbon particles. These carbon particles were quickly taken up by Type I alveolar cells but did not access the interstitium until 12 hours postinstillation (Adamson and Bowden, 1978). Unlike O₃, which reacts rapidly with the lung surfactant layer, PM may reside in the lungs for days before it is cleared (Pepelko, 1987), causing prolonged irritation and inflammation. Such retention of PM may play a role in the different patterns of hypothermic response caused by the two toxicants.

Experimental models of reported risk factors for PM exposure (cold-acclimated, pulmonary inflammation, and pulmonary inflammation and hypertension) were shown to potentiate the observed hypothermic response. The magnitudes of the maximum decreases in T_{CO} and HR seen in normal animals exposed to 2.5 mg ROFA (2.2°C; 95 bpm) were significantly increased in the cold T_a animals (3.5°C; 175 bpm), the O₃-preexposed animals (3.8°C; 125 bpm), and the MCT-treated animals (3.1°C; 120 bpm). This exacerbation of PM toxicity elicited by animal models of cardiopulmonary disease supports findings of epidemiological studies which suggest that humans with similar diseases may be at greater

risk for PM morbidity and mortality. However, these dose-related changes in T_{CO} and HR, which may be specific to either rodents and/or the high dosages administered suggest that the rodent physiology/metabolism is different at each dose.

The increased hypothermic response in cold-acclimated animals may be at least partially attributable to an increased "driving force". Thus, the increased magnitude of the difference between T_{CO} and T_a improves the efficiency of hypothermic effectors resulting in a larger decrease in T_{CO} . Similarly, HR in cold-acclimated rats may range from 50-100 bpm higher than rats acclimated to 22°C depending on the state of wakefulness and level of activity and also has a greater potential for change.

While the driving force for the hypothermic response is obviously greater for the animals housed at 10°C T_a , it is unclear why the O_3 preexposed animals responded so severely, particularly in light of studies showing rapid adaption to repeated ozone exposure and attenuated hypothermic response (Watkinson *et al.*, 1995). O_3 can increase permeability of lung tissue, which increases the fluid and constituents in the lung capable of radical formation via Fenton-type reactions. O_3 can also inhibit mucociliary clearance, thereby increasing the exposure time to larger particles.

The reported epidemiological association between increased cardiac symptoms and increased PM exposure in humans is supported by the results of the present study. Godleski (1996) has recently presented evidence of T wave alternans in dogs exposed to PM, an effect reported to be a predictor of fatal arrhythmias. The present study demonstrated increases in the frequency and severity of arrhythmic events following ROFA instillation. The

induced arrhythmias ranged in severity from changes in R-R interval and occasional premature beats to serious conduction defects and skipped beats. The imposed risk factors potentiated these events, with the greatest effects seen in the MCT-treated animals, although even control animals from this group sometimes exhibited spontaneous arrhythmias.

Whether the ROFA-induced arrhythmias in the rat are relevant to the human condition remains unclear. Arrhythmias and bradycardia of a similar nature have been reported in rats following ozone (Arito *et al.*, 1990) and nitrogen dioxide exposure (Tsubone *et al.*, 1982). Tsubone *et al.* (1982) attenuated these effects by atropine injection, suggesting the bradycardia was caused by increased vagal tone. Many of the specific arrhythmic events elicited by ROFA instillation were reproducible by intravenous infusion of acetylcholine in anesthetized rats (Addendum A), strengthening the hypothesis that the observed hypothermic response may be partially mediated by the parasympathetic nervous system. This preliminary study of the mechanism(s) of this response demonstrated Mobitz type II AV node blocks and premature ventricular contractions following methacholine or acetylcholine injected via the jugular vein of saline- and ROFA- treated rats. In one subject, acetylcholine infusion elicited a severe conduction disturbance for a seven second period, during which the heart was unable to generate a single beat. This evidence suggests the possible existence of a central nervous system component of PM toxicity, however, it may be specific to rodents and have little or no role in the reported mortality and morbidity in humans.

Lethality was only observed in the MCT-treated group and appeared to be consistent with either 1) a relatively slow failure of the myocardium due to the presence of pulmonary

edema and consequent hypoxemia leading to myocardial ischemia, as shown by severe ST-segment area depression (figure 14), or 2) an abrupt failure of the heart due to a conduction-related fatal arrhythmia (figure 15). The latter scenario was common to most lethalties, and was characterized by a relatively stable rhythm with considerable conductive "noise" or myocardial irritability. These scenarios concur with two predominant hypotheses of why cardiac hospital admissions are positively associated with increased PM levels. Misdiagnosis of heart failure which is actually secondary to edema and subsequent hypoxemia was suggested by Bates (1992) and is consistent with the first lethality scenario. Sudden unexpected death from heart failure may also be associated with hypoxemic conditions (Barr, 1994; Kiely et al. 1995) and appears consistent with the latter hypothesis.

The ECG abnormalities in lethalties characterized by severe cardiac irritability (figure 15) are distinguishable from the specific arrhythmias typical of those brought on by acetylcholine infusion (figure 16). Because such ECG "noise" was not observed in surviving animals, the mechanism of mortality in MCT rats may be due to a different mechanism than that of the hypothermic/parasympathetic response. It is clear, however, that these cardiac abnormalities are important components of the observed PM toxicity.

Hypothetically, ROFA and its constituents can affect the myocardium directly via translocation through the bloodstream, or indirectly by its effects on the lungs. Surface-complexed transition metals, especially Fe, V, and Ni, are known cardiotoxins, however, their presence in the bloodstream has yet to be reported. Cytokine production and release into the bloodstream has been reported following inhalation of particles (Oberdörster, 1995),

and increases in cardiac macrophages have been observed following exposure to oil fly ash (Godleski, 1996). Whether these two observations are related mechanistically and whether or not they contribute to the arrhythmias reported in the present study are unknown. Decreased gas exchange brought on by PM-induced inflammation may be severe enough in respiratory disease patients to cause hypoxemia which may create observable effects on cardiac function. Similarly, a slight, otherwise innocuous decrease in blood oxygen level may lead to regional ischemia in coronary artery disease patients. It is quite probable, given the varied compositions of regional PM and the panoply of suggested risk factors for PM toxicity, that all of these mechanisms, as well as others as yet unknown, may play a role in the epidemiologically observed morbidity and mortality

There are several methodological aspects of the present study that should be considered. Previous studies have been criticized regarding the environmental relevance of the high doses used to elicit responses from subjects. While 2.5 mg ROFA is low in comparison with several other animal studies, it is three orders of magnitude greater than exposures causing mortality in humans. However, recent reports of occupational settings with fly ash levels exceeding 3 mg/m³ suggest that humans may be exposed to doses in this range (Hauser *et al.*, 1995). While sample size and individual variation are important factors, the observed response of MCT-treated rats administered 0.25 mg ROFA is encouraging. The T_{CO} of untreated subjects decreased only slightly following instillation of 0.25 mg ROFA, whereas the MCT-compromised animals demonstrated increased mortality

and severe decreases in T_{CO} .

An important drawback of the MCT pulmonary hypertension model is the variability of pathogenesis for individual rats. One lethality occurred before ROFA instillation and, for obvious reasons, was excluded from the study. Slight hypothermia in MCT-treated rats in the absence of ROFA was found to be predictive of death within roughly 24 hours, and several rats in the present study had temperatures 1°C below the group average just prior to instillation. The experimental design of the present study presumed a homogeneous response to the MCT treatment, however, in future studies it may be preferable to use more individual diagnoses to assess the extent of pulmonary hypertension before exposure to ROFA. While there remain several caveats to the MCT model of pulmonary hypertension as it compares to the human situation (Heath, 1992), the results of the present study suggest that it provides a useful and sensitive model for studying ROFA toxicity.

The percentages of lethality were not significantly dose-related which is most likely attributable to both the low subject number and the nonhomogeneous pathogenesis for MCT. A more elaborate lethality study with greater sample size might create a more statistically sound dose response curve.

An important caveat for all of these compromised animal models is the time required for full development of the specific pathology. Human diseases such as hypertension and COPD require years to develop, while the models used in the present study develop over periods ranging from 18 hours to two weeks. These modeled pathologies cannot produce

the degree of lung obstruction and elasticity found after years of fibrosis. Also, alterations to the heart muscle and arteries of rats cannot match those changes which occur over a human lifetime. However, it should also be noted that human disease often fails to conform to the definitions of the diagnosis, and that these animal models serve primarily to highlight susceptible subgroups and not to establish individual mechanisms for each human disease.

With respect to edema and mortality, the animals of the present study responded much more severely in terms of mortality and gross pathology to MCT administration than has been reported in the literature for the same dosage. It is unclear whether this exacerbation was a result of surgical procedures, anesthetic use, or housing conditions. The inducing effect of pentobarbital on P450 isozymes may play an important role in modulating MCT biotransformation to its toxic metabolite, MCTP. Bruner *et al.* (1986) demonstrated a potentiation of the MCT toxicity in rats pretreated with phenobarbital, a known P450 inducer, and an attenuation of MCT toxicity in rats pretreated with SKF-525A (a compound which inhibits P450 expression). Pentobarbital potentiation of MCT has not been specifically studied, but it is known to increase levels of multi-function oxidases such as the P450 isozymes and therefore is of concern when examining the results of this study. A pilot experiment (Addendum B), conducted to determine the effect of pentobarbital on the MCT toxicity, demonstrated increased lung weight and nonsignificant increased mortality in the group administered both pentobarbital and MCT over all other groups (saline/saline, pentobarbital/saline, saline/MCT). While not definitive, these results suggest a possible

synergistic effect of pentobarbital pretreatment on MCT pulmonary toxicity.

Conclusions

The results of this study support with the associations observed epidemiologically between increased levels of ambient PM and excess daily mortality in humans. The compromised animal models used in this study demonstrated an exacerbation of the effect of ROFA on cardiovascular and thermoregulatory parameters, further supporting the use of such models to assess the plausibility of PM-induced morbidity and mortality in the human population. The most sensitive model in this study, the MCT-induced pulmonary hypertension model, showed potentiated cardiac effects and impaired survivability following administration of ROFA. Additionally, analysis of ROFA-induced ECG changes in MCT-treated animals suggests that lethality may occur by one of two mechanisms: 1) a slow, progressive myocardial ischemia possibly due to decreased gas exchange in the inflamed and edematous lung, or 2) an abrupt failure of the heart due to a conduction-related fatal arrhythmia. As both mechanisms involve cardiac function, these data support epidemiological findings of cardiovascular morbidity and mortality.

ROFA exposure caused decreased T_{CO} and HR, and increased the frequency of cardiac arrhythmic events in normal and compromised rat models. The induced hypothermic response was similar in many ways to that for ozone and chlordimeform

exposure although ROFA differed from these toxins by the elicitation of an additional delayed response. Acetylcholine infusion in rats was able to induce arrhythmias and bradycardia similar to that observed following ROFA instillation, suggesting that the central nervous system may play an important role in the rodent response to toxic agents. Further investigation into the nature of the observed cardiac arrhythmias, hypothermic response, and the relationship between the two is essential for a better understanding of xenobiotic toxicity in rodents.

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Addendum A

Acetylcholine (Ach) Preliminary Study

Two rats (60 d male Sprague-Dawley), one which had been instilled 48 hours prior with 2.5 mg ROFA and one which had been instilled with saline, were anesthetized with urethane and cannulated via the right jugular vein. Radiotelemeters were attached subcutaneously and used to collect ECG signals. Ach (0.5 mg/mL) was administered through the cannula in 3 x 0.1 ml increments, followed by 0.2 ml, 0.4 ml, and 0.5 ml infusions. An average time of two minutes was allowed between infusions. ECG tracings were printed on a chart recorder (Astro-Med, West Warwick, RI).

Acetylcholine infused intravenously induced a variety of arrhythmias (figure 17), consisting predominantly of Mobitz type II AV block, the duration of which appeared dose-related, and premature contractions similar to those observed in ROFA instillation experiments. This response was elicited in both the ROFA-instilled and the saline-instilled rats. The saline-treated animal suffered a severe tachyarrhythmia following the initial dose of Ach. T_{CO} was not measured in this pilot study as the animals were anesthetized. These results suggest that bradycardia induced by xenobiotics may be mediated by the parasympathetic nervous system, however, further research will be required to properly assess the relationship between xenobiotic exposure, vagal conduction, and hypothermia. Determination of this mechanism is important for proper physiological modeling of toxicokinetics and extrapolation to human risk assessment.

Addendum B

Alterations in MCT-induced Hypertension by Pretreatment with Sodium

Pentobarbital

Barbiturates, commonly used as anesthetics for animal surgery, can induce P450 isozymes. As MCT was used to produce pulmonary hypertension in rats that had previously undergone surgery in which sodium pentobarbital was used, this pilot study sought to examine the possibility that sodium pentobarbital potentiated the MCT-induced pulmonary hypertension. Sprague-Dawley rats (60 d) were administered pentobarbital (n=8; 50 mg/kg, *ip*) or saline (n=8; 3 cc/rat, *ip*), then injected with MCT (60 mg/kg, *ip*) or saline (3 cc, *ip*) after eleven days. MCT was given to half (n=4) of the pentobarbital-dosed animals and half (n=4) of the saline-dosed animals. All subjects remained in the temperature, humidity, and light controlled animal care facility, housed two per cage for fourteen days at which time they were sacrificed. Samples collected included serum enzymes, RV/LVS, and lung and body weights. Data were collected and averaged in groups and analyzed by Student's t-test.

Compared to saline pretreated animals, animals administered pentobarbital exhibited increased lung weights (LW/BW; $p < 0.15$) and mortality (25%; small n precludes p statistic) but not right ventricular hypertrophy in MCT treated animals. RV/LVS and LW/BW were elevated in MCT animals compared to saline injected

controls. Pentobarbital in the absence of MCT did not affect the monitored parameters. Serum biomarkers were elevated on an individual basis primarily in MCT-treated rats, but due to considerable variance within the experimental population these observations were not significant. These results suggest that pentobarbital, a known inducer of hepatocellular P450 isozymes, may exacerbate the pulmonary damage caused by MCT.

Diagram of Single Electrocardiographic Complex Showing Individual Waves and Intervals

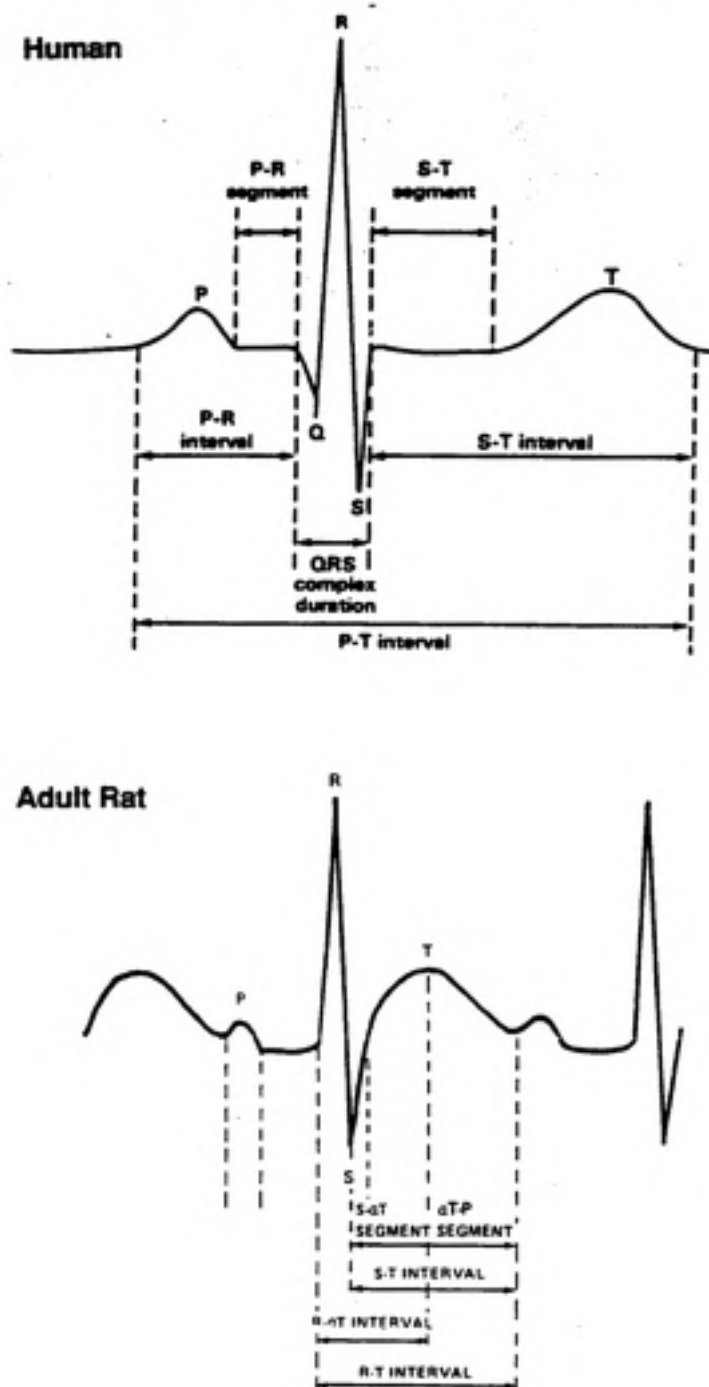


Figure 1. Typical ECG complex for human and rat showing important diagnostic intervals and wave durations. Note the isoelectric S-T segment and initial Q-wave in the human signal which are absent in the rat. Other major differences include increased rate and sloping P-wave in the rat ECG.

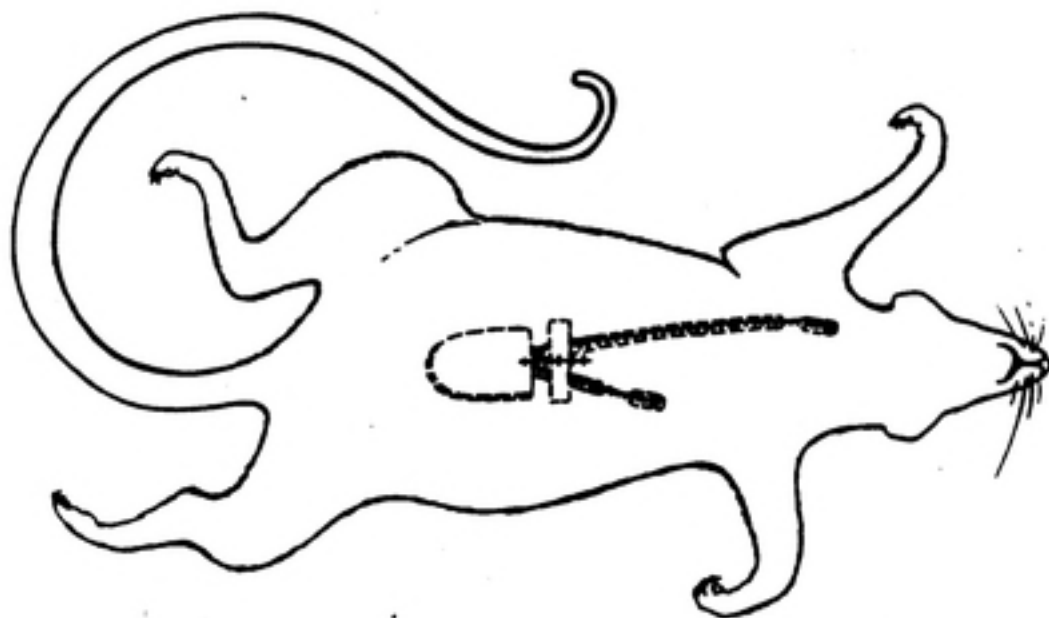


Figure 2. Surgical placement of radiotelemetry transmitter. Transmitter ECG leads were secured in the subcutaneous region while the body of the transmitter was placed within the peritoneal cavity.

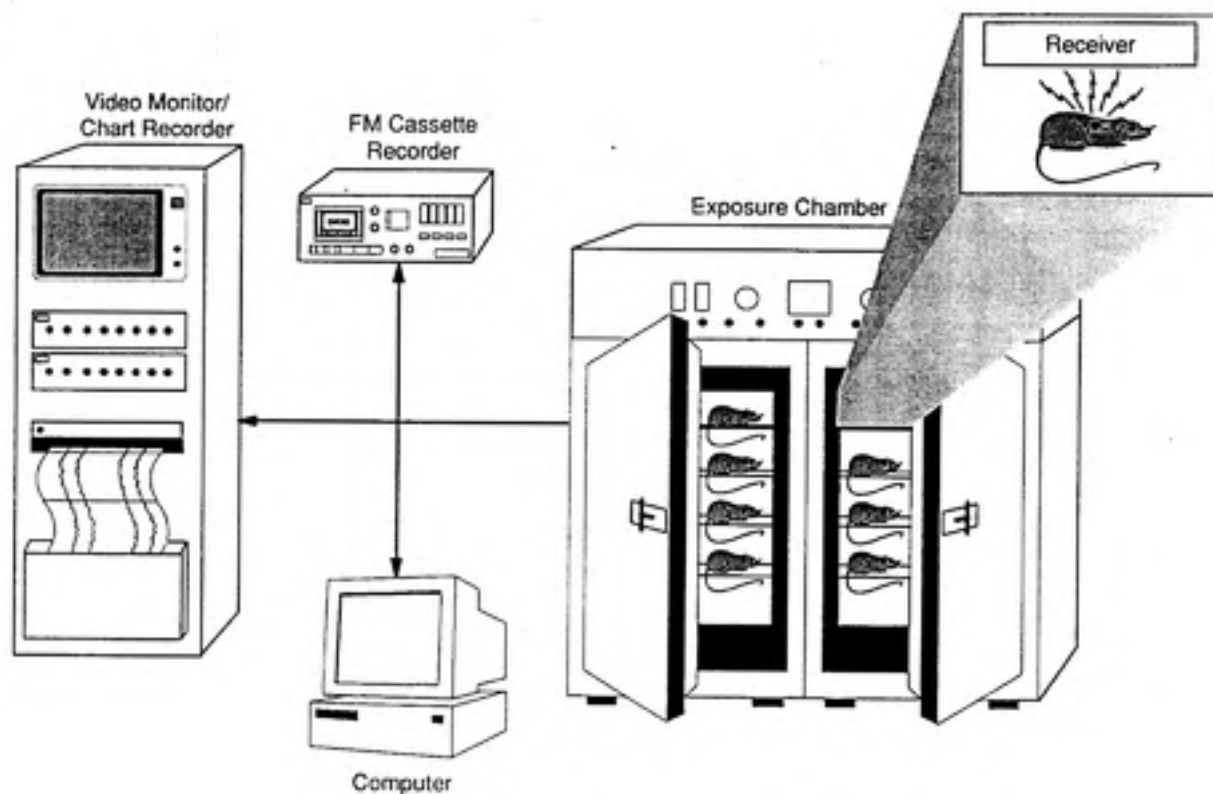


Figure 3. Diagram of data acquisition system. Each animal housed within the temperature-, humidity-, and light-controlled exposure chamber was implanted with a radiotransmitter which sent information on HR, T_{CO} , and ECG to individual receivers. Each receiver relayed signals to the computer to acquire HR and T_{CO} data, to the chart recorder for ECG printouts, and to the tape recorder to save certain ECG samples for later component analysis.

Figure 4. Thermoregulatory response to ROFA in rats housed at normal ambient temperature (21–24°C). Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 4

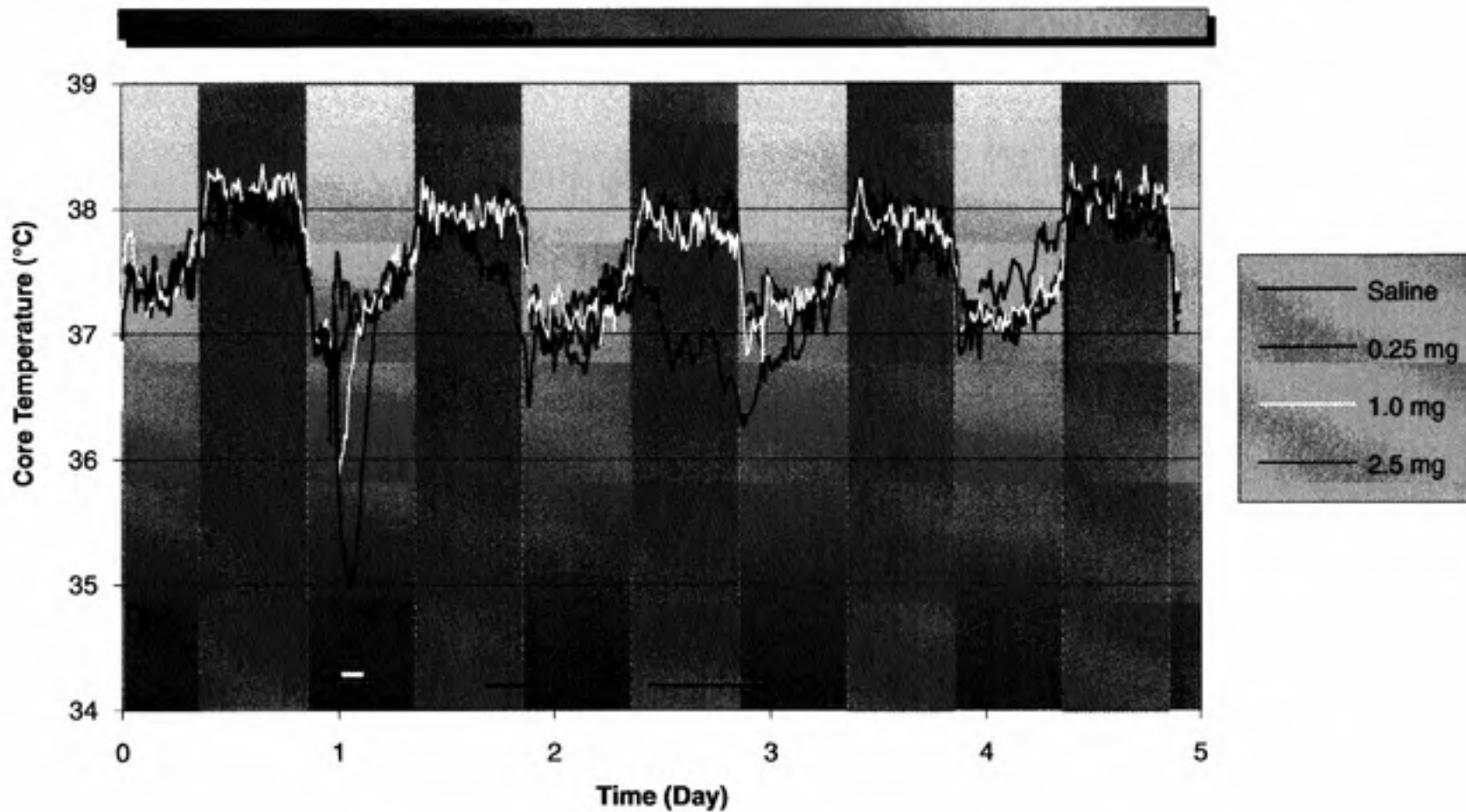


Figure 5. Thermoregulatory response to ROFA in rats housed at cold ambient temperature (10–14°C). Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 5

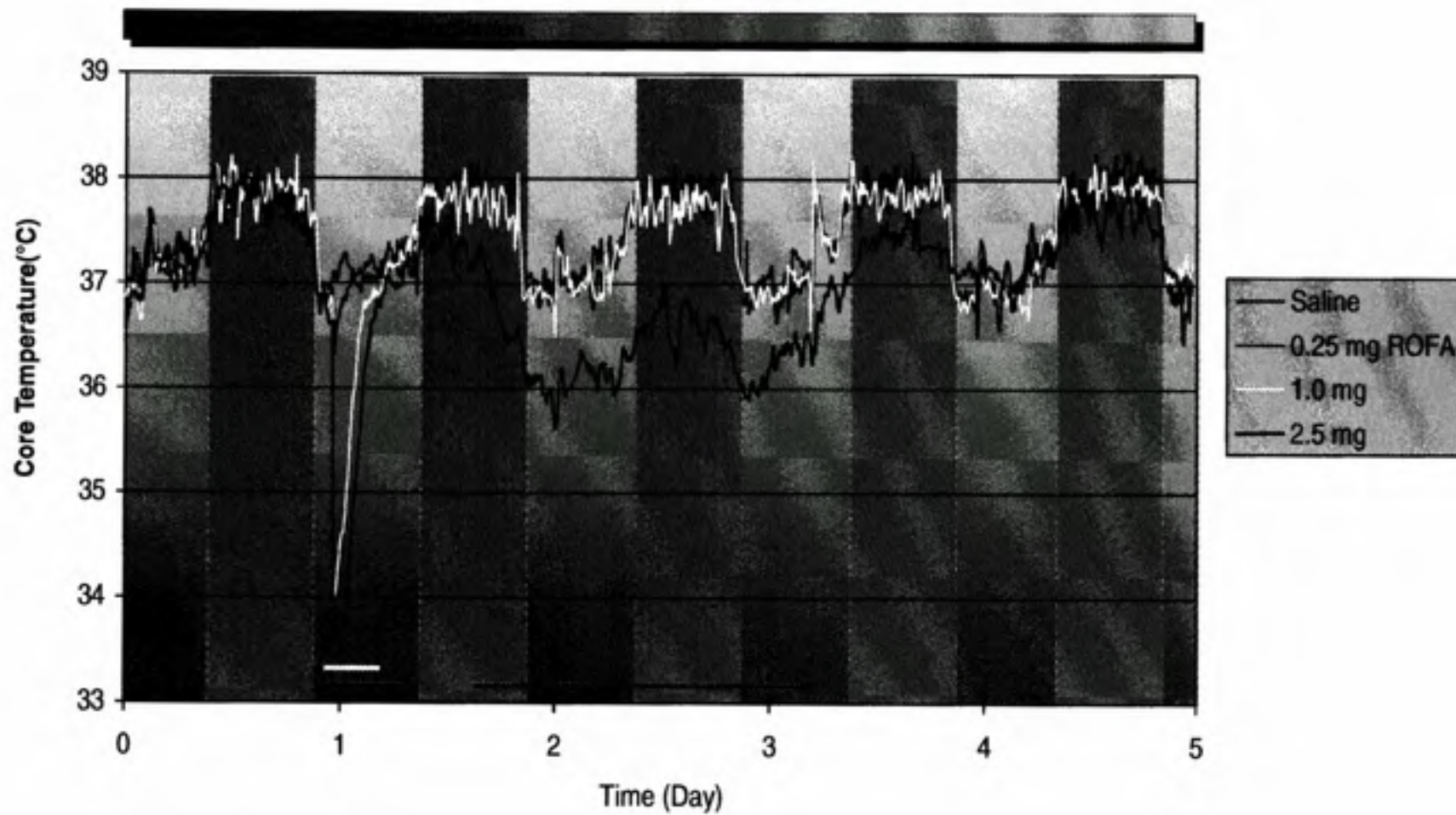


Figure 6. Thermoregulatory response to ROFA in rats with O₃-induced pulmonary inflammation. Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 6

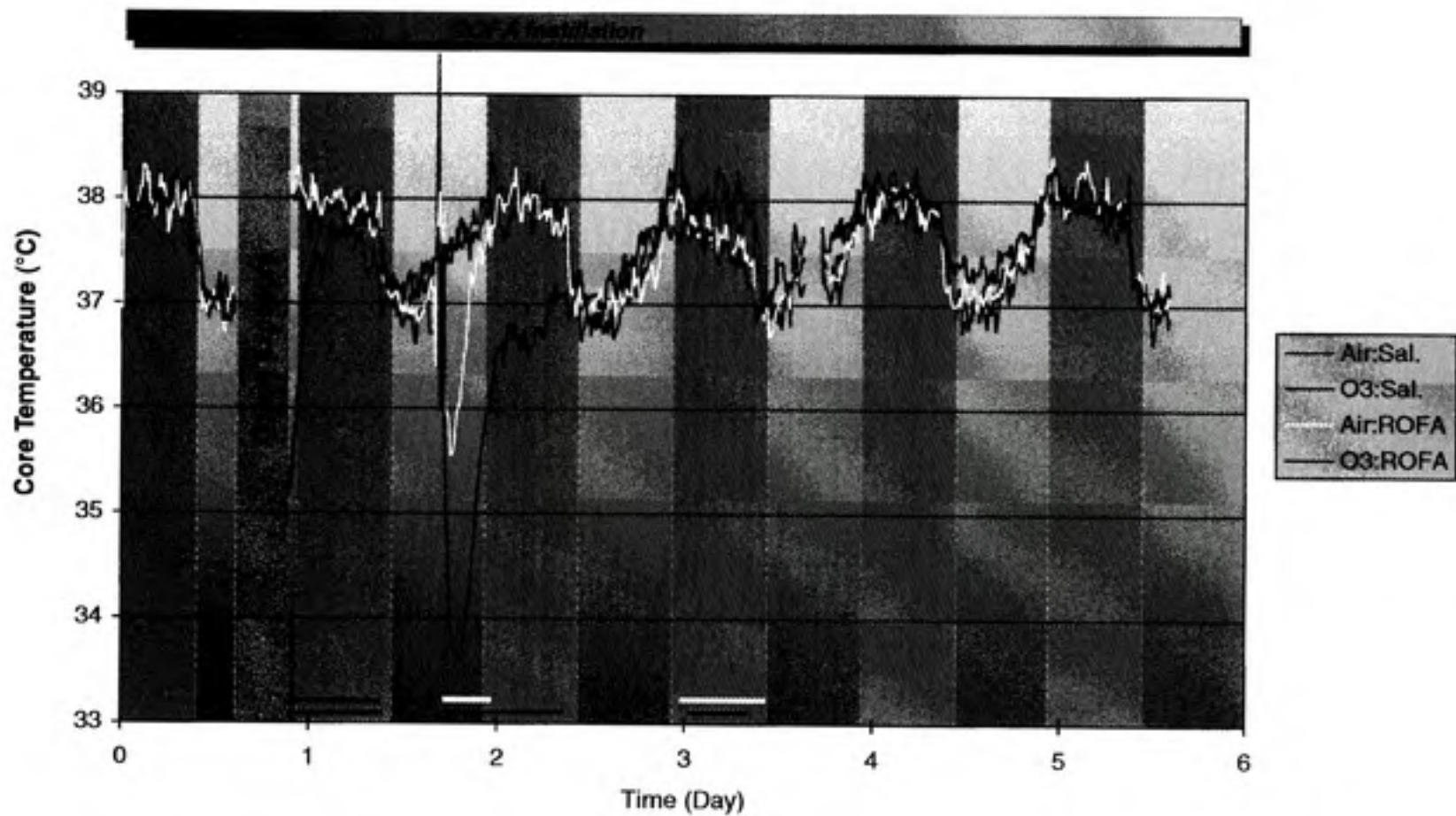


Figure 7. Thermoregulatory response to ROFA in rats with MCT-induced pulmonary hypertension and inflammation. Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. No tests of significance were performed due to lethality.

Figure 7

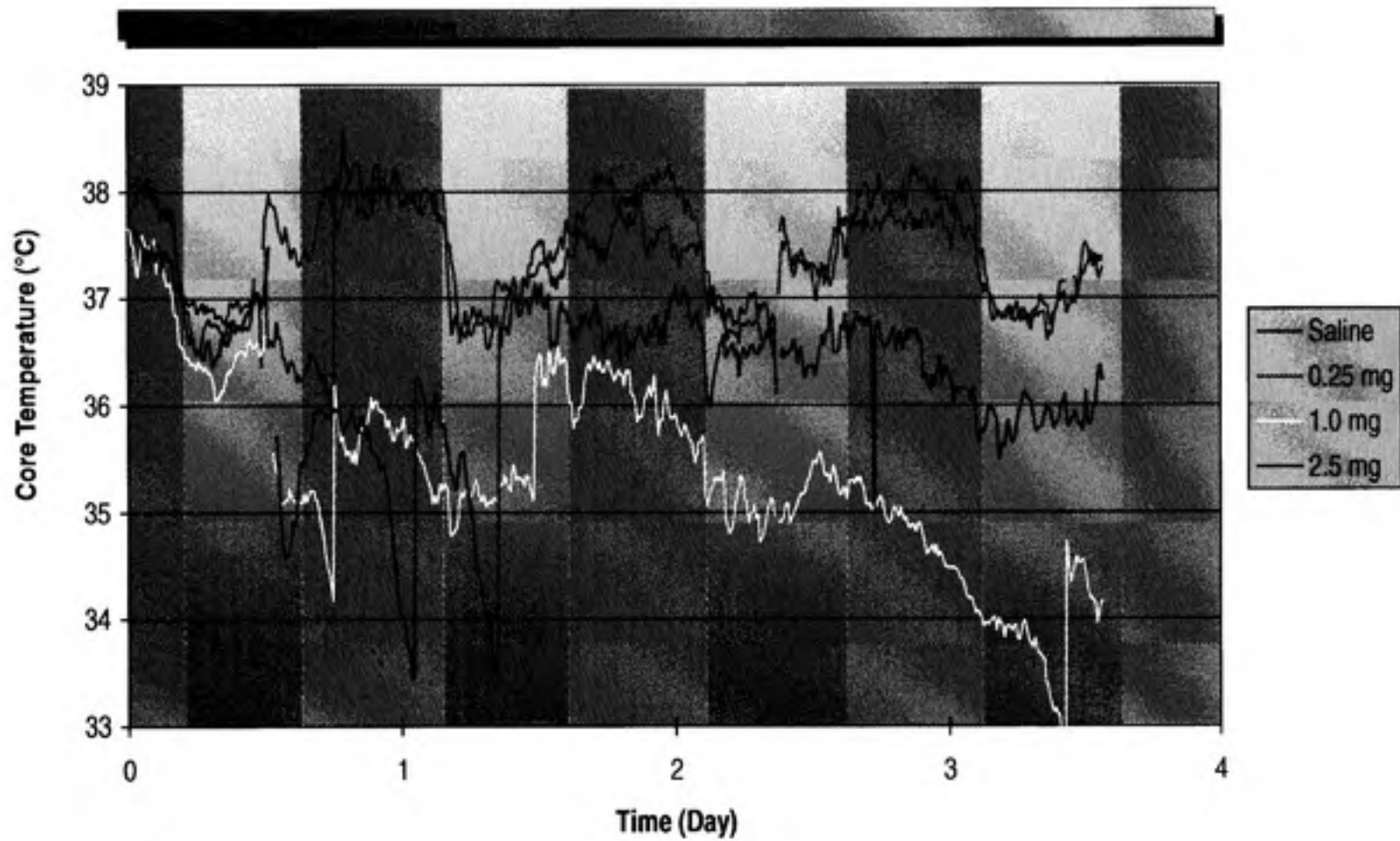


Figure 8. Cardiovascular response to ROFA in rats housed at normal ambient temperature (21-24°C). Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 8

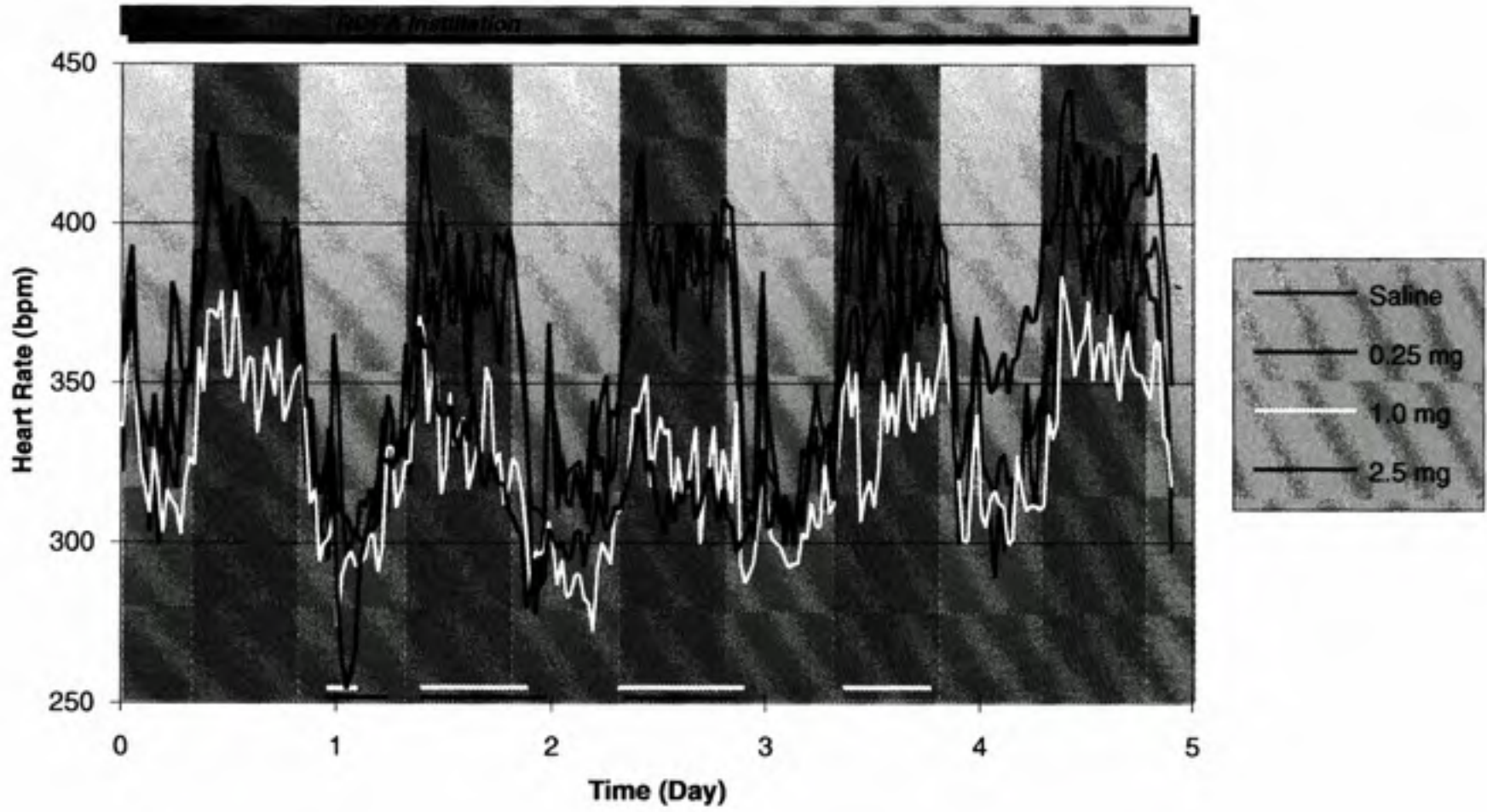


Figure 9. Cardiovascular response to ROFA in rats housed at cold ambient temperature (10–14°C). Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 9

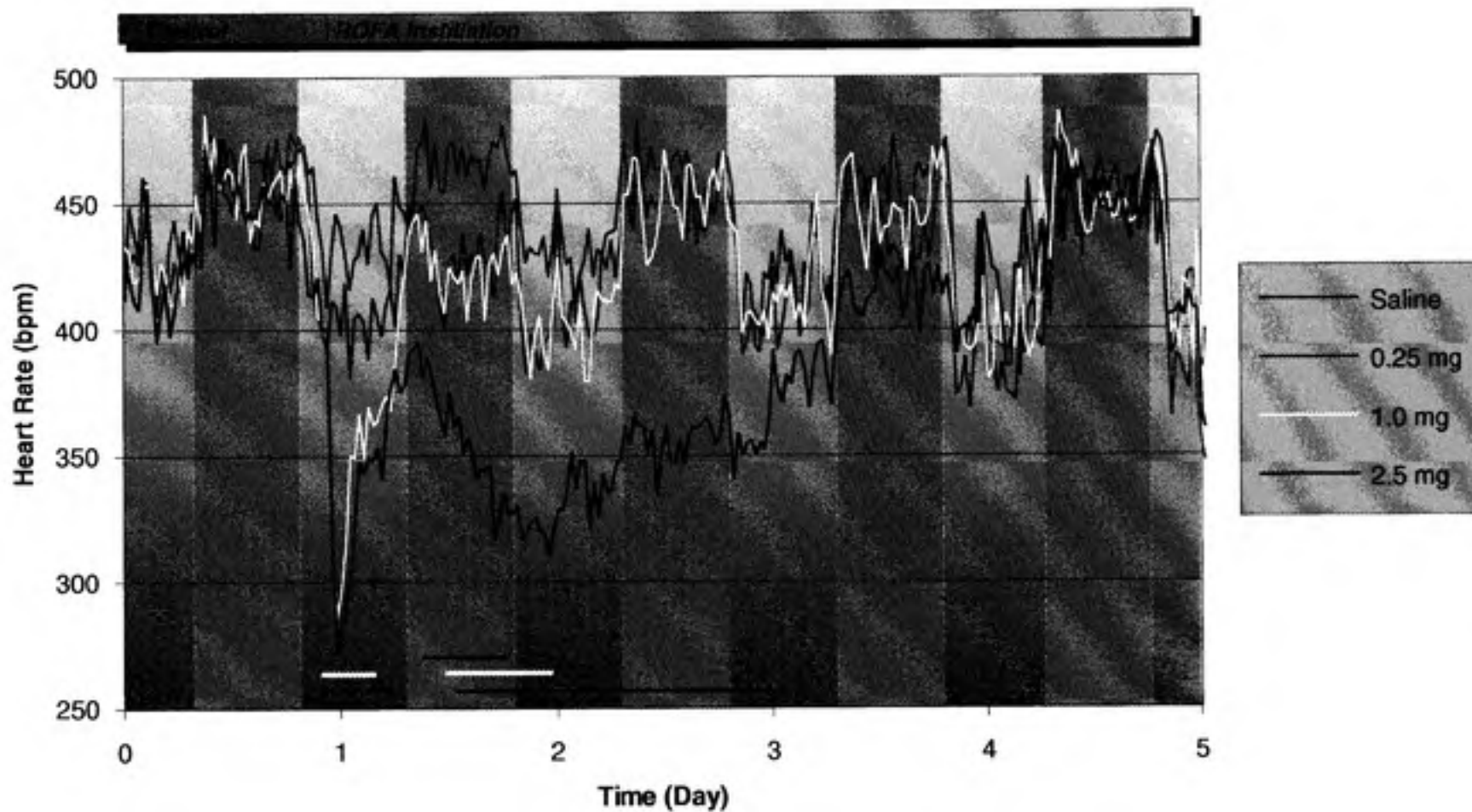


Figure 10. Cardiovascular response to ROFA in rats with O₃-induced pulmonary inflammation. Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. Horizontal bars at bottom indicate periods of significant difference from control (Tukey's ANOVA; $\alpha=0.05$).

Figure 10

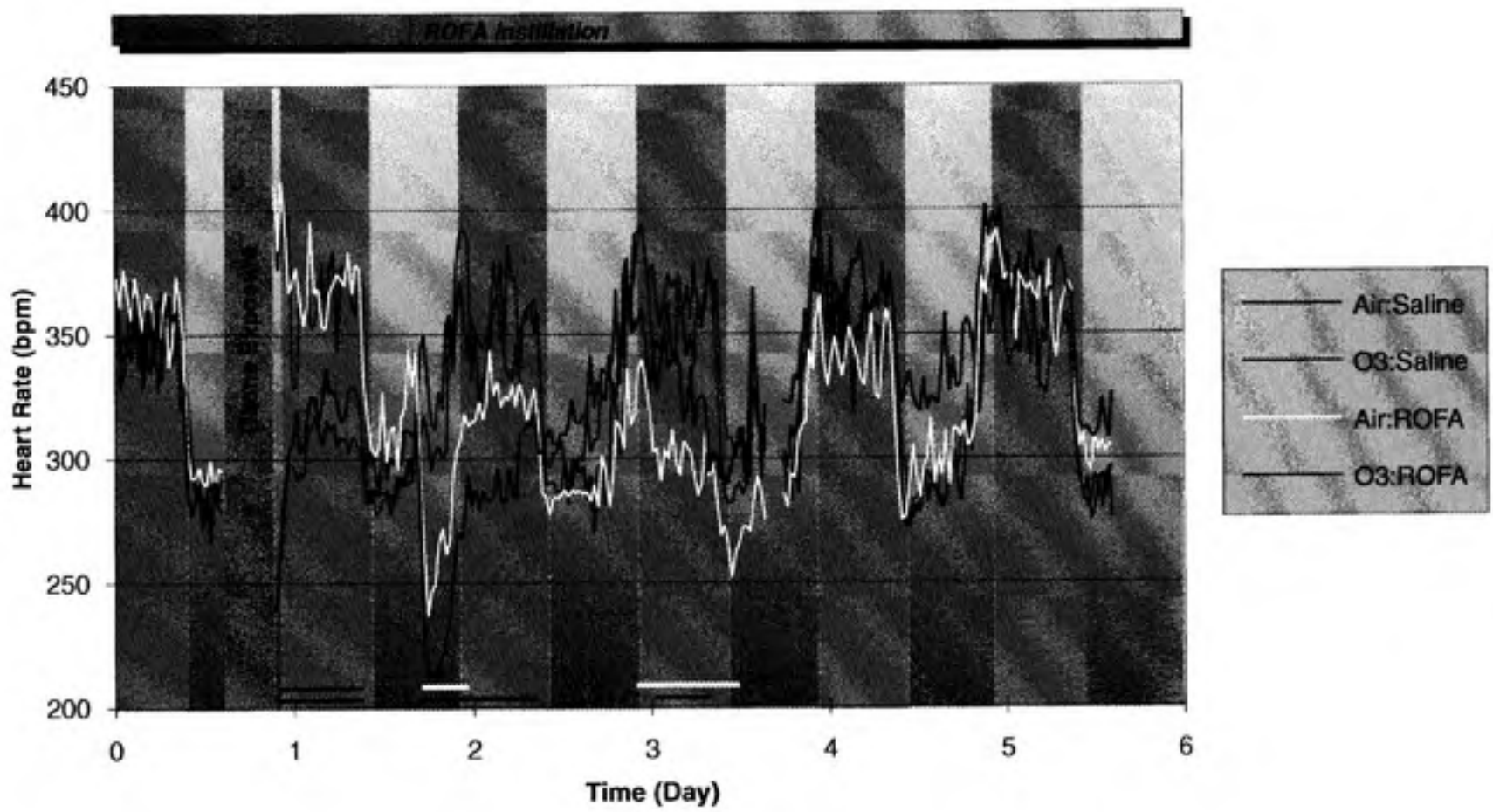
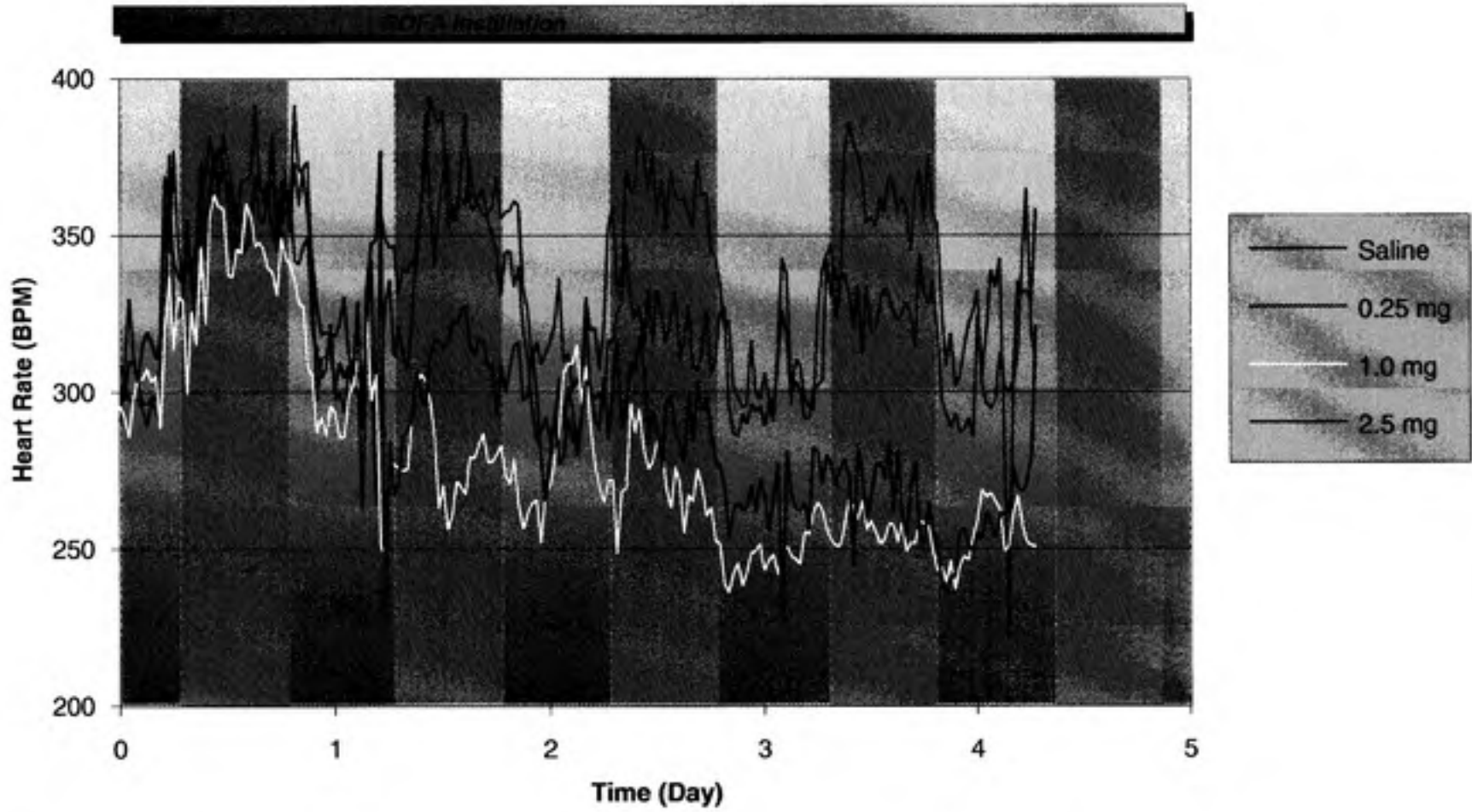


Figure 11. Cardiovascular response to ROFA in rats with MCT-induced pulmonary hypertension and inflammation. Data presented are group mean values acquired at ten minute intervals and averaged to half hour time points. Solid gray vertical bands indicate dark periods in the exposure chamber. No tests of significance were performed due to lethality.

Figure 11



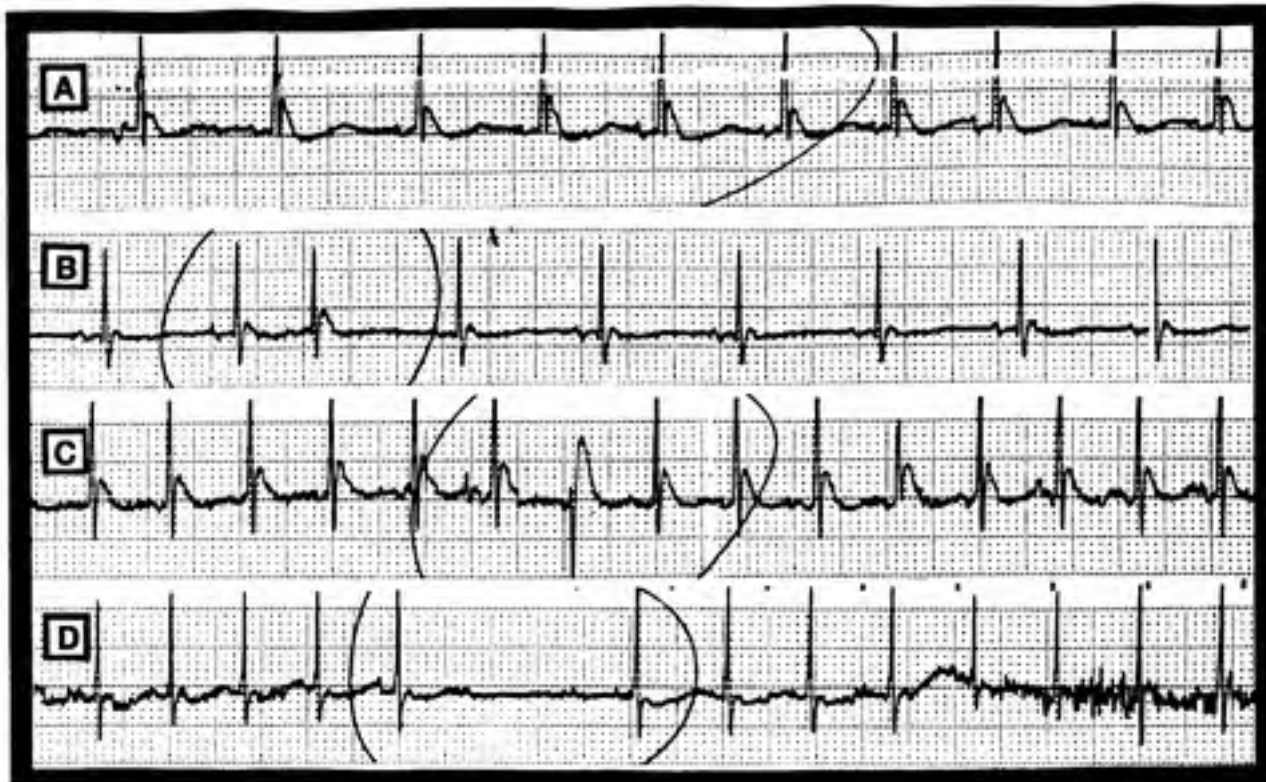


Figure 12. Examples of less serious arrhythmias observed following intratracheal instillation of ROFA: A. R - R interval changes. B. Premature atrial contractions. C. Premature ventricular contractions. D. Single skipped beat (AV block).

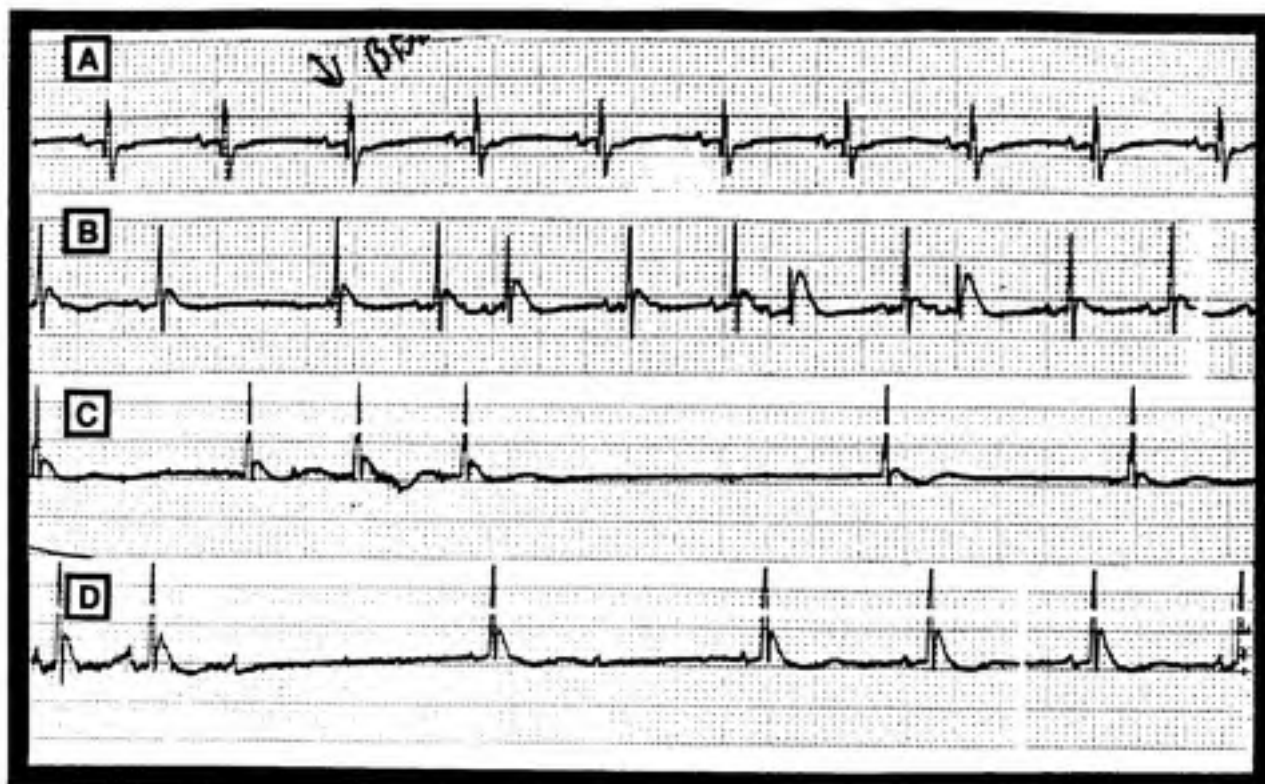
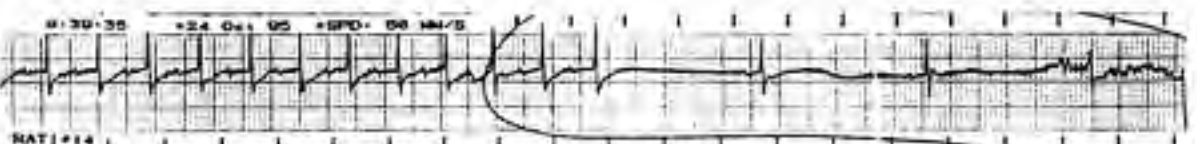
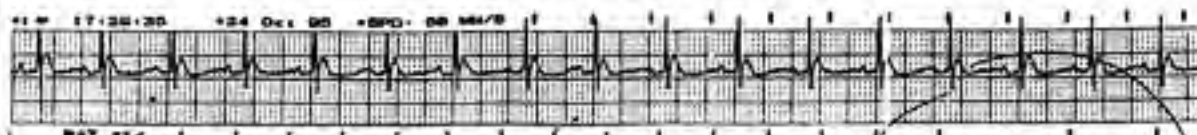


Figure 13. Examples of more serious cardiac arrhythmias observed following intratracheal instillation of ROFA: A. Bundle branch block. B. Multiple premature contractions. C. and D. Multiple skipped beats.

Figure 14. First lethality scenario: gradual cardiac failure due to progressive hypoxemia. Note marked depression of ST segment, severe bradycardia, and eventual asystole. Strip recording of medium dose MCT-treated animal. Each row represents a four second sample, with a ten minute interval between most samples.

RAT #14 - MONOCROTALINE PLUS ROFA (1.0 mg, IT)



RAT #14 - MONOCROTALINE PLUS ROFA (1.0 mg, IT) Continued

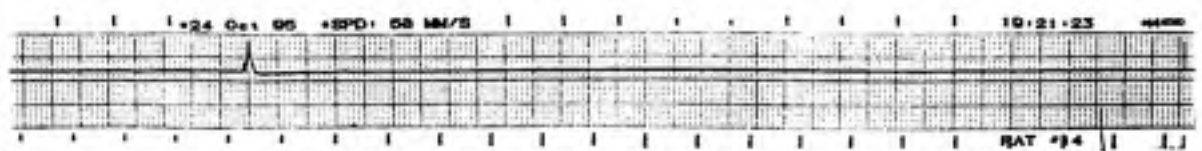
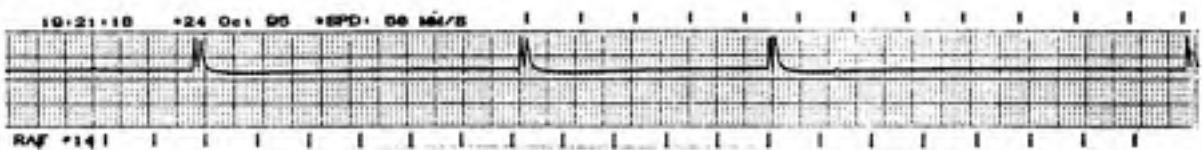
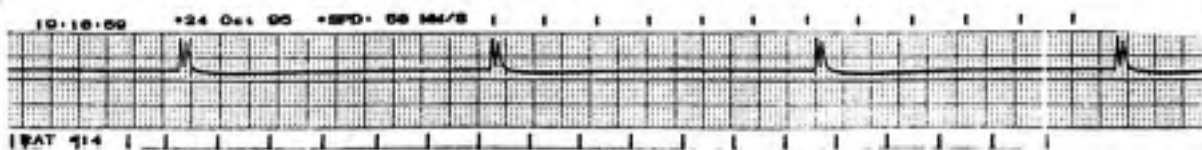
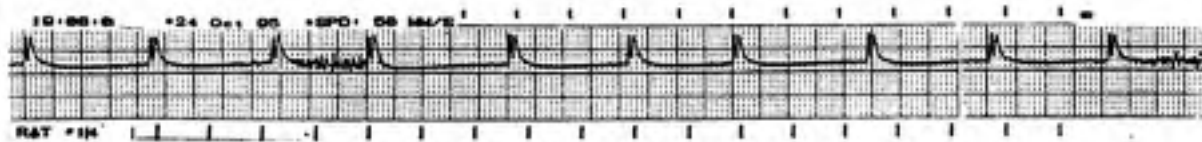
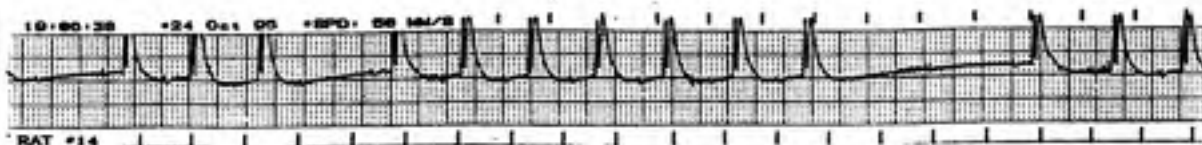
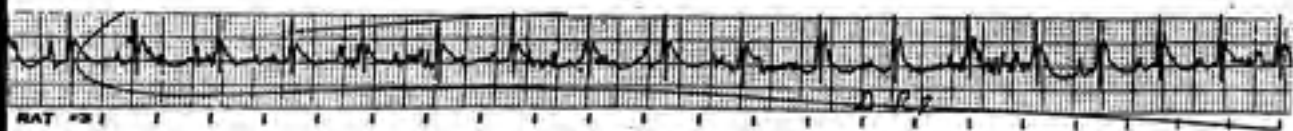


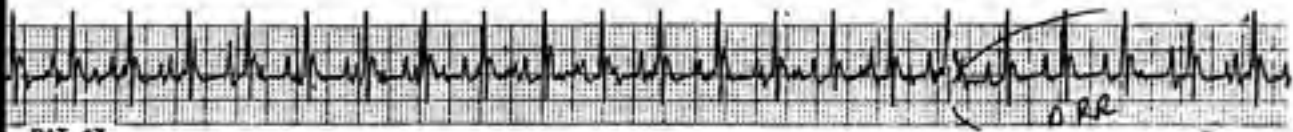
Figure 15. Second lethality scenario: induction of abrupt fatal conduction-related arrhythmia. Note apparently normal ECG with associated "noise" which may be caused by ectopic stimulation or myocardial conduction defects. Heart failure is sudden, with no prior indication of ST segment depression. Strip recording of low dose MCT-treated animal. Each row represents a four second sample, with a ten minute interval between most samples.

RAT #3 - MONOCROTALINE PLUS ROFA (0.25 mg, IT)



RAT #2

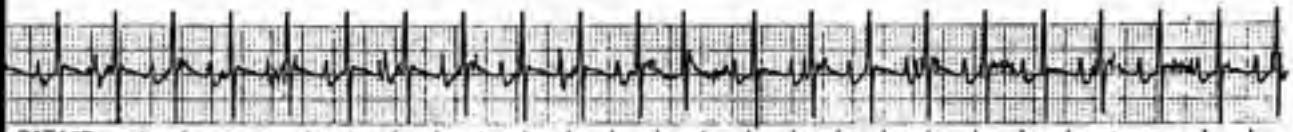
RAT #2



RAT #3



RAT #3



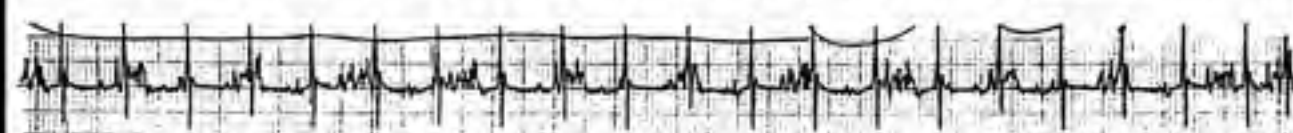
RAT #3



RAT #3



RAT #3



RAT #3



RAT #3



RAT #3

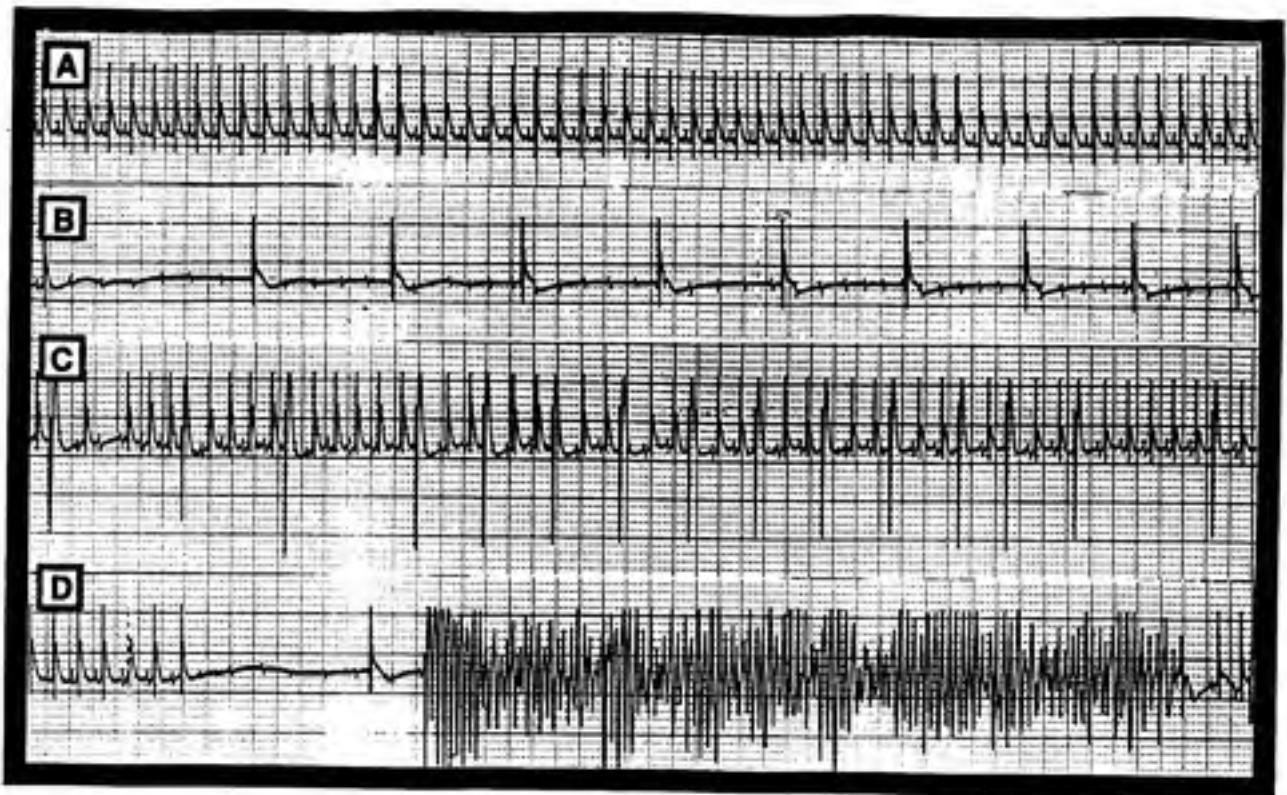


Figure 16. Acetylcholine-induced arrhythmias in Sprague-Dawley rats.
A. Normal ECG prior to ACh infusion. B. Mobitz type II AV block.
C. Premature ventricular contractions. D. Severe tachyarrhythmia.

Table 1. Gas Chromatographic Analysis of ROFA Constituents

Mineral Ash Analysis:

Sodium oxide	7.72%
Magnesium oxide	17.59%
Aluminum oxide	0.94%
Silicon dioxide	2.61%
Sulfite	44.04%
Potassium oxide	0.32%
Calcium oxide	2.53%
Titanium oxide	0.13%
Ferric oxide	5.13%
Phosphorus pentoxide	0.33%
Vanadium pentoxide	5.82%
Nickel oxide	3.66%
Manganese dioxide	0.04%
Lead oxide	0.07%

Organic Analysis

Carbon	1.20%
Hydrogen	1.29%
Nitrogen	<0.50%

Endotoxin content 2.50 pg/mg

Mass mean aerodynamic diameter

1.95 ± 0.18 µm

Geometric mean

2.19 ± 0.22

Dose:	Subject	Day 1	2	3	4
Control (Saline)	1	1	a,a		
	8	1,1,r			
	11	1,bb			bb
	15				
Low (0.25 mg)	2	bb			
	5	3:1,2			
	10	2,1,1	a,a		1,r
	16	2,2,1,2			
Medium (1.0 mg)	3	1,1,2:1,1,3:1:1,1	2,2		1
	6	2:1,2,2,2,1:1:1,1,1,1,3,r, 2 3,1,1,1,1,1,1,1,1,2,2		1	1
	12	bb		v,v,v	v x 34
	13	1,2:1,2:1,1,2:1,2,r,1,2	1:1,		bb,bb
High (2.5 mg)	4	1,1,1,1,1,2,1:1	1,1:1,2		
	7	1,1,1,1,1,1,1,1,3:1,r,bb,v v,v,2,r	a		v
	9	2:1,1,1,2,2	1,1,v,r,2	1,1	1
	14	2:r,r			

Table 2. Arrhythmic events observed in rats housed at normal ambient temperature (21-24°C). Data were derived from chart recorder printouts and tabulated for each subject. Arrhythmias are indicated by the following abbreviations:

- | | |
|-------------------------|---|
| r: R-R interval change | v: Premature ventricular contraction |
| bb: Bundle branch block | a: Premature atrial contraction |
| bc: Bradycardia | af: Atrial flutter |
| bi: Bigeminy | #: Skipped beats; consecutive arrhythmias are combined by a colon (:) |

Dose:	Subject	Day 1	2	3	4
Control (Saline)	2				
	5				
	10				bb
	16	2,bb			
Low (0.25 mg)	4				2:1:a:av
	7	1	2		2:1,1
	9				v
	14	1,π	v		
Medium (1.0 mg)	1	πA:1:0:1,v,bi,v,2	π		
	8	bi,3,2,π,3:2,1,3:1:1,2,2 1,1,1,1,1,1,1,1,1,2,2	π	π,π	
	11	2:0:2,1,1,1,1,1,1,1,1,1,1, 3:1:1,2:1,2/af	π,1	2	
	15	a,a,a,a,a,aaetc			
High (2.5 mg)	3	π,π,1,π,π,1,2:1,2:1:1,π,2, 1:1,3,2,1,2:1	2,π,π,1,bb,bb,bb	a	bb,bb
	6	1,π,π,2:1,2	2:1,1,π,π,1		
	12	1,3,af,2,π,1	1,π,2,bc,π	π	
	13	1:1:0:1,1,v,bc,π,1,1:1:1, 2:1:1,2:1,π,π,bc,2,2,bc,2	π,π,bc,π,bc,π,π,bc,bc	π,π,π	

Table 3. Arrhythmic events observed in rats housed at cold ambient temperature (10–14°C). Data were derived from chart recorder printouts and tabulated for each subject. Arrhythmias are indicated by the following abbreviations:

- | | |
|-------------------------|---|
| r: R-R interval change | v: Premature ventricular contraction |
| bb: Bundle branch block | a: Premature atrial contraction |
| bc: Bradycardia | af: Atrial flutter |
| bi: Bigeminy | #: Skipped beats; consecutive arrhythmias are combined by a colon (:) |

Dose:	Subject	Day 1	2	3	4
Control (Saline)	4	v		r,v,v	v,v,v,v,b
	9				
	15	1,1,r,1,r,1	r,v,r,r,1	v,r,r	v,b,b, b
Low (0.25 mg)	3	r, r, r, r, 1, r, r, v, DEAD (10/24 19:41)			
	6	1,1	a, a, a, 1, 1, 1, r, 1	2, 2, r, 2:1:1, 2	v, 2, 1, 2:1, 1
	12	1, 1, 3:2:1:1	r, 1, 2:1, r, 3:1, 1, 2:1, 1, r, 1, 2, r, 2, 2:v:2, 1, r, 1, r, 1, r, r, a, r	2:1, r, 2:1, 2:1, r, r, 1:1, 1, 3:1, 2:1:1, r	r, 2:1, v, 2:2:r, v, 2:1, 2:1, 2:r, r, r, 1, r, r, r, 1, 2, r, a, 2, 1, 1
	18			1	
Medium (1.0 mg)	2	3:2:1:1:1, 1, 2, 2, 2:1:1:1, r, r, v, r	r, r, r, r, r, DEAD (10/25 15:31)		
	5	1, 2:1, 2, 1, 1, 1, 1, R, 1, R, 2, 1, 1, 2:1	1, 1, 1, 2:1, 1, 1:1, 1, 1, 1, r, 3:2:1, 1, r, r, 1, 1, 1, r, r, r, 1, r, v, 1, r, 1, r, r, r	r, v:v:1, r, r, a	v, r, r, r, r, r, r, r, r, r, r
	10	1, 1:1, r, r, r, r, r, a, r, r, r	r, r, r, r, r		r, r, DEAD (10/27 13:41)
	14	r, 2:2:2v, r, DEAD (10/24 19:00)			
High (2.5 mg)	1	a, 1, r, 2, 1, 1, r, 1, 1, 1, r, r, r, r		r, r	r, r, r
	8	a, a, a, a, r, a, 1, 1, 1, 1, a, r, r, r, DEAD (10/25 9:41)			
	11	DEAD (10/25 01:01)			
	13	2, 1, 2	r	r, r, r, a, r, r, r, r, a, r, r, v, v, v, v, r, r, r, r, r, r, r, r	

Table 5. Arrhythmic events observed in rats with MCT-induced pulmonary hypertension and inflammation. Data were derived from chart recorder printouts and tabulated for each subject. Arrhythmias are indicated by the following abbreviations:

- | | |
|-------------------------|---|
| r: R-R interval change | v: Premature ventricular contraction |
| bb: Bundle branch block | a: Premature atrial contraction |
| bc: Bradycardia | af: Atrial flutter |
| bi: Bigeminy | #: Skipped beats; consecutive arrhythmias are combined by a colon (:) |