Project 1:

Relative Toxicity of Air Pollution Mixtures

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ABSTRACT – Project 1

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b. Title: Relative Toxicity of Air Pollution Mixtures
c. Investigators: PI: John Godleski, Co-PI: Petros Koutrakis
d. Institution: Harvard School of Public Health, Boston, MA
e. Project Period and Location: 2010-2015, Boston MA
f. Project Cost: $2 Million
g. Project Summary:

1) Objectives and hypotheses: Project 1, an inhalation toxicological animal exposure study, will investigate the relative toxicity of different component concentration combinations of air pollution mixtures. These components include both particles and gases that are emitted directly from sources (primary species) or are formed in the atmosphere through a series of reactions that are predominantly photochemical (secondary species). Using a novel integration of our ambient particle concentrator and photochemical chamber technologies to generate realistic mixtures, we will test these specific hypotheses: (i) secondary gaseous pollutants formed from the photochemical oxidation of Boston ambient gases can induce biological responses; (ii) aging Boston concentrated ambient particles (CAPs) in the photochemical chamber enhances their toxicity; (iii) toxicological effects of photochemically aged CAPs are exacerbated by co-exposure to ozone and other secondary gases; and (iv) mixture composition and toxicity exhibit inter- and intra-seasonal variability due to changes in source emissions and weather conditions.

2) Experimental approach: Toxicity will be assessed in Sprague-Dawley rats by changes in 1) in vivo oxidant response, 2) blood pressure, 3) measures of inflammation, and 4) vascular blood flow/resistance. Three concurrent exposures groups (Sham, Control Exposure and Exposure) will allow us to control for the variability in CAPs composition. With this design, there can always be a direct comparison between two exposure mixtures on every exposure day, making it possible to determine which mixture is more toxic. In studies of vascular blood flow/resistance using a crossover design, each animal will have multiple exposures that will include each of the three types, including baseline measurements. This will permit for control of inter-subject variability in the biological response. Exposure atmospheres will be chemically and physically characterized using a broad array of measurement techniques for CO, NOx, O3, PM, BC, particle count and size distribution, EC/OC, elemental composition, sulfate, formaldehyde, acetaldehyde and VOCs. For the biological effects observed during each exposure, inter-group differences will be assessed using multi-way analysis of variance. To determine the effect of PM composition on biological response, linear regression models containing exposure concentrations as predictors will be fitted to each response outcome measure. Multiple pollutant linear regressions will be used to assess the independent effects of multiple pollution components on biological response.

3) Expected Results: The proposed study will differentiate the health effects of components of multi-pollutant exposure mixtures. We expect to add to our understanding of the exposure-response relationship, the interaction between particulate matter and photochemical gases, and the extent to which the resultant products exert toxicity. The biological outcomes assessed in this Project focus on responses important in oxidant initiation of pulmonary inflammation, and important functional measures of vascular and cardiovascular health.

Key words: Air pollution, concentrated ambient particles, vascular blood flow and resistance, inflammation, blood pressure, reactive oxygen species, oxidant response, atmospheric photochemistry
1. OBJECTIVES/HYPOTHESES:

Components of air pollution mixtures include both particles and gases that are emitted directly from sources (primary species) or are formed in the atmosphere through a series of reactions that are predominantly photochemical (secondary species).\(^1\) To the overarching hypothesis of Project 1, and the Center as a whole, is that the toxicity of air pollution mixtures can be related to specific component concentrations, but some combinations of components in mixtures are more toxic than others. Testing this overarching hypothesis is a challenging task that will require the collective efforts of many research groups. Here we propose to advance current understanding by examining how the addition or subtraction of individual or multiple components influences mixture toxicity. In particular we focus on toxic effects defined by changes in blood pressure, *in vivo* oxidant response, inflammatory parameters, and vascular blood flow/resistance. Using novel approaches to generate exposures of realistic pollutant mixtures, we will test four specific hypotheses:

1) **Secondary gaseous pollutants formed from the photochemical oxidation of Boston ambient gases can induce biological responses.** These gaseous species include O\(_3\), which is the most abundant atmospheric oxidant, as well as a spectrum of reactive species including nitrogen compounds, peroxides, organic radicals, polar organic compounds, and others. We further postulate that such biological effects are not solely O\(_3\)-related, but can also be induced by the concomitant reactive gaseous species;

2) **Aging of Boston Concentrated Ambient Particles (CAPs) in the photochemical chamber enhances their toxicity.** Many of our Center studies have reported toxic effects of Boston CAPs.\(^4\)\(^-\)\(^1\)\(^6\) Now we postulate that further photochemical aging of CAPs renders them more toxic by altering their organic components and adding secondary PM. Secondary PM includes many reactive species including mono- and di-carboxylic acids, aldehydes and other polar organic compounds, sulfuric acid, nitrates, and organic radicals. Photochemical oxidation may alter the organic component of CAPs, forming various polar derivatives more reactive than their parent compounds, as well as organic radicals and peroxides, which may enhance CAPs toxicity;

3) **Toxicological effects of photochemically aged CAPs are exacerbated by co-exposure to ozone and other secondary gases.** Mixtures of secondary gases, secondary PM, and aged CAPs may elicit increased toxic effects. More complex exposure mixtures will produce greater responses. And;

4) **Both mixture composition and toxicity exhibit inter- and intra-seasonal variability due to changes in source emissions and weather conditions.** Some variability of mixture toxicity may be explained in part by changes in mixture composition.

**Objectives:**

1) **Test each of the 4 specific hypotheses by generating different types of multi-pollutant mixtures:** We will combine two exposure-generation technologies developed by our ongoing and previous Centers including: i) the Harvard Fine Particle Concentrator (HFPC) that is used to generate CAPs for inhalation studies;\(^1\)\(^7\)\(^-\)\(^1\)\(^9\) and ii) the Toxicological Evaluation of Realistic Emission Source Aerosol (TERESA) exposure system that includes a photochemical chamber to simulate atmospheric aging of source emissions.\(^2\)\(^0\)\(^-\)\(^2\)\(^2\) For this study, we will use the TERESA photochemical chamber on the output of the HFPC to form secondary particles and gases from ambient air and to age the CAPs. Central to this effort is the use of a *parallel-plate membrane*
denuder to remove gaseous species from the generated pollutant mixtures. This device will make it possible to examine the effects of particles and gases combined and separately.

2) Expose an animal model to well characterized air pollution mixtures: Our exposure plan features three concurrent types of exposures (Sham, Control Exposure and Exposure) in a design that allows us to control for the variability in CAPs composition and for inter-subject variability in biological response. For example, to test the effects of aging CAPs we will perform three concurrent exposures: filtered air sham (Sham); CAPs (Control Exposure); and aged CAPs (Exposure). Using the effect of aging can be examined because we can control the inherent variability of CAPs toxicity.

3) Characterize the physico-chemical properties of exposures: Particle and gas exposures will be characterized using continuous measurements of PM, size distribution, BC, Particle Number, CO, NO, NOx and O3, along with integrated measurement of PM, sulfate, trace elements, EC/OC, water soluble OC, formaldehyde and acetaldehyde.

4) Assess biological effects: We will test the biological hypothesis that exposures to these air pollution mixtures will lead to varying degrees of increased in vivo oxidant response in the lungs that induce systemic inflammation and cardiovascular responses including increased blood pressure and changes in vascular reactivity. We will employ well-established assessments we have used with Sprague-Dawley rats in our CAPs and TERESA studies which will allow us to: i) measure in vivo oxygen radical activity in the heart and lung; ii) assess markers of pulmonary and systemic inflammation; and iii) monitor blood pressure. In addition, we will use a novel assessment of vascular reactivity using fluorescent microspheres to measure vascular flow and resistance in a panel of vital organs. And;

5) Perform statistical analysis: We will estimate associations between exposure and biologic response parameters using multi-way ANOVA techniques, univariate analyses and, longitudinal methods. These analyses will allow us to test our hypotheses regarding the toxicity of different multi-pollutant mixtures and their components. Our analysis will focus on identifying the characteristics of these mixtures that are mostly responsible for their toxic effects including: individual components, combinations of components, formation processes or source types.

1.2 Background and Introductory Information

1.2.1 Northeast air pollution mixture: As discussed in the Exposure Core, most of the air pollution in the Boston area comes from sources located outside Massachusetts. These include large urban centers, power plants, industries and biogenic sources most of which are located in the continental USA. Pollution is transported to the Boston area by prevailing southern, southwestern, western and northwestern winds. The regional component can often account for a large fraction of ambient pollution locally, but emission from local sources such as traffic-derived pollution, off-road mobile source emissions, seasonal space heating (distillate oil, gas, wood), marine aerosol, seasonal biogenic, and local industrial emissions also contribute. Because air pollution is such a complex mixture, with respect to its origin, composition, and physical properties, it is a long-term challenge to assess what components, properties, or sources are responsible for air pollution impacts on human health.

1.2.2 Linking health outcomes with air pollution: Laboratory inhalation studies have a critical role because of their ability to generate specific exposures and directly measure health outcomes. A significant advance in air pollution toxicology has been to study the effects of exposure to real ambient particulate pollution. We developed the HFPC that is used to conduct inhalation
toxicological assessments of responses to particulate matter (PM). The HFPC permits us to increase the concentrations of northeast urban ambient fine particle mass by a factor of 30-40 for our studies. Concentrator studies from our Center in Boston and other research groups worldwide have substantially contributed to the PM health effects field.

Our concentrator studies rely upon sensitive laboratory assessments to define acute responses to ambient particulates. In vivo oxidant measurements have been used in our CAPs and TERESA studies measuring oxygen radical activity in the heart and lung. We and many others (e.g., 31-33) have shown that CAPs increases pulmonary and systemic inflammation. Increases in blood pressure have been shown with Boston CAPs as well as CAPs in other cities in studies of our Center. A novel assessment of vascular reactivity in the heart using fluorescent microspheres to measure vascular flow and resistance has been developed in our laboratory and will be used in this project.

There has been intense interest recently in the effects of exposures to emissions from specific sources. To date toxicological approaches have focused on exposures to emissions from combustion sources, such as cigarette smoke, diesel exhaust, gasoline vehicles, and wood burning smoke. All these studies have used only primary emissions. Because particulate and gaseous source emissions undergo many transformations once released into the atmosphere, it is likely that secondary and primary pollutants from individual sources will exhibit different toxicities. Thus, it is critical to investigate the relative toxicity of source-specific primary and secondary particles and gases.

Another important recent advance in exposure studies has been the development of systems to simulate atmospheric chemical reactions and expose animals to particles resulting from aged source emission. Our Toxicological Evaluation of Realistic Emission Source Aerosol (TERESA) studies have investigated the toxicity of primary and secondary emissions from mobile sources (funded by our ongoing EPA PM Center) and power plants (funded by EPRI and the previous Center). In our TERESA studies we have developed technologies to sample source emissions, form secondary particles inside a photochemical chamber, and expose animals to both primary and secondary particles. The TERESA approach can readily be adapted to investigate other combustion sources, and the effects of mixtures of primary and photochemically aged gases and particles. Here, we propose to combine ambient particle concentrator and TERESA exposure technologies to explore how atmospheric chemical reactions may modify ambient particles.

1.2.3 Toxicity of photochemically aged gases and particles: Recent research has shown that O3 and other photochemical gases may play an important role in the toxicological response to ambient pollution. Sexton et al. have shown that secondary gases produced from photochemical oxidation of typical ambient VOCs (or hazardous air pollutants such as 1,3-butadiene) can enhance the toxicity of the parent compounds beyond the effects of comparable concentrations of O3 and NOx alone. Studies of human respiratory epithelial cells showed that the effects of a photochemically oxidized mixture persisted even after the O3 was removed by titration with NO, suggesting contributions from other components. The effects exceeded those of a synthetic mixture of the identified products (including acrolein, acetaldehyde, formaldehyde, furan, and O3) at the same concentration. However, because the photochemical aging in these studies was performed in the absence of PM, the interactions of such gases and particles in exposure and toxicity remain to be determined. It is also important to determine the relevance of these in vitro studies to inhalation exposure in appropriate in vivo subjects, as we propose here.
1.2.4 PM and O₃ interaction in biological outcomes: Understanding the interactions of particles and gases (specifically O₃) in generating biological responses remains the subject of much interest. Many studies have tested the combined effects of O₃ and PM (reviewed in 60). Many important gaps remain, but laboratory exposure studies have shown evidence of synergy between PM and O₃ in humans and animals. However, CAPs plus O₃ does not have greater effects on some cardiovascular functions such as heart rate variability compared to CAPs alone. The focus of this project is to not only explore the simple combination of CAPs and O₃, but to assess the interactions between CAPs, O₃, and the resulting products of photochemistry on measures of toxicity.

1.2.5 Ties to other Projects and Cores: Our studies will strongly complement the Center’s 3 proposed Cohort Studies (Projects 2, 3, and 4), which focus on cardiovascular and central nervous system outcomes. A potential mechanism for pollution-related decreases in cognitive and neurobehavioral functions is decreased vascular perfusion. In this project, we will directly measure vascular perfusion and vascular resistance in the hearts and brains of animals using microspheres, a powerful approach for assessing vascular flow. Microspheres distribute to all organs permitting flow measurements in any vascular bed in relationship to exposure or other parameters. Also, both our animal toxicology study and the three Cohort Studies will assess pollution-related pulmonary and systemic inflammation. Our toxicology study will thus help us to understand the biological mechanisms underlying associations observed within the Cohorts between outcomes and the exposure mixtures.

1.3. Progress Report and Preliminary Results

1.3.1 TERESA Exposure Studies

1.3.1.1 Coal power plants: Our TERESA technologies have been used successfully in field studies at three coal fired power plants with distinctly different emissions control, resulting in distinctly different SO₂ and NO concentrations. During these studies, we: 1) optimized stack sampling methods to prevent condensation and PM loss from the hot and humid emissions; 2) developed a photochemical chamber to age primary emissions for animal exposures; 3) developed and validated denuder technology to remove excess gaseous components from the exposure atmosphere; and 4) developed a state-of-the-art mobile toxicology laboratory. During these field investigations, we tested several exposure scenarios including: primary PM alone (P); primary PM plus secondary acid sulfate (PO); primary PM plus secondary neutralized sulfate (PON); primary PM plus secondary organic aerosol and acid sulfate (POS) and; primary PM plus secondary organic aerosol and neutralized sulfate (PONS); secondary acidic sulfate only with no primary PM (O) and; secondary organic aerosol with secondary acidic sulfate and no primary PM (OS). We exposed male Sprague-Dawley rats to these mixtures, and measured biological responses including continuously monitored pulmonary function, post-exposure measurement of pulmonary and cardiac oxidative stress, pulmonary inflammation and injury, and blood chemistry and cytology. To further assess cardiovascular effects, we repeated the most complex exposure scenarios in a rat model of myocardial infarction and monitored cardiac function using implanted telemeters. Overall, we found that the TERESA aerosols from power plant emissions were less toxic than CAPs. These findings are summarized in Table 1. We found that the most significant responses occurred with the more complex oxidized scenarios studied, supporting our Hypothesis 2, that photochemically aged particles are more toxic than primary particles.
Table 1: Summary of the statistically significant biological outcomes from the coal power plant TERESA studies.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Summary of Statistically Significant Biological Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO</td>
<td>Only effect was a breathing pattern change to rapid shallow breathing</td>
</tr>
<tr>
<td>POS</td>
<td>Increases in in vivo chemiluminescence of the heart, abrogation of the change in breathing pattern with PO; Increases in total cell count and macrophage number on broncho-alveolar lavage and; Increases in premature ventricular heart beats in the myocardial infarction model</td>
</tr>
<tr>
<td>PONS</td>
<td>Decreases in peak expiratory air flow and expiratory flow at 50%; Increases in in vivo chemiluminescence of the lung and; Increases in total cell count, polymorphonuclear leukocyte number, and macrophage number on broncho-alveolar lavage</td>
</tr>
</tbody>
</table>

1.3.1.2 Mobile Source Emissions: We have successfully adapted the TERESA approach to investigate vehicular emission sources in a laboratory pilot study and in a northeastern highway tunnel. Our adaptation of the TERESA approach for mobile source emissions is described in Section 4 of the Engineering Core. During the laboratory development study for vehicular emissions, we optimized our technique for formation of secondary particle mass from the dilute tailpipe emissions of a single car. Because there was very little PM in the car exhaust, we used an inert seed aerosol particle for the secondary PM to condense on. We then conducted pilot exposures using normal male Sprague-Dawley rats, and measured pulmonary and cardiac oxidative stress using in vivo chemiluminescence as well as bronchoalveolar lavage to assess pulmonary inflammation. For these exposures, a sample of Mount St Helen’s Ash (MSHA) was used as the seed aerosol, because in our experience it is toxicologically inert.\(^6\) Two scenarios were tested, diluted car exhaust with MSHA (Primary PM), and diluted car exhaust with MSHA irradiated with UV light to produce secondary PM. The exposure results are summarized below in Table 2:

Table 2: Pilot exposure study with car exhaust diluted to 5 ppm CO (at 50 min residence time).

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Seed PM (μg/m(^3))</th>
<th>PM (μg/m(^3))</th>
<th>EC/OC (μg/m(^3))</th>
<th>[\text{In Vivo Chemiluminescence}]</th>
<th>Total BAL Cells (x10(^6))</th>
<th>Total BAL PMNs (x10(^5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary PM</td>
<td>298*</td>
<td>298</td>
<td>N/A</td>
<td>No heart or lung difference from control Lung: 2.3±1.3</td>
<td>No difference from control 0.4±0.8</td>
<td>No difference from control 0.0±0.1</td>
</tr>
<tr>
<td>Secondary PM</td>
<td>142*</td>
<td>212</td>
<td>0.5/6.9</td>
<td>Significant lung ↑↑↑ from control and Primary PM</td>
<td>Significant ↑↑ from control and Primary PM</td>
<td>Significant ↑ from control and Primary PM</td>
</tr>
</tbody>
</table>

*MSHA: Mt St Helens Ash (from the Bates Ridge site), a toxicologically and chemically inert particle
** Significant outcome differences from primary PM, p<0.05

These pilot exposures were small scale, using small numbers of exposed and sham rats (n=8), but show that secondary PM from car exhaust can significantly increase oxidant radicals in the lung, increase total BAL cells, and increase BAL polymorphonuclear leukocytes (PMNs) over sham controls (p=0.005, p=0.001, and p=0.05, respectively). Most importantly, there were significant differences between primary and secondary PM for all of these outcomes (p<0.05) even with slightly less mass in the secondary PM exposure, thus further supporting our specific hypotheses.
1.3.2.3 TERESA approach with ambient gases and particles: When the TERESA technology is integrated with the ambient fine particle concentrator, we will use ambient air rather than source emissions in the photochemical chamber. We performed feasibility testing with Boston ambient air to confirm that the concentration of oxidizable gaseous species present in ambient air is still sufficient to produce O$_3$ and secondary PM when photochemically aged in our reaction chamber. Results from a typical day (evolution of the particle size distribution and accumulation of O$_3$ – see figures below) support the validity of this approach.

1.3.3 Microsphere studies in dogs: Microspheres have been used as measures of organ vascular perfusion in numerous studies of both large animals$^{16,24,25,67}$ and rodents.$^{68-71}$ Our laboratory has pioneered the use of this approach to study cardiac blood flow and coronary vascular resistance in relationship to CAPs exposures. Our results show that this method is sensitive enough to detect changes in response to single 5-hr exposures. We found that acute exposure to CAPs decreases myocardial blood flow during subsequent coronary artery occlusion in chronically instrumented dogs. The decrease in myocardial blood flow was most pronounced in tissue within or near the ischemic zone and was accompanied by increases in coronary vascular resistance.$^{16}$ This finding has important, direct relevance to the potential influence of pollutant exposure on the size, severity, and chances of survival of an acute myocardial infarction. Our observation that CAPs exposure increases coronary vascular resistance during ischemia suggests an important role for PM-induced coronary vasoconstriction. This is consistent with the findings by Brook et al.$^{72}$ that a 2-hr exposure to CAPs and O$_3$ in healthy volunteers decreased brachial artery diameter, but not endothelium-dependent vascular reactivity. Also, we have previously shown by histology that increased vasoconstriction occurs acutely in pulmonary vessels of CAPs-exposed rats$^{11}$ and that chronic ambient exposure of mice results in both pulmonary and coronary vasoconstriction.$^{73}$ Thus, we now propose to further test the hypothesis that PM-induced alterations in vascular structures is associated with decreased blood flow and increased vascular resistance in the heart and potentially in the vasculature of all major organs including the brain.

1.3.4 Microspheres in rats: As presented above, we have considerable experience with microsphere methodology in the dog,$^{16,24}$ but we needed to adapt the surgical techniques for chronic rat experiments. In a number of preliminary studies, we have been able to chronically implant catheters in the left ventricle and thoracic aorta for perfusion and sampling, respectively, and connect these to two subcutaneous vascular access ports implanted subcutaneously in the
posterior intrascapular area. Data Sciences blood pressure telemeters will be implanted abdominally. We have found that rats with abdominally implanted telemeters routinely live for a year or more without any adverse effect of the telemeter. During this time, the telemeter will remain operational if it is off between experiments. After implantation of the vascular access ports, the rats fully recover within two weeks. The cardiac port is used to inject microspheres. The aortic port is used to obtain a blood sample while the microspheres are injected into the heart. Both the injection and the sample are done with a calibrated pump so that both the injection rate and the sample rate are constant. We have routinely used 7 different colors obtained from IMT Stason laboratories. Microspheres can be injected at weekly intervals to measure vascular perfusion and vascular resistance in every vascular bed of the animal. The blood pressure measurement is used to calculate vascular resistance.

2. APPROACH/ACTIVITIES

2.1 Overall study design: The figure below presents our exposure generation plan. We will first produce CAPs using our HFPC and introduce them into our photochemical chamber to simulate the reactions that occur naturally in the atmosphere. These reactions will result in the formation of O₃ and other photochemical gases, secondary PM, and photochemically aged CAPs.

Project 1: Experimental Conceptual Schematic
Secondary gases can be removed or reduced using our non-selective denuder, or alternatively they can remain components of the exposure mixture. Likewise, the photochemically aged CAPs and secondary PM can also be removed, leaving only the secondary gases. The exposure scenarios that we will use to test our hypotheses are detailed below in Section 2.4.3.

Our exposure plan, described in detail in section 2.4.4 below, features three concurrent exposures (e.g., Exposure Mixture, Control Exposure Mixture, Filtered Air Sham). We will use a crossover design that allows us to control for: 1) variability in CAPs composition and concentration; and 2) inter-subject variability in biological response. Subsequently, these exposures will be repeated with co-exposure to controlled concentrations of O₃ and other photochemical gases. Because northeastern aerosol has significant differences in seasonal composition, a full crossover study will be done during winter and summer to test each of the study hypotheses. Animals used in the crossover studies will have implanted hardware for: 1) telemetric monitoring of blood pressure and heart rate; and 2) injection of fluorescent microspheres for vascular perfusion assessment. In addition, normal rats will be exposed during each experiment for measurement of in vivo chemiluminescence and other acute biological outcomes.

2.2 Exposure system: We will integrate our HFPC with the exposure technology developed for the TERESA studies. The TERESA exposure technology is a complex system designed to sample source emissions, simulate atmospheric photochemistry, generate an exposure, expose laboratory animals, characterize the exposure, and determine the biological outcomes of exposure. Key elements of this exposure system include the photochemical reaction simulation system, and the parallel plate membrane denuder, detailed in the Engineering Core, Section 3.2.

2.3 Planned Exposures: The HFPC and photochemical chamber will be interfaced to produce exposures, consisting of the mixtures of components described below and summarized in Table 3 below. The scenarios are designed to represent four components of ambient air pollution (ambient PM, ambient gases, secondary PM (SPM), secondary gases (SG) including O₃) separately and in combination. Comparisons of biological outcomes among these exposure scenarios will enable us to ascertain the relative contribution of each component to the toxicity of ambient air pollution. To facilitate these comparisons, we have developed the following novel exposure plan that will control for the inherent variability of CAPs composition. Our exposure plan features three concurrent exposures, including two exposed groups and a filtered air exposure sham group. The responses are thus always directly comparable for two scenarios (mixtures) and sham control for every experiment. This means, for example, that the response to photochemically aged CAPs is assessed concurrently with the response to CAPs alone.

<table>
<thead>
<tr>
<th>Year/Seasons</th>
<th>Exposure Mixture</th>
<th>Control Exposure Mixture</th>
<th>Sham/Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 1 Winter/Summer</td>
<td>CAPs + SPM* (no Gases)</td>
<td>CAPs Only (no gases or SPM)</td>
<td>Filtered clean air</td>
</tr>
<tr>
<td>Year 2 Winter/Summer</td>
<td>CAPs + SPM+ SG**</td>
<td>CAPs + SPM (no gases)</td>
<td>Filtered clean air</td>
</tr>
<tr>
<td>Year 3 Winter/Summer</td>
<td>CAPs + SPM+ SG</td>
<td>SG Only (no CAPs or SPM)</td>
<td>Filtered clean air</td>
</tr>
<tr>
<td>Year 4 Summer</td>
<td>CAPs + SPM+ O₃</td>
<td>CAPs + SPM (no gases)</td>
<td>Filtered clean air</td>
</tr>
</tbody>
</table>

*SPM=Secondary Particles (including photochemical aging of CAPs); **SG=Secondary Gases
In general, significant variability in ambient pollution concentrations and composition occur due to the contributions of sources and weather conditions. It is expected that there will be day-to-day variability in composition and concentration, as we have observed in many of our CAPs studies. We also expect seasonal variability as discussed previously (biogenic emissions, space heating, regional scale photochemistry, etc.). Because of these seasonal differences in source contributions, each of the scenarios will be conducted during warm and cold seasons (Table 3).

Based on our previous concentrator studies and preliminary studies using secondary PM formed from photochemical oxidation of ambient air, we expect that CAPs concentrations will be 300-350 μg/m³ on average. We estimate that photochemically aged CAPs + SPM will add an average of 40 μg/m³ to the CAPs concentration, varying according to the concentrations of VOCs and NOx in ambient air. Likewise, the amount of O₃ formed will vary depending on the VOCs and NOx concentrations in the ambient air. In our preliminary studies with ambient air, we found O₃ concentrations of 120-350 ppb downstream of the photochemical chamber. Because the effects of O₃ at high concentrations have already been studied extensively, we will dilute the chamber output, if necessary, to keep the O₃ exposure concentration to < 120 ppb. The series of exposures described above will enable us to test our 4 specific hypotheses (Section 1) by comparing responses as indicated below in Table 4.

**Table 4:** Comparisons to address specific hypotheses (presented above in Section 1)

<table>
<thead>
<tr>
<th>Specific Hypothesis</th>
<th>Comparison(s) to Evaluate Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SG* and O₃ from oxidation of AG** have toxic effects</td>
<td>Year 3, Control Exposure Mixture vs. Sham</td>
</tr>
<tr>
<td>2.a CAPs have toxic effects</td>
<td>Year 1, Control Exposure Mixture vs. Sham</td>
</tr>
<tr>
<td>2.b SPM*** has toxic effects</td>
<td>Year 1, Exposure Mixture vs. Sham</td>
</tr>
<tr>
<td>2.c CAPs toxicity is enhanced by photochemical oxidation</td>
<td>Year 2, Control Exposure Mixture vs. Sham</td>
</tr>
<tr>
<td>3.a SPM effects are exacerbated by O₃</td>
<td>Year 4, Exposure vs. Control Exposure Mixture</td>
</tr>
<tr>
<td>3.b SPM effects are exacerbated by SG including O₃</td>
<td>Year 2, Exposure vs. Control Exposure Mixture</td>
</tr>
<tr>
<td>3.c SG/O₃ exacerbate effects of SPM more than O₃ only</td>
<td>Year 4 Exposure vs. Control Exposure Mixture compared to Year 2 Exposure</td>
</tr>
<tr>
<td>3.d Effects: SPM/SG/O₃ &gt; SPM/O₃ &gt; SG &gt; SPM &gt; CAPs</td>
<td>All Exposures, Longitudinal Analysis</td>
</tr>
<tr>
<td>4.a SPM effects differ in summer vs winter</td>
<td>Year 1, Exposure Mixture Summer vs. Winter</td>
</tr>
<tr>
<td>4.b SPM toxicity depends on composition of CAPs and AG</td>
<td>All Summer and Winter Exposures</td>
</tr>
</tbody>
</table>

*SG=Secondary Gases; AG=Ambient Gases; SPM=Secondary Particles (includes photochemically aged CAPs)

Although our hypotheses follow a step-wise logical plan of increasing complexity, our strategic plan takes these hypotheses out of turn for several reasons. First, we need to establish the relative toxicity of CAPs and SPM in order to effectively compare scenarios that may generate greater or lesser responses. Secondly, we can apply the results of some studies to more than one hypothesis. For example, we will test Hypothesis 3 (toxicological effects of photochemically aged CAPs are exacerbated by co-exposure to O₃ and other secondary gases) during Year 2 by comparing the
Exposure Mixture (photochemically aged CAPs with secondary gases) to the Control Exposure Mixture (photochemically aged CAPs with secondary gases removed). We will test Sub-hypothesis 3a, that effects of photochemically aged CAPs may be exacerbated by O₃, during Year 4, by comparing the Exposure Mixture (photochemically aged CAPs with the secondary gases removed and O₃ added) with the Control Exposure Mixture (photochemically aged CAPs with the gases removed). Sub-hypothesis 3b, that secondary gases including O₃ will increase the effects of exposure to photochemically aged CAPs more than will O₃ without the other secondary gases, will be tested by comparing responses in Year 2 to those in Year 4. We will evaluate the extension of hypothesis 3, that mixtures containing greater numbers of oxidized components (i.e., more complex mixtures) are more toxic than those containing fewer oxidized components. Within the successive years covering this relatively short span, a given summer or winter should be comparable to other summers of the study, respectively.

2.4 Animal exposures design: Our planned design, including Exposure, Control-Exposure, and Sham-Exposure will enable us to control for the inherent variability in CAPs concentration and composition. During the two seasons tested (summer, winter) we will use a crossover exposure design for blood pressure and vascular reactivity outcomes. Repeated exposures of individual animals will allow us to control for variability between subjects, as each animal will be included at least once in the Exposed, Control Exposed, or Sham groups. The crossover design for each exposure (Year 1: summer, winter, etc.) is summarized below in Table 5. Three groups of 6 rats, each implanted with Telemeters and hardware for injecting microspheres and sampling cardiac output (Section 2.4.6) will be exposed in the rotating pattern indicated (Table 5). Each group will have a total of 5 exposures. This will result in 15 Exposures, 15 Control Exposures, and 10 Sham exposures per group, and animal exposure day totals of 45 Exposure, 45 Control Exposure, and 30 Sham per study.

**Table 5:** Treatment Plan: Implanted rats for crossover study and normal rats for acute exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat</th>
<th>Microsphere Color</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>1</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>CL</td>
<td>S</td>
<td>E</td>
</tr>
</tbody>
</table>

E: Exposure Mixture; CE: Control Exposure Mixture; S: Filtered Air Sham Exposure
Only 5 microsphere colors (A, B, C, D, and E) are shown in Table 5. Not included in Table 5 are the initial and final baseline color determinations which are needed in order to internally control all flow measurements. Therefore, a total of 7 colors will be used in each animal. In addition, to those animals in the crossover studies, 4 acute normal rats will be exposed each day, and used in the studies of acute outcomes listed below in Table 6, including in vivo oxidant response and measures of inflammation. All animals will be exposed in individual whole body plethysmograph chambers.

2.5 Toxicological Assessments: Toxicological assessments to be performed are summarized in Table 6 for the exposure studies listed in Table 4. Methods are detailed in sections 2.4.5.1-7.

Table 6: Toxicology plan – Normal Rats (each scenario from Table 3)

<table>
<thead>
<tr>
<th>Exposure Type</th>
<th>Evaluation Method</th>
<th>Effect Assessed</th>
<th>Specific Parameters</th>
<th>Measurement Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Day (Acute)</td>
<td>Organ Chemiluminescence</td>
<td>Pulmonary, Cardiac Oxidative Stress</td>
<td>\textit{In vivo} whole organ chemiluminescence of heart and lung</td>
<td>Immediately post exposure</td>
</tr>
<tr>
<td></td>
<td>Blood Chemistry and Blood Cytology</td>
<td>Systemic Markers of Inflammation</td>
<td>Total white cell counts, differential profiles, platelets, ebc, cytokines (interlekin-6), atrial natriuretic peptide, vasoactive mediator endothelin-1</td>
<td>Immediately post exposure</td>
</tr>
<tr>
<td></td>
<td>Histopathology and Morphometry</td>
<td>Heart, Lung, Brain, &amp; Other Organs</td>
<td>3 Randomly selected slices of fixed heart, lung, brain, and other tissue assessed for inflammation, injury, and vasoconstriction</td>
<td>Immediately post \textit{in vivo} chemiluminescence</td>
</tr>
<tr>
<td>All Exposures</td>
<td>Non-invasive Plethysmography BUXCO</td>
<td>Pulmonary Function</td>
<td>Peak inspiratory flow, peak expiratory flow, tidal volume, minute volume, respiratory frequency, end inspiratory pause, end expiratory pause, derived parameter PenH</td>
<td>Continuously during exposure</td>
</tr>
<tr>
<td>Crossover Studies (Chronic)</td>
<td>Microspheres</td>
<td>Vascular blood Flow/Resistance</td>
<td>Microsphere quantification in organs, vascular perfusion rates; with BP vascular resistance</td>
<td>Post exposure injections</td>
</tr>
<tr>
<td></td>
<td>Blood Pressure Telemetry (DSI)</td>
<td>Blood Pressure and Cardiac Function</td>
<td>Systolic and diastolic pressure; Heart rate, heart rate variability</td>
<td>Continuously during exposure and injections</td>
</tr>
</tbody>
</table>

2.5.1 Pulmonary function testing: We will measure breathing parameters on 6 animals from each group each day to assess acute pulmonary irritant responses in calibrated whole body plethysmography chambers with an automated software system (Buxco Electronics, Sharon, CT). After 10 min of animal acclimatization, respiratory parameters will be measured continuously throughout exposure. Measurements will be calculated from flow changes in pressure transducers connected to the plethysmographs. During exposures, breathing patterns are continuously measured and analyzed breath-by-breath to derive breathing rate, tidal volume, minute ventilation, as well as inspiration and expiration times, peak inspiratory and expiratory flows, and end inspiratory and expiratory pauses. These parameters are used to estimate the parameters Penh (Penh = t_e/RT –1; where RT is the relaxation time derived from the expiratory cycle), Inspiratory Ventilatory Drive (IVD = TV/t_i), and Inspiratory Duty Cycle (IDC = t_i/t_i+t_e), measures indicative of bronchoconstriction and respiratory effort. Changes in respiratory parameters have provided important evidence of pollution-induced health effects.

2.5.2 Blood pressure and pulse rate: These will be monitored with our DSI telemetry system. Physiotel PA-C40 transmitters will be surgically implanted in the abdomen of the rat at least 2
weeks before thoracic hardware implantation. The pressure transmission catheter will be secured in the iliac/femoral artery. Systolic, diastolic, mean blood pressure, heart rate, and heart rate variability (HRV) will be obtained from the continuous arterial pressure signal. Increases in blood pressure and changes in heart rate and HRV indicate significant cardiovascular health effects. Rats with these implanted devices will be used in multiple exposure experiments in a crossover design with at least one week between exposures for any animal.

2.5.3 Regional blood flow and vascular resistance in multiple organ systems: Regional blood flow and vascular resistance will be evaluated with a fluorescent microsphere technique. Briefly, polystyrene microspheres containing one of seven distinct fluorescent dyes are injected into the left ventricle of the heart. Injected microspheres become lodged within a given capillary bed. Because the number of occluded capillaries is small, local vascular resistance is not altered. Regional blood flow is proportional to the number of microspheres trapped in the region of interest. A reference blood sample withdrawn at a known rate allows calculation of regional blood flow in ml/min. A chronic indwelling injection catheter will be positioned in the left ventricle, and a chronic indwelling withdrawal catheter will be placed in the aorta at the time of surgery. Both are connected to subcutaneous vascular access ports as shown in section 1.2.3. For injections, microspheres (15 µm, IMT Stason Labs, CA) will be diluted to 1x10^5 in 0.5 ml of saline solution and injected over a 30-second period. Up to 100,000 microspheres can be used per injection in rats. Reference blood samples will be withdrawn using a dual-piston syringe pump at a constant rate of 0.3 ml per 30 seconds through the aortic vascular access port. After all experiments are completed, the rats will be sacrificed and the organs excised and sectioned. We will dissect the brain into its major anatomic areas and separately analyze the cerebral cortex, midbrain and thalamus, cerebellum, and medulla and brain stem. The heart will be dissected into the left ventricle, right ventricle, and septum. The kidneys, adrenals, a lobe of the liver, the spleen, pancreas, and right lower lobe of the lung will be collected. All tissues will be sent to IMT Stason laboratories for analyses. All other tissues from the animal will be saved in 10% formaldehyde. Each sample will be weighed (W in mg) and digested in a strong base. Microspheres are recovered and quantified. Regional blood flow (Q in ml/min/g) will be calculated from fluorescence measurements of each tissue sample (I_{sample}) and the reference blood samples (I_{ref}). Since arterial pressure will be monitored throughout the procedure, we will also be able to calculate regional resistance (flow/mean arterial pressure) as we have reported.

2.5.4 In vivo chemiluminescence: Spontaneous chemiluminescence of the lung and heart surface is a sensitive measure of reactive oxygen species (ROS) in tissues. We will monitor ROS using a photon counter with a photomultiplier responsive in the range 300-900 nm. Rats will be anesthetized (Nembutal 50 mg/kg body weight), connected to an animal ventilator, and the chest opened to expose the lungs and heart. Measurements will be performed within 10-20 min in a light-tight chamber kept at 32-36°C. We will shield the heart or lung using aluminum foil while measuring the other organ in order to record both measurements in the same animal. Emission will be expressed as counts per second per unit of lung or heart surface (cps/cm^2) as we have previously published.

2.5.5 Blood collection: One day prior to exposure, 0.2-0.5 ml of blood will be drawn under sterile conditions from the tail vein of each animal. Serum and plasma will be prepared, stored at -20°C, and used for comparisons with post-exposure levels of circulating mediators (e.g., endothelin and IL-6). Immediately after 6 hr exposure and completing the chemiluminescence measurements, 3
ml blood will be collected by cardiac puncture, and a portion used for complete blood count and peripheral blood cell differential. Half of the remaining sample will be saved as plasma and half as serum for analyses of circulating mediators. After blood collection, the animals will be given an overdose of pentobarbital to harvest tissues for morphological studies.

2.5.6 Mediator analyses in blood: Blood Endothelin-1, atrial naturetic peptide (ANP), and IL-6 will be analyzed. All standard peptides, antibodies, and assay reagents will be obtained from a commercial radio immunoassay kit for rat ANP (Peninsula). For endothelin-1 and plasma IL-6 assays we will use commercial kits (rat endothelin-1 ELISA kit, Assay Designs; Quantikine rat IL-6 ELISA, R&D Systems).

2.5.7 Histopathologic and morphometric assessments: Morphometric assessments will quantify vasoconstriction of vessels in heart and lung tissues. The trachea will be tied off and the lung removed. The lungs will be fixed by intratracheal fixation in situ at a constant pressure of 20 cm H2O with 2% glutaraldehyde in 0.085 M cacodylate buffer, 0.05% CaCl2, pH 7.4. The lungs and heart will be removed and stored in fixative at 4°C. Morphometry will be carried out as described in our previous studies.9

2.6 Characterization of exposures: We will use a variety of continuous and integrated methods to characterize the exposures generated. Many of these methods are described in detail in the Engineering Core, and are briefly listed here. Particle and gas exposures will be characterized using continuous measurements of PM (TEOM), size distribution (APS, SMPS), BC (Aethalometer), particle count (CPC), CO, NO, NOx and O3, along with integrated measurement of PM (gravimetric), sulfate (Ion Chromatography), trace elements (X-Ray Fluorescence), EC/OC (Thermal/Optical Reflectance), water soluble OC and formaldehyde and acetaldehyde (HPLC). Sampling will be conducted at four locations indicated on the schematic diagram (Section 2.4.1): 1) concentrator inlet (ambient air); 2) concentrator outlet/photochemical chamber inlet; 3) outlet of the photochemical chamber (aged emissions) and; 4) at the point of exposure. The first two locations will be used to monitor the effectiveness of the HFPC, looking specifically at the enrichment of PM2.5 mass to verify that the concentrator performance. The second and third locations will provide information on the effectiveness of the photochemical chamber, specifically by monitoring changes in gas and particle concentrations and size distribution. The fourth sampling location will be used for exposure characterization, with the sampling location and conditions as comparable to the exposure conditions as possible.

2.7 Statistical methods and data analysis: The exposure experiments use a partial factorial design. Outcomes of interest on each animal will include chemiluminescence, histological outcomes assessing inflammation, pulmonary breathing rate, volumes, and flow measures, and blood parameters. In these studies, subjects are randomized to receive the Exposure Mixture, Control Exposure Mixture, or Sham Exposure either within a straightforward factorial structure or a more complicated cross-over design. To analyze these data, we will conduct multiple analyses that use exposure metrics of increasing sensitivity. First, we begin by using (potentially multi-way) ANOVA techniques that treat exposure as a treatment. That is, we will assess overall differences between Exposure, Control Exposure, or Sham Exposure responses (i.e., a binary exposure covariate). Second, to assess univariate associations between mixture mass and component levels and health, we will conduct single-pollutant analyses in which a separate regression model is fitted using biologic response as the dependent variable and either PM mass, number, single particle component or single gas as the exposure metric. Third, to confirm univariate findings, we will conduct multiple pollutant analyses to investigate joint effects of multiple mixture
components. For the studies of blood flow and resistance, we will employ longitudinal methods to estimate associations between exposure and biologic parameters in the proposed cross-over design. Briefly, data from each animal is collected during two baselines (one at the beginning and the end of the experiment) and 5 exposures. Each animal will be exposed to each of filtered air (Sham), control exposures (e.g., CAPs), and exposures (e.g., aged CAPs).

Let $Y_{ij}$ be a biologic endpoint recorded for animal $i$ at time $j$. We will use models of the form:

$$Y_{ij} = \beta_0 + \beta_{1 \text{sham}ij} + \beta_{2 \text{expo}ij} + \beta_{3 \text{c} \text{exp}oij} + \beta_4 j + u_i + \epsilon_{ij} \quad (1)$$

where $\text{sham}ij$, $\text{expo}ij$ and $\text{c} \text{exp}oij$ are indicator variables equal to 1 if subject $i$ receives sham, exposure or controlled exposure, respectively, at time $j$, and $\beta_4 j$ is a linear term corresponding to the time (order) of measurement. The model compares the mean outcome under each exposure condition (sham, controlled exposure, exposure) to that measured under baseline conditions. We will consider interaction terms that allow the effect of exposure to vary over time. If necessary we will also consider more general forms for the effect of time and more flexible correlations structures than implied by random subject-specific intercepts. We have used this class of models to analyze similar microsphere data in a study of CAPs effects in dogs.\(^{16}\)

We will continue to use a multi-tiered strategy that successively incorporates more refined exposure measures to assess the relative contributions of different pollutants to observed health effects.\(^{26,79}\) Furthermore, we will extend the above model to use concentrations of individual or multiple pollutants, depending on the specific hypothesis of interest. For a given pollutant, let $\text{conc}ij$ denote the concentration of that pollutant for animal $i$ at exposure occasion $j$. The model becomes:

$$Y_{ij} = \beta_0 + \beta_{1 \text{sham}ij} + \beta_{2 \text{conc}ij \text{expo}ij} + \beta_{3 \text{conc}ij \text{c} \text{exp}oij} + \beta_4 j + u_i + \epsilon_{ij}, \quad (2)$$

Fitting this model yields estimates of an exposure-response slope under both exposure and controlled exposure conditions, and yields a test of whether these slopes are equal to one another. This test will assess the evidence that exposure increases the toxicity of particular air pollution components. Extension to non-linear forms for the exposure-response function or multi-component models is straight forward.

2.7.1 **Power calculations:** We estimated the power we will have to detect realistic effect sizes for four primary outcomes using the study design presented in Table 5. The primary outcomes used were organ chemiluminescence (CL) and three main respiratory parameters: respiratory frequency (F), peak expiratory flow (PEF) and expiratory flow at 50% (EF50). We assumed effect sizes observed in our previous studies:\(^{4,74,79}\) 1) 1.7 fold increase for CL; 2) 28 breaths/min for F; 3) -0.37 ml/s for EF50; and 4) -4.6 ml/s for PEF. We assumed residual variances equal to estimates from these earlier analyses. The estimated power to detect these effects for any one exposure setting versus sham is 93, 84, 89 and 95% for CL, F, EF50, and PEF, respectively, suggesting the proposed sample sizes will provide adequate power to detect exposure scenario effects.

2.8 **Animal Subjects:** Animals will be maintained and studied in accordance with the National Institutes of Health (NIH) guidelines for animal care and use in research. The proposed protocols have already been approved by the Harvard Medical Area Standing Committee on Animals. Rat strain selected for this project is Sprague-Dawley, used most successfully in our prior CAPs studies. Our School is accredited by the American Association for the Accreditation of
Laboratory Animals Care and meets the NIH standards as set forth in the “Guide for the Care and Use of Laboratory Animals,” DHEW Publication No. [NIH] 78-23 Revised.

2.9 **Timeline:** Table 3 shows the timeline for the proposed project, which will begin during Year 1 of our Center, and conclude after the summer of Year 4. Two Exposure studies will be conducted each year, one during the winter and the other during the summer. Each study will consist of 15 exposure days, leaving sufficient time between exposure campaigns for analysis. The second half of Years 4 and 5 will be used to complete data analysis and integrate the results.

3. **EXPECTED RESULTS:** These studies will differentiate the effects of different components of multi-pollutant mixtures on toxicological outcomes, using a novel and sophisticated controlled-exposure plan that controls both variability in exposure to ambient particles and variability of response between subjects. Using ambient particles and gases will allow us to generate realistic exposure mixtures that are toxicologically relevant to public health. Exposure mixtures include CAPs, photochemically aged CAPs, O₃ and other photochemical gases, separately and in combination. The sequence of exposures, progressively adding or subtracting components of the mixture, will provide insight into the role of each component in producing biological responses. Inherent variability in the concentration and composition of CAPs and other components of the exposure mixture will provide insight into the shape of exposure-response relationships. We expect to advance understanding of interactions between PM and photochemical gases in health outcomes, addressing a critical knowledge gap. The biological outcomes assessed in this Project include responses important in oxidant induced pulmonary inflammation, and functional vascular/cardiovascular health responses that can also contribute to neurocognitive toxicities and impairments. Our Project strongly complements the other Center Projects, which comprise human epidemiological studies that are focused on many of the same outcomes but cannot directly assess the underlying molecular or morphological outcomes critical to understanding pollutant toxicity and mechanisms of such vascular and cardiovascular responses.

4. **GENERAL PROJECT INFORMATION:** Dr. Godleski will be the Project PI. He is an experimental Pathologist with extensive experience in inhalation toxicology studies of CAPs and TERESA exposures. He will be responsible for aspects of this study that pertain to the biological outcomes. Dr. Godleski will be assisted by Co-PI Dr. Koutrakis, who will be responsible for design of the exposure scenarios. Drs. Lawrence and Wolfson will assist Dr. Koutrakis with the technical and administrative aspects of this project. Dr. Lawrence is an atmospheric chemist who will be responsible for the HFPC and the reaction chamber, generation and characterization of exposures. Dr. Edgar Diaz will be responsible for animal exposures and biological assessments. Finally, Dr Brent Coull, Center Biostatistician, will participate in the data analysis. **Facilities:** The HFPC and Inhalation Toxicology laboratory will be available for use in this study, including all the instrumentation and equipment for generating and characterizing CAPs exposures. The photochemical chamber and denuders designed, built, and used in the TERESA studies funded by the existing PM Center, will also be available for this study.
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