Combined Exposure to Ultrafine Particulate Matter and Ozone:

Characterization of Particulate Deposition, Lung Oxidant Stress, and Heart Injury

California Air Resources Board Contract# 17RD011

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PM Effects Cardiovascular System

- Epidemiologic associations between PM & cardiac events
  - **Mortality** (Harvard Six Cities, 1993)
  - **Arrhythmias** (Peters et al., 2000)
  - **Myocardial Infarction** (Peters et al., 2001)
  - **Stroke** (Women's Health Initiative, 2007)
  - **Heart Failure** (ACS cohort, 2008)
- Higher incidence of PM-related cardiac events
  - Mature populations with pre-existing CVD
  - Exposures to heavily trafficked roadways
    - PM size & composition
    - Gaseous pollutants
      - Ozone (O_3)

PM Effects Cardiovascular System

- Animal studies have examined the pulmonary and cardiovascular effects of acute PM and O_3 exposure adolescent rodent with and without hypertension.
- No animal studies have used mature adult animals with established CVD to mimic the exposure of elderly adults with CVD.

Present Study

- **Objective:** Examine the effects of acute exposure to ultrafine particulate matter (UFPM), ozone (O_3), and UFPM combined with O_3 in mature adult spontaneous hypertensive (SH) rats with preexisting CVD.
  - This study examined banked lung and heart tissues that were collected previously
    - CARB Contract# 13-311: Co-exposure to PM and O_3: Pulmonary C fiber and platelet activation in decreased HRV (Principal Investigator: Fern Tablin).

Research Design

1. **Rodent Model of CVD**
   - Spontaneous hypertensive
     - Genetic variant of WKY
   - Mature adults (~50 wko)
     - ↑Cardiac remodeling
       - Left ventricular hypertrophy
       - Fibrosis
     - ↑Arrhythmias
2. **Combined pollutant exposure atmosphere** (Wong et al. 2018)
   - Filtered Air
   - UFPM - Simulate traffic-related exposure
     - Premixed flame-generated ultrafine particles (UFPM)(70 nM; 250 ug/M^3)
     - PAH enriched
   - O_3
     - 1.0 ppm
   - UFPM+O_3

3. **Exposure protocol**
   - 1h Baseline, FA
   - 6h Exposure
   - 8h Recovery, FA
4. **Methods**
   - ECG telemeters implanted +2wks prior to exposure.
   - Blood collected post recovery.
   - Lungs airway fixed with 10% formalin at 20 cm H2O
   - Hearts fixed in 10% formalin.

STATISTICAL ANALYSIS

1. **Exposure-Related Differences**
   - Nonparametric Kruskal-Wallis test with Dunn-Bonferroni post hoc comparisons
     - Distinguished by strain
2. **Strain-Related Differences**
   - Nonparametric Mann-Whitney test

***p*-Values ≤ 0.050 considered significant**

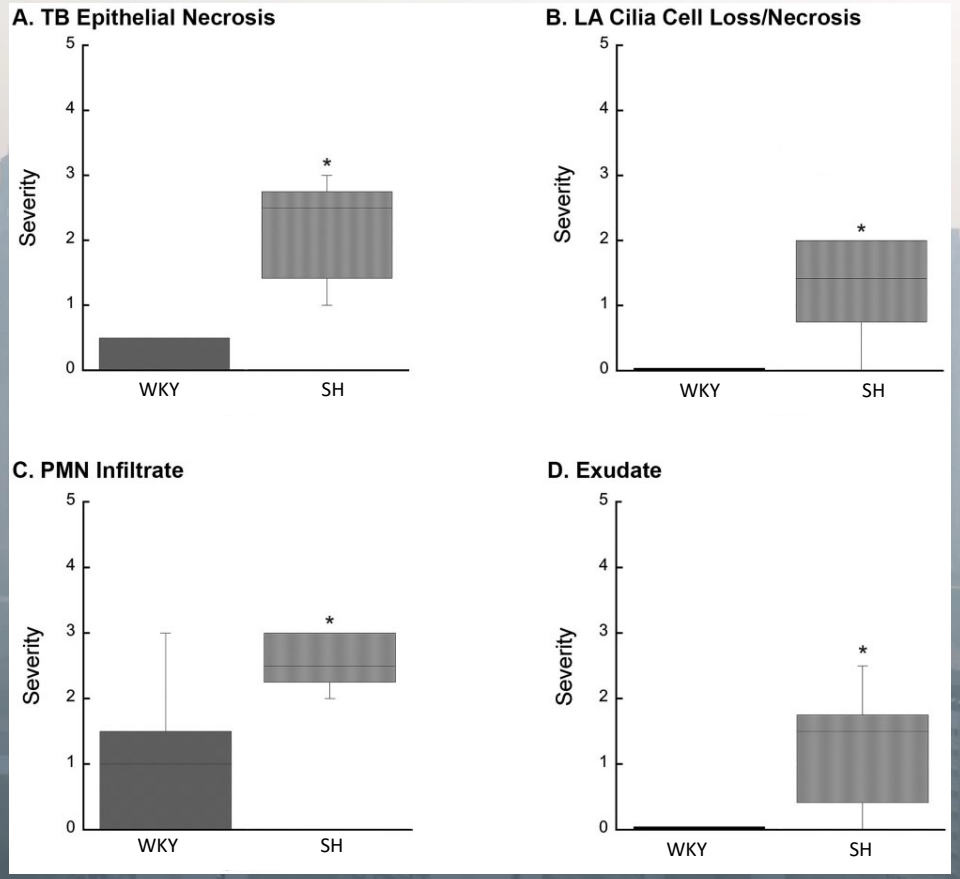
Previous Observations:

- **O_3 reacts with polycyclic-aromatic hydrocarbons** (PAHs) contained in combustion-derived UFPM to produce a potentially more toxic profile of oxidant-derived reaction products.

UFPM		UFPM+O_3	
Compound	Abundance (ng/m^3)	Compound	Abundance (ng/m^3)
2-methylbiphenyl	114.68	3-methylbiphenyl	73.12
3-methylbiphenyl	64.30	2-methylbiphenyl	35.87
2-methylnaphthalene	35.86	**Pyrene**	**30.35**
1,3+1,6+1,7 dimethylnaphthalene	23.78	4-methylbiphenyl	20.70
Naphthalene	**18.73**	Anthrone (p)	19.32
1-methylnaphthalene	17.51	1+2ethylnaphthalene	19.31
Naphthalene (p)	**15.37**	Coronene (p)	17.94
2,6+2,7 dimethylnaphthalene	14.22	Benzo[*b*]chrysene (p)	16.56
2-methylnaphthalene (p)	13.84	7,12-dimethylbenz[*a*]anthracene (p)	13.80
1+2ethylnaphthalene	12.61		
Fluorene	11.54		

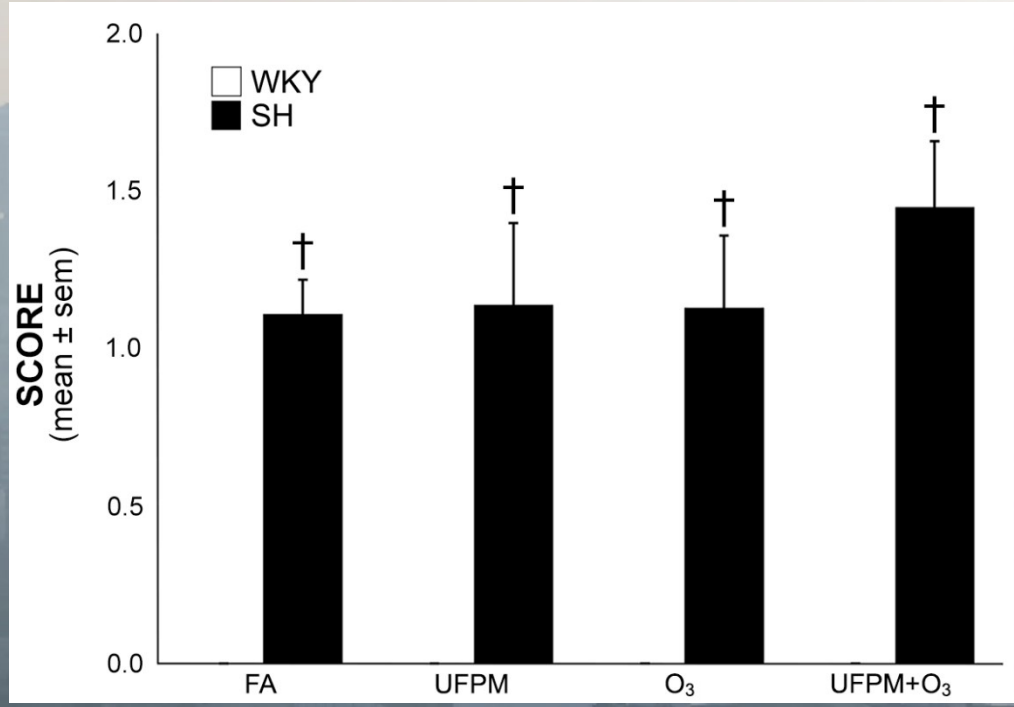
Previous Observations:

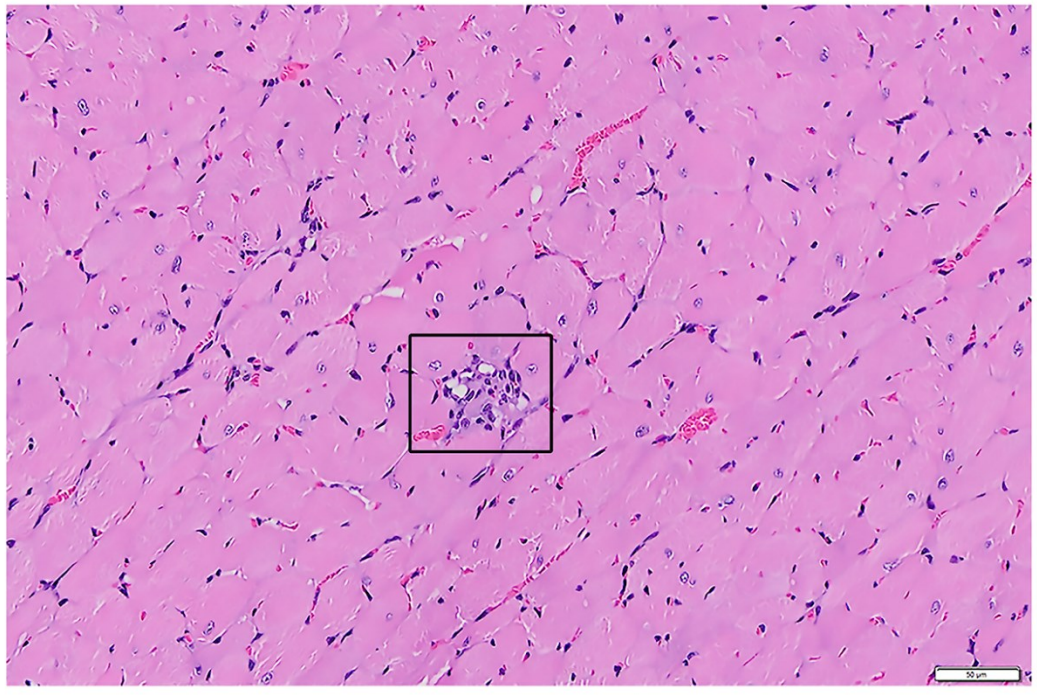
- Mature adult animals with CVD are more susceptible to oxidant injury induced by O_3 compared to age matched animals without CVD as indicated by greater inflammation, airway injury and alterations in HRV.



Previous Observations:

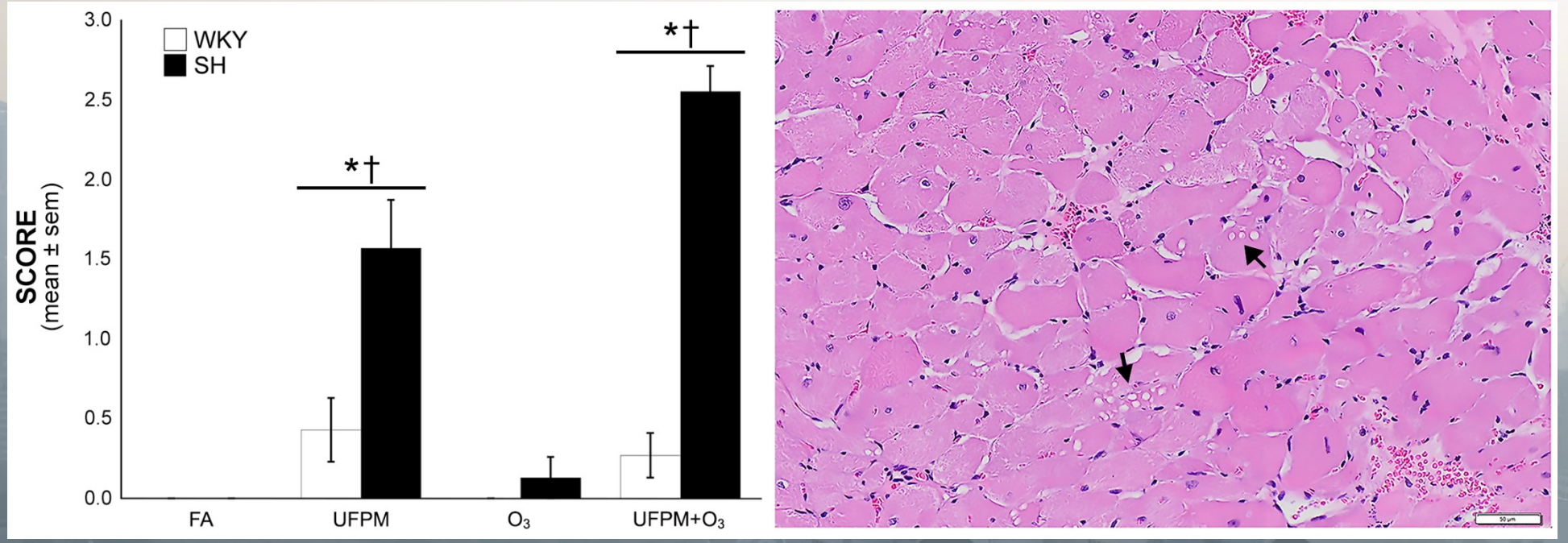
- Mature SH rats with CVD had elevated ventricular focal organized necrotic lesions compared to Wistar controls that were independent of exposure.





Previous Observations:

- Mature SH rats with CVD exposed to the **UFPM and UFPM+O_3** atmospheres developed **acute myocardial necrosis**.



What are the factors that contribute to this UFPM and UFPM+O3 induced acute myocardial injury?

Previous Observations:

- The **UFPM+O_3** atmosphere induced significantly **greater changes in airway injury, and inflammation** in mature adult SH and WKY rats.

	WKY				SH			
Parameter	FA (n = 10)	UFPM (n = 8)	O_3 (n = 9)	UFPM+O_3 (n = 11)	FA (n = 12)	UFPM (n = 10)	O_3 (n = 8)	UFPM+O_3 (n = 12)
Macrophage Infiltrate	0.5 ± 0.2	0.8 ± 0.2	1.3 ± 0.3	2.8 ± 0.2$^{*,**,\#}$	0.2 ± 0.1	0.6 ± 0.3	0.8 ± 0.2	2.6 ± 0.2$^{*,**,\#}$
Edema	0.4 ± 0.2	0.3 ± 0.1	0.9 ± 0.3	3.7 ± 0.1$^{*,**,\#}$	0.2 ± 0.1	0.0	2.3 ± 0.4$^{*,**,\#\#}$	3.5 ± 0.3*,**
PMN Infiltrate	0.6 ± 0.2	0.1 ± 0.1	1.0 ± 0.4	3.8 ± 0.1$^{*,**,\#}$	0.3 ± 0.2	0.4 ± 0.3	2.4 ± 0.3$^{*,\#\#}$	4.0 ± 0.2*,**
TB Epithelial Necrosis	0.0	0.0	0.4 ± 0.3	3.8 ± 0.1$^{*,**,\#}$	0.1 ± 0.1	0.0	2.2 ± 0.3$^{*,**,\#\#}$	3.7 ± 0.2*,**
Exudate	0.0	0.0	0.3 ± 0.2	3.7 ± 0.2$^{*,**,\#}$	0.0	0.1 ± 0.1	1.2 ± 0.3$^{\#\#}$	3.5 ± 0.3*,**
LA Cilia Cell Loss/Necrosis	0.8 ± 0.2	0.0	0.2 ± 0.1	3.8 ± 0.2$^{*,**,\#}$	0.1 ± 0.1	0.0	1.3 ± 0.3$^{**,\#\#}$	3.1 ± 0.3*,**

Note: Standard, paraffin-embedded, histopathologic sections of lung tissue were evaluated by a board-certified veterinary pathologist for lesions in the large airways, terminal bronchiolar/alveolar duct regions, alveolar parenchyma and vasculature and assigned a severity score (0-5). Normal Wistar-Kyoto rat (NW); Spontaneously Hypertensive rat (SH); filtered air (FA); ultrafine particulate matter (UFPM); ozone (O_3); ultrafine particulate matter combined with ozone (UFPM+O_3); polymorphonuclear leukocyte (PMN); terminal bronchiole (TB); large airway (LA); intravenous fibrin-neutrophil aggregates (IVFNA); the Kruskal-Wallis test was used to calculate the differences between exposure groups by strain and significant *p*-values ($p < 0.05$) from Dunn-Bonferroni post-hoc test are shown above. Values are shown as the means ± SEM by exposure group and strain.

* Significant compared to FA within same strain exposure groups.

** Significant compared to UFPM within same strain exposure groups.

$^{\#}$ Significant compared to O_3 within same strain exposure groups.

$^{\#\#}$ Significant difference between NW and SH within same strain exposure groups.

Previous Observations:

- The O_3 and UFPM+O_3 atmospheres induced significant alterations in HRV parameters, and cardiac arrhythmias in mature adult SH and WKY rats.
- Increased NN interval, RMSSD, HF power, AVB number and severity are consistent with increased parasympathetic outflow.
- Increased in LF power, LF/HF ratio, PVCs and decrease in approximate entropy are consistent with increased sympathetic outflow.
- **Conclusion: The cardiac arrhythmias and alterations in HRV observed in this study are the result of coactivation of the parasympathetic and sympathetic nerves that supply the heart.**

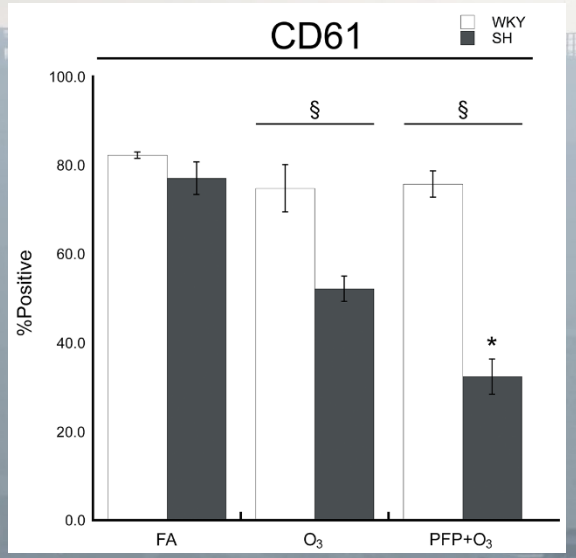
Previous Observations:

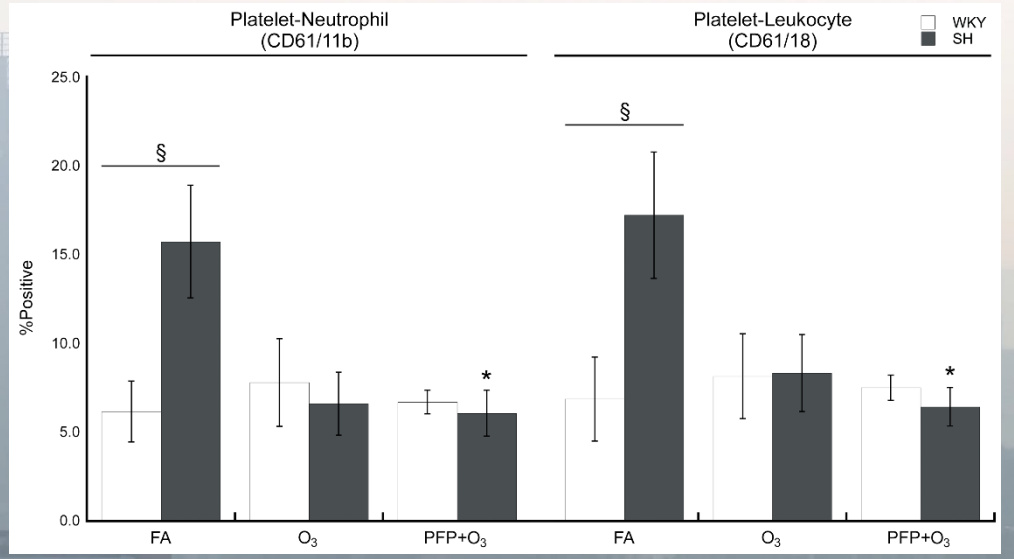
- The **UFPM and UFPM+O_3 atmosphere induced** changes in platelet levels, platelet-white blood cell aggregates and lung venous thrombi in the SH rats. These changes are suggestive of a **pro-coagulant and pro-inflammatory systemic vascular environment**.

CD61: Major platelet integrin $\alpha_{2b}\beta_{3a}$

- Exposure ↓$CD61^+$ platelets
  - ↑**Platelet activation**
    - CD62P: α granule
    - CD63: lysosomal granule

↑**Fibrin stabilized aggregates in tissues**

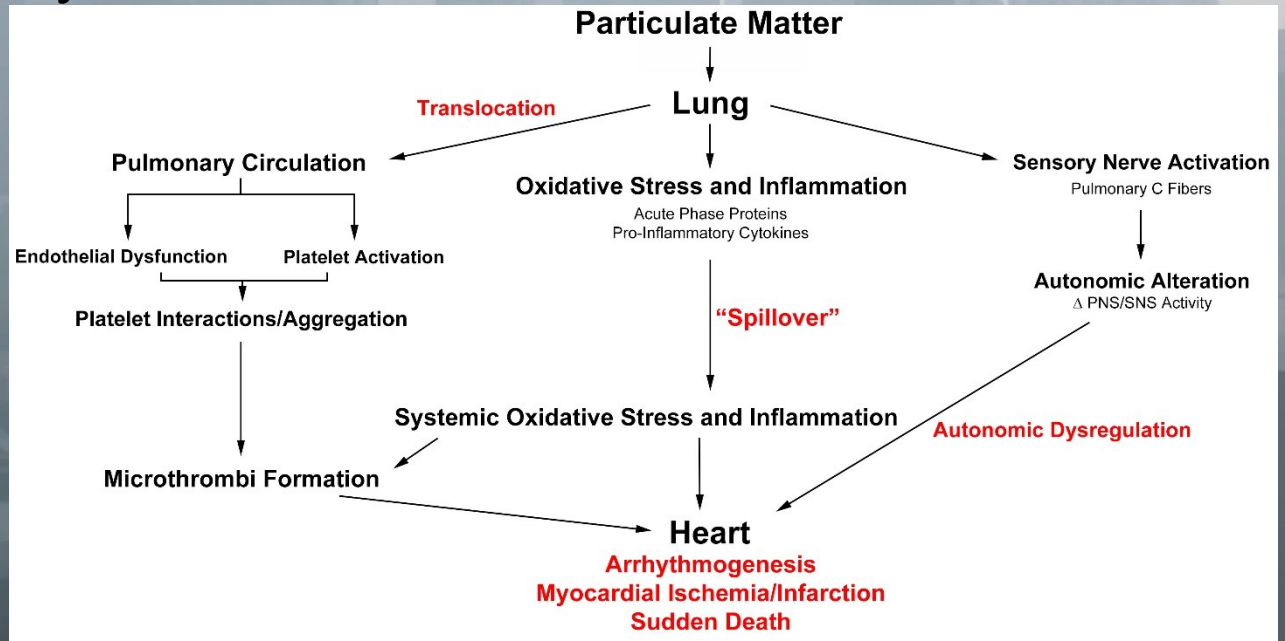




	WKY				SH			
Parameter	FA (n = 10)	UFPM (n = 8)	O_3 (n = 9)	UFPM+O_3 (n = 11)	FA (n = 12)	UFPM (n = 10)	O_3 (n = 8)	UFPM+O_3 (n = 12)
Lung IVFNA	0.0	0.8 ± 0.4	0.9 ± 0.5	0.8 ± 0.2	0.6 ± 0.2	1.9 ± 0.4*	1.9 ± 0.5	1.3 ± 0.2
Lung Intravenous Thrombus	0.0	1.8 ± 0.4	0.8 ± 0.4*	1.3 ± 0.4	0.4 ± 0.2	1.0 ± 0.4	0.4 ± 0.3	0.9 ± 0.3

Conclusion From Previous Study :

- Integrated response to the UFPM+O_3 is initiated in the lung, results in downstream hematological and autonomic nervous system responses:
  - Increased platelet activation
  - Parasympathetic and sympathetic nervous system coactivation
  - Cardiac arrhythmias
  - Myocardial injury in mature adult SH rats with CVD.



Objectives of Current Study

Hypothesis:

- ***O_3 enhances the biologic potency of UFPM*** *by promoting ROS production at the particle surface.*
  - *Increasing cellular antioxidant expression*
- ***Increased oxidant sensitivity of mature adult SH rats*** *makes them more susceptible to microthrombi formation and myocardial damage.*
  - *Platelet-leukocyte and platelet-neutrophil interactions*.

Objective 1. Define the effect of O_3 on the local relationship between particle deposition and cellular oxidative stress within the airways.

Rationale: UFPM+O_3 induced greater airway injury and inflammation in WKY and SH rats. Particles and cellular antioxidant expression were investigated by examining their spatial proximity and organization.

Approach: Evaluate the spatial proximity and organization of particles and cellular markers of oxidant stress and metabolism

Methods: Lung tissue double-stained for Nrf2-dependent phase II antioxidants hemeoxygenase-1 (HMOX1) and superoxide dismutase-1 (SOD1) were examined with enhanced darkfield microscopy complemented with a hyperspectral imaging system.

Objective 1. Define the effect of O_3 on the local relationship between particle deposition and cellular oxidative stress within the airways.

TABLE 2
Histopathological Measures of the Lung

	WKY				SH			
Parameter	FA	UFPM	O_3	UFPM+O_3	FA	UFPM	O_3	UFPM+O_3
HMOX1	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.4	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.4
SOD1	1.2 ± 0.4	1.3 ± 0.5	1.5 ± 0.4	1.6 ± 0.3	1.2 ± 0.3	1.2 ± 0.6	1.1 ± 0.4	1.2 ± 0.4
Fibrin-Stabilized Microthrombi								
Category I	0.0	1.0 ± 0.2*	0.4 ± 0.2	1.2 ± 0.2*†	0.1 ± 0.1	1.9 ± 0.3*§	0.9 ± 0.3	2.5 ± 0.3*†§
II	0.0	0.7 ± 0.2*	0.0	0.6 ± 0.2*†	0.1 ± 0.1	1.1 ± 0.3*	0.5 ± 0.2	1.6 ± 0.2*†§
III	0.0	0.0	0.0	0.1 ± 0.1	0.0	0.4 ± 0.2	0.0	0.6 ± 0.2*†§

Note: Sections of the lung were evaluated histologically for microthrombi; or immunohistochemically for HMOX1, a marker of oxidative stress, or SOD1, a marker of superoxide metabolism, within the airway epithelium. Lung sections contained approximately three to five airways, and the number of HMOX-1 and/or SOD1-positive cell clusters within the airway epithelium were counted and recorded per airway section. Microthrombi scores (scale: 0 to 5) were given based on number of aggregates observed per category per lung section for each animal. Values are shown as the means ± SEM within strain-related exposure groups. *p*-Values ≤ 0.05 were considered significant. *Significant difference compared to FA within strain-related exposure groups. †Significant difference compared to O_3 within strain-related exposure groups. §Significant difference between strains within exposure group.

Objective 2. Characterize UFPM+O_3 impact on microthrombi formation.

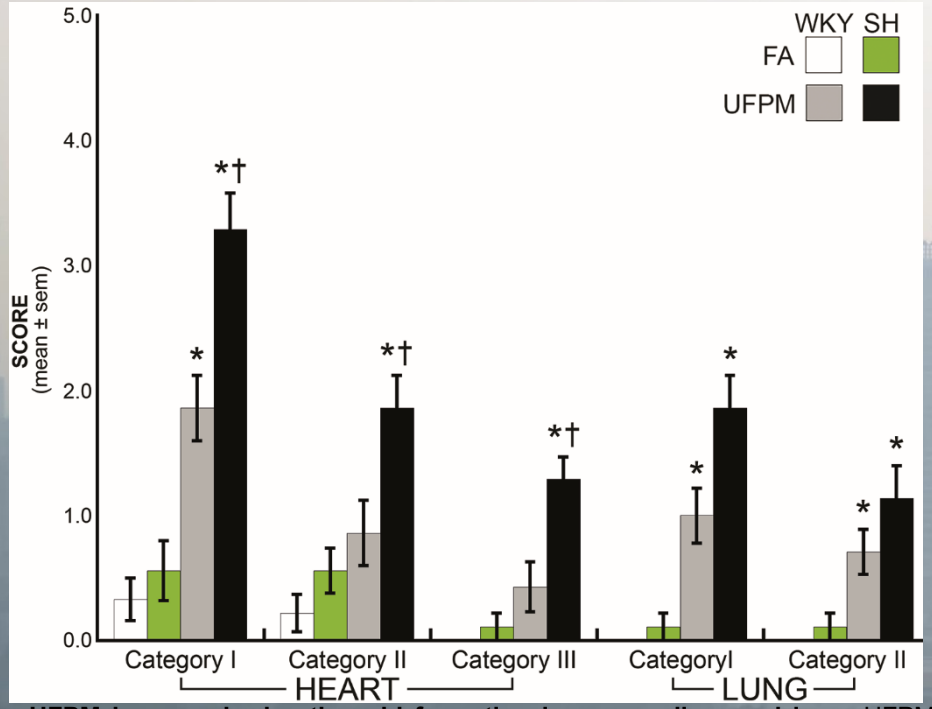
Rationale: UFPM+O_3 induced changes in platelet activation, and platelet-white blood cell aggregation.

Approach: Examine fibrin-stabilized aggregates in lung and myocardial tissue using a phosphotungstic acid-hematoxylin (PTAH) stain.

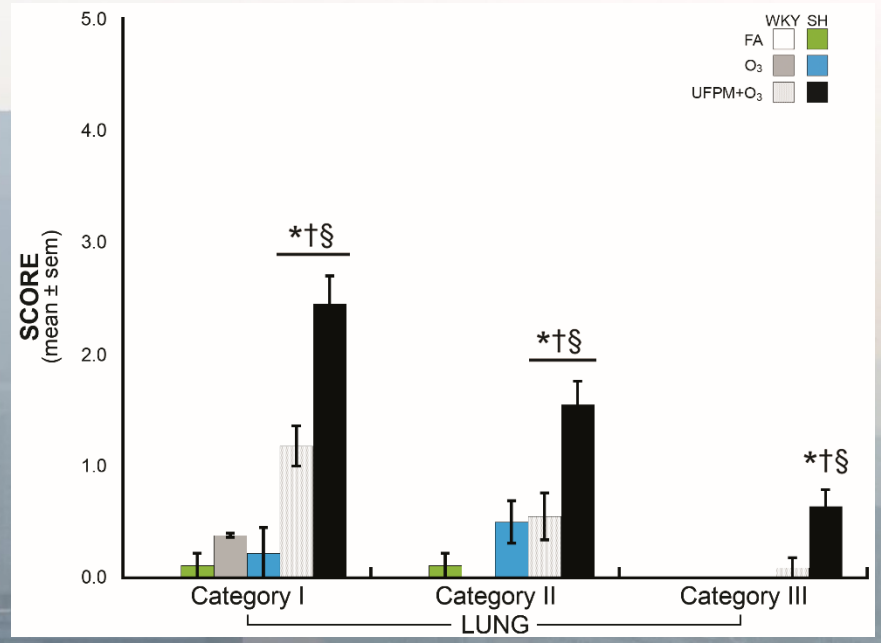
FIBRIN-STABILIZED AGGREGATES

1. **PTAH-stained heart and lung**
   - Category I: A small aggregate within an arteriole or artery
   - Category II: An aggregate fully occluding an arteriole
   - Category III: A large, multifocal aggregate within an artery
2. **Semi-quantitative scores**
   - 0 – None
   - 1 – One to three
   - 2 – Four to six
   - 3 – Seven to nine
   - 4 – Ten to twelve
   - 5 – Thirteen or more

Objective 2. Characterize UFPM+O_3 impact on microthrombi formation.



UFPM increased microthrombi formation in myocardium and lung. UFPM increased microthrombi in the heart and lung compared to FA. *p*-Values ≤ 0.05 were considered significant. *Significant difference compared to FA within strain-related exposure group. †Significant difference between strains within exposure-related groups.

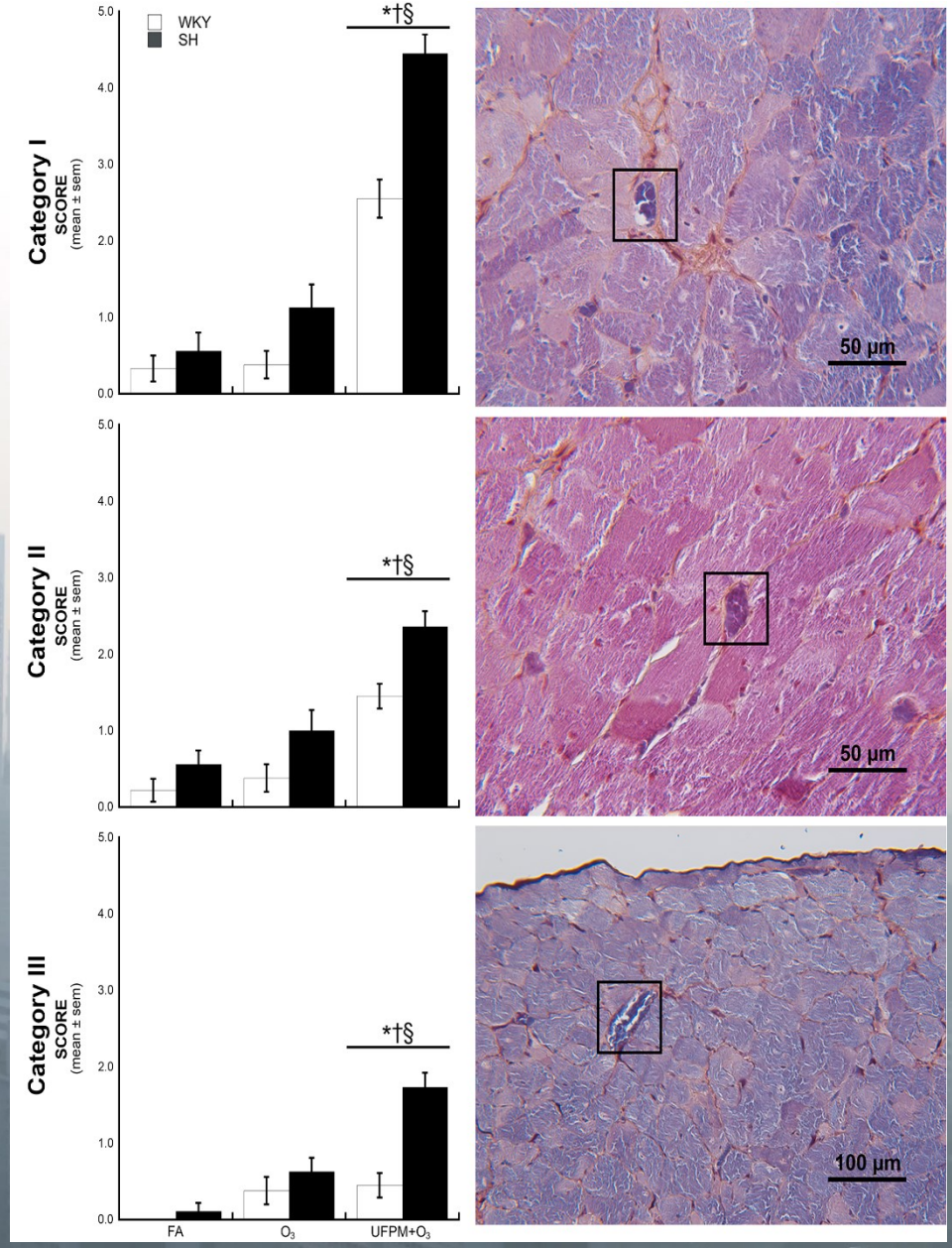


UFPM+O_3 increased microthrombi formation in lung. UFPM+O_3 increased microthrombi compared to FA and compared to O_3. *p*-Values ≤ 0.05 were considered significant. *Significant difference compared to FA within strain-related exposure groups. †Significant difference compared to O_3 within strain-related exposure groups. §Significant difference between UFPM+O_3-exposed WKY and SH rats.

Objective 2. Characterize UFPM+O_3 impact on microthrombi formation.

UFPM+O_3 increased microthrombi formation in myocardium. Right panel: Representative images of category I-III fibrin-stabilized aggregates observed in left ventricular region of a UFPM+O_3-exposed SH rat. Left panel: *Significant difference compared to FA within strain-related exposure groups. †Significant difference compared to O_3 within strain-related exposure groups. §Significant difference between UFPM+O_3-exposed WKY and SH rats.

Consistent with exposure induced ↓ in $CD61^+$ platelets and platelet-WBC aggregates.



Objective 3. Define UFPM+O_3 impact on myocardial injury.

Rationale: Mature adult SH rats exposed to UFPM or UFPM+O_3 displayed **acute myocardial injury**, including **acute cellular necrosis**, and **hypercontractility**.

Approach: To determine ischemia contributed to **myocardial injury** the cellular expression of **JunB**, an early marker of myocardial ischemia, and **Nur77**, a marker of nonapoptotic cell death were examined.

Cardiac fibrosis (CF), a marker of **chronic injury associated with CVD progression** was examined using Masson's trichrome staining.

Objective 3. Define UFPM+O_3 impact on myocardial injury.

TABLE 3
Histopathological Measures of the Myocardium

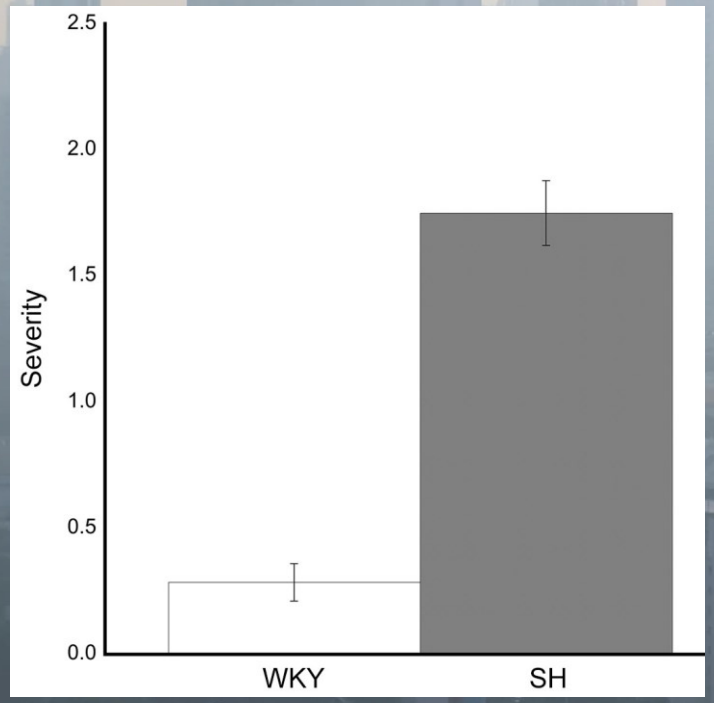
	WKY				SH			
Parameter	FA	UFPM	O_3	UFPM+O_3	FA	UFPM	O_3	UFPM+O_3
§Fibrosis	0.2 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.3 ± 0.1	1.9 ± 0.3	1.7 ± 0.3	1.8 ± 0.2	2.0 ± 0.2
Nur77	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3 ± 0.1
JunB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4 ± 0.2
Fibrin-Stabilized Microthrombi								
Category I	0.3 ± 0.2	1.9 ± 0.3*	0.4 ± 0.2	2.6 ± 0.3*	0.6 ± 0.2	3.3 ± 0.3*§	1.1 ± 0.3	4.5 ± 0.3*†§
II	0.2 ± 0.2	0.9 ± 0.3	0.4 ± 0.2	1.8 ± 0.2*	0.6 ± 0.2	1.9 ± 0.3*§	1.0 ± 0.3	2.4 ± 0.2*†§
III	0.0	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.1 ± 0.1	1.3 ± 0.2*§	0.6 ± 0.2	1.7 ± 0.2*†§

Note: Sections of the heart were evaluated histologically for interstitial fibrosis, microthrombi; or immunohistochemically for Nur77, nonapoptotic death marker, or JunB, myocardial ischemia marker. Myocardial fibrosis scores (scale: 0 to 5) were assigned based on extent and severity of lesions observed. The number of Nur77 and/or JunB-positive cell clusters within the left ventricle and interventricular septum of a heart section were counted and recorded per section. Microthrombi scores (scale: 0 to 5) were given based on number of aggregates observed per category per lung section for each animal. Values are shown as the means ± SEM within strain-related exposure groups. *p*-Values ≤ 0.05 were considered significant. *Significant difference from FA within strain-related exposure groups. †Significant difference from O_3 within strain-related exposure groups. §Significant difference between strains within exposure group (overall for IF).

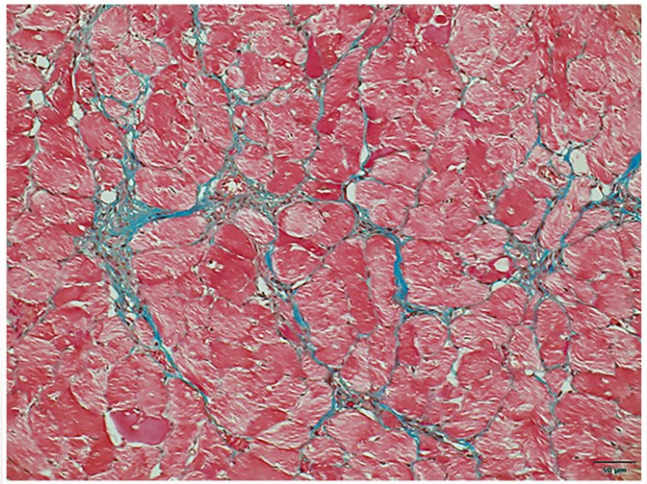
Objective 3. Define UFPM+O_3 impact on myocardial injury.

Scoring Criteria

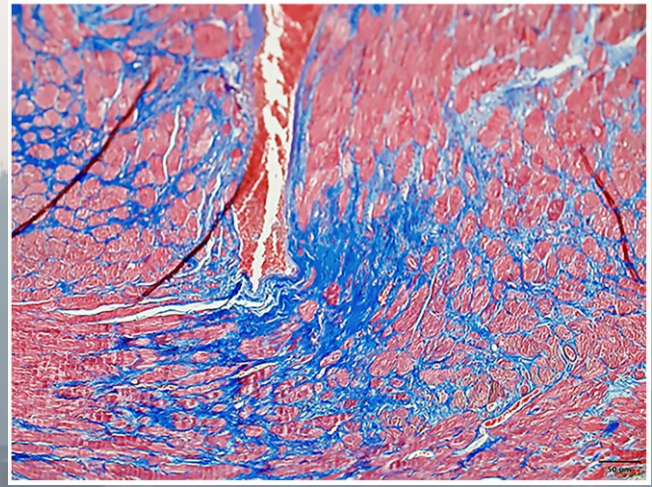
Score		Description
0	-	No interstitial expansion
1	-	Minimal multifocal interstitial expansion
2	-	One or more large networks of mild interstitial expansion
3	-	One to three networks of mild interstitial expansion with minimal replacement fibrosis



Dissecting Fibrosis



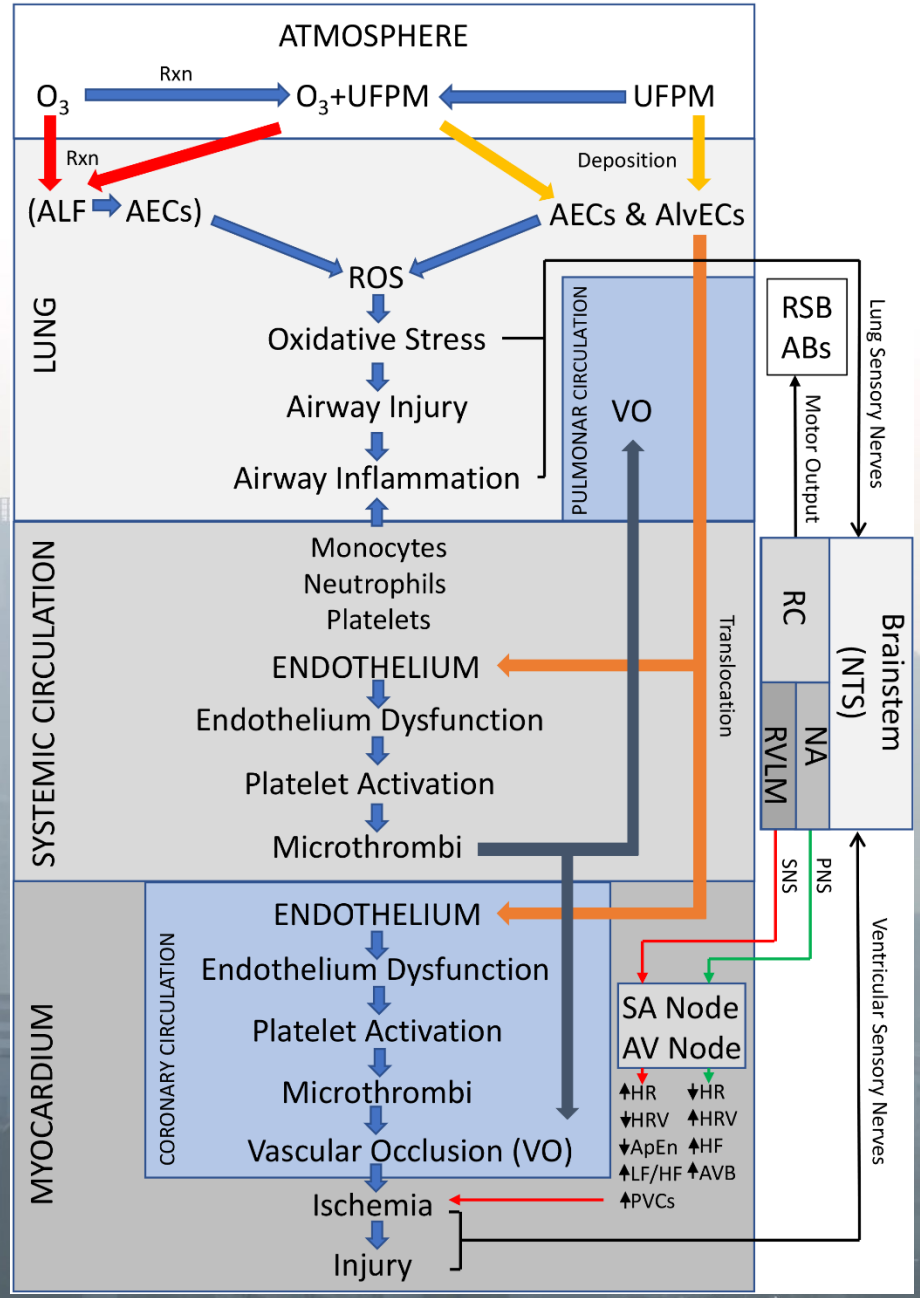
Replacement Fibrosis



Consistent with chronic myocardial stress and the previous observation of increased focal organized necrosis in mature adult SH rats.

Integration of Results

Integration of results of the current study (CARB# 17RD011; PI Schelegle) and those from our previous study (CARB# 13-311; PI Tablin): Abbreviations: ABs, augmented breaths; AECs, airway epithelial cells; ALF, airway lining fluid; AlvECs, alveolar epithelial cells; ApEn, approximate entropy; AVB, atrioventricular block; AV Node, atrioventricular node; HR, heart rate; HRV, heart rate variability; HF, high frequency; LF/HF, ratio of low to high frequency; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; PNS, parasympathetic nervous system; PVCs, premature ventricular contractions; RSB, rapid shallow breathing; RC, respiratory center; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; Rxn, reaction; SNS, sympathetic nervous system; and VO, vascular occlusion.



CONCLUSIONS

1. **Exposure to UFPM and UFPM+O_3 promotes the formation of microthrombi in heart and lung.**

2. **Mature adult SH rats are more susceptible to thrombotic effects of air pollution**
   - Strain related ↑ in circulating platelet-WBC aggregates consistent with CVD
   - Strain related ↑ in sensitivity to oxidant stress.
   - Strain related ↑ in chronic myocardial injury and fibrotic remodeling

CONCLUSIONS

Individuals with preexisting CVD are at greater risk of suffering from adverse lung and heart effects of oxidant and ultrafine particulate pollution supporting the notion that they should be considered a susceptible population in the standard setting process.

Collaborations and Funding

Schelegle Laboratory

- Dr. Emily Wong
- William Walby

- Dr. Fern Tablin, UCD SVM
- Dr. Dennis Wilson, UCD SVM
- Dr. Ronald Lee, UCD SVM
- Dr. Joshua Stern, UCD SVM
- Dr. Laura Van Winkle, CHE and UCD SVM
- Dr. Rachel Reader, CNPRC and UCD SVM

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Agreement No:
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QUESTIONS?