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Gestational Vulnerability to Ozone Air Pollution - A Placental Story

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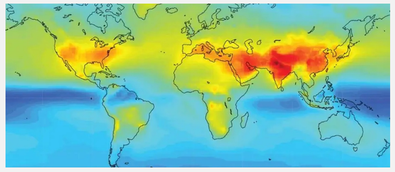
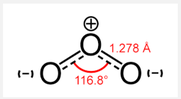
Introduction

- Ambient air pollution accounts for an estimated **4.2 million premature deaths** annually worldwide per the World Health Organization
- **About 99%** of the global population inhales air below World Health Organization standards
- Ozone forms reactive oxygen species (ROS) that may lead to **cellular damage and organ system dysfunctions**
- Ozone exposure may increase **dangerous neonatal health outcomes**
- Literature shows ROS in bloodstream may lead to **fetal vulnerability during pregnancy via the placenta.**
- Placental and fetal development **changes in susceptibility over time** and may lead to negative gestational outcomes such as **pre-eclampsia**.
- **Different layers of placenta** could reveal fetal/maternal origin for responses





Methods

- Pregnant Sprague-Dawley rats were exposed once to **0.3 ppm of ozone or filtered air** via whole-body inhalation (using OREC ozone generator) at gestational day 10 (GD10) or GD20 for 4 hours while control received a sham filtered air exposure at both time points. At term, GD21, amniotic fluid and tissues were collected for further analysis.
- **Amniotic fluid proteomics** was conducted to determine differences in the amniotic fluid to identify potential protein biomarkers associated with fetal development and other relevant features.
- **Optimization of primary antibodies** was conducted by creating 3 wells of two sample placental tissues per slide that are prepared and marked with primary then secondary antibodies specific to the target protein of interest - each well contains a different dilution of antibody that will yield different fluorescence under the microscope. Signal to noise ratios were calculated and obtained for all new antibodies.
- **Initial trial for antibody staining:** Placental tissues were 4% PFA fixed and blocked in goat blocking buffer. CD80 (Biolegend 600053), CD206 (Santa Cruz SC-376323), Connective Tissue Growth Factor (CTGF-Abclonal A11456) and other primary antibodies were matched with Alexaflour and FITC conjugated secondary antibodies and images were collected at 20x on a Zeiss Axio Imager .M2 using Zen software.

Results

Protein	GD10 0.3PPM	q value GD10 0.3PPM	GD20 0.3PPM	q Value GD20 0.3PPM
	Fold Change ±SEM		Fold Change ±SEM	
Osteopontin	+0.65 ±0.1999	0.0331	-1.70 ±0.1849	0.0448
Galectin-1	+3.78 ±0.4450	0.0025	No change	
MMP-2	No Change		-0.88 ±0.3036	0.0269
TIMP-1	+0.83 ±0.1894	0.0309	No change	
TIMP-2	+0.93 ±0.1179	0.0024	No change	
E-cadherin	+0.69 ±0.1722	0.0082	No change	
Pkm1	+0.61 ±0.1831	0.0314	-1.59 ±0.3101	0.0049
Pkm2	No change		-4.09 ±0.4546	0.0004
Superoxide Dismutase 1	No change		-0.63 ±0.2808	0.0492
Catalase	No change		-3.07 ±0.6915	0.0141
Collagen, Type I, Alpha 2	+0.60 ±0.2313	0.0148	-1.07 ±0.2851	0.0307
Vascular Cell Adhesion Molecule	No change		-2.09 ±0.1579	0.0128
Tissue Factor Pathway Inhibitor	-1.39 ±0.5199	0.0314	-2.22 ±0.5297	0.0025
Connective Tissue Growth Factor	+0.65 ±0.2296	0.0314	No change	
Sex Hormone Binding Globulin	No Change		+2.67 ±0.2516	1.28 E-05
Rack1	No Change		+4.30 ±0.2715	7.68 E-08

Table 1: Proteomic Targets of Focus: Significant fold changes within the amniotic fluid proteome, correcting for multiple measures to an FDR of 5%, were filtered to focus on proteins with larger fold change and a known role in vascular function and remodeling or antioxidant activity. From this table, targets **(SOD1, VCAM, and CTGF) were selected for future follow-up studies to investigate placental tissue as a potential source**.

A

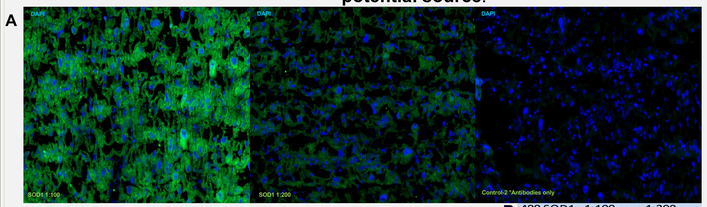


Figure A-B: **Results from SOD 1 Antibody Optimization:** Images show SOD1 and DAPI staining with a stronger signal for SOD1 at 1:100 than 1:200 or the control group with secondaries only. Signal to noise calculations were obtained to confirm correct dilution for antibodies, which confirmed SOD1 was optimized at a dilution of 1:100. This was repeated for all antibodies in our study.

B

488 SOD1	1:100	1:200
Noise 1	1694.89	921.04
Noise 2	2124.151	1660.94
Noise 3	2593.625	1229.22
average	2137.555	1270.4
Signal 1	6369.54	2449.41
Signal 2	6074.65	3044.4
Signal 3	7309.126	2516.12
average	6584.439	2669.977
sig:nois	3.08	2.101682

C

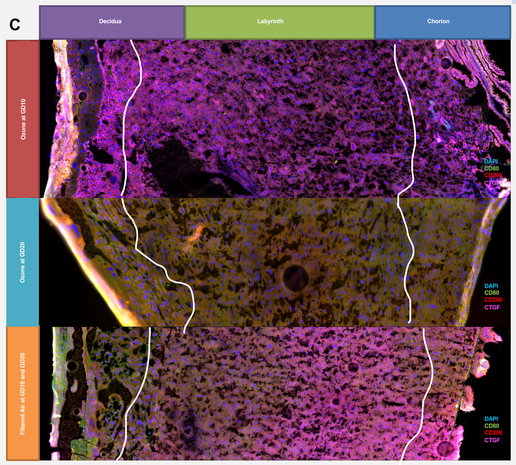


Figure C: Experimental Antibody Staining Trial: Antibody staining set 1 was conducted across ozone and filtered air experimental groups. Images show an example of a well co-stained with **DAPI, CD80, CD206, and CTGF**. In **GD10** there was an **apparent increase** in **CTGF** along with stronger protein expression in the maternal decidual layer. In **GD20** there was an apparent **decrease** in **CTGF** and increase in **CD80** along with strong expression in the maternal decidual layer. Follow-up studies will be conducted to replicate antibody staining with additional analysis across placenta lamina.

Discussion & Future Directions

- GD10 ozone exposure led to an increased influence on **growth factors**, such as **CTGF**, and **extracellular matrix factors** such as vascular cell adhesion molecule 1 **(VCAM-1)** and vascular endothelial cadherin **(VE-cadherin)** that are also indicated in studies involving pre-eclampsia. GD20 ozone exposure led to an increase in **antioxidative** factors such as **SOD-1** and **catalase.** This provided grounds for targeted histological analysis.
- Antibody optimization was successful in providing proof that SOD-1 had better signal to noise at a dilution of 1:100 than 1:200 providing a much better image. Optimizations were repeated across multiple antibodies to ensure best image will be produced under the microscope.
- CTGF was elevated in the GD10 ozone exposed group and is a well-known indicator of pre-eclampsia having implications in the stimulation of proliferation, angiogenesis, migration, ECM production, cell attachment, and cell survival.
- M1 and M2 macrophages were also tested due to their relevance after oxidative stress. CD80, an M1 macrophage known to exhibit pro-inflammatory qualities was elevated in the maternal decidua in both GD10 and GD20 groups compared to the filtered air group. CD206, an M2 macrophage, which has anti-inflammatory properties was elevated in the GD20 timepoint but was lower in the GD10 timepoint. **Future studies aim to replicate antibody staining across an n=5.**
- Future studies will involve continuing to co-stain and co-image placental tissues to obtain an n=5. Additional quantification of mean fluorescence intensity will be tabulated across decidual, labyrinth, and chorionic placenta lamina and results will be assessed using ANOVA with post-hoc testing for group differences. Expected outcomes will demonstrate the relationship between prior amniotic fluid proteomic findings and effects within the placenta as a potential source of changes to investigate time-dependent gestational effects on placental vulnerability. Outcomes will demonstrate the **relationship between amniotic fluid proteomic findings and fetal/maternal response** as viewed through the placenta

Section A (Prepared in Goat Block)				
Channel	**Primary Antibody**	**Company**	**Lot number**	**Host Species/Isotype**	**Dilution**
488g	Superoxide Dismutase 1	Abclonal	A0274	Rb	1:100
Section B (Prepared in Goat Block)				
Channel	**Primary Antibody**	**Company**	**Lot number**	**Host Species/Isotype**	**Dilution**
488g	E-Cadherin	DSHB	rr1-s	Ms-IgG1	1:10
568r	VCAM	DSHB	P8B1-s	Ms-IgG2b	1:15
680l	VE-cadherin	Abclonal	A12416	Rb	1:50
Section C (Prepared in Goat Block)				
Channel	**Primary Antibody**	**Company**	**Lot number**	**Host Species/Isotype**	**Dilution**
488g	CD80 (M1)	Biolegend	600053	Rat-IgG2a	1:100
568r	CD206 (M2)	Santa Cruz	sc-376232	Ms-IgG2a	1:50
680l	CTGF-Connective Tissue Growth Factor	Abclonal	A11456	Rb	1:100

Acknowledgements

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