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The Potential Role of Oxidative Stress in Reduced Uterine Perfusion Pressure Vascular Reactivity

Abigail J. Gindlesberger
ajg96@zips.uakron.edu

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Abigail Gindlesberger

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Abstract

Preeclampsia is a hypertensive disease of pregnant women that is known to cause detrimental physiological complications to both the mother and fetus. The hypertension hypothesized to result from endothelial dysfunction may be improved therapeutically by increasing Vascular Endothelial Growth Factor ligand concentration and it's Vascular Endothelial Growth Factor Receptor 2 (VEGF and VEGFR2). Together, this receptor pathway may help overall vasodilation of key blood vessels linking the mother and fetal placental unit, allowing for increased clinical pathologies of both. Previous studies have also linked increased reactive oxygen species (ROS) production in preeclamptic pregnancies to the damaged endothelial tissues of the placenta. This specific investigation will use RUPP model to measure the effect of increased VEGF and VEGFR2 expression on ROS production in placental tissues, measured using lucigenin-enhanced chemiluminescence (LECL). These results may help further characterize ROS role in hypertensive pregnancies physiological demise, and help indicate whether VEGFR may serve as a potential therapeutic in treating preeclampsia.

Introduction

The hypertensive disease of pregnancy known as preeclampsia is responsible for morbidity, perinatal death and preterm deliveries worldwide (Uzan et. al, 2011). The clinical signature of preeclampsia includes edema, renal dysfunction, and fetal growth restriction (Facemire, 2009). It has been hypothesized that the observed hypertension results ultimately from endothelial cell dysfunction. This dysfunction induces damaging vascular changes such as decreased vasodilation, increased vasoconstriction and

vasospasms (Crews et. al, 2000). Decreased blood flow to the fetal placental unit arises from the renal and cardiovascular changes known to be associated with the disease. Vascular dysfunction and nutrient deficiencies often occur from the positive feedback loop (Maynard, 2008). Oxidative stress may also arise as a result of any of the observed defects.

VEGF and VEGFR2 are thought to play a significant role in vascular homeostasis. As a vasodilator, the ligand VEGF binds VEGFR2 and potentially activates endothelial nitric oxide synthase (NOS) (Crews et. al, 2000). Together, the two perform significant roles in uterine remodeling, vasodilation and placental development (Facemire, 2009).

In this study, the surgical Reduced Uterine Perfusion Pressure (RUPP) pregnant rat model is used because of its similarities in utero-placental under-perfusion of preeclampsia in humans. The RUPP model has replicated the maternal hypertension, vascular dysfunction and fetal demise commonly observed in the preeclamptic pathology of humans in a previous investigation (Reho et. al, 2011). After two weeks of gestation, a surgical procedure is performed to diminish uterine blood flow approximately 40%. Specifically, small clips are clamped around the abdominal aorta and the uterine-ovarian arteries (Crews et. al, 2000). This decreased blood flow causes the decreased uterine perfusion, and therefore deprives the fetus of a sufficient blood volume. This cascade adds to the maternal hypertension, as the mother's physiology must then work harder to provide the fetal-placenta with sufficient blood, oxygen and nutrients (Crews et. al, 2000).

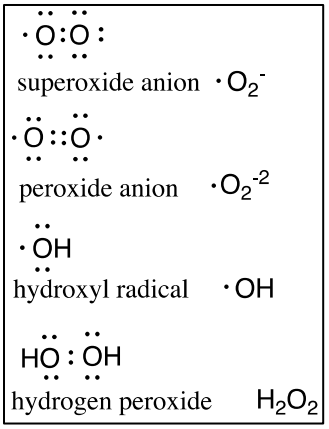

Figure 1: Example reactive oxygen species. Created using ChemDraw Professional, Version 16.0, PerkinElmer Informatics.

Oxidative stress has previously been investigated for its role in the pathophysiology of preeclampsia. Production of free radicals could impact pregnancy by altering the normal growth and function of the placental unit (Burton, 2009). Free oxygen radicals have the potential to oxidize fatty acids in lipids and amino acids in proteins, damaging cells and causing overall organ dysfunction (Sies, 1985). Potential cell ROS are seen in Figure 1. Increased oxidative stress in hypertensive pregnancies is thought to play a major hand in the initiation of degenerative preeclamptic physiology because of the ROS damage to the endothelium of placenta (Draganovic, 2016).

Dr. Ramirez's lab has demonstrated that vasoconstriction occurred as a result of increased oxidative stress in RUPP models while investigating the effects of ascorbic acid, a compound with antioxidant properties (Ramirez et. al, 2011). The aim of the overall project is to examine whether overexpression of VEGFR2 improves the utero-placental blood flow and the maternal and fetal clinical pathologies. The experimental schematic can be seen in Figure 2. Placental tissues have been suggested in a previous study to contribute to the overproduction VEGFR; therefore contributing to the overall excessive vascular permeability observed in preeclamptic physiology (Grummer, 2009). Decreased perfusion via altered vascular reactivity may confer placental oxidative stress in the RUPP model. The specific aim of this branch of the investigation is analyzing ROS production in RUPP model placentas with and without increased VEGFR2 expression.

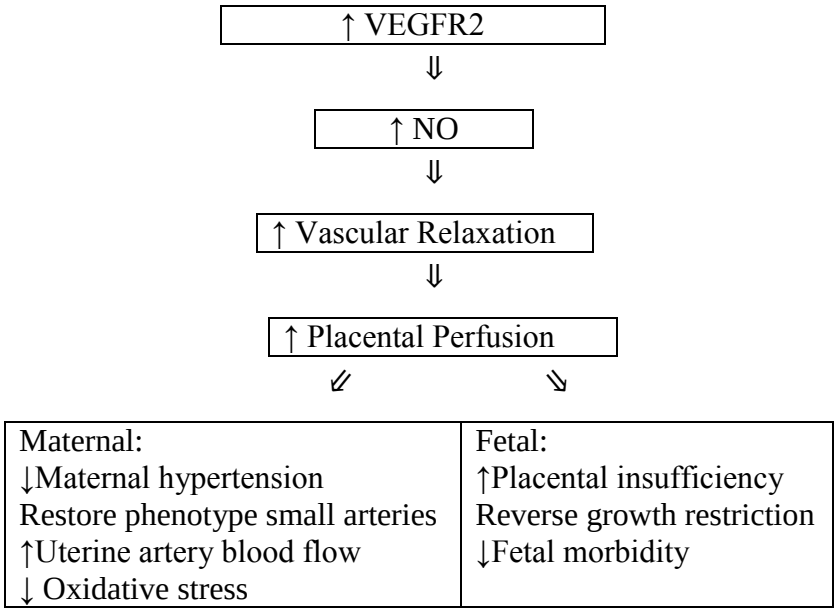

Figure 2: The approach to investigate the mechanism of VEGF signaling by selectively overexpressing VEGFR2 in the treated RUPP model.

ROS production will be used as an indicator of overall pathology. A decrease may be linked to clinical improvement or impairment of the receptor expression. To measure the levels of oxidative stress in the rat's placental tissues, a luminometer will be used. This tool will measure the emission of light from the chemical reaction between lucigenin (bis-*N*-methylacridinium nitrate) and superoxides present in the placental homogenate. LECL serves as one of the most convenient and sensitive approaches for ROS detection (Yamazaki, 2011). Using this method, superoxides can be quantitatively monitored by the luminometer by collecting reactive light units (RLU) produced by the reaction of superoxide with lucigenin (Yamazaki, 2011). A detailed depiction of the chemical reaction of lucigenin with a superoxide is seen in Figure 3.

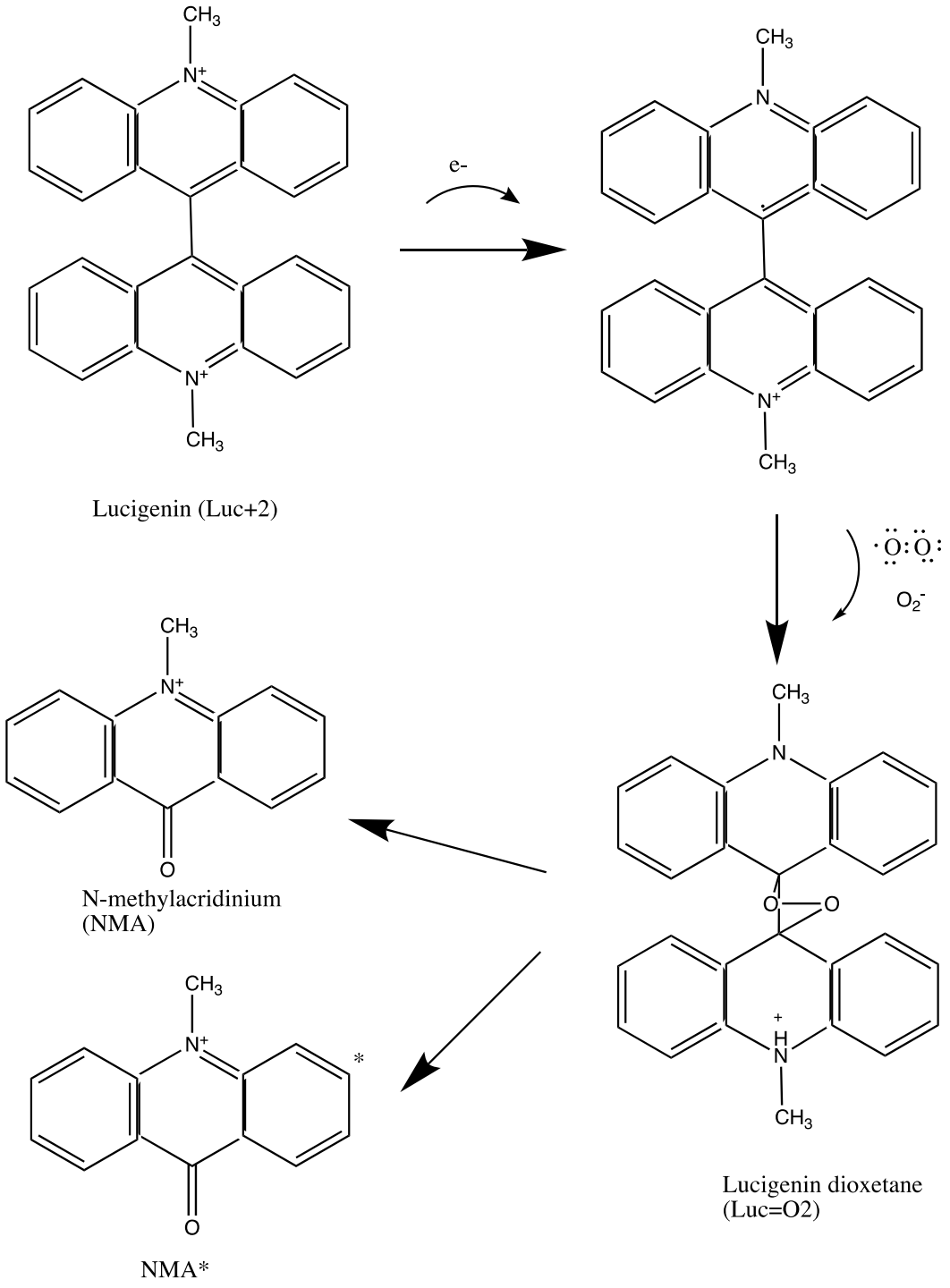

Figure 3: Hypothesized reactions undergone in luminometer between lucigenin and a superoxide in order to generate chemiluminescence (Lu, 2006). NMA* = NMA + *photon*. Created using ChemDraw Professional, Version 16.0, PerkinElmer Informatics.

Upon interaction with the superoxide, the lucigenin will undergo a single electron reduction, forming a cation radical. The radical quickly binds to the superoxide, yielding lucigenin dioxetane as a brief intermediate. Lucigenin dioxetane undergoes an internal rearrangement, creating a N-methylacridone (NMA) molecule, and a NMA radical ion (NMA*) (Lu, 2006). Chemiluminescence occurs as a result of NMA* relaxing from its excited state, returning to the ground state where it emits light at 470 nm. This relaxation is then detected and interpreted by the luminometer as RLU's (Vásquez-Vivar, 1997).

A baseline with no homogenate sample will be run for control, and a superoxide baseline with lucigenin and a homogenate sample is run for comparison to the treated groups, treated with NADPH oxidase and tiron respectively.

NADPH oxidase is a flavoprotein that enzymatically facilitates the single electron reduction of oxygen while using NADPH is oxidized to complete the redox reaction. The end product is a superoxide anion. The NADPH oxidase has previously been suggested as a major source of cellular superoxides in rabbit vascular tissues (Vásquez-Vivar, 1997). Therefore, we would expect the NADPH treated sample to cause a spike in superoxides, and therefore increased RLU's collected by the luminometer. Tiron, who's chemical structure can be seen in Figure 4, has a radical scavenging mechanism that is hypothesized to occur by ROS via direct oxidation (Taiwo, 2008). Upon oxidation, the reduced Tiron molecule will create a hydroquinone.

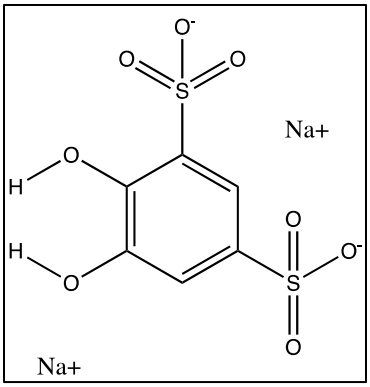

Figure 4: Chemical structure of Tiron, a superoxide radical scavenger. Created using ChemDraw Professional, Version 16.0, PerkinElmer Informatics.

Therefore, we expect a decrease in lucigenin chemiluminescence in the Tiron-treated sample (Münzel, 1995). Three different experimental groups were proposed to be analyzed using luminometer technology- SHAM, RUPP and RUPP treated with the nanoparticles. We hypothesize that overexpression of VEGFR2 will increase blood flow in the maternal-placental unit, and thus lower oxidative stress. If this holds true, then we should see decreased oxidative stress signals in our data collected from the treated RUPP model (Grummer, 2009).

Materials and Methods

Animal Maintenance

All animal studies were approved by IACUC at the University of Akron .The rats used in the investigation were handled and housed in the vivarium of the University of Akron. Two lab technicians and one trained medical veterinarian handled the animals on a daily basis. The animals were observed for illness and any adverse effects from experimental treatments. Records of their health were logged twice a week. Medical documentation was updated and available on each of the rat's cage card when in the lab. Standard Operating Procedures from the IACUC were followed in all aspects.

Buffer Preparation

1 L of 10X HEPES buffer was first prepared using 83 g of NaCl, 3.50 g of KCl, 1.60 g of KH_2PO_4, 2.88 g of $MgSO_4*7H_2O$, 3.68 g of $CaCl_2*2H_2O$, and 23.83 g of HEPES. The buffer was brought to pH 7.37 by using 1 M NaOH. 10X HEPES was then diluted with DI water tenfold to make 1X HEPES followed by the addition of 0.5 g of

glucose and the pH was checked again. Before incorporation into lucigenin assay samples, 1X HEPES was heated for ten minutes at 37 °C, re-evaluated and the pH was adjusted to 7.37 again to ensure it as optimal physiological comparison. The pH was measured over the course of experiments to ensure it remained stable. 10X HEPES was stored for several weeks, however 1X HEPES was freshly prepared before each use.

Preparation of protein samples

Placental tissues were isolated from frozen rat placenta collected six-ten months prior to experimentation. Each sample was homogenized prior to the measurement of protein concentration by Bradford Assay. The preparation entailed collecting ~0.3 g tissue and adding 1X HEPES buffer (pH ~7.37) at 0.1 g/mL. An example can be seen in Sample Calculation 1.

$$tissue\ weight = 0.327\ g$$

$$\frac{0.327\ g}{0.1} = 3.27\ mL$$

$$3.27\ mL - \ 0.327\ mL = 2.943\ mL\ 1X\ HEPES\ buffer\ needed$$

Sample Calculation 1: Above is an example of the equations used to find the appropriate buffer volume needed to make the homogenate.

The protein solution was kept on ice for the remainder of preparation. Approximately 50 μL of protease inhibitor (100x) was added before homogenizing until smooth liquid was observed. Homogenate was then spun in an IEC Centra CL2 centrifuge at 3000 RPM for six-eight minutes. The protein supernatant was collected and

the cell pellet was disposed. Protein was stored at -80 °C until assayed. SHAM placenta was used to create the first set of data. RUPP placenta without treatment of VEGF was to be used the second time, while RUPP placenta treated with VEGF was to be used for the final data set.

Measurement of Protein Concentration in Placental Homogenate Samples

A standard Bradford assay purchased from Sigma was run each day tissues were assayed to generate a standard curve. This curve was used to find the protein concentration in each of the homogenate samples analyzed based on their recorded absorbance. The amount of protein present in each sample is relative to the amount of RLU's measured using the lucigenin assay. Six standard samples were prepared, with a total volume of 3000 μL, and measured in a Spectrometer at 595 nm.

The first sample contained (0 μL BSA + 2400 μL DI water + 600 μL Bradford Reagent). The second sample contained (3.9 μL BSA + 2397 μL DI water + 600 μL Bradford Reagent). The third sample contained (7.5 μL BSA + 2391μL DI water + 600 μL Bradford Reagent). The fourth sample contained (15 μL BSA + 2385 μL DI water + 600 μL Bradford Reagent). The fifth sample contained (22.5 μL BSA + 2376 μL DI water + 600 μL Bradford Reagent). The sixth sample contained (30 μL BSA + 2370 μL DI water + 600 μL Bradford Reagent). Four placental samples were prepared with (200 μL HMG + 1800 μL DI + 600 μL Bradford) and measured as well. Their measurements can be seen in Table 1. The absorbance measurements from four representative protein samples were averaged and plotted on the standard curve, seen in Figure 5. R^2 values were obtained to ensure standards accuracy.

Measurement of ROS Production

The lucigenin assay was used to measure chemiluminesence exhibited by superoxides formed via NADPH oxidase, and to confirm specificity by the ROS scavenger, Tiron. The protocol for the Turner Biosystems luminometer called for the use of lucigenin at 100 μmol/L. Once properly diluted, the lucigenin stock was covered in foil when being stored in the freezer, and was carefully added to samples in a dark room to prevent interaction with light waves. Once in the samples, each bullet tube that contained lucigenin was covered in foil in order to prevent potential light wave interaction. Before use, the pH of 1X HEPES buffer was adjusted to 7.37, incubated at 37 °C for ten minutes, then re-examined to ensure proper pH prior to the beginning of the analysis. A baseline sample was prepared with (400 μL 1X HEPES buffer + 0.6 μL lucigenin) before being placed in luminometer. Fifteen RLU collections were taken. Ideally, this should reflect the lowest value because no homogenate or protein was present in the sample.

A superoxide baseline sample was prepared with (100 μL HMG + 400 μL 1X HEPES buffer + 0.6 μL lucigenin). The sample was incubated in the water bath for three minutes at 37 °C. A stimulated superoxide sample was prepared using 10mM NADPH (100 μL HMG + 400 μL 1X HEPES buffer + 0.6 μL lucigenin + 10 μL of 10 mM NADPH), and incubated in a water bath at 37 °C for three minutes. The addition of NADPH was to produce ROS in an NADPH oxidase-dependent manor, causing a spike in the signal detected in this sample. After preparation, the bullet tube was placed in the luminometer and fifteen RLU collections were taken.

A final sample was prepared with 100 μM/L Tiron (100 μL HMG + 400 μL 1X HEPES buffer + 0.6μL lucigenin + 50 μL Tiron) and incubated in a water bath at 37 °C

for three minutes. After incubation, the bullet tube was placed in the luminometer and fifteen RLU's were collected.

The parameters of the luminometer were preset to the lucigenin protocol. Reactive light unit (RLU) measurements for each sample were taken constantly every five seconds until fifteen measurements were recorded.

Statistics

In order to normalize the data, the RLU's were to be divided by the concentration of protein found in the homogenate sample used during that particular trial. This data could then be used in a T-test and ANOVA to compare ROS measurements between experimental groups.

Results and Discussion

In order to standardize our luminometer measurements, we first normalized our protein concentrations for each of our placental samples. The measurements can be seen in Table 1.

Table 1: Absorbance values for standards used in the Bradford assay.

Absorbance's Collected to Generate Standard Curve		
µL in Assay	**µg in assay**	**Abs (595nm)**
Standard Samples		
3000	0	0
3000	390	0.046
3000	750	0.09
3000	1500	0.148
3000	2250	0.217
3000	3000	0.261
Unknown Samples		
2600	61.44	0.566
2600	56.78	0.524
2600	61.00	0.562
2600	61.33	0.565
Average protein concentration	60.14	

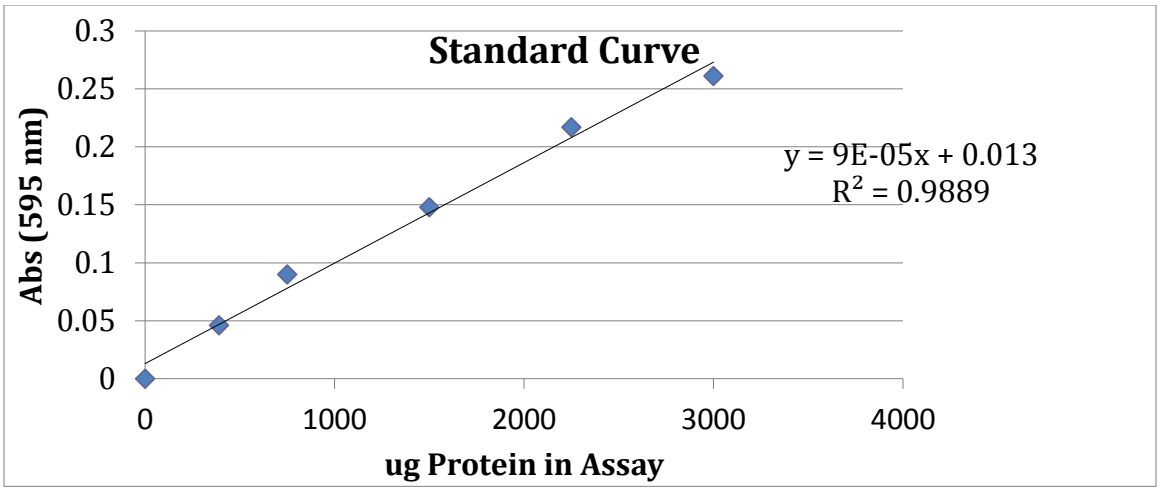

Figure 5: Standard curve developed using six standards and the Bradford Assay protocol. Absorbances were recorded at 595 nm, and the linear fit equation generated was used to find the protein concentrations of the four homogenate tissue samples. The R^2 value indicates a relatively linear fit, making it a suitable equation to find protein concentrations of unknown samples.

The standard curve was corrected with an absorbance reading of 0 for a protein concentration of 0. The linear fit equation $y = 9 * 10^{-5}x + 0.013$ (y = mx + b) was used to find the unknown samples protein concentrations. The absorbances at 595 nm were plugged in for y, and solved for x in order to find protein concentration in the sample. The corresponding arithmetic can be seen in Sample Calculation 2. Four homogenate samples were made so their protein concentrations could be averaged, yielding an average protein concentration of 60.14 μg/μL.

$$y = 9 * 10^{-5}x + 0.013$$

$$0.566 = 9 * 10^{-5}x + 0.013$$

$$x = 6144.44\ ug/mL$$

$$\frac{6144.44\ ug}{mL} * 10 = \frac{61444.4\ ug}{mL} = 61.44\ ug/uL$$

Sample Calculation 2: Linear equation used to find protein concentrations of unknown placental tissues, created by the standard curve seen in Figure 5. Because the homogenate samples were diluted 10 fold, this had to be factored into the final protein calculation.

Once the protein concentrations were found, the homogenate samples were taken to the luminometer to measure RLUs. Fifteen data points were collected for each of the four experimental conditions, using two different protein samples. The normalized data is shown in Figure 6.

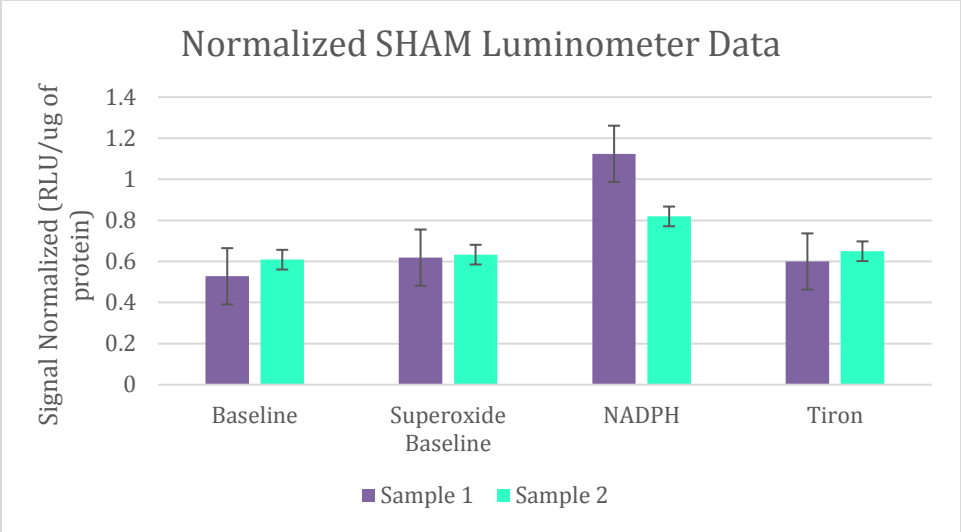

Figure 6: Measurement of superoxide levels in placental tissue isolated from SHAM animals. Data was normalized by dividing RLU signal strength by concentration of protein found within the sample. The baseline was developed using a chemiluminesence assay and compared to a positive control (superoxide baseline). The NADPH-treated samples were stimulated to produce ROS and spike RLU production, while the Tiron-treated samples were meant to scavenge the superoxide anions, decreasing RLU signal.

At this point, only SHAM placental tissues were tested and not the VEGF experimental groups. Each of the two samples tested in the luminometer were collected from SHAM placental tissues. The fifteen replicate measurements for each condition were averaged and later normalized by dividing them by the protein concentration found in that particular sample. This allowed for the comparison of both SHAM samples, despite their differences in protein concentration. The baseline sample was meant to act as a control to compare to the superoxide baseline that contained the homogenate sample. The baseline should have the lowest lucigenin stimulation because it contained no homogenate, and therefore we would expect the superoxide baseline to have higher RLU signal. Although the sample 1 superoxide baseline appeared to have a higher signal than

the baseline, the error bars demonstrate there was no significant difference between the superoxide baseline and lucigenin baseline. Both NADPH-stimulated samples showed an increase in RLU production, as predicted. Similarly, the data reflect that the Tiron did act as a superoxide scavenger, seeking out ROS and preventing the lucigenin from interacting with the free electrons. This led to an overall decrease in RLU production in comparison to the NADPH treated samples**.**

Conclusion

The detrimental capability of oxidative stress in hypertensive pregnancies has been an important topic of study to medical professionals worldwide (Draganovic, 2016). This investigation was meant to test the oxidative stress levels between three experimental groups- SHAM, RUPP, and RUPP models with increased VEGFR2 expression. Both RUPP models represented preeclamptic pathology (Reho et al., 2011). RUPP treated with LTP nanoparticles were hypothesized to increase VEGFR2 expression. The SHAM models were used as a control to compare ROS levels of normal pregnant rats with those in a hypertensive state (Reho et al., 2011).

We would predict that the RUPP models would exhibit higher levels of oxidative stress due to the decreased uterine perfusion, vasoconstriction and hypertensive physiology when compared to the untreated SHAM models (Ramirez, 2015). Treatment of LTP nanoparticles was meant to increase VEGFR2 expression. Decreased VEGFR2 expression is linked to preeclampsia pathology (Grummer, 2009). Treatment of rat's experimental preeclampsia is predicted to increase VEGF signaling and enhance the

overall uterine perfusion. We would expect to see a decrease in oxidative stress when analyzing these tissues samples (Grummer, 2009).

During the time of writing, the experiment was still continuing. Only data collected from the SHAM group was recorded to this point, and some difficulties were experienced in the process. Only two protein samples produced adequate RLU measurements, because for all other samples the baselines did not generate accurate measurements. Without proper baseline measurements, no valid comparisons could be made to the experimental samples. Several obstacles prevented the collection of multiple adequate data sets, including the failure to ensure proper pH before testing for ROS by the luminometer. Improper pH may have denatured the protein samples before processing in the luminometer. In an attempt to correct this problem, all homogenate samples and buffers were re-made, ensuring proper pH every step of the way before testing the entire experimental sample. Unfortunately, this did not seem to correct the inaccurate baseline readings obtained. A new lucigenin sample was ordered and the stock was re-made to correct any discrepancies that may have come from it, as it is a very light-sensitive chemical and could have been another cause of the inaccurate baselines. Even with fresh lucigenin reagent, RLU readings were still not as predicted. In addition, the Turner Biosystems luminometer may have been responsible for inaccurate RLU measurements, as it had not been calibrated since our use in the experiment.

Although there were two SHAM homogenate samples that aligned with what was predicted to happen, there was not enough significant evidence to support our hypothesis at this time. More SHAM samples should be analyzed, and the other experimental groups should be tested as well to draw accurate conclusions from the total data. Further

investigation the cross-talk between VEGF signaling and ROS production could help find potential pharmaceuticals and therapeutics to combat preeclampsia, bettering the lives of the mother and the fetus.

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Safety Appendix

Dr. Ramirez and his graduate students taught safety training. The use of Personal Protective Equipment was used to minimize risk of injury while dealing with chemicals. Lab gloves and safety glasses also reduced risk of incidence. Appropriate LUC-0-ING protocol was followed when using the luminometer in reference to the Turner Biosystems manual. Salts for making HEPES buffer were handled with care, and cautious handling of the 1 M NaOH was used when pHing the buffer. pH waste was dumped in a specified container and excess buffer was diluted and disposed of down the drain. No injuries or major spills occurred in the laboratory.