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Cardiovascular Tissue Remodeling of Preeclampsia

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Honors Research Project

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Abstract

Preeclampsia is a hypertensive disorder of pregnancy that leads to a multitude of problems for the mother and fetus during and after pregnancy. While the effects on the mother's body after pregnancy are often overlooked, preeclamptic mothers are prone to cardiovascular diseases from the great deal of stress experienced through the duration of a preeclamptic pregnancy. The associated high blood pressure causes cardiac muscle to work a great deal harder in an attempt to successfully perfuse tissues adequately. This increase in prolonged stress leads to remodeling of the cardiovascular tissues-significantly increasing the risk of heart issues in life beyond the term of the preeclamptic pregnancy. Efforts to improve cardiac oxidative stress via Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) treatment are analyzed in this study.

Introduction

Preeclampsia is a hypertensive disease of pregnancy that occurs in 3 to 5 % of pregnant women worldwide (Young, Levine, & Karumanchi, 2010). Preeclampsia is the leading cause of maternal and neonatal morbidity and mortality --deeming it a vitally important disease to study. The disease is characterized by onset hypertension including (systolic blood pressure greater than or equal to 140 mm Hg or diastolic blood pressure greater than or equal to 90 mm Hg) and proteinuria (300 mg or greater in a 24-hour urine specimen) (Young, Levine, & Karumanchi, 2010).

This disease leads to a multitude of problems for the mother and fetus throughout the pregnancy. The mother’s body experiences pathology extending greater than the duration of the pregnancy. While the effects on the mother’s body after pregnancy are often overlooked, preeclamptic mothers are prone to cardiovascular diseases. The tissues of the body, especially

cardiovascular tissues, experience a great deal of stress through the duration of a preeclamptic pregnancy. The associated high blood pressure causes cardiac muscle to work a great deal harder in an attempt to successfully perfuse tissues adequately. This increase of prolonged stress creates a remodeling of the cardiovascular tissues—significantly increasing the risk of heart issues in life beyond the term of the preeclamptic pregnancy.

To efficiently study preeclampsia pathology, the animal model of a rat is used. Rats are excellent models of the human pregnancy due to the hemochorial placentation. Maternal rats show paralleling adaptation to pregnancy in renal, cardiovascular, and endocrine systems that closely models human gestation. Rats are advantageous due to their short gestational periods, inexpensive cost and accessibility.

Reduced Uterine Perfusion Pressure (RUPP) model includes a surgically placed silver clip around the abdominal aorta and the uterine-ovarian arteries of pregnant rats on day 14 of gestation. This procedure results in a 40% reduction in uterine blood flow and the rats experience similar pathology parameters associated with preeclampsia.

Evidence of maternal tissue remodeling is observed with preeclamptic pregnancies and can be studied further with RUPP model rats. This remodeling of the cardiovascular tissue, specifically remodeling of the left ventricular, is characterized by increased collagen deposition. This increased collagen deposition will lead to an increasing wall thickness and mass of the left ventricle of the heart. The mitral valve allows blood to flow properly from the left atrium to the left ventricle. With this increased tissue mass and thickness, a mitral valve prolapse is likely to occur (Glesby, 1989). The leaflets bulge causing improper closing and leading to regurgitation (Kolibash, et al., 2004).

Regurgitation and back low leads to a lowered cardiac output. Blood that is supposed to be moving out of the heart and through circulation is now flowing back into the heart. Inadequate perfusion of tissues will occur, leading to a positive feedback loop of increasing the workload on the heart in an attempt to resolve the lowered blood supply. This remodeling occurring in women who have had preeclamptic pregnancies can potentially lead to compromised cardiac health.

Our lab has been studying the possibility that optimization of the VEGF signaling pathway via nanoparticle injection with plasmid for VEGF receptor 2 may be a potential treatment for the vasoconstriction and hypertension associated with RUPP. We posit that VEGFR2 treatment will increase the release of nitric oxide—a vasodilator. This in turn, will aid in vasodilation of the blood vessels and allow an increase in blood flow to uterine tissues, thereby normalizing mean arterial pressure. If blood pressures can return to a lowered "normal" state and overall cardiac output can increase, the negative effects of cardiovascular tissue remodeling can be reduced. Creating a way to return to homeostatic range in terms of blood flow through the heart and tissues, can improve overall cardiac health. This should correlate to females who have had preeclamptic experiences.

Very recent work by our collaborator Rouzbeh Amini suggests that pregnancy produces significant alterations to collagen structure leading deformation of the mitral valve (Pierlot, Lee, Amini, Sacks, & Wells, 2014). The increased tissue thickening of the mitral valve leads to improper closing and regurgitation (Kolibash, et al., 2004). Our data plus Amini's would suggest that during pregnancy a proper change in protein expression must occur in order for the maternal heart to function properly. The inefficient function furthers the positive feedback of improper valve function and low cardiac output leading to increased vasoconstriction and tissue stress. This protein expression may be compromised in pathological hypertensive pregnancies

associated with altered vascular remodeling such as preeclampsia. Finally, the detrimental changes may be normalized with our experimental increase in VEGFR2 signaling.

Increasing the functioning amount of VEGF receptor 2 to initiate a vasodilatory state could reduce the hypertensive and oxidative stress of the heart tissue. We will address the impact of RUPP rat pregnancy on cardiac tissue oxidative stress with and without VEGFR2 nanoparticle treatment. We hypothesize that hearts from untreated pregnant RUPP dams will show indices of increased oxidative stress, indicative of detrimentally altered cardiac function and perfusion.

Materials and Methods

Animal Maintenance:

Timed Female Sprague- Dawley Rats were obtained for the lab at 10-12 weeks old and are purchased from Hilltop Lab Animal. These rats were housed at The University of Akron Main Campus vivarium. The rats were spilt into two groups: RUPP (experimental) and SHAM (control). On day 14, rats underwent surgery of either RUPP or SHAM depending on their group placement. The rats were euthanized on gestational day 22 and tissue samples were collected to be analyzed. Samples not immediately studied were flash frozen and stored at -80℃.

VEGFR2 Nanoparticle Treatment:

Previous studies using LTP-pDNA nanoparticles have provided a successful, nonviral alternative delivery of therapeutic genes in vivo to treat diseases (Ditto, et al., 2013). These nanoparticles are nontoxic and do not illicit an immune response. We hypothesize that treatment with LTP nanoparticles encapsulated DNA encoding for VEGFR2 is a viable method to

upregulate available VEGF receptors; therefore, improving vasodilatory capacity of tissue through nitric oxide production.

Measurement of Oxidative Stress in Tissue Samples:

Obtaining quantitative oxidative stress results in tissues allows us to determine the extent of tissue damage caused by RUPP animals—modeling preeclampsia. Increased tissue damage can lead to heighten tissue remodeling including the tissue of interest—the mitral valve. Tissue repair is a process involving inflammation, proliferation, and remodeling of tissue (Piantoni et al., 2010). The body's heightened inflammation response to oxidative stress increases remodeling of such tissues to neutralize the damage and get back to homeostasis.

Reactive oxygen species (ROS) created by tissue stress and damage have an extremely short-half life; therefore, making it incredibly challenging to measure directly (Dhalla, Hill, & Singal, 1996). However, the byproducts of the damage reactions caused by oxidative stress are maintained in tissue levels for extended durations. This extended timeframe makes the byproducts ideal for measuring oxidative stress that the tissues have undergone.

The oxidative stress tests performed are 8-Isoprostane Elisa Kit (Cayman Chemical Item No. 516351), TBARS Assay Kit (Cayman Chemical Item No. 10009055), and Superoxide Dismutase Assay Kit (Cayman Chemical Item No. 706002). Two groups (RUPP rats and control group) results are compared to determine the extent of the oxidative stress caused the RUPP model—modeling the preeclampsia disease conditions.

Tissue Homogenization for Protein Analysis:

Biological tissue samples of left ventricles were homogenized in order to extract proteins, DNA, RNA, and other small molecules from the cells, so these smaller components of the cell were able to be studied and quantified. Tissue samples gathered from the RUPP model rats and control group were homogenized prior to running the three protein analysis tests: 8-Isoprostane Elisa, TBARS Assay, and Superoxide Dismutase Assay.

The homogenization buffer was prepared by combining the following chemicals together. (Add 0.1 M phosphate buffer, pH 7.4 containing 1mM EDTA and 0.0005% BHT). We obtained 100 mg of sample tissue (heart or placenta). One milliliter of homogenization buffer was added into a capped, locking, microfuge tube along with 100 mg of sample tissue.

The sample mixture was homogenized using the BioSpec Stainless Steel BioPulverizer with Hammer (Item No. 59014 N 316). After homogenization, the sample was centrifuged at 8,000 x g for 10 minutes to pellet particulate matter. Supernatant was removed using a pipette and transferred into a clean, new microfuge tube. An aliquot of this supernatant was reserved for use in the protein assay.

The homogenized mixture was used to complete necessary protein analysis. Samples that were not assayed immediately were stored at -80 degrees C in the presence of 0.005% BHT. (The medium used to dilute the standard was the same medium used to dilute the saved samples.)

Storing Kits & Tissues Between Assays:

All three kits need to be stored at -20 degrees Celsius and used before the expiration date. Tissues had to be stored at -80 degree Celsius after rats were euthanized and in between experiment if multi-day experiments were running.

8-Isoprostane Assay Kit:

Tissues sampled were prepared following the protocol for Tissue Homogenization found above. Following the 8-Isoprostane Assay Kit provided instruction book, homogenized tissue samples were purified using the SPE purification protocol (page 19).

The assay was performed, ELISA buffer, 8-Isoprostane ELISA standard (both provided in the kit) were added to the NSB wells. Samples of the purified and homogenized tissues were added to the NSB wells following the dilution standards provided on page 23. The ELISA AChE Tracer and Antiserum were added to the wells last. The plates were incubated for 18 hours at 4 degrees Celsius before the results could be developed and analyzed.

The results were analyzed by calculating the B/Bo value (Standard Bound/ Maximum Bound) values. These values are multiplied by 100 to obtain the %B/Bo value needed to plot the standard curve. The standard curve of %B/Bo for standards S1-S8 vs. 8-Isoprostane concentration was plotted using a linear 4- parameter logistic fit.

Logit(B/Bo)= ln[B/Bo/(1-B/Bo)]

Using the equation above, the data was plotted as logit (B/Bo) vs log concentrations of 8-Isoprostane to perform a linear regression fit.

TBARS Assay Kit:

Tissues sampled were prepared following the protocol for Tissue Homogenization found above. Following the TBARS Assay Kit provided instruction book, homogenized tissue samples were prepared, and experimental plates were set up using the protocol (page 9-10).

The assay was performed as stated in the TBARS Assay Kit book and plates are read with the plate reader value set at an absorbance of 530 nm. The average absorbance of each standard and sample was calculated and recorded. The MDA value for each sample from the standard curve was determined.

$$\boldsymbol{MDA\ (uM)} = [\frac{(\boldsymbol{corrected\ absorbance - y\ intercept})}{\boldsymbol{slope}}]$$

Superoxide Dismutase Assay:

Tissues sampled were prepared following the protocol for Tissue Homogenization found above. Following the Superoxide Dismutase Assay Kit provided instruction book, homogenized tissue samples were prepared, and experimental plates were set up using the protocol (page 14).

The 96-well plate was mixed carefully by shaking for a few seconds, covered and incubated. The plate was incubated for 30 minutes at room temperature and then read with absorbance plate reader at 440-460 nm.

Average absorbance was calculated for each standard and sample. The linearized SOD standard rate (LR) was plotted as a function of final SOD Activity (U/ml). The SOD activity of each sample was calculated using the equation below. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

$$\boldsymbol{SOD}\ \left(\frac{\boldsymbol{U}}{\boldsymbol{ml}}\right) = \left[\left(\frac{\boldsymbol{sample\ LR - y\ intercept}}{\boldsymbol{slope}}\right) \times \frac{\boldsymbol{0.23\ ml}}{\boldsymbol{0.01\ ml}}\right] \boldsymbol{x\ sample\ dilution}$$

- *Note: 0.23/0.01 is the factor for converting from U/ml in well to U/ml in 10 ul added to 230 ul well volume*

Expected Results

The expected results would include higher than standard level amounts of the three tests (8-Isoprostane, TBARS, and SOD) in some combination. An increase in one or multiple of these tests would correlate to the expected stress of the tissues causing high levels of oxidative stress.

Preeclampsia leads to a decreased perfusion of the tissues—which can lead to compromised fetal and maternal health. The body will try to maintain homeostatis by increasing blood pressure and begin perfusing tissues adequately. This causes increased stress on tissues of the heart and can be viewed as oxidative stress by markers of 8-Isoprostane, TBARS, and SOD (Glesby, 1989).

8- Isoprostane Assay:

Isoprostanes are non-enzymatic eicosanoids that are produced by random oxidation of tissue phospholipids by oxygen radicals (Wolfram, Oguogho, Palumbo, & Sinzinger, 2005). Tissues that have experienced heightened oxidative stress will have isoprostane markers. The body's heightened inflammation response to oxidative stress is correlated with increased level of isoprostanes (Dhalla, Hill, & Singal, 1996).

The expected results would include a significant increase in 8- Isoprostane levels of heart tissues from RUPP rats when compared to SHAM rats. The SHAM rats would have relatively

low levels of 8-Isoprostane due to minimal tissue stress and appropriate perfusion throughout gestation. Whereas the RUPP group has undergone intense tissue stress which presents as oxidative stress.

TBARS Assay:

Thiobarbituric acid reactive substances (TBARS) are a product formed as the byproduct of lipid peroxidation—a process heightened during tissue damage (Dhalla, Hill, & Singal, 1996). This test measures levels of Malondialdehyde (MDA) which is a naturally occurring product of the lipid peroxidation (Piantoni et al., 2010).

The results of this test include comparisons of the SHAM and RUPP groups' absorbance spectrum of MDA concentration to the standard curve. The expected results would include a significant increase in MDA concentration from the RUPP rats' tissue vs the SHAM tissue. This increase in MDA would imply an increase in lipid peroxidation occurring from the stress placed on the tissues.

Superoxide Dismutase Assay:

This assay quantifies the enzymatic activity of superoxide dismutase within the tissues. The three types of SOD measured include (Cu/Zn, Mn and FeSOD); however, do not appropriately measure the mitochondrial levels of MnSOD. The activity of SOD and oxidative stress levels have a direct relationship (Deel, et al., 2007).

The expected results would include a significant increase in SOD activity measured in RUPP rats vs SHAM rats. The rats in the RUPP group would be experiencing higher levels of oxidative stress; therefore, would show increased levels of superoxide dismutase in an attempt to alleviate such stress. The SHAM group would not show elevated SOD activity levels from the

standard curve as the enzymatic activity would have no need to be heightened—as oxidative stress levels should be relatively normal.

VEGFR2 Nanoparticle Treatment:

Completing the experiment with RUPP rats receiving the VEGF receptor 2 treatment, it would be expected for the oxidative stress levels in each assay to return to normal levels—similar to the SHAM group.

Discussion

We would expect that RUPP rats would experience heightened levels of oxidative tissue damage causing significantly higher than normal levels of oxidative stress. RUPP rats have lower tissue perfusion leading to increased heart rate to maintain a homeostatic mean arterial pressure. RUPP rats experience decreased cardiac output which leads to increased oxidative stress. Oxidative stress is caused by the imbalance between production and accumulation of reactive oxygen species (ROS). When completing the experiment, the expected results would include at least one, possibly up to all three assays showing an increase in oxidative stress. However, there is the possibility of one test showing increased levels without the others and vice versa. This is due to the angle the assay test is using to determine the tissue's oxidative stress level. For example, 8-Isoprostane quantifies oxidative stress by increased inflammatory pathways while TBARS assay is using lipid peroxidation pathway byproducts. The levels of oxidative stress must be measured using byproducts as the half-life of free radicals makes them an inadequate factor to measure.

RUPP rats could show increased levels in the 8-Isoprostane and TBARS test; however, not in the SOD assay. The SOD assay measures the activity of the enzyme responsible for eliminating super oxides within the tissue. There is a possibility of this enzyme being ineffective due to a mutation or inability to function properly with the pathology. If body's response is to increase transcription and translation of this enzyme during increasing oxidative stress, but the enzyme is not functioning properly; the activity levels would not show as increased in the assay.

Another alternate possibility would include an increase in SOD levels and 8-Isoprostane in RUPP rats; however, normal range shown in the TBARS test. This outcome could be explained by the type of oxidative stress the rats' tissue is experiencing. RUPP rats have decreased perfusion leading to decreased cardiac output resulting in increased oxidative stress. If the body is not increasing lipid peroxidation pathways but is still experiencing oxidative stress through increased inflammatory pathways, this could explain the variation in the test results.

RUPP rats treated with VEGF receptor 2 nanoparticles are expected to have decreased indices of oxidative stress. This is due to increased vasodilatory function stemming from restored nitric oxide production. Increased nitric oxide production causing vasodilation will result in increased cardiac output, and increased tissue perfusion. Treatment can have incredible results to the maternal preeclampsia pathology. This is due to the increased vasodilation caused by NO production would improve blood flow to perfuse tissues adequately. The feedback cycle of constantly increasing tissue stress to maintain an efficient cardiac output would not be necessary. The dilated blood vessels would increase flow, increasing stroke volume; therefore, the heart would not need to increase pumping rate to increase cardiac output. Treatment would allow for tissue perfusion and cardiac output to return closer to homeostasis.

Allowing the heart rate and pressure to return back to SHAM or normal physiological levels would decrease the amount of oxidative stress on the heart tissues. This would eliminate the intense damage to the tissues and lower the amount of remodeling occurring. If heart tissue is undergoing lower amounts of remodeling, the valves could remain at functioning size and shape. Physiologically normal valves of the heart would allow normal function to be maintained; therefore, decreasing the maternal risk of cardiovascular damage following a preeclamptic pregnancy.

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