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Oxidative Stress Analysis of Placental Tissue in the RUPP Pregnant Rat

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Honors Research Project

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Abstract

Preeclampsia is a disease associated with a vasoconstrictive phenotype due to disrupted signaling in the vascular endothelial growth factor (VEGF) relaxation pathway. The purpose of these studies is to determine the level of oxidative damage that is occurring in the placenta of the reduced uterine perfusion pressure (RUPP) rat, which serves as a common model for preeclampsia. RUPP and SHAM placenta tissue will be examined for oxidative stress as an indicator of tissue damage associated with the pathologically induced ischemia. Oxidative stress in the placental tissue will be measured by performing the following oxidative stress assays: 8-isoprostane test, TBARS assay, and SOD assay kit. In addition, it will be assessed whether this damage is reversible with uterine injection of L-tyrosine polyphosphate (LTP) nanoparticles that encode for VEGF2 receptors. We further hypothesize that improved VEGF signaling via receptor upregulation may ameliorate vascular signaling which would lead to improved utero-placental perfusion. Ultimately improved blood flow to the uterine circulation may decrease measurable levels of oxidative stress.

i. Introduction

Preeclampsia is defined as a hypertensive disorder that occurs during pregnancy and is one of the leading causes of maternal and fetal mortality (Hansson, Naav, & Erlandsson, 2015). Currently the only known cure for preeclampsia is premature delivery of the baby. Preeclampsia is thought to be caused by defective remodeling of maternal spiral arteries that leads to inadequate perfusion of placental tissues (Reho et al., 2012). Hypoxia and oxidative stress occur in the placental tissues as a result of this reduction in perfusion (Hansson et al., 2015). Studies show that antioxidant levels are reduced in preeclamptic women and that biomarkers of oxidative

stress can be seen in the systemic circulation and placenta of preeclamptic pregnant women (Hansson et al. 2015). Eventually symptoms, such as hypertension and proteinuria, that are associated with preeclampsia manifest in the pregnant woman. One defining feature of preeclampsia is maternal and feto-placental endothelial dysfunction, in which the balance between vasoconstrictive and vasodilatory pathways is disrupted (Giachini et al., 2017). One important vasodilatory pathway utilized by the body and of particular importance in preeclampsia pathogenesis is the vascular endothelial growth factor (VEGF) pathway. VEGF binding to VEGFR2 receptors signals an increase in nitric oxide (NO) production. The synthesis and release of nitric oxide (NO) is critical for maintaining adequate blood flow to tissues in pregnant women. In addition, NO also displays anti-inflammatory and antioxidant properties in blood vessels (Warrington et al., 2013). Scientific studies show that there is decreased endothelial NO synthase function in women with preeclampsia (Warrington et al., 2013). Thus, the VEGF pathway may be a possible target for relieving the pathology seen in women with preeclampsia.

The reduced uterine perfusion pressure (RUPP) pregnant rat has been chosen as the model that best replicates the symptoms and pathology seen in preeclampsia (Reho et al., 2012). For example, the RUPP pregnant rat shows reduced uterine and placental blood flow and the uterine arteries show a more constrictive phenotype (Reho et al., 2012). The overarching goal of this experiment is to examine the effects of increasing VEGF signaling in the RUPP pregnant rat. L-tyrosine polyphosphate (LTP) nanoparticles that encode for VEGFR2 receptors will be injected into the uterine wall myometrium of RUPP pregnant rats. This new technique should increase the amount of VEGFR2 receptors in RUPP rats, allowing for increased VEGF signaling. Increased VEGF activation should lead to an increased production of NO and thus promote

angiogenesis and vasodilation (Eddy, Bidwell, & George, 2018). Therefore, improvements are expected to be seen in maternal and fetal outcomes.

The specific question that will be examined in this study is determining the level of oxidative damage that is occurring in the rat placental tissue and determining whether this damage is reversible with increased VEGF signaling. Various oxidative stress analysis tests will be performed to measure the level of oxidative stress that is occurring in rat placental tissue of both RUPP and SHAM rats either treated with or without LTP nanoparticles containing DNA encoding for VEGFR2. In addition, determining the mechanism of oxidative stress in RUPP rats will allow one to elucidate the mechanism of oxidative stress in preeclamptic women.

Two of the tests that will be used to determine lipid oxidation are the 8-isoprostane test and the TBARS test to measure malondialdehyde. The 8-isoprostane ELISA kit measures levels of 8-isoprostane, an eicosanoid that is produced when phospholipids in tissue are oxidized. 8-isoprostane has been found to act as a renal vasoconstrictor and therefore we expect to find significantly higher levels of 8-isoprostane in the RUPP rat's placental tissue compared to the SHAM rat. The TBARS (Thiobarbituric Acid Reactive Substances) Assay kit is a method used to test for an end product of lipid peroxidation. This end product, malondialdehyde (MDA), reacts with TBARS and thus allows for quantification of MDA using a plate reader. Because oxidative stress leads to the production of unstable lipid hydroperoxides we also expect to find larger amounts of MDA in RUPP rats' placental samples compared to SHAM rats. Previous studies have shown that RUPP rats show increased levels of oxidative stress markers including 8-isoprostane and malondialdehyde (MDA) (Sedeek et al. 2008). The goal of this experiment is to determine whether the LTP nanoparticle therapy will be effective in reducing the levels of 8-isoprostane and MDA found in the placenta of the RUPP rats.

Finally, the Superoxide Dismutase assay kit (SOD) will be used to measure levels of SOD activity in the placenta. Superoxide dismutase describes a class of metalloenzymes that catalyze the conversion of superoxide anion (O_2^-) into oxygen and hydrogen peroxide (Hansson et al. 2015). SOD activity is necessary for prevention of oxidative stress. We expect SOD activity to be lower in the placenta of RUPP rats compared to SHAM rats as previous studies have shown (Sedeek et al. 2008). We hypothesize that treatment with LTP nanoparticle therapy encoding VEGFR2 will increase SOD activity in the placenta of the RUPP rats.

ii. Materials and Methods

Animal Maintenance and Care

Timed female Sprague-Dawley rats were obtained (Hilltop Lab Animal, PA) and housed in the vivarium at the University of Akron. Experiments were followed according to the protocols approved by the IACUC. On day 14 of gestation rats underwent surgery (sham or reduced uterine perfusion pressure surgery). On the same day RUPP rand SHAM dams were injected with LTP nanoparticles containing DNA encoding VEGFR2 or blanks in the myometrium of the uterine wall. On day 22 of gestation the rats were euthanized. The placenta will be removed from the organism and placental weight will be documented.

Tissue Homogenization and Oxidative Stress Assays:

Placental tissue will be homogenized as described below. If the tissue homogenate is not assayed the same day it will be kept in the -80 ºC freezer for storage.

The first oxidative stress that is to be performed is the 8-Isoprostane ELISA Kit (Cayman Chemical Item No. 516351). Placental samples need to be prepared before the oxidative stress

analysis tests is performed. The placental tissue samples will be manually homogenized as described by the 8- Isoprostane ELISA Kit Manual by using a BioSpec Stainless Steel BioPulverizer with Hammer. After following the SPE Purification protocol the assay will be performed as described by the 8-Isoprostane Elisa Kit manual and the plate will be incubated for 18 hours at 4 ºC. The plate will be developed with Ellman's Reagent and read with a plate reader at a wavelength between 405-420 nm. A standard curve will be formed by plotting $\%B/B_o$ versus 8-isoprostane concentration and performing a linear regression fit. The equation obtained from the standard curve will be used to determine the 8-isoprostane concentration in each sample.

The second oxidative stress to be performed is the Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Cayman Chemical Item No. 10009055). Placental tissue will be homogenized by following the protocol from the TBARS Assay kit for tissue homogenates with the BioSpec BioPulverizer. The assay will be performed as described by the TBARS Assay kit manual and plates will be read with a plate reader at an absorbance of 530 nm. The MDA standard curve will be formed by plotting absorbance as a function of MDA concentration. Finally, the standard curve will be used to determine the concentration of MDA in M for each sample.

The third oxidative stress to be performed is the Superoxide Dismutase Assay Kit (Cayman Chemical Item No. 706002). Placental tissue will be homogenized following the protocol in the Superoxide Dismutase Assay Kit using the BioSpec BioPulverizer. The assay will be performed as described by the Superoxide Dismutase Assay Kit manual, and plates will be read with a plate reader at an absorbance of 450 nm. The linearized SOD standard rate will be plotted as a function of final SOD activity to obtain a standard curve. The linear regression

equation obtained from the standard curve will be used to calculate the SOD activity for each placental sample.

iii. Expected Results

Due to unexpected events with the COVID-19 pandemic the oxidative stress analysis tests were unable to be carried out. Instead the expected results will be discussed.

8-Isoprostane:

We expect to find significantly higher levels of 8-isoprostane in the RUPP rat's placental tissue compared to the SHAM rat. Previous studies have shown that RUPP rats show increased levels of oxidative stress markers including 8-isoprostane (Sedeek et al., 2008). Furthermore, we expect that LTP nanoparticle therapy will be effective in reducing the pathological levels of 8-isoprostane found in the placenta of the RUPP rats. RUPP rats treated with the LTP nanoparticle therapy may even have 8-isoprostane levels that are comparable to the SHAM rats.

Thiobarbituric Acid Reactive Substances (TBARS):

We expect to find significantly higher levels of malondialdehyde (MDA) in the RUPP rats' placental tissue samples compared to the SHAM rats. Previous studies have shown that RUPP rats show increased levels of oxidative stress markers such as MDA (Sedeek et al., 2008). Furthermore, we expect that LTP nanoparticle therapy will be effective in reducing the pathological levels of MDA found in the placenta of the RUPP rats. RUPP rats treated with the LTP nanoparticle therapy may even have MDA levels that are comparable to the SHAM rats.

Superoxide Dismutase:

We expect superoxide dismutase (SOD) activity to be significantly lower in the placenta of RUPP rats compared to SHAM rats. Previous studies have shown that renal cortical SOD activity was significantly decreased in RUPP rats compared to controls (Sedeek et al., 2008). Therefore, we expect to see similar findings in RUPP rat placental tissue. We expect that treatment with LTP nanoparticle therapy encoding VEGFR2 will increase SOD activity in the placenta of the RUPP rats.

iv. Discussion

Oxidative stress is involved in the pathology of many diseases, such as preeclampsia, and thus it is important that researchers are able to accurately measure biomarkers of oxidative stress (van 't Erve et al., 2017). The focus of this research is to gain further insight into the oxidative stress that occurs in the placenta of preeclamptic women. Measurement of 8-isoprostane, a prostaglandin F_2 –type compound formed from free radical peroxidation of arachidonic acid, is essential because it serves as biomarker for oxidative stress caused by lipid peroxidation (van't Erve et al., 2017). Placentas obtained from preeclamptic women had significantly higher levels of 8-isoprostane than normal placentas (Walsh et al., 2000). This means that lipid peroxidation is pathologically increased in the placentas of women with preeclampsia. In addition, previous studies have shown that 8-isoprostane acts as a vasoconstrictor in rat smooth muscle tissue (Janssen et al., 2001). Thus, pathologically increased 8-isoprostane levels in preeclamptic women may in turn cause decreased perfusion to oxygen deprived tissue by restricting blood flow. The RUPP rat serves as an excellent model of preeclampsia because it mimics several pathological features that are seen in preeclamptic women such as the increased placental

oxidative stress (Li et al., 2012). For example, placentas obtained from RUPP rats also have increased levels of 8-isoprostane (1.9 ± 0.4 ng/g RUPP tissue compared to 0.8 ± 0.1 ng/g normal tissue) (Sedeek et al., 2008). Thus, we also expect to see a significant increase in 8-isoprostane levels in the RUPP rats compared to the SHAM rats. It is expected that LTP nanoparticle therapy, the injection of LTP nanoparticles containing DNA encoding VEGFR2 in the myometrium of the uterine wall, will be effective in reducing the pathological levels of 8-isoprostane found in the placenta of the RUPP rats. This will signify that the RUPP rats treated with LTP nanoparticles have increased amount of VEGF receptors in the cell membrane of their placental tissue. Furthermore, it would support the theory that increasing VEGF receptors leads to an increase in VEGF cell signaling. An increase in VEGF signaling could lead to an increase in NO production that promotes vasodilation in the RUPP rat placenta, thus explaining the observed decrease in oxidative stress. However, an increase in VEGF signaling may also likely involve other mechanisms of cell signaling pathways that decrease oxidative stress.

Malondialdehyde (MDA) is another end product of lipid peroxidation that is caused by increases in reactive oxygen species (Yoneyama et al., 2002). Plasma MDA levels are significantly elevated in preeclamptic women compared to women with normal pregnancies (Yoneyama et al., 2002). Similarly, malondialdehyde levels in placental tissue have been found to be increased in RUPP rats (6.9 ± 0.6 μmol/g RUPP tissue vs. 3.9 ± 0.4 μmol/g tissue) (Sedeek et al., 2008). Thus, we expect to see significant increases in MDA levels in the RUPP rats placental tissue compared to the SHAM rats. While increased MDA levels in the placenta does not directly mean tissue damage is occurring in the fetus, it still signifies that there is some sort of pathology in the RUPP rat due to increased oxidative stress. It is expected that LTP nanoparticle therapy will be effective in reducing the pathological levels of MDA found in the

placenta of the RUPP rats. This will also support the hypothesis that increased VEGF signaling allows the RUPP rat to better deal with increased oxidative stress. Furthermore the results may even show that RUPP rats treated with the LTP nanoparticle therapy have MDA levels that are comparable to the SHAM rats.

The last indicator used to measure oxidative stress in tissues is measurement of superoxide dismutase (SOD) activity. SODs are metalloenzymes that decrease the concentrations of harmful superoxide anion free radicals (Younus, 2018). Thus, measurement of SOD activity is a reflection of the cell's natural ability to fight off oxidative stress. Under normal conditions, free radicals generated in endothelial cells are kept at relatively low concentrations because they are neutralized by antioxidant compounds (Taravati & Tohidi, 2018). Studies have shown that women with preeclampsia have a significant decrease in total antioxidant capacity which includes a significant decrease in enzymes such as SOD (Taravati & Tohidi, 2018). Therefore, pregnancies complicated by preeclampsia have decreased capacity to deal with free radicals and oxidative stress. Likewise previous studies have shown that renal superoxide dismutase activity is decreased in RUPP rats (1.2 ± 0.1 units/mg RUPP protein vs. 1.6 ± 0.1 units/mg protein) (Sedeek et al., 2008). Thus, we also expect to see significant decreases in SOD activity in the RUPP rats placental tissue compared to the SHAM rats. Previous studies show that VEGF signaling increases expression of manganese SOD (Mn-SOD) in endothelial cells through the activation of NADPH oxidase (Abid et al., 2004). Thus it is possible that LTP nanoparticle therapy will be effective in increasing SOD activity in the placenta of the RUPP rats by increasing VEGF signaling pathways. If there is increased SOD activity in RUPP rats treated with LTP nanoparticle therapy it will also further support why there would decreased lipid peroxidation in placental tissue.

If one finds that oxidative stress levels are reduced the next step would be to determine whether this correlates with alleviation of the symptoms seen in the RUPP rats. Will the RUPP rats treated with LTP nanoparticle therapy encoding VEGFR2 have physiological improvements compared to RUPP rats without the therapy? One would possibly expect to see improvements in the RUPP rat's pups such as an increased litter size and an increase in pup size. One would also need to determine whether improvements are also seen in the RUPP rat itself such as whether there are decreases in mean arterial pressure. The next step would be to further elucidate how increased VEGF signaling improves the RUPP rat's response to oxidative stress and determine the key cell signaling pathways that are activated. Ultimately, the end goal of this study is to determine whether increased VEGF receptor signaling will lead to improvements in preeclamptic women. If increased VEGF receptor signaling leads to a decrease in placental oxidative stress and improvements in the health of both the fetus and the mother, then LTP nanoparticle therapy encoding VEGFR2 receptors should be examined as a possible treatment option for pregnancies complicated by preeclampsia.

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