THE IMPACT OF REDUCTIONS IN UTERINE PERFUSION PRESSURE ON UTERINE ARTERIAL REACTIVITY IN GRAVID RATS AND L-TYROSINE POLYPHOSPHATE NANOPARTICLES AS A POTENTIAL IN VIVO GENE DELIVERY DEVICE

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THE IMPACT OF REDUCTIONS IN UTERINE PERFUSION PRESSURE ON
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POLYPHOSPHATE NANOPARTICLES AS A POTENTIAL \textit{IN VIVO} GENE
DELIVERY DEVICE

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ABSTRACT

Reductions in uterine perfusion pressure are thought to be a central component of the pathological pregnancy disease, preeclampsia. Preeclampsia is a hypertensive disorder of pregnancy characterized by vascular dysfunction and end organ underperfusion and is the leading cause of maternal and fetal morbidity and mortality in the United States and throughout the world. Uterine arterial reactivity and structural mechanics during preeclampsia are poorly understood and likely contributed to the pathophysiology of the maternal hypertension and altered fetal growth demonstrated in this disease. The first aim of this dissertation project was to characterize the impact of reductions in uterine perfusion pressure on maternal and fetal pregnancy outcomes. Maternal hypertension and fetal morphometrics have been examined in response to reductions in uterine perfusion pressure. The second aim of this research was to characterize the vascular behavior and structural biophysical mechanics of resistance-caliber uterine arteries in response to reductions in uterine perfusion pressure. Vascular behavior was examined using a pressurized arteriograph where myogenic reactivity, agonist induced vasodilation and vasoconstriction, and passive structural mechanics were assessed. The third aim of this research involved testing the efficacy of a novel L-tyrosine based gene delivery device. L-tyrosine polyphosphate (LTP) nanoparticles have demonstrated promise as a potential intracellular gene delivery device aimed at
therapeutic avenues however; the in vivo efficacy of the delivery vehicle has been unknown. We aimed to prove the concept that LTP nanoparticles encapsulated with plasmid DNA would be efficacious as an *in vivo* gene delivery device in the rat uterus.

Chronic reductions in uterine perfusion pressure resulted in maternal hypertension and severe fetal growth restriction in pregnant rats suggesting an integral role of uterine perfusion pressure on maternal and fetal responses to the pathology. Uterine artery reactivity was found to be altered towards a constrictive phenotype in response to chronic reductions in uterine perfusion pressure with increased myogenic reactivity and decreased agonist induced vasodilation. Structural parameters of resistance-caliber uterine arteries were unaltered in response to the pathology while biophysical mechanical properties of the uterine arteries were altered. Distensibility was altered in isolated resistance-caliber uterine arteries isolated from reductions in uterine perfusion pressure gravid rats suggesting a potential contributing factor to the vascular dysfunction described above. Nanoparticles formulated from L-tyrosine polyphosphate successfully delivered plasmid DNA *in vivo* to the rat uterine tissue. These data suggest that uterine arterial reactivity is altered in response to reductions in uterine perfusion pressure and that LTP nanoparticles encapsulated with plasmid DNA may have potential as a delivery platform for therapies aimed at diseases of the uterus and uterine vasculature.
DEDICATION

This dissertation is dedicated to my parents, John William Reho and Suzann Sanitra Marshall, and to my sister, Casey Suzann Reho. Family is the only constant we have in this world. Their love, guidance, and support make everything in this life worthwhile.
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CHAPTER I
PREAMBLE, INTEGRATION & SPECIFIC AIMS

Preamble

This dissertation focused on a disease of pregnancy that is the leading cause of both maternal and fetal death throughout the world. Preeclampsia is a disease of pregnancy where the etiology of the disease is currently unknown. Preeclampsia kills 76,000 mothers and over 500,000 infants annually and it is estimated that the costs due to this disease are about 7 billion dollars every year. Equally troubling is the fact that the incidence of preeclampsia has risen ~40% over the past 7 years (Gilbert et al., 2010). This concern has driven a substantial amount of research to elucidate not only the etiology of the disease, but also the pathophysiology and possible therapeutic avenues.

Preeclampsia results in increased systemic blood pressure, altered renal function, hyperuricemia and altered vascular hemodynamics. Specifically in terms of altered vascular function, uterine artery reactivity is altered in preeclampsia however the mechanism by which this occurs is currently unknown. A portion of infants born to preeclamptic mothers have low birth weight and an enhanced predisposition to cardiovascular disease later in life possibly indicative of an altered uterine circulatory environment. Understanding the vascular mechanisms of the uterine circulation during preeclampsia may help develop therapies aimed at treating the uterine vasculature which may improve maternal and fetal pregnancy outcomes.
Reductionist biological approaches to pathologies associated with pregnancy have been fruitful. However the emphasis on translational therapeutic approaches that may have efficacy for human pregnancy is currently lacking. The advent of non-viral gene delivery has brought about possible therapies at the molecular level that can be performed safely and with relatively low immunogenic effects. Long term vascular therapy through non-viral gene delivery may provide a therapeutic avenue to the treatment of this disease of pregnancy. These with its combination of vascular functional analysis, biomedical gene delivery engineering, and unique animal modeling provides a mechanistic integration ranging from the whole animal down to the genomic level. This dissertation also emphasizes the collaboration of an interdisciplinary team of biologists and biomedical engineers to meet the demands to answer complex questions related to this disease of pregnancy.

Integration

Preeclampsia research has been copious. However, the ultimate cause of the disease remains unknown. A general consensus among investigators is that reductions in uterine perfusion, resulting in placental ischemia, play a central role of the pathogenesis and subsequent pathology of the disease. In preeclampsia, the maternal endothelium is altered leading to a vascular state characterized by vasospasm and vasoreactivity that favors a more constrictive phenotype. Additionally, within the uterine circulation, these studies will examine the impact of reductions in uterine perfusion pressure on uterine arterial reactivity in pregnant rats.
Potential therapies aimed at the vascular level may be advantageous in the treatment of vascular disorders such as preeclampsia. Delivery methods of genetic material have been slowed due to immunogenic effects of many of the viral based vectors as well as the instances of cytotoxicity associated with many non-viral polymer delivery devices. These studies present a novel gene delivery device formulated from L-tyrosine based polymers and examines its potential as an in vivo gene delivery device.

The purpose of this study was to accomplish two separate research goals. The first was to characterize uterine arterial reactivity and mechanics in response to reductions in uterine perfusion pressure in pregnant rats. The second goal was to characterize L-tyrosine polyphosphate nanoparticles as an in vivo gene delivery device to the rat uterus. To accomplish these study goals, the following specific aims and hypotheses were formulated.

Specific Aims

Aim 1: Characterize maternal and fetal pregnancy outcomes in pregnant rats with chronic reductions in uterine perfusion pressure (RUPP) compared to SHAM control.

Hypothesis 1: RUPP pregnancies will be associated with increased maternal mean arterial pressure (MAP) and decreased fetal morphology (i.e. decreased fetal and placental weights, crown-rump length, and head circumference) compared to control.
Aim 2: Characterize resistance-sized uterine artery reactivity and passive structural mechanics in pregnant rats with chronic reductions in uterine perfusion pressure (RUPP) compared to SHAM control.

Hypothesis 1: The hypothesis to be tested is that isolated resistance-caliber uterine arteries from RUPP rats will display a more vasoconstrictive phenotype with increased myogenic reactivity and decreased dilator (metacholine and VEGF) responsiveness compared to control.

Hypothesis 2: Isolated RUPP rat resistance-caliber uterine arteries will exhibit altered passive structural mechanics compared to control.

Aim 3: Demonstrate the efficacy of LTP-pDNA nanoparticles as a gene delivery device in vivo in the rat uterus.

Hypothesis 1: LTP-pDNA nanoparticles will transfect the rat uterus in vivo.
CHAPTER II
BACKGROUND

Pregnancy

Human pregnancy results in profound changes in maternal organ system homeostasis to confer growth and support for the developing fetus. Pregnancy alters the maternal cardiovascular, renal and uterine systems among others with an emphasis on providing adequate nutrient and oxygen exchange to the developing fetus which ultimately affects maternal health. The following sections of this background chapter will examine many of the key aspects of the physiological adaptations to pregnancy and how pathology during pregnancy in the specific case of preeclampsia alters these systems.

Cardiovascular adaptation to pregnancy

The cardiovascular system adapts to the gravid physiological state through various hemodynamical mechanisms. Plasma volume expansion is increased by an estrogen mediated effect on an activated renin-angiotensin system (Magness and Rosenfeld, 1989) that leads to a reversible left ventricular hypertrophy due to increased ventricular filling pressure at a given blood volume (Clapp and Capeless, 1997). This ventricular hypertrophy aids in the increased cardiac output seen in pregnancy through
increases in end diastolic volumes and atrial dimensions (Robson et al., 1989). Cardiac output is also increased in part due to a significant decrease in systemic vascular resistance (Clapp and Capeless, 1997) through reduced arterial diameters of resistance-sized arteries downstream in the arterial tree which leads to increased arterial compliance (Poppas et al., 1997) and therefore an overall decrease in arterial blood pressure. The decrease in systemic vascular resistance is also likely due to the development of the low resistance uteroplacental circulation in parallel with other systemic organs.

**Uterine Adaptation to Pregnancy**

The gravid uterus undergoes significant hypertrophy and stretch to accommodate the developing fetus. In addition to this increase in size, there is a significant increase in uterine blood flow to deliver nutrients and oxygen to the developing fetus (Metcalfe et al., 1955). The increase in uterine blood flow is accompanied by a significant decrease in uterine vascular resistance (Osol and Mandala, 2009) through both extensive uterine vasculature remodeling and the establishment of the placenta.

**Rodent Model of Pregnancy**

Physiological responses to pregnancy between rodents and humans are similar in many aspects and the gravid rat provides several advantages compared to other pregnant animal models. Of course, the relatively short gestational period of the gravid rat (~22 days) allows for the successful study of a number of different animals within a manageable time period. Both rodents and humans possess a hemochorial form of placentation. This type of placentation allows for low intraplacental pressure in the
intravillous (human) and intralabyrinthine (rodent) fetal vessels to avoid compression and blood flow reduction (Moll et al., 1975). Both species also exhibit comparative increases in uterine blood flow from a non-pregnant to gravid state (Moll et al., 1975). Human uteroplacental blood flow increases nearly 10 fold from control levels (Metcalfé et al., 1955). Similar results have been found in the gravid rodents which increase uterine blood flow ~10 fold (Bjellin et al., 1975; Bruce, 1976). Pregnant rats also exhibit similar increases in glomerular filtration rate and effective renal plasma flow with concomitant decreases in renal vascular resistance which is crucial to accommodate the volume expansion throughout pregnancy (Conrad, 1984). Additionally, cardiac output is increased with a parallel fall in total systemic vascular resistance in humans (Lees et al., 1967).

**Preeclampsia**

Preeclampsia is a pathological disease of pregnancy that affects 5-10% of all human pregnancies (George and Granger, 2011). This disease is the leading cause of maternal and fetal morbidity and mortality throughout the United States and likely throughout the world as data from third world countries is becoming known. Ominously, the incidence of preeclampsia has risen 40% in the past 8 years and is primarily a disease of primagravidas (Roberts et al., 2003).

Preeclampsia presents as maternal hypertension, proteinuria (indicating kidney damage) and some instances of peripheral edema as the clinical diagnosis. It is a heterogeneous disorder as there are varying degrees of severity afflicting the pregnant women. The maternal hypertension and proteinuria remit after parturition suggesting an
integral role of the feto-placental unit in the disease. Furthermore hydatiform molar pregnancies have demonstrated that it is likely the presence and failure of the placenta and not the fetus that causes preeclampsia (Page, 1939). Delivery of both the fetus and placenta remains the only known cure for the disease (George and Granger, 2011).

The etiology and subsequent pathophysiology is poorly understood in preeclampsia. However, the prevailing hypothesis is that abnormal placentation resulting from shallow trophoblastic invasion of the maternal spiral arteries (Zhou et al., 1993) leads to a reduction in uteroplacental perfusion pressure. This reduction in uteroplacental perfusion pressure leads to placentally derived signals altering the maternal vascular system to increased perfusion pressure and thus blood flow (George and Granger, 2011). This response is appropriate under acute conditions but a vicious cycle of placental signals from reduced perfusion pressure altering the maternal vascular endothelium leading to maternal vasospasm, hypertension, and end organ underperfusion as well as instances of fetal demise.

**Cardiovascular system during preeclampsia**

The cardiovascular system undergoes significant changes during preeclampsia as compared to normal pregnancy. In preeclampsia, the cardiovascular hemodynamics are altered where cardiac output is decreased due to a potential decrease in total blood volume (Zamudio et al., 1993) and an increase in total peripheral resistance (Visser and Wallenburg, 1991). The increase in total peripheral resistance is attributed to a decrease in vascular vasodilation as well as a decrease in arterial compliance (Hibbard et al., 2005). Vascular reactivity is increased in preeclampsia as pressor responses to
angiotensin II and norepinephrine are increased compared to normotensive pregnancy (Gant et al., 1973; Nisell et al., 1985). Alterations in inflammatory, angiogenic, and oxidative stress pathways are thought to cause a global endothelial damage and is especially evident in the characteristic glomerular endotheliosis in the preeclamptic kidney (Jeyabalan and Conrad, 2007). These pathways can lead to decreases in nitric oxide production and nitric oxide synthase activity in the endothelium (Sladek et al., 1997). Overall, these factors lead to the maternal hypertension and end organ underperfusion demonstrated in preeclampsia (Khalil and Granger, 2002).

**Reduced Uterine Perfusion Pressure (RUPP) Gravid Rat Model**

The chronic reduction in uterine perfusion pressure (RUPP) rat model reduces blood flow to the feto-placental unit by ~40% in late pregnancy (Crews et al., 2000) and was originally developed by Abitbol (Abitbol, 1982). This result is similar to the reduction in uterine blood flow seen in preeclamptic women during the third trimester via Doppler velocimetry (Konje et al., 2003). This surgical technique yields placental underperfusion causing many characteristics similar to that of human preeclampsia (Sholook et al., 2007). This model yields a characteristic underperfusion to preeclampsia by placing a silver clip (0.203 mm ID) around the abdominal aorta. Uterine blood flow adaptively increases through the utero-ovarian during rodent pregnancy (Nienartowicz et al., 1989), therefore two additional silver clips (0.106 mm ID) are placed around the utero-ovarian arteries below the ovary and above the first segmental uterine artery.

Some of these hallmarks include a significant rise in mean arterial pressure by ~20 mmHg (Alexander et al., 2001), decreases in glomerular filtration rate and effective
renal plasma flow indicating renal dysfunction (Alexander et al., 2001), increases in inflammatory cytokines (LaMarca et al., 2005, Gadonski et al., 2006, LaMarca et al., 2007) and alterations in nitric oxide (NO), which may be similar to the human form of the disease (Alexander et al., 2001, Alexander et al., 2004). Renal proteinuria has also been shown in the animal model (Alexander et al., 2001) although this is not an entirely consist finding (Ramirez et al., 2011).

Vascular reactivity of resistance-sized mesenteric and renal arteries is detrimentally altered in the RUPP pregnancies compared to control (Ramirez et al., 2011; Reho et al., 2011). Furthermore, these changes may represent similarities seen in the maternal circulation of preeclamptic women. These include a decreased relaxation response to acetylcholine in subcutaneous arteries (Blaauw et al., 2005) which is similar to aortic strips in the RUPP (Crews et al., 2000). These studies also indicated that the endothelium is the major regulator of vascular relaxation due to the fact that both studies found no significant relaxation response change to sodium nitroprusside (SNP), an NO analog that exhibits only autacoid functions. Our laboratory has demonstrated that a dysfunction exists in NO mediated vascular responses in isolated resistance-sized mesenteric and renal interlobar arteries (Ramriez et al., 2011). Uterine arteries from RUPP animals exhibit altered responsiveness on a wire myograph possibly due to impaired NO (Anderson et al., 2005). Additionally we have described altered NO mediated vascular reactivity in resistance-sized uterine arteries studied via pressure arteriography (Reho et al., 2012). Certain facets of the NO system have been found to be attenuated in this model and L-arginine supplementation has shown to slightly improve
vascular behavior in blood pressure regulation and placental weights (Alexander et al., 2004).

Figure 2.1 Schematic of the reduced uterine perfusion pressure (RUPP) surgical model in pregnant rats.

_Uterine artery reactivity in pregnancy and in preeclampsia_

Adequate perfusion pressure to the uteroplacental unit is essential to maternal hemodynamics and ultimately fetal health. The vascular reactivity of uterine arteries within the uteroplacental unit plays a large role in the regulation of this perfusion pressure. Human studies have demonstrated a prominent role for enhanced vasodilation in uterine arteries (Kubliciene et al., 1997; Nelson et al., 1998) with nitric oxide playing
a preeminent role in this process (Kublickiene et al., 1997; Nelson et al., 2000). Studies in animal models of pregnancy have also demonstrated the role of nitric oxide in the enhanced vasodilation associated with pregnancy (Ni et al., 1997; Cooke and Davidge, 2003). Potential mechanisms associated with this enhancement of nitric oxide signaling include increased DNA synthesis in the uterine artery (Keyes et al., 1997), estrogen enhancement (Storment et al., 2000), and decreased sensitivity to vasoconstrictors such as angiotensin II (St. Louis et al., 2001) and serotonin (Mandala et al., 2002).

A prominent mechanism regulating uterine arterial reactivity in pregnancy is the myogenic response. Nitric oxide has been demonstrated to regulate myogenic reactivity in rat uterine arteries (Veerareddy et al., 2002). Additionally, signaling through the MEK/ERK pathway (Xiao et al., 2006) and channels such as the voltage-gated potassium channel (Telezhkin et al., 2008) and large conductance calcium activated potassium channel (Hu et al., 2011) have been implicated in the regulation of the myogenic response in uterine arteries during pregnancy.

Women with preeclampsia experience reductions and/or reversals in uteroplacental blood flow (Ekici et al., 1996; Lau et al., 2007; Zahumensky, 2009) with intrauterine growth restriction affecting 1/3 of all preeclamptic pregnancies (Chappell and Morgan, 2006). The alterations in uteroplacental blood flow suggest a potential role of uterine artery dysfunction in preeclampsia. Isolated arterial studies have demonstrated increased myogenic reactivity and decreased vasodilation in myometrial arteries from preeclamptic women (Kublickiene et al., 2000; Ong et al., 2003). The potential role of dysfunctional endothelial NO is this pathway has been also demonstrated in subcutaneous
fat vessels (McCarthy et al., 1993; Knock and Poston, 1996), omental resistance arteries (Pascoal et al., 1998), and resistance myometrial arteries (Ashworth et al., 1997).

Animal studies have furthered mechanisms of vascular reactivity during preeclampsia pathology. A high altitude model of chronic hypoxia during pregnancy revealed decreases in DNA synthesis (Rockwell et al., 2000) and interestingly increases in endothelial nitric oxide synthase mRNA (Xiao et al., 2001) likely as a compensatory mechanism. Myogenic reactivity of uterine arteries in increased in pathological pregnancy models such as maternal undernutrition (Veerareddy et al., 2004; Hemmings et al., 2005) and reduced uterine perfusion pressure (Reho et al., 2012). Vascular reactivity to vasoconstrictors are also increased in L-NAME treated pregnant rats (Lu et al., 2008) and transgenic RAS rats (Verlohren et al., 2008) while vasodilation is decreased to VEGF (Itoh et al., 2002), acetylcholine (Anderson et al., 2005), and methacholine (Reho et al., 2012).

_Uterine artery remodeling during pregnancy and preeclampsia_

Pregnancy is associated with a dramatic reduction in uterine vascular resistance with a parallel increase in uterine blood flow. The primary determinants of this decrease in uterine resistance and thus increased perfusion are a combination of vascular reactivity within the uterine circulation and vascular remodeling influences. Uterine artery remodeling during pregnancy is a complex, highly dynamic process that is still poorly understood. During normotensive pregnancy, uterine arteries undergo outward hypertrophic remodeling to increase luminal cross sectional area to allow for increased uterine blood flow (Osol and Mandala, 2009). In outward remodeling, circumferential
luminal diameter increases. Uterine intraluminal arterial diameters have been demonstrated in increase in humans (Palmer et al., 1992) and rodents (Nienartowicz et al., 1989) as well as a variety of other species (van der Heijden et al., 2005; Keyes et al., 1997). Hypertrophic remodeling in rats occurs through a combination of processes involving cellular hypertrophy, through an increase in smooth muscle cell mass and protein content (Annibale et al., 1990), and cellular hyperplasia, by an increase in smooth muscle cell division (Cipolla et al., 1994). Additionally, axial growth of uterine arteries occurs during pregnancy (Cipolla et al., 1994) which positively correlates with the number of viable pups in rodent pregnancy (Osol et al., 2009).

Physical characteristics of the vessel wall are also poorly understood because of the plasticity of the vessel wall in response to genetic and environmental signals (Martinez-Lemus et al., 2009). In uterine arteries during pregnancy, Osol and colleagues have demonstrated increased distensibility of arteries likely due to decreases in collagen (Griendling et al., 1985) and elastin (Page et al., 2002) which may influence vascular capacitance of these vessels. Localized influences on vascular remodeling have been demonstrated to occur as opposed to systemic signals in unilateral horn ligation studies (Fuller et al., 2009). Mechanisms of uterine artery remodeling include vascular effects of estrogen such as increased DNA synthesis (Makinoda et al., 1986) and nitric oxide interactions with matrix metalloproteinases (Dumont et al., 2007; Grandas et al., 2009) and shear stress increases in endothelial nitric oxide synthase production (Tuttle et al., 2001).

Uterine artery remodeling during preeclampsia is largely unknown in women as these arteries are not readily accessible. However, Ong and colleagues have reported that
uterine biopsy myometrial uterine arteries from preeclamptic women display inward eutrophic remodeling indicative of hypertension (Ong et al., 2005). Studies of hypertension during pregnancy in animal models are also lacking. Endothelial nitric oxide synthase deficient knockout mice (Nos3 -/-) demonstrated decreased radial and medial cross sectional area of uterine arteries compared to wild type (van der Heijden et al., 2005). Osol (2009) and Barron (2010) report that chronic infusion of L-NAME (nitric oxide synthase inhibitor) attenuates the outward hypertrophic remodeling of uterine arteries during pregnancy. Additionally this same research group recently reported increases in uterine artery collagen and elastin and a decrease in matrix metalloproteinase 2 in L-NAME treated pregnant rats suggesting a possible mechanism preventing the vascular remodeling (Hale et al., 2011). Indeed, Myers (2005) and Raffetto (2008) report alterations in matrix metalloproteinase levels in preeclamptic women. Chronic hypoxia during pregnancy induced by gestational altitude incubation altered distensibility and stress-strain parameters of uterine arteries indicative of reduced uteroplacental blood flow (Mateev et al., 2006). Uterine artery remodeling in response to reductions in uterine perfusion pressure (a hallmark of preeclampsia), however, is currently unknown.

**VEGF and the VEGF 2 receptor**

Vascular endothelial growth factor (VEGF) is a growth factor in the sub-family of platelet-derived growth factors of cysteine knot factors. Alternative exon splicing of the VEGF gene has produced four prominent isoforms (VEGF-A, VEGF-B, VEGF-C, VEGF-D) with VEGF-E (viral) and VEGF-F (snake venom) not found endogenously in
mammals. The primary isoform of VEGF is VEGF-A which is involved in numerous cellular functions such as cellular migration, proliferation, permeability through fenestrations, and vascular function through nitric oxide dilation (Ferrara et al., 2005). VEGF-B is primarily involved in embryonic angiogenesis (Olofsson et al., 1996) and VEGF-C and VEGF-D in the lymphatic vasculature (Nagy et al., 2002; Karkkainen et al., 2002).

A prominent role for VEGF signaling during pregnancy has been demonstrated with VEGF knockout mice as these animals die as fetuses after parturition (Carmeliet et al., 1999). VEGF is primarily regulated by hypoxia via hypoxia induced factor (HIF-1) (Semenza, 2002). A role for VEGF has been established in pregnancy as vascular homeostasis is maintained by VEGF and to support angiogenesis in the uteroplacental unit (Karumanchi and Epstein, 2007). Deficiencies in VEGF have been implicated in preeclampsia resulting in poor vasculogenesis of the placenta and for the proteinuria and glomerular endotheliosis characterized in the disease in both humans and rodents (Ahmad and Ahmed, 2004; Maynard et al., 2003).

The VEGF 2 receptor (VEGFR-2) is a member of the receptor tyrosine kinase family and is also known as kinase insert domain containing region (KDR; in humans) and fetal liver kinase-1 (Flk-1; in rodents). The receptor consists of a seven immunoglobulin-life extracellular domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. VEGFR-2 is expressed on endothelial cells and is a co-receptor with heparin-sulfate proteoglycans and the neuropilins. VEGFR-2 is involved in numerous cellular functions such as endothelial cell survival, cellular proliferaton and permeability, actin reorganization, and nitric oxide release. Expression
is primarily regulated by hypoxia as the VEGFR-2 gene contains a hypoxia response element with HIF-2α (Elvert et al., 2003). The importance of the VEGFR-2 has been demonstrated in embryonic knockouts and VEGFR-2 knockout mice die in utero at Day 8.5 due to vascular disorganization and failed blood island formation (Sakurai et al., 2005).

Signaling pathways underlying the VEGFR-2 have been further elucidated in recent years. Early studies by Kroll and Waltenberger established VEGFR-2 as an activator of endothelial nitric oxide synthase (1997, 1998) and nitric oxide release (1999) in aortic endothelial cells. Subsequent studies have provided a critical role of VEGF-A stimulation (Kou et al. 2005), mitogen activated protein kinase (D’Angelo et al., 1995), and the ERK-Akt pathway (Endo et al., 2003). VEGFR-2 has been identified in uterine vascular endothelial cells (Zaitseva et al., 2004). Recently, a critical role of VEGFR-2 in the regulation of systemic blood pressure has been demonstrated through a connection with endothelial nitric oxide synthase expression and nitric oxide activity (Facemire et al., 2009).

**Gene Delivery Vectors**

Gene delivery vectors derived from viruses have been used primarily as a means of gene delivery because they are uniquely effective at transfecting host cells and sequestering this host’s cellular machinery to produce a desired end product (i.e. the transcription and translation of a viral gene). In addition to their ability to successfully transfect cells, their ability to target specific cells has made them an attractive vector for the delivery of genes particularly in disease states affected by defective alleles. Viruses
have the ability to transport across the plasma membranes of cells and evade lysosomes (Latchman, 2001) which have made them a prime candidate for delivery vehicles. However, clinical success of viral gene delivery vectors is lacking and the severe immune responses associated with viral gene delivery vectors have raised numerous safety concerns (Anderson, 1998). These concerns towards the protein coat of the viral capsid leading to immunogenic responses (Anderson, 1998) have been a primary concern due to the risk of possible viral recombination (Akinc et al., 2005). The increased risk of cancer from viral vectors (Woods et al., 2006) and fatalities associated with viral gene delivery vectors (Hollon, 2000) have raised serious safety points. Additionally, viral vectors have exhibited small loading capacities, cellular toxicity, and possess a high manufacturing cost. Thus their effectiveness as a potential gene delivery device for human therapy is currently lacking.

The advent of the development of non-viral gene delivery vectors came in response to the failed clinical trials associated with viral gene delivery vectors. Specifically, the design of vectors without using viruses (to prevent immunocompromising) but still achieving virus-like efficacy (Hollon, 2000). Two primary forms of non-viral gene delivery have been used as an alternative to viral vector based gene delivery. Condensation of DNA conjugated with cationic polymers or lipids or encapsulation of the genetic material within a degradable polymeric based device (Prokop et al., 2002) or a combination of both (Ditto et al., 2009). Condensed DNA conjugated to cationic materials has shown utility in vitro, but have shown limited efficacy as a gene delivery device in vivo due to cellular toxicity (Pouton and Seymour, 2001) and quick clearance rates within the body prior to successful transfection (Itaka et
Degradable polymers have been utilized to control the release of the genetic material from the polymeric device to provide more sustained release of DNA that may lead to enhanced transfection of the desired cells.

**Biodegradable Particles**

In order for more sustained and possibly more efficient transfection of the desired cellular target, biodegradable microparticles and nanoparticles have been utilized to circumvent the cellular toxicity and rapid clearance problems associated with cationic polymers and lipids (Prokop *et al*., 2002; Csaba *et al*., 2006). Biodegradable microparticles have been reported to successfully transfect tissues *in vivo* at a controlled rate (Yun *et al*., 2004; Duvvuri *et al*., 2005). However, the transfection efficiency is quite low possibly due to the fact that microparticles can not be endocytosed by cells therefore the DNA is being released extracellularly and having to be transported across the cell membrane by other means. Theoretically, much of the DNA may be lost at this stage due to the microparticles inability to enter the cell. Therefore, microparticles on a smaller scale (i.e. nanoparticles) may provide a better platform to enter the cell and release its genetic material inside the cell.

Nanoparticles as a gene delivery vehicle provide many advantages to efficient and sustained delivery of genetic material to cells. Specifically, nanoparticles provide the potential for intracellular delivery of their genetic material because of their nanoscale size which is easily internalized via endocytosis into cells (Desai *et al*., 1997). Additionally, nanoparticles can be manipulated by the addition of structural moieties as a potential cellular targeting mechanism (Ditto, 2010). While research into nanoparticles as a gene
delivery device is still early in its development, nanoparticles have shown promising results in vitro as an intracellular gene delivery device (Ditto et al., 2009). Research is further warranted into this emerging field gene delivery engineering (Ditto et al., 2009).

*L-tyrosine polyphosphate*

Amino acids are the building blocks of proteins and provide a unique opportunity to engineer delivery vehicles with the capabilities of drug/gene encapsulation and controlled release. Amino acids, however, are fused into protein structures via strong peptide bonds which make their utility in native form for delivery vehicles difficult. However, chemical modification of the amino acid backbone has shown utility in engineering amino acid base drug/gene delivery devices (Ditto et al., 2009).

Specifically, L-tyrosine provides a prime amino acid for chemical modification because of its biological compatibility and non-toxicity and physically because of its solubility (sen Gupta and Lopina, 2002). Chemical modification of the amino acid backbone involves the integration of non-peptide groups into the molecule. Specifically, L-tyrosine is modified via two separate blends of the molecule. First, desaminotyrosine is linked to the amino acid backbone via a peptide bond to the amine group of L-tyrosine (sen Gupta and Lopina, 2005). Desaminotyrosine was chosen because it structurally and chemically resembles L-DOPA, which is a natural metabolite of L-tyrosine through the catecholamine synthesis pathway via tyrosine hydroxylase (Nagatsu, 1995). The use of desaminotyrosine allows natural enzymatic degradation pathways to break down the delivery vehicle. The other chemical modification to the L-tyrosine backbone consists of linking phosphates (poly) to the R group via phosphoester bonds. The linking of the
monomers desaminotyrosine and phosphate groups confer solubility and can be easily shaped into spherical/circular arrangements as well as easily allowing it to be degraded through multiple mechanisms (sen Gupta and Lopina, 2005). Phosphoester bonds replace a carbonyl carbon with a phosphorous and this allows for hydrolysis of this linkage to occur due to its instability (sen Gupta and Lopina, 2005). Additionally, a hexyl ester bond to the carboxyl terminus of the L-tyrosine amino acid aids in the prevention of extra side products (sen Gupta and Lopina, 2005; Ditto et al., 2009). Polymerization of repeating units of the monomers yields L-tyrosine polyphosphate (LTP) which is classified as a pseudo (poly) amino acid (sen Gupta and Lopina, 2005) and have been applied to prepare devices for biomedical applications (Ditto et al., 2009; Hindi et al., 2009).
Figure 2.2 Structure of L-tyrosine polyphosphate (LTP)

*L-tyrosine Polyphosphate Nanoparticles*

Nanoparticles provide a unique platform for the design of gene delivery vehicles as their size is generally under 1 micron, which has been shown to be easily internalized by most cells (Desai et al., 1997), and they can be manipulated to provide sustained and controlled release of genetic material (Ditto et al., 2009). L-tyrosine polyphosphate is an ideal polymer for the design of nanoparticles as this polymer can easily be manipulated into nanoparticle size by standard emulsion and solvent evaporation techniques and because this polymer has a degradation profile of ~7 days *in vitro* which is well within
the life span of most cells (Ditto et al., 2009). LTP nanoparticles incorporate polyethylene glycol grafted to chitosan into the surface of the nanoparticles. This aids the stabilization of the emulsion and enhances biocompatibility by preventing plasma protein absorption to the nanoparticle surface (Ditto et al., 2009).

The biodegradation of the nanoparticles is a distinct advantage in developing a gene delivery vector. LTP degrades over the course of 7-10 days due to unstable and highly hydrolytic phosphoester bonds in the LTP polymer backbone (Ditto et al., 2009) and this has also been shown in LTP formulated films (sen Gupta and Lopina, 2002; sen Gupta and Lopina, 2005). Most nanoparticles formulated with poly(lactic-co-glycolic acid) (PLGA) have taken months for degradation and release of genetic materials (Duvvuri et al., 2005; Chen et al., 2006). Thus, genetic products released from many PLGA nanoparticles cannot adequately sustain transfection of cells due to the release products exceeding the lifetime of most cells. Also the degradation products of LTP (such as phosphates and alcohols) results in no appreciable change to localized pH (Ditto, 2010). However, poly-ester based nanoparticles formulated from PLGA and polycaprolactone (PCL) produce acidic byproducts by degradation of polyesters with this polymeric structure (Witt et al., 2000; Ara et al., 2002) and this can lead to immunogenic responses within the tissue (Ara et al., 2002). Conversely, LTP nanoparticles have demonstrated non-cytotoxicity in a cell line of human dermal fibroblasts (Ditto et al., 2009) and did not result in the activation of antigen-presenting cells in vitro suggesting that LTP nanoparticles can be internalized without eliciting much of an inflammatory response (Jawyn, 2011).
As a potential intracellular gene delivery device in vitro LTP nanoparticles have shown efficacy in a cell line of human dermal fibroblasts (Ditto et al., 2009). Recently, the in vivo efficacy of LTP nanoparticles has been tested. LTP nanoparticles encapsulated with silver carbine compounds has shown efficacy as a drug delivery device in a cystic fibrosis mouse model (Hindi et al., 2009). Additionally, drug loaded LTP nanoparticles have been demonstrated to enhance therapies aimed at a variety of clinically relevant bacterial and fungal pathogens (Leid et al., 2011). While these in vitro and in vivo studies are intriguing and further the potential use of LTP nanoparticles as a delivery device, the potential of LTP nanoparticles as an in vivo gene delivery device remains unknown.
Figure 2.3 Schematic depiction of an LTP-pDNA nanoparticle for gene delivery. LTP: L-tyrosine polyphosphate; PEG: polyethylene glycol; pDNA: plasmid DNA; LPEI: linear polyethylinimine.

**DNA-LPEI complexes**

Successful gene delivery using naked plasmid DNA (pDNA) without a carrier or delivery device has consistently been low (Ditto *et al.*, 2009). This is likely due to the fact that pDNA is easily degraded within blood plasma by enzymes and pDNA does not
readily cross the plasma membrane of cells due to its negative charge (Akinc et al., 2005). Therefore, cationic polyplexes have been engineered to create nanoscale assemblies of nucleic acids (small enough to enter the cell) with a carrier polymer to enhance transfection of the nucleic acid. Of particular interest has been the use of linear polyethylenimine (LPEI) as the polymer portion of the cationic polyplex. LPEI is a cationic polymer that has been shown to stabilize the emulsion formulation of LTP nanoparticles (Ditto et al., 2009). In addition, LPEI also protects the pDNA from shear forces during the nanoparticle formulation and is involved in cellular transport of the pDNA once within the cell (Ditto et al., 2009). Specifically, LPEI buffers the pH of endosomes by altering the osmotic gradient of the organelle and this allows the pDNA-LPEI complex encapsulated in the LTP nanoparticles to avoid degradation within the cell prior to its diffusion within the cytoplasm and ultimate arrival at the nucleus ("proton sponge theory") (Akinc et al., 2005).

LPEI has also exhibited enhanced transfection of cells compared to uncomplexed pDNA and can be easily manipulated with the nucleic acid to control for size by variation of ionic strength and concentration of formulation solutions (Ditto et al., 2009). In spite of the enhanced transfection effects of pDNA-LPEI complexes, high amounts of LPEI has been associated with increased cellular toxicity and can be clear quickly from the circulation (Watson et al., 2005) therefore not providing a viable option as a potential in vivo gene delivery device by itself. Encapsulation of pDNA-LPEI cationic polyplexes within LTP nanoparticles has shown promise as a potential intracellular gene delivery device (Ditto et al., 2009). Plasmid DNA conjugated to LPEI release from LTP
nanoparticles has shown enhanced transfection compared to uncomplexed pDNA and has exhibited low cytotoxicity in a cell line of human dermal fibroblasts (Ditto et al., 2009).

Figure 2.4 Structure of linear polyethylenimine (LPEI)

Conclusions

Preeclampsia is a devastating disease of pregnancy where alterations in uterine artery function and structure may play a role. Uterine blood flow and vascular resistance are directly regulated by uterine artery size and reactivity. Additionally, reductions in uterine perfusion pressure are involved in the pathophysiology of preeclampsia. Uterine artery reactivity, remodeling, and biophysical characteristics in response to reductions in uterine perfusion pressure are currently unknown therefore proper model selection is crucial to studying the effects of reduced uteroplacental perfusion. The uterine vascular bed is crucial to the maintenance and well-being of the fetus and to the overall health of the mother and should be explored. Insights into functional reactivity and structural
mechanics of these arteries may properly further avenues of research into how these arteries contribute to the pathology of preeclampsia.

Understanding the vascular mechanisms of the uterine circulation in normal and RUPP pregnancies may provide a foundation for the development of novel therapies aimed at the vascular level and the treatment of this disease. L-tyrosine polyphosphate nanoparticles possess a rapid release of their encapsulation and may provide a delivery vehicle for genetic material capable of altering vascular function. This delivery vehicle with its rapid release, non-cytotoxic nature, and targeting application may provide efficacy in the treatment of vascular disorders associated with preeclampsia and possibly other disease states.
CHAPTER III

CHRONIC REDUCTIONS IN UTERINE PERFUSION PRESSURE: IMPACT ON MATERNAL AND FETAL PREGNANCY OUTCOMES

Acknowledgements


Introduction

The etiology of preeclampsia is unknown, but is has been well established that the central role in the pathogenesis of the disease is a reduction in placental perfusion pressure (Roberts et al., 1989). The reduction in perfusion pressure to the placenta likely results in placental ischemia and has been shown to have a multifaceted effect on both the mother and the fetus. In the mother, placentally-derived signals damage the maternal endothelium (Roberts et al., 1989) and result in altered cardiovascular hemodynamics and increased arterial pressure (Chesley, 2009). In the fetus, placental ischemia from reduced uterine perfusion pressure can result in fetal hypoxia, growth restriction,
neurological deficits, and potential cardiovascular problems in adulthood (Barker et al., 1989; Bell et al., 1999). While research into the mechanisms associated with preeclampsia has been copious; no animal model to date truly represents the pathology except for the human female (Granger, 2007).

Preeclampsia is a heterogeneous disease with women experiencing a wide range and varying degrees of the pathology. For instance, fetal growth restriction occurs in 1/3 of all preeclamptic pregnancies in the presence of gestational hypertension (Chappell and Morgan, 2006) indicating reduced uterine perfusion is one of many factors involved in fetal growth restriction and possibly maternal blood pressure alterations. Animal modeling of this disease has taken numerous forms. Pharmacological models such as L-NAME induced hypertension (Yallampalli et al., 1993) and sFlt-1 overexpression during pregnancy (Maynard et al., 2003) have resulted in the characteristic phenotypes associated with preeclampsia. However, these models failed to represent the central component of the disease in humans namely the reduction in uterine perfusion pressure. Granger and colleagues developed the reduced uterine perfusion pressure pregnant rat model (RUPP) as an animal model of a preeclampsia-like pathophysiology (Crews et al., 2000) first described by Abitbol (1982). Historically, chronic reductions in uterine perfusion pressure in gravid rats have been demonstrated to increase mean arterial pressure and decrease fetal weight (Alexander et al., 2001). While reductions in uterine perfusion pressure seem prominent in the etiology of preeclampsia, its role in maternal blood pressure increases and altered fetal morphometrics is not fully elucidated. Furthermore, fetal morphometrical analyses on very late gestation fetuses (close to term) have not been performed. Indeed, previous studies in rats have demonstrated an increase
in growth the last two days of pregnancy (Langley-Evans et al., 1996) and therefore
detail fetal morphometrics is in order.

Concerning the RUPP model, there are two specific goals of this investigation 1) to
establish the RUPP pregnant rat model in our laboratory as a preeclampsia-like animal
model, 2) characterize maternal and fetal pregnancy outcomes in these animals. These
goals are designed to test the following hypotheses: 1) Chronic reductions in uterine
perfusion pressure will result in hypertension in gravid rats, 2) Maternal pregnancy
outcomes and fetal morphology will be detrimentally altered in RUPP rats compared to
SHAM operated controls. This will provide maternal and fetal characteristics that will be
used in subsequent experiments to assess the efficacy of the RUPP pathology as well as
parameters to determine therapeutic potential.

Materials and Methods

The purpose of this study was to demonstrate the effects of chronic reductions in
uterine perfusion pressure on pregnancy outcomes in both the mother and the fetuses.
This study will utilize two separate treatments consisting of SHAM surgery (n=10) and
reduced uterine perfusion pressure (RUPP) surgery (n=10).

Animals

Virgin, Sprague-Dawley rats 8-12 weeks old (200-250g) were housed singly
under standard cage conditions with food and water available ad libitum. Female rats
were mated overnight with a male rat and the presence of sperm on a vaginal smear was
used to designate Day 1 of pregnancy (full term 22-23 days). All protocols were
reviewed and approved by the Institutional Animal Use and Care Committee (IACUC) at the University of Akron (Reho et al., 2011; Reho et al., 2012).

Surgical Manipulation

Animals were subjected to surgical procedures on Day 14 of gestation. The RUPP surgical procedure has been described in detail elsewhere (Crews et al., 2000). Briefly, a midline incision was made to isolate the abdominal aorta. A silver clip (0.203 mm ID) was placed around the abdominal aorta below the renal arteries and above the iliac artery bifurcation. Blood flow increases to the uterine arcade adaptively during pregnancy through the uterine-ovarian arteries (Nienwartowicz et al., 1989), thus silver clips (0.106 mm ID) were placed around the uterine-ovarian arteries to reduce this response. This surgical manipulation reduces blood flow to the uteroplacental unit by ~40% (Crews et al., 2000). SHAM animals underwent the same surgery but did not receive the silver clips. Animals that completely resorbed all pups were excluded from the study (Reho et al., 2011; Reho et al., 2012).

Maternal Pregnancy Outcomes

On Day 21 of gestation, maternal body weights were determined prior to termination. The animals were then subjected to mean arterial pressure (MAP) analysis. The animals were similarly anesthetized with 2.5% Sodium Pentothal (50mg/kg IP; EJ Lily, Indianapolis, IN) and immediately surgically implanted with a carotid artery catheter (0.015in x 0.030in OD). The catheter was attached to a pressure transducer through a Doppler BioAmp (Crystal Biotech, Cincinnati, OH) and a 20 minute MAP
measurement was recorded. The data was collected and analyzed by computer data acquisition software (DATAQ, Akron, OH). At the conclusion of the MAP measurement, hematocrit was taken via the retro-orbital technique (Riley, 1960).

**Conceptus Morphometrics**

After termination and tissue collection, the uterus was exteriorized and fetal pups were counted and weighed along with placental weight. Litter sizes and fetal resorptions were also counted at this time. Fetal morphological measurements were taken as crown to rump lengths (cm), head circumference (cm), head area (cm²) using calipers. Head circumference was calculated using the circumference equation of a circle where circumference (C) = \( \pi \times d \) where d is equal to the diameter of the circle. Head area was determined by the equation for area of a circle. Area (A) = \( \pi \times r^2 \) where r is the radius of the circle. Further measurements of feto-placental ratios and ponderal indices were also calculated. Feto-placental ratios were determined with the following equation: fetal weight (g)/placental weight (g). Ponderal indices were determined by the following equation: (fetal weight(g)/length (cm³)) x 100.

**Statistical analysis**

Data are expressed as means ± SEM. Maternal mean arterial pressure, maternal weight, and fetal morphometrics were analyzed using a one way ANOVA and a Bonferroni post-hoc test. A Kruskal-Wallis one way ANOVA on ranks was used where applicable. Linear regression and Pearson’s correlation were used where applicable. Student’s t-test was used and statistical significance was accepted if p<0.05.
Results

Mean arterial pressure (MAP) was significantly elevated in RUPP mothers (Figure 3.1) compared to SHAM treated animals (F=21.9; p<0.001). Hematocrit (Figure 3.2) was significantly increased in RUPP animals compared to SHAM (F=6.1; p<0.05) rats.

Table 3.1 represents maternal pregnancy outcomes in response to reductions in uterine perfusion pressure. As expected, maternal weight was significantly reduced in RUPP (F=14.8; p<0.01) animals compared to SHAM. Similarly, percent weight gain was reduced in RUPP (F=13.1; p<0.01) mothers compared to SHAM from gestational Day 14 to Day 21. Gravid uterus weight was significantly reduced in RUPP mothers compared to SHAM (F=14.1; p<0.01) rats. Similarly, RUPP significantly reduced litter size compared to SHAM (F=14.3; p<0.01). Fetal resorptions were increased in RUPP rats compared to SHAM (F=21.6; p<0.001).

Figure 3.3 represents typical images of fetus and placentas from SHAM and RUPP pregnant rats. Table 3.2 represents fetal pregnancy outcomes associated with chronic reductions in uterine perfusion pressure. RUPP (F=59.4; p<0.001) rats demonstrated a significant reduction in placental weights compared to SHAM control. Fetal weights were reduced in RUPP (F=21.5; p<0.001) rats compared to SHAM rats. The feto-placental ratio (placental efficiency) was significantly reduced in RUPP rats compared to SHAM (F=4.7, p<0.05) a finding that mimics human preeclampsia (Gilbert et al., 2012). Crown to rump length was significantly reduced in fetuses from RUPP mothers compared to SHAM (F=9.5; p<0.05) confirming previous results from Joyner
and colleagues in Day 19 fetuses from RUPP mothers (Joyner et al., 2007). However, the ponderal index, a measure of leanness in fetuses, was unaltered between SHAM and RUPP fetuses suggesting that the RUPP is a model of late onset preeclampsia compared to early onset (Walker et al., 2003). Another measurement of fetal morphology is head circumference and head area. RUPP fetuses experienced a significant reduction in head circumference (F=9.9; p<0.01) and head area (F=10.4; p<0.01) compared to fetuses from SHAM operated rats.

Linear regression analysis of mean arterial pressure to litter size (Figure 3.4) demonstrated a positive correlation in RUPP (R²=0.764; F=16.2; p<0.01) pregnant rats. SHAM animals demonstrated a trend toward a correlation (R²=0.551; F=3.5; p=0.09). Similarly, a correlation was demonstrated in comparing mean arterial pressure to fetal resorptions (Figure 3.5) in RUPP (R²=0.585; F=7.1; p<0.05) pregnant rats. A correlation was also found comparing mean arterial pressure to fetal weight (Figure 3.6) in RUPP (R²=0.880; F=36.6; p<0.01) pregnant rats.
Figure 3.1 Mean Arterial Pressure (mmHg) for SHAM and RUPP pregnant rats on Gestational Day 21 (means, ± SEM). Mean arterial pressure was significantly increased in RUPP rats compared to SHAM (*p<0.05).
Figure 3.2 Hematocrit for SHAM and RUPP pregnant rats on Gestational Day 21 (means, ± SEM). Hematocrit (expressed as percentage of plasma to red blood cells) was significantly increased in RUPP rats compared to SHAM (p<0.05).
### Table 3.1 Maternal pregnancy outcomes of SHAM and RUPP pregnant rats (means, ± SEM).  *p<0.05

<table>
<thead>
<tr>
<th>Maternal Pregnancy Outcomes</th>
<th>Treatment</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>RUPP</td>
</tr>
<tr>
<td>Maternal Body Weight (g)</td>
<td>411.8 ± 17.7</td>
<td>*331.8 ± 10.9</td>
</tr>
<tr>
<td>Percent Body Weight Gain</td>
<td>19.6 ± 1.3%</td>
<td>*6.9 ± 3.1%</td>
</tr>
<tr>
<td>(Gestational Day 14 – Day 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravid Uterus Weight (g)</td>
<td>87.7 ± 10.8</td>
<td>*34.6 ± 9.0</td>
</tr>
<tr>
<td>Litter Size</td>
<td>13.8 ± 1.4</td>
<td>*4.8 ± 1.9</td>
</tr>
<tr>
<td>Fetal Resorptions</td>
<td>0.3 ± 0.1</td>
<td>*10.2 ± 2.1</td>
</tr>
</tbody>
</table>
Figure 3.3 Typical images of fetuses and placentas from SHAM and successful RUPP rats. A) SHAM fetus, B) RUPP fetus, C) SHAM placenta, D) RUPP placenta.
Table 3.2 Fetal pregnancy outcomes of SHAM and RUPP pregnant rats (means, ± SEM).

*p<0.05
Figure 3.4 Linear regression for maternal mean arterial pressure (mmHg) and litter size from SHAM and RUPP pregnant rats. As mean arterial pressure increases, litter size decreases in RUPP pregnant rats ($R^2=0.764; F=16.2; p<0.01$). SHAM demonstrated a trend toward decreased litter size ($R^2=0.304; F=3.5; p=0.09$).
Figure 3.5 Linear regression for maternal mean arterial pressure (mmHg) and fetal resorptions from SHAM and RUPP pregnant rats. As mean arterial pressure increases, fetal resorptions increase in RUPP ($R^2=0.585; F=7.1; p<0.05$) pregnant rats.
Figure 3.6 Linear regression for maternal mean arterial pressure (mmHg) and fetal weight from SHAM and RUPP pregnant rats. As mean arterial pressure increases, fetal weight decrease in RUPP ($R^2=0.880$; $F=36.6$; $p<0.001$) pregnant rats. SHAM demonstrated a trend toward decreased fetal weight ($R^2=0.386$; $F=3.1$; $p=0.1$).
Discussion

The results of this investigation support the hypotheses that chronic reductions in uterine perfusion pressure alter maternal and fetal pregnancy outcomes that provide similarities to preeclampsia. The major findings of this study include 1) RUPP pregnancy demonstrated a significant rise in maternal blood pressure compared to SHAM control (~20 mmHg). 2) Maternal pregnancy outcomes were altered in RUPP pregnant rats compared to SHAM. 3) Fetuses from RUPP rats displayed growth restriction compared to SHAM with morphological decreases in length and overall size.

Elevated maternal blood pressure is a hallmark of the pathology associated with preeclampsia, which is a human disease. Previous studies from our laboratory (Ramirez et al., 2011; Reho et al., 2011) and this current study, confirm that chronic reductions in uterine perfusion pressure results in hypertension in pregnancy (~20 mmHg increase). This increase in blood pressure from RUPP rats altered a number of maternal parameters indicative of an adverse pregnancy environment associated with the pathology. Hematocrit was significantly increased in gestational Day 21 RUPP rats to a similar degree demonstrated in preeclamptic women (Golboni et al., 2011) compared to SHAM control. This finding likely reflects the increased arterial pressure effect on the vascular wall and could also indicate increased inflammatory pathways. Indeed, Heilmann (1981) found a positive correlation between hematocrit and mean arterial pressure during preeclampsia. Vessel walls and perfusion endpoint tissues likely absorb more blood plasma leading to hemoconcentration which may ultimately influence flow properties of the blood itself (i.e. viscosity) (Heilmann et al., 1981). As vasoconstriction and increased arterial pressure are common facets of the preeclampsia vascular pathology, the increased
hematocrit in connection with the reduced vessel radius likely can lead to altered perfusion hemodynamics.

Maternal body weight and percentage weight gain were significantly reduced in RUPP rats compared to control. Accordingly, gravid uterus wet weights and litter size were reduced in RUPP rat compared to SHAM animals while the RUPP pathology also led to severe reductions in viable fetuses. These data suggest that the pathology associated with the RUPP model possibly leads to reduction in the stimulus of the ischemia response (i.e. the ischemic placenta) potentially as a compensation mechanism to combat the increased arterial pressure.

Preeclampsia is a heterogeneous disease as women experience varying degrees of the pathology. Fetal growth restriction occurs in 1/3 of all preeclamptic pregnancies (Chappell and Morgan, 2006) and is likely due to the degree of reduction in uterine perfusion pressure, the timing of the uterine insult as well as the maternal cardiovascular response. Previous data from our lab has demonstrated severe reductions in fetal weight in response to chronic reductions in uterine perfusion pressure (Ramirez et al., 2011; Reho et al., 2011). The current study confirmed our laboratories previous results and expanded into morphological measurements of the fetal weight reduction. Fetal and placental weights were reduced in RUPP pregnant rats compared to SHAM. The feto-placental ratios (a measurement of placental efficiency) were reduced in RUPP rats compared to SHAM, a finding confirming recently published data from Gilbert and colleagues (Gilbert et al., 2012). Additionally, the crown rump length of fetuses (a measurement of overall length) was reduced in RUPP fetuses compared to SHAM confirming previous data from Day 19 RUPP fetuses (Joyner et al., 2007). This study
has also demonstrated the positive correlation of mean arterial pressure with successful pregnancy outcome measurements such as litter size, fetal resorption, and fetal weights. As the severity of the hypertension increases in the rats, the number of viable fetuses and size of the fetuses decreases further demonstrating the role of hypertension in the pathology associated with reduced uterine perfusion pressure.

This investigation found no significant differences between RUPP or SHAM fetuses for the ponderal index measurement. Ponderal index is a measure of the overall leanness in a fetus/newborn and can be used to assess the in utero environment characteristics of development during late pregnancy (Walker et al., 2003). In preeclampsia, the ponderal index is reduced however; in our animals we did not demonstrate any changes. Thus reductions in uterine perfusion pressure alone are not sufficient to lead to ponderal index changes and the RUPP model is likely a model of late-onset preeclampsia as opposed to early onset.

Interestingly, fetal head circumference and head area were decreased in RUPP rats compared to SHAM fetuses. Kajantie and colleagues have demonstrated that preeclampsia is associated with decreased head circumference in fetuses (Kajantie et al., 2009). Recently, the increases in the soluble VEGF 1 receptor variant (sFlt-1) associated with preeclampsia have been negatively correlated with fetal head circumference (Kulkarni et al., 2011). The RUPP model has been demonstrated to exhibit increases in sFlt-1 in response to placental ischemia (Gilbert et al., 2007). Measurements of sFlt-1 were not taken in this study to make correlation with fetal morphology. Future studies will be designed to investigate this potential mechanism.
In conclusion, we have successfully established the reduced uterine perfusion pressure (RUPP) animal model of a preeclampsia-like pathology in our laboratory. The current study established maternal and fetal parameters that can be used to assess the maternal and fetal pregnancy outcome. Reductions in uterine perfusion pressure detrimentally altered many maternal pregnancy parameters such as mean arterial pressure, hematocrit and body weight gain. Fetal pregnancy parameters are also detrimentally altered such as an overall decrease in fetal weight, length, and head size. Taken together it’s clear that reductions in uterine perfusion pressure play an integral role in the successful outcomes of pregnancy and these outcomes can be used as endpoints for pregnancy success and potential end point therapeutic markers for the study of reductions in uterine perfusion pressure.
CHAPTER IV

ALTERED VASCULAR REACTIVITY OF RESISTANCE-CALIBER UTERINE ARTERIES FROM PREGNANT RATS WITH REDUCED UTERINE PERFUSION PRESSURE

Acknowledgement

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Introduction

Reductions in uterine perfusion pressure are thought to be a central component in the pathogenesis of preeclampsia. Preeclampsia is a heterogeneous disease that results in
maternal hypertension, vascular dysfunction, and end organ underperfusion (Chesley, 2009). A major contributing factor to these characteristics is endothelial dysfunction, which is a hallmark of preeclampsia (Roberts et al., 1989), with alterations in vasoconstrictive and vasodilatory pathways compared to normal pregnant women (Sankaralingam et al., 2006). These alterations in the functional reactivity of vasculature may lead to the end organ underperfusion and placental ischemia characterized in preeclampsia (Reho et al., 2012).

The reduced uterine perfusion pressure (RUPP) gravid rat is an animal model of preeclampsia that mimics several characteristics of the vascular dysfunction associated with preeclampsia (Khalil and Granger, 2002). Studies using the RUPP model have demonstrated detrimentally altered vascular myogenic responses in the mesenteric and renal circulations (Ramirez et al., 2011) as well as decreased vasodilatory (Ramirez et al., 2011; Reho et al., 2011) and increased vasoconstrictive pathways (Anderson et al., 2006). Furthermore, mechanisms of intracellular calcium (Chen and Khalil, 2008) and endothelial nitric oxide (Ramirez et al., 2011; Reho et al., 2011) may contribute to the vascular dysfunction noted in these animals. Studies within the uterine circulation of RUPP animals are currently unknown with only one study to date by Anderson and colleagues (2005). This study used wire myography setting to investigate uterine arcuate arteries from RUPP rats where vasoconstriction was found to be increased (Anderson et al., 2005). While these arteries are important to the understanding of the vascular regulation within the uterine circulation, uterine arteries further downstream within the uterine arcade may provide more mechanistic regulation through resistance changes within the uteroplacental circulation (Reho et al., 2012).
It is clear that reductions in uterine perfusion pressure are central to the pathogenesis of preeclampsia. However, the role of these reductions in uterine perfusion pressure on the functional reactivity of resistance-sized uterine arteries is currently unknown (Reho et al., 2012). Functional arterial reactivity parameters such as myogenic reactivity as well as vasodilatory and vasoconstrictive capabilities of uterine arteries can provide an \textit{in vitro} assessment of the functional state of the uterine resistance vasculature. Therefore, the hypotheses to be tested in this investigation are that 1) chronic reductions in uterine perfusion pressure in pregnant rats will result in increases in myogenic reactivity of resistance-sized uterine arteries compared to SHAM control. Additionally, we hypothesize that RUPP will result in decreases in vasodilation of the uterine arteries indicative of a defective endothelial nitric oxide response.

**Materials and Methods**

Eight to twelve week old Sprague Dawley female rats were used for this investigation. Animals were housed in pairs in standard polyethylene cages (46 x 20 x 20cm) with stainless steel tops. Heated treated wood chip bedding (PJ Murphy, Montville, NJ) and standard rat chow (Teklad Rodent, Indianapolis, IN) and water were provided \textit{ad libitum}. The animals were on a 12hr light (07:00-19:00)/dark (19:00-07:00) cycle with a constant temperature of 23-25°C and ~40% relative humidity. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Akron (Reho et al., 2012).
Mating

Females were placed with males overnight and the presence of sperm on a vaginal smear was determined as Day 1 of pregnancy (22-23 day gestation). After successful mating, animals were then singly housed for the duration of their pregnancy.

Surgery Manipulation

On Day 14 of gestation, animals were subjected to RUPP or SHAM surgery as previously described in detail by our lab (Ramirez et al., 2011; Reho et al., 2011) and others (Crews et al., 2000). For the RUPP surgical procedure, a midline incision was made in the abdomen and the abdominal aorta was exposed below the renal arteries and above the iliac bifurcation. A silver clip (0.203mm ID) was placed around the abdominal aorta. Due the adaptive response of the uterine arcade to this reduction in uterine perfusion (Nienartowicz et al., 1989), the uterine-ovarian arteries were clipped with 0.106mm ID silver clips below the ovary and before the branching of the first pup in the uterus (Reho et al., 2012).

Termination

Animals were terminated on Day 21 of gestation by an overdose of 2.5% Sodium Pentothal. All animals where the surgery resulted in complete resorption of fetuses were excluded from the study.
Pressure Arteriograph

The uterus and mesentery were placed into an ice cold physiological HEPES buffered saline solution consisting of the following concentrations (mmol/L): sodium chloride 142, potassium chloride 4.7, magnesium sulfate 1.17, calcium chloride 2.5, potassium phosphate 1.18, HEPES 10, dextrose 5.5 (Toot et al., 2011). The pH of the solution was adjusted to 7.4 with 1M sodium hydroxide. Resistance-sized uterine arteries (200-300 μm) were isolated from the third branch of the main uterine artery (or the second branch depending on availability). Resistance-sized mesenteric arteries (200-300 μm) were also isolated. Arteries were cleaned of adipose and connective tissue debris and mounted between two glass microcannulas in a pressure arteriograph bath chamber (CH/1/SH; Living Systems, Burlington, VT). Any residue blood within the vessel was flushed out by the proximal cannula and the distal cannula was occluded. Pressure was maintained at 60 mmHg for a 45 minute equilibration period with fresh, heated (37°C) HEPES buffer added. The artery was then conditioned to stretch by increasing the pressure from 60 to 100 mmHg and then returning to 60 mmHg for an additional 15 minute equilibration period with fresh HEPES buffer added to the bath. (Reho et al., 2012).

Myogenic Reactivity

Arteries were preconstricted to 70-85% of their initial diameter with the α-adrenergic agonist phenylephrine. This small of constriction eliminates tone differences between arterial preparations (Gandley et al., 1997) and has been demonstrated to optimize the myogenic response (MacPherson et al., 1991).
Myogenic reactivity was calculated as a percent change in diameter from the initial arterial diameter at 20 mmHg using the following equation: 
\[
\left( \frac{D_x - D_{20}}{D_{20}} \right) \times 100
\]
where \( D_x \) represent the intraluminal diameter at a specific transmural pressure and \( D_{20} \) represents the intraluminal diameter at 20 mmHg. This equation can be used to indicate the constrictive or dilatory properties of the blood vessel in response to changes in intraluminal pressure. A positive percent change is indicative of dilation, while a negative percent change is indicative of constriction. Increased myogenic reactivity is represented by a net zero or negative percent change in arterial diameter from its initial diameter at 20 mmHg (Reho et al., 2012).

**Methacholine Vasodilation**

Arteries were preconstricted to 50% of their initial diameter with phenylephrine. Uterine arteries were then exposed to cumulative concentrations of the endothelium dependent vasodilator methacholine (1nmol - 3μmol). Similarly mesenteric arteries were exposed to cumulative concentrations of methacholine (5nmol - 1μmol). Arterial responses were expressed as percent dilation in response to phenylephrine constriction. Methacholine dilation curves were repeated in the presence of L-NMA to block nitric oxide synthase production (Reho et al., 2012).

**VEGF Vasodilation**

Arteries were preconstricted to 50% of their initial diameter with phenylephrine. Uterine arteries were then exposed to cumulative concentrations of vascular endothelial growth factor (VEGF) (0.05nmol – 9nmol). Arterial responses were expressed as percent...
dilation in response to phenylephrine constriction. VEGF dilation curves were repeated in the presence of L-NMA to block nitric oxide synthase production (Reho et al., 2012).

*Sodium Nitroprusside Vasodilation*

Arteries were preconstricted to 50% of their initial diameter with phenylephrine. Uterine arteries were exposed to cumulative concentrations of the endothelium independent vasodilator sodium nitroprusside (1nmol - 3μmol). Arterial responses were expressed as percent dilation in response to constriction (Reho et al., 2012).

*Phenylephrine Constriction*

Uterine arteries were exposed to increasing cumulative concentrations of phenylephrine (0.01μmol to 3μmol). Mesenteric arteries were exposed to increasing cumulative concentrations of phenylephrine (0.1μmol – 7μmol). Data were analyzed as percent constriction and then normalized for percent maximum constriction (Reho et al., 2012).

*U-46619 Constriction*

Uterine arteries were exposed to increasing cumulative concentration of U-46619, a thromboxane receptor agonist (1nmol - 3μmol). Mesenteric arteries were also exposed to increasing cumulative concentrations of U-46619 (1nmol - 3μmol). Data were analyzed as percent constriction and then normalized for percent maximum constriction (Reho et al., 2012).
**Statistical Analysis**

Sigma Stat and Sigma Plot software were used for the statistical and graphical representation of this data (SYSTAT, San Jose, CA). Arterial bioassays were analyzed using a two way repeated measures ANOVA with a post-hoc Bonferroni test. EC$_{50}$ values were analyzed with a one way ANOVA. Statistical significance was accepted if $p<0.05$.

**Results**

Myogenic reactivity of resistance-caliber uterine arteries was increased in RUPP rats ($F=6.6; p<0.05$) compared to SHAM (Figure 4.2.A). Similarly, myogenic reactivity of resistance-sized mesenteric arteries was increased in RUPP rats ($F=40.1; p<0.001$) compared to SHAM (Figure 4.2.B). Preincubation of uterine arteries from SHAM pregnant rats with N-methyl-L-arginine (L-NMA) resulted in a significant increase in myogenic reactivity ($F=8.7; p<0.05$) compared to untreated arteries. The mesenteric circulation was similar in that L-NMA preincubation significant increased myogenic reactivity of resistance mesenteric arteries ($F=18.5; p<0.01$). Preincubation of uterine (Figure 4.3.A) and mesenteric (Fig 4.3.B) arteries from RUPP rats with L-NMA resulted in no significant change in myogenic reactivity as these arteries responded to pressure increases with vasoconstriction.

Agonist induced vasodilation with methacholine, an endothelium dependent vasodilator, was significantly decreased in uterine arteries (Figure 4.4.A) from RUPP animals ($F=9.8; p<0.05$) compared to SHAM. Similarly, resistance-sized mesenteric arteries from RUPP rats (Figure 4.4.B) also demonstrated a significant decrease in
methacholine induced vasodilation compared to SHAM control (F=5.5; p<0.05). Preincubation of uterine arteries from SHAM pregnant rats (Figure 4.5.A) with N-methyl-L-arginine (L-NMA) resulted in a significant decrease in methacholine induced vasodilation (F=8.4; p<0.05) compared to untreated arteries. The mesenteric circulation in SHAM rats (Figure 4.5.B) was similar in that L-NMA preincubation significant increased myogenic reactivity of resistance mesenteric arteries (F=8.3; p<0.05). Preincubation of uterine (Figure 4.6) and mesenteric (Fig 4.7) arteries from RUPP rats with L-NMA resulted in no significant change in methacholine induced vasodilation as these arteries responded similarly to untreated arteries. Agonist induced vasodilation to VEGF (Figure 4.8) was significantly reduced in uterine arteries from RUPP rats (F=30.3; p<0.01) compared to SHAM control. Preincubation of uterine arteries from SHAM pregnant rats (Figure 4.9.A) with N-methyl-L-arginine (L-NMA) resulted in a significant decrease in VEGF induced vasodilation (F=12.6; p<0.05) compared to untreated arteries. Preincubation of uterine (Figure 4.9.B) arteries from RUPP rats with L-NMA resulted in no significant change in VEGF induced vasodilation as these arteries responded similarly to untreated arteries. Endothelium-independent vasodilation with sodium nitroprusside (SNP) was unaltered in uterine arteries from RUPP and SHAM animals (Figure 4.10).

Vasoconstriction to the α-adrenergic agonist phenylephrine (Figure 4.11) and the thromboxane agonist U-46619 (Figure 4.12) were unaltered between RUPP and SHAM animals for both uterine and mesenteric arteries. Effective concentration to constrict the blood vessel to 50% of the total diameter (EC$_{50}$) with phenylephrine (Table 4.1) was unaltered in RUPP uterine arteries compared to SHAM as was found in uterine arteries in
response to U-46619. No differences were found in EC$_{50}$ for mesenteric arteries (Table 4.2) in response to phenylephrine or U-46619.
Figure 4.1 Typical image of a gravid uterine horn. The arrow refers to the type of uterine artery (third order branch) isolated for the arteriograph experiments.
Figure 4.2 Myogenic reactivity of resistance-caliber uterine (A) and mesenteric (B) arteries from RUPP and SHAM pregnant rats. RUPP significantly increased myogenic reactivity in uterine and mesenteric arteries compared to SHAM. *p<0.05
Figure 4.3 Myogenic reactivity of resistance-caliber uterine arteries preincubated with N-methyl-L-arginine (L-NMA) from SHAM (A) and RUPP (B) animals. Uterine arteries from SHAM rats incubated with L-NMA displayed increased myogenic reactivity compared to untreated arteries. Uterine arteries from RUPP animals incubated with L-NMA did not differ in response. *p<0.05
Figure 4.4 Myogenic reactivity of resistance-caliber mesenteric arteries preincubated with N-methyl-L-arginine (L-NMA) from SHAM (A) and RUPP (B) animals. Mesenteric arteries from SHAM rats incubated with L-NMA displayed increased myogenic reactivity compared to untreated arteries. Mesenteric arteries from RUPP animals incubated with L-NMA did not differ in response. *p<0.05
Figure 4.5 Methacholine induced vasodilation of resistance-caliber uterine (A) and mesenteric (B) arteries from RUPP and SHAM pregnant rats. RUPP significantly decreased methacholine vasodilation in uterine and mesenteric arteries compared to SHAM. *p<0.05
Figure 4.6 Methacholine induced vasodilation of resistance-caliber uterine arteries preincubated with N-methyl-L-arginine (L-NMA) from SHAM (A) and RUPP (B) animals. Uterine arteries from SHAM rats incubated with L-NMA displayed decreased methacholine induced vasodilation compared to untreated arteries. Uterine arteries from RUPP animals incubated with L-NMA did not differ in response. *p<0.05
Figure 4.7 Methacholine induced vasodilation of resistance-caliber mesenteric arteries preincubated with N-methyl-L-arginine (L-NMA) from SHAM (A) and RUPP (B) animals. Mesenteric arteries from SHAM rats incubated with L-NMA displayed decreased methacholine induced vasodilation compared to untreated arteries. Mesenteric arteries from RUPP animals incubated with L-NMA did not differ in response. *p<0.05
Figure 4.8 Vascular endothelial growth factor (VEGF) induced vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats. RUPP significantly decreased VEGF vasodilation in uterine arteries compared to SHAM. *p<0.05
Figure 4.9 VEGF induced vasodilation of resistance-caliber uterine arteries preincubated with N-methyl-L-arginine (L-NMA) from SHAM (A) and RUPP (B) animals. Uterine arteries from SHAM rats incubated with L-NMA displayed decreased VEGF induced vasodilation compared to untreated arteries. Uterine arteries from RUPP animals incubated with L-NMA did not differ in response. *p<0.05
Figure 4.10 Sodium nitroprusside (SNP) induced vasodilation of resistance-caliber uterine arteries from RUPP and SHAM pregnant rats. No differences in response were noted.
Figure 4.11 Phenylephrine induced vasoconstriction of resistance-caliber uterine (A) and mesenteric (B) arteries from RUPP and SHAM pregnant rats. No difference between treatment groups was noted.
Figure 4.12 U-46619 induced vasoconstriction of resistance-caliber uterine (A) and mesenteric (B) arteries from RUPP and SHAM pregnant rats. No difference between treatment groups was noted.
Table 4.1 Effective concentration of phenylephrine and U-46619 to constrict the uterine arteries to 50% of the total diameter (EC$_{50}$) (means, ± SEM).
Table 4.2 Effective concentration of phenylephrine and U-46619 to constrict the mesenteric artery to 50% of the total diameter (EC$_{50}$) (means, ± SEM). *p=0.06
Discussion

This is the first report of the functional reactivity of resistance-caliber uterine arteries from pregnant rats with reduced uterine perfusion pressure (RUPP) (Reho et al., 2012). The major findings of this investigation are that 1) myogenic reactivity is increased in resistance-caliber uterine arteries from RUPP rats compared to control, 2) there is a decreased vasodilation response (methacholine and VEGF) of the uterine arteries from RUPP rats that may be due to deficits in vascular nitric oxide, 3) agonist induced vasoconstriction is unaltered between RUPP and SHAM uterine arteries. These findings support our hypothesis of an increased vasoconstrictive phenotype of resistance-caliber uterine arteries in response to chronic reductions in uterine perfusion pressure (Reho et al., 2012). The findings of this investigation also support the hypothesis that vascular nitric oxide is altered in uterine arteries in response to RUPP (Reho et al., 2012).

The vascular myogenic response is an intrinsic property of the vasculature that involves the integration of all three layers of the blood vessel (i.e. the endothelium, tunica media, and adventitia) (Mulvany, 2002). This mechanism is the active response of the blood vessel to changes in transmural pressure and can be indicative of resistance within an arterial arcade. Myogenic reactivity was increased in uterine arteries isolated from RUPP pregnant rats compared to SHAM controls (Reho et al., 2012) a finding that was also demonstrated in the mesenteric circulation (Ramirez et al., 2011; Reho et al., 2011). These data indicate an enhanced constrictive phenotype of uterine arteries in response to RUPP and may be a potential mechanism for the maternal hypertension and fetal growth restriction noted in this animal model (Chapter III).
Vascular nitric oxide is a prominent vasodilator of resistance arteries during pregnancy and deficits in nitric oxide have been implicated in the pathology associated with preeclampsia (Roberts et al., 1989; Sladek et al., 1997). As myogenic reactivity of uterine arteries was increased in RUPP animals, we sought to investigate the role of vascular nitric oxide in the mediation of this response. Blockade of nitric oxide with N-methyl-L-arginine (L-NMA; a nonspecific nitric oxide synthase inhibitor) resulted in a significant increase in myogenic reactivity of uterine arteries from SHAM pregnant controls but not in uterine arteries from RUPP animals. These data suggest an integral role for nitric oxide in mediation of the myogenic response in these arteries from RUPP pregnant rats. This finding also extended into the mesenteric circulation suggesting the potential for whole animal endothelial dysfunction (Ramirez et al., 2011).

Myogenic reactivity is determined by the balance of vasoconstriction and vasodilation responses. We thus sought to investigate whether vasodilation, vasoconstriction or both were altered in response to reductions in uterine perfusion pressure and what the role of nitric oxide was in these responses. Previous studies from our laboratory have demonstrated that resistance-caliber mesenteric arteries exhibit decreased endothelium dependent vasodilation in response to reductions in uterine perfusion pressure (Ramirez et al., 2011; Reho et al., 2011). Additionally, vasodilation responses of large conduit arteries are reduced in RUPP rats (Crews et al., 2000). These data suggest a potential for whole animal nitric oxide deficits as L-arginine supplementation to RUPP rats decreased blood pressure and improved fetal growth (Alexander et al., 2004). Furthermore, these data suggest a potential for improvement of blood flow within the uteroplacental circulation as fetal weight was improved. Thus, we
sought to determine if altered vasodilation was also found in the uterine circulation by investigating two separate vasodilators. Methacholine and vascular endothelial growth factor (VEGF) induced vasodilation were significantly decreased in resistance-caliber uterine arteries from RUPP pregnant rats compared to SHAM controls (Reho et al., 2012). Blockade of nitric oxide synthase with N-methyl-L-arginine (L-NMA) significantly reduced the vasodilation response of uterine arteries to both methacholine and VEGF in SHAM controls but not in uterine arteries from RUPP pregnant rats. These data suggest that endothelial mediated vasodilation in resistance-caliber uterine arteries is decreased in response to the RUPP pathology (Reho et al., 2012). To confirm this hypothesis, we subjected the uterine arteries to vasodilation with the endothelium independent vasodilator sodium nitroprusside. Sodium nitroprusside (SNP) is a nitric oxide donor that exhibits only autocoid functions and can be used to assess whether there are any differences in the cellular machinery of the uterine artery nitric oxide dilatory response. Uterine arteries from RUPP and SHAM pregnant rats responded similarly to SNP suggesting that the nitric oxide pathway in the uterine arteries is intact while the defect likely lies within the endothelial signaling to the vascular smooth muscle (Reho et al., 2012) possibly indicating a damaged endothelium.

As the uterine arteries from RUPP pregnant rats exhibited increased myogenic reactivity and decreased vasodilation responses we also investigated agonist induced vasoconstriction in these arteries. Previous studies using wire myographs on mesenteric and uterine arteries from RUPP animals have demonstrated an enhanced vasoconstrictive response of these arteries to phenylephrine and angiotensin II (Anderson et al., 2005; Anderson et al., 2006). However, our laboratory has demonstrated no differences in
vasoconstriction to phenylephrine in resistance mesenteric arteries following RUPP
(Ramirez et al., 2011) and this is likely due to vascular technique (Wire myograph versus
pressure arteriograph) (Dunn et al., 1994). Vasoconstrictive responses to phenylephrine
(α-adrenergic receptor agonist) and U-46619 (a thromboxane mimetic) were similar in
uterine and mesenteric arteries from RUPP and SHAM pregnant rats suggesting no
differences in agonist induced vasoconstriction in these animals. This confirms a
previous report from our laboratory in the mesenteric circulation (Ramirez et al., 2011).

In closing, this is the first report of the impact of reductions in uterine perfusion
pressure on uterine arterial reactivity in pregnant rats (Reho et al., 2012). Our study
demonstrates that vascular myogenic reactivity is increased in resistance-caliber uterine
arteries from RUPP animals as well as a decrease in agonist induced vasodilation of these
arteries. Agonist induced vascular constriction is intact and unaltered in uterine arteries
from these animals. These data suggest that reductions in uterine perfusion pressure alter
the vascular responses of the uterine circulation towards a vasoconstrictive phenotype
that may be involved in the mechanism of high blood pressure and fetal growth
restriction noted in this animal model and potentially in preeclampsia.
CHAPTER V

VASCULAR TONE AND PASSIVE STRUCTURAL MECHANICS OF RESISTANCE-CALIBER UTERINE ARTERIES FROM PREGNANT RATS WITH CHRONIC REDUCTIONS IN UTERINE PERFUSION PRESSURE

Acknowledgements

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Introduction

Pregnancy is associated with profound alterations in the uterine hemodynamics. It is these alterations that ensure proper blood flow to the fetoplacental unit and ultimately result in a successful pregnancy outcome. In pregnant women, uterine blood flow increases 10 fold to 750 mL/min at term (Bader et al., 1968). Similarly in the pregnant rat, uterine blood flow nearly doubles by midgestation and reaches a 14 fold increase at its peak compared to nonpregnant levels (Bruce, 1976; Ahokas et al., 1983).

The increase in uterine blood flow demonstrated during pregnancy is likely due to a decrease in vascular resistance within the uterine arcade thereby increasing perfusion (Osol and Mandala, 2009). This increase in perfusion can be achieved through functional
alterations of the uterine arteries such as a reduction in intrinsic tone and the localized
production of vasodilators leading to a larger arterial diameter (Osol and Mandala, 2009).
In addition to functional changes, uterine arteries also undergo extensive structural and
mechanical remodeling during pregnancy resulting in low resistance high capacitance
vessels and thereby increasing blood flow within the uterine circulation (Mandala and
Osol, 2012).

The remodeling of uterine arteries during pregnancy involves numerous
mechanisms. The smooth muscle cells of uterine arteries undergo significant
hypertrophy and hyperplasia (Cipolla and Osol, 1994) and small uterine arteries remodel
outward to allow for a larger diameter for perfusion (Osol and Mandala, 2009). Research
has also demonstrated a role for nitric oxide in the structural and mechanical remodeling
of uterine arteries as L-NAME (nitric oxide synthase inhibitor) attenuates uterine artery
remodeling (Barron et al., 2010). Accordingly, studies from our laboratory has
demonstrated that chronic reductions in uterine perfusion pressure result in deficits in
nitric oxide regulation of arterial reactivity (Ramirez et al., 2011; Reho et al., 2011). As
nitric oxide is altered in this animal model of preeclampsia, these data indicate a potential
for uterine artery structural and mechanical remodeling to occur as a result of the
hypertensive pathology. In the hypertensive human disorder of pregnancy, preeclampsia,
placental perfusion is reduced (Chesley, 2009) and inhibition of trophoblastic spiral
artery remodeling has been demonstrated to reduce placental perfusion in pregnant rats
(Verlohen et al. 2010). Recently reductions in uterine perfusion pressure have been
demonstrated to result in increased uterine artery resistance in pregnant rats (Tam Tam et

77
These data suggest a potential for uterine artery structural and mechanical remodeling to occur in response to hypertension in pregnancy.

Currently, however, the impact of reductions in uterine perfusion on structural and mechanical properties of uterine arteries is unknown as mechanistic studies have not been performed in preeclamptic women. Additionally, the impact of reductions in uterine perfusion in pregnant rats on uterine artery tone, structural, and mechanical parameters is unknown (Reho et al., 2012). Therefore, the purpose of this study is to determine vascular tone, structural and mechanical properties of resistance-caliber uterine arteries after chronic reductions in uterine perfusion pressure in late gestation pregnant rats. The hypothesis to be tested is that chronic reductions in uterine perfusion pressure will increase vascular tone and alter structural and mechanical parameters of resistance-caliber uterine arteries in pregnant rats.

Materials and Methods

Eight-twelve week old Sprague Dawley female rats (200-250g) were used for this investigation. Rats were placed with a male overnight and the presence of sperm on a vaginal smear designated Day 1 of pregnancy (Full term 22-23 days). Animals were singly housed for the duration of the experiment. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Akron (Reho et al., 2012).
**Surgical Manipulation**

Pregnant rats were subjected to reduced uterine perfusion pressure (RUPP) or SHAM surgery of Day 14 of gestation as described in detail elsewhere (Crews *et al*., 2000; Ramirez *et al*., 2011; Reho *et al*., 2011). Briefly, animals were anesthetized with 2.5% isoflurane in 100% oxygen at a flow rate of 1L/min. A midline incision was made and the abdominal aorta was partially occluded with a silver clip (0.203 mmID). The uterine-ovarian arteries were also partially occluded with silver clips (0.106 mmID). SHAM animals underwent the same surgery but did not receive the silver clips. All animals that resorbed all of their fetuses were excluded from the study.

**Uterine and Mesenteric Arteries**

Resistance caliber uterine arteries (200-300 μm) were isolated from the third branch of the main uterine artery (or second branch depending on availability). Resistance caliber mesenteric arteries (second-order; 200-300 μm) were isolated from a 5-10cm section distal to the pylorus. Arteries were cleaned of debris and mounted in a pressurized arteriograph (Living Systems, Burlington, VT) as previously described (Ramirez *et al*., 2011; Reho *et al*., 2011). The arteries were mounted in a HEPES buffered saline solution consisting of the following: sodium chloride 142, potassium chloride 4.7, magnesium sulfate 1.17, calcium chloride 2.5, potassium phosphate 1.18, HEPES 10, dextrose 5.5, and the pH was adjusted to 7.4 (Toot *et al*., 2011; Toot *et al*., 2012).
**Vascular Tone**

Vascular tone was determined by comparing active to passive diameters under varying intraluminal pressures using the following equation: \[1-(D_{\text{pass}}-D_{\text{act}})/D_{\text{pass}}]*100. In this equation, \(D_{\text{pass}}\) represents the luminal diameter at a specific pressure under passive conditions; \(D_{\text{act}}\) represents the luminal diameter at a specific pressure under active conditions. Active conditions were determined during myogenic reactivity assays (described in Chapter IV) and therefore were similarly preconstricted.

**Passive Structural Mechanics**

Upon complete of the active diameter investigation, arteries were investigated for passive structural and mechanical parameters in a calcium free HEPES buffer in the presence of 0.1 mM EGTA and 0.1 mM papaverine (Toot et al., 2012). The intraluminal pressure was held constant at 60 mmHg and the vessel was equilibrated in this buffer for 30 minutes. The luminal diameter and vessel wall thickness were measured with an electronic filar (Lasico, Los Angeles, CA) and expressed in micrometers. Wall:lumen ratios were calculated: \(\omega/\Omega_{\text{inner}}, \omega\) is the vessel wall thickness and \(\Omega_{\text{inner}}\) is the luminal diameter. Cross sectional wall area was calculated as follows: \(\pi r^{2}(\omega + \Omega_{\text{inner}}) - \pi r^{2}\) \((\Omega_{\text{inner}})\) where \(\omega\) is the vessel wall thickness and \(\Omega_{\text{inner}}\) is the luminal diameter. In addition to structural measurements, mechanical measurements of the vessels were also assessed. Wall tension was calculated with the following equation: \(T=P \times r\) where tension (\(T\)) is equal to the intraluminal pressure (\(P\); measured in dynes/cm\(^2\); 1 mmHg = 1333.2 dynes/cm\(^2\)) multiplied by \(r\) (radius; converted to cm). Wall stress was calculated with the following equation (assuming the arterial wall is uniform), and is
expressed in dynes/cm$^2$: $T/\omega$, where $T$ is equal to the intraluminal pressure (expressed in dynes/cm$^2$) multiplied by the luminal radius and $\omega$ is the vessel wall thickness diameter.

Wall stress values were multiplied by $10^5$ to reduce scientific notation. Distensibility of the arteries was calculated using the following equation: $[(\Omega_x - \Omega_{0mmHg})-1] \times 100$. In this equation, $\Omega_x$ is the intraluminal diameter at a specific transmural pressure and $\Omega_{0mmHg}$ is the intraluminal diameter at 0 mmHg. The distensibility data is presented as percent distensibility (Cipolla et al., 1994; Reho et al., 2012; Toot et al., 2012).

Statistics

Intrinsic tone, structural and mechanical measurements were analyzed using a one way ANOVA. Passive distensibility was analyzed with a two way repeated measures ANOVA and a Bonferroni post hoc test. Student’s t-test was used where applicable. Statistical significance was accepted with a $p<0.05$.

Results

Resistance-caliber uterine arteries from RUPP pregnant rats displayed a significant increase in pressure-induced tone (Figure 5.1 A) compared to SHAM pregnant rats ($p<0.05$; for all pressures analyzed). Similarly, resistance-sized mesenteric arteries from RUPP pregnant rats demonstrated an increase in pressure-induced vascular tone (Figure 5.1 B) compared to SHAM ($p<0.05$; for all pressures analyzed).

Distensibility, calculated a percent distensibility, was significantly decreased in resistance-sized uterine arteries from RUPP pregnant rats compared to SHAM ($F=14.5$;
p<0.001) (Figure 5.2 A). No differences were found in resistance-sized mesenteric arteries from RUPP and SHAM pregnant rats (Figure 5.2 B).

No differences were found for uterine or mesenteric artery passive arterial diameter, wall thickness or wall:lumen ratios for RUPP and SHAM pregnant rats (Table 5.1). Additionally, no differences were found for lumen area and cross sectional wall area of uterine and mesenteric arteries from SHAM, RUPP and Occluder pregnant rats (Table 5.2). Mechanical parameters of circumferential wall tension and wall stress were also analyzed and found to demonstrate no differences in uterine and mesenteric arteries from RUPP and SHAM pregnant rats (Table 5.3).
Figure 5.1 Average percent tone of resistance-caliber uterine arteries (A) and mesenteric arteries (B) from RUPP and SHAM pregnant rats (means, ± SEM). Resistance-caliber uterine arteries (A) from RUPP (*p<0.05) rats displayed increased tone compared to SHAM. Resistance-caliber mesenteric arteries (B) from RUPP (*p<0.05) rats displayed increased tone compared to SHAM rats.
Figure 5.2 Percent distensibility of resistance-caliber uterine (A) and mesenteric (B) arteries from RUPP and SHAM pregnant rats (means, ± SEM). Distensibility was significantly decreases in resistance caliber uterine arteries (A) from RUPP rats compared to SHAM (*p<0.05). No differences were found in percent distensibility of resistance-caliber mesenteric arteries (B).
<table>
<thead>
<tr>
<th></th>
<th>Uterine Arteries</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passive Diameter (µm)</td>
<td>SHAM</td>
<td>231.4 ± 7.8</td>
<td>283.7 ± 10.6</td>
<td>303.8 ± 11.4</td>
<td>314.3 ± 12.1</td>
<td>323.4 ± 14.1</td>
<td>326.6 ± 13.8</td>
<td>332.1 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>270.3 ± 21.9</td>
<td>298.5 ± 23.1</td>
<td>312.8 ± 23.2</td>
<td>318.7 ± 23.6</td>
<td>325.3 ± 24.1</td>
<td>329.1 ± 23.8</td>
<td>333.6 ± 23.6</td>
</tr>
<tr>
<td>Wall Thickness (µm)</td>
<td>SHAM</td>
<td>28.4 ± 1.7</td>
<td>24.2 ± 1.7</td>
<td>22.0 ± 1.6</td>
<td>20.2 ± 1.3</td>
<td>18.9 ± 1.5</td>
<td>17.5 ± 1.5</td>
<td>17.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>27.5 ± 3.4</td>
<td>23.5 ± 2.8</td>
<td>22.2 ± 2.7</td>
<td>19.6 ± 1.9</td>
<td>17.8 ± 1.4</td>
<td>16.9 ± 1.3</td>
<td>16.4 ± 1.3</td>
</tr>
<tr>
<td>Wall: Lumen</td>
<td>SHAM</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>0.10 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Mesenteric Arteries</td>
<td>20</td>
<td>242.2 ± 13.1</td>
<td>279.4 ± 12.1</td>
<td>298.3 ± 11.9</td>
<td>305.7 ± 12.5</td>
<td>314.0 ± 12.1</td>
<td>319.6 ± 10.8</td>
<td>323.1 ± 10.4</td>
</tr>
<tr>
<td>Passive Diameter (µm)</td>
<td>SHAM</td>
<td>224.1 ± 9.8</td>
<td>263.0 ± 8.7</td>
<td>285.0 ± 6.3</td>
<td>294.2 ± 5.3</td>
<td>302.4 ± 5.2</td>
<td>308.5 ± 5.7</td>
<td>312.3 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>231.1 ± 9.8</td>
<td>298.4 ± 3.2</td>
<td>260.8 ± 2.3</td>
<td>23.3 ± 2.7</td>
<td>22.3 ± 2.7</td>
<td>19.9 ± 2.4</td>
<td>19.6 ± 2.4</td>
</tr>
<tr>
<td>Wall Thickness (µm)</td>
<td>SHAM</td>
<td>29.2 ± 2.2</td>
<td>24.8 ± 1.8</td>
<td>21.8 ± 1.5</td>
<td>19.9 ± 1.3</td>
<td>17.6 ± 1.2</td>
<td>16.3 ± 1.1</td>
<td>15.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>33.1 ± 2.0</td>
<td>29.8 ± 2.0</td>
<td>26.0 ± 2.3</td>
<td>23.3 ± 2.7</td>
<td>22.3 ± 2.4</td>
<td>19.9 ± 2.4</td>
<td>19.6 ± 2.4</td>
</tr>
<tr>
<td>Wall: Lumen</td>
<td>SHAM</td>
<td>0.13 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>0.15 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

Table 5.1 Structural parameters of resistance-caliber uterine and mesenteric arteries from SHAM and RUPP pregnant rats (means ± SEM). No differences were noted between treatment groups.
Table 5.2 Mechanical parameters of resistance-caliber uterine and mesenteric arteries from SHAM and RUPP pregnant rats (means ± SEM). No differences were noted between treatment groups.

<table>
<thead>
<tr>
<th>Arteries</th>
<th>Wall Tension (dynes/cm)</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine</td>
<td>SHAM</td>
<td>308 ± 10</td>
<td>756 ± 28</td>
<td>1215 ± 45</td>
<td>1676 ± 64</td>
<td>2155 ± 93</td>
<td>2612 ± 110</td>
<td>3320 ± 139</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td>360 ± 29</td>
<td>796 ± 61</td>
<td>1251 ± 93</td>
<td>1699 ± 126</td>
<td>2168 ± 161</td>
<td>2632 ± 191</td>
<td>3336 ± 236</td>
</tr>
<tr>
<td>Wall Stress</td>
<td>SHAM</td>
<td>1.1 ± 0.1</td>
<td>3.3 ± 0.3</td>
<td>5.8 ± 0.4</td>
<td>8.7 ± 0.6</td>
<td>12.1 ± 1.0</td>
<td>15.9 ± 1.2</td>
<td>20.2 ± 1.4</td>
</tr>
<tr>
<td>(dynes/cm²)</td>
<td>RUPP</td>
<td>1.5 ± 0.3</td>
<td>3.7 ± 0.5</td>
<td>6.3 ± 0.9</td>
<td>9.7 ± 1.4</td>
<td>13.1 ± 1.7</td>
<td>16.6 ± 2.0</td>
<td>21.8 ± 2.6</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>SHAM</td>
<td>323 ± 17</td>
<td>745 ± 32</td>
<td>1193 ± 48</td>
<td>1630 ± 67</td>
<td>2093 ± 80</td>
<td>2557 ± 86</td>
<td>3231 ± 103</td>
</tr>
<tr>
<td>Arteries</td>
<td>Wall Tension (dynes/cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>299 ± 13</td>
<td>701 ± 23</td>
<td>1140 ± 25</td>
<td>1569 ± 28</td>
<td>2016 ± 34</td>
<td>2468 ± 46</td>
<td>3123 ± 55</td>
</tr>
<tr>
<td></td>
<td>RUPP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Wall Stress</td>
<td>SHAM</td>
<td>1.3 ± 0.1</td>
<td>3.4 ± 0.4</td>
<td>6.0 ± 0.6</td>
<td>9.0 ± 0.8</td>
<td>13.2 ± 1.1</td>
<td>17.3 ± 1.2</td>
<td>22.7 ± 1.4</td>
</tr>
<tr>
<td>(dynes/cm²)</td>
<td>RUPP</td>
<td>1.0 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td>4.8 ± 0.6</td>
<td>7.3 ± 0.7</td>
<td>10.0 ± 1.1</td>
<td>13.8 ± 1.4</td>
<td>17.7 ± 1.8</td>
</tr>
</tbody>
</table>

Intraluminal Pressure (mmHg)
Table 5.3 Area parameters of resistance-caliber uterine and mesenteric arteries from SHAM and RUPP pregnant rats (means ± SEM). No differences were noted between treatment groups.
Discussion

This is the first study to examine structural and mechanical parameters of resistance-caliber uterine arteries from pregnant rats with chronic reductions in uterine perfusion pressure (RUPP). The results of these investigations support the hypothesis that resistance-caliber uterine arteries in response to reductions in uterine perfusion pressure display altered passive mechanical parameters compared to normal pregnant controls. The major findings of this investigation include 1) increased arterial tone in RUPP rats resistance-caliber uterine arteries compared to SHAM control 2) Decreased distensibility of uterine arteries from RUPP pregnant rats compared to SHAM controls.

Intrinsic vascular tone is the inherent constriction of a blood vessel in response to the activation of the smooth muscle by circulatory, mechanical and innervation signals. The arteriograph techniques used in these studies results in the removal of influences from vascular nerves and the circulatory milieu (Dunn et al., 1994), thus our studies are a response of the mechanical stretch of pressure induced vascular tone. Uterine arteries from RUPP pregnant rats displayed a significant increase in vascular tone compared to SHAM pregnant rats. This finding was also similar to the mesenteric circulation indicating a potential for whole animal vascular tone alterations. The differences in pressure induced vascular tone are likely due the altered nitric oxide release from the endothelium (Gandley et al., 1997). The altered nitric oxide release is likely due to endothelial dysfunction as demonstrated from our previous experiments (Ramirez et al., 2011; Reho et al., 2012) as well as Chapter IV of this dissertation. Additionally, Tam and colleagues have recently reported an increase in uterine artery resistance in response to the RUPP pathology (Tam Tam et al., 2011). This may be due to an increase
in vascular tone of the uterine arteries in response to the pressure stimulus as demonstrated in this study.

Passive arterial distensibility is a measure of vascular compliance and indicative of extracellular matrix volume, components such as collagen and elastin, and fiber orientation (Mateev et al., 2006; Wagenseil and Mecham, 2009). Studies of rat uterine arteries during pregnancy have demonstrated a decrease in elastin content of the ECM (Page et al., 2002). The decrease in elastin content may prevent elastic recoil in the blood vessels and allow for increased distensibility (Gardiner et al., 2001). Additionally, decreases in vascular collagen have been demonstrated in resistance arteries during rat pregnancy (Mackey et al., 1992) and may also contribute to increases in vascular distensibility and compliance. In this study, distensibility of resistance-caliber uterine arteries was significantly reduced in RUPP pregnant rats compared to SHAM. This may be contributed to by an alteration in collagen and elastin ratios in the extracellular matrix of these arteries such that a decrease distensibility of the arteries resulted. Indeed, collagen has been demonstrated to be increased (2x) in umbilical vessels from preeclamptic women compared to normotensive controls (Bankowski et al., 1996). Additionally, small decreases have been noted in elastin content of umbilical vessels from preeclamptic patients (Bankowski et al., 2003). This shift in the ratio of collagen to elastin likely results in increased stiffness of vessels in response to hypertension in pregnancy and the resulting placental ischemia that occurs. Our laboratory (Ramirez et al., 2011) has demonstrated a significant role of vascular nitric oxide in the mediation of vascular function during pregnancy induced hypertension and this may play a role in the regulation of collagen and elastin content in the vasculature. Nitric oxide has been shown
to be a positive regulator of matrix metalloproteinase expression and activity (Drapier and Bouton, 1996) and deficits in nitric oxide may be a regulatory mechanism of MMP function in preeclampsia. Matrix metalloproteinase 2 (MMP-2), a major collagenolytic enzyme, is decreased in L-NAME induced hypertension during pregnancy (Barron et al., 2010) potentially leading to an accumulation of collagen in the uterine vessels and potentially decreasing arterial distensibility. Furthermore this decreased distensibility may be a potential mechanism for the decreased fetal weight in RUPP demonstrated in Chapter III.

Structural parameters of uterine arteries from RUPP and SHAM pregnant rats did not result in any appreciable changes to passive arterial diameter, wall thickness or wall to lumen ratios of the arteries. Vessel luminal area and cross sectional wall area of resistance-sized arteries were also unaltered in response to reductions in uterine perfusion pressure. Additionally, mechanical parameters of the arteries such as circumferential wall tension and wall stress were similar between all the treatment groups. Taken together, these data suggest that reductions in uterine perfusion pressure and the associated reductions in vascular nitric oxide (NO) together may not themselves be capable of altering these specific vascular remodeling parameters in resistance-caliber uterine arteries. Additionally, the pressure arteriograph system may not be sensitive enough to distinguish very small changes in vessel wall volume which may have important impacts on mechanical function. However, Hale and colleagues recently reported that chronic NO inhibition (through L-NAME infusion) starting at gestational Day 10 altered the characteristics outward remodeling of uterine arteries during pregnancy (Hale et al., 2011). Thus it is likely that vascular NO, along with other
mechanisms such as angiogenesis, and the timing of the NO reductions, as well as the maternal vascular response to the insult, lead to alterations into the myriad of vascular remodeling mechanisms that occur during pregnancy. Additionally the time course of our investigation (i.e. gestational Day 14 – Day 21) may not have been adequate time for the structural remodeling of the uterine arteries to occur.

In conclusion, this study is the first report of the impact of reductions in uterine perfusion pressure on structural and mechanical parameters of resistance-sized uterine arteries from pregnant rats (Reho et al., 2012). We demonstrated in this study increases in pressure induced vascular tone of uterine arteries from RUPP rats as well as decreases in uterine arterial distensibility from these animals. Additionally, tone was also altered in resistance-sized mesenteric arteries with several other structural and mechanical measurements not being altered. These data suggest that reductions in uterine perfusion pressure may alter some of the biophysical mechanical properties of resistance-caliber arteries in vivo and may be a potential mechanism by which altered perfusion pressure leads to the preeclampsia-like pathology and fetal growth restriction.
CHAPTER VI

NANOPARTICLES FORMULATED FROM L-TYROSINE POLYPHOSPHATE AS A POTENTIAL NON-VIRAL INTRACELLULAR GENE DELIVERY DEVICE: EFFICACY IN VIVO IN THE RAT UTERUS

Introduction

Gene therapy has been proposed as a therapeutic modality for a variety of diseases including cardiovascular related pathologies (Baker, 2002). The primary gene delivery devices used have been viral based vectors because they are particularly effective at transfecting cells with nucleic acids due to their unique cellular characteristics and their ability to manipulate the host’s cellular machinery. However, primary immunological responses, high cost, and possible deadly viral recombination are complications that come with the use of viral vectors (Cristiano, 1998). Recently, non-viral gene delivery devices have been investigated to circumvent the immunogenic and cellular toxicity associated with viral delivery vectors. One promising form of non-viral gene delivery devices currently being investigated is through the use of condensed DNA encapsulated within nanoparticles (Ditto et al., 2009). Nanoparticles provide unique structural characteristics such as size (<1 micron), smooth and spherical morphology, and the ability to be adapted for various cellular targeting applications through chemical modifications of structural moieties. Accordingly, eukaryotic cells are able to easily
endocytose nanoparticles due to their small size being less than 1 micron (Desai et al., 2002). However, biocompatibility and biodegradation are important factors in the development of nanoparticles destined for systemic use. Thus these characteristics must be incorporated into the polymeric design to be essential to the efficacy of the gene delivery device for an *in vivo* application (Ditto et al., 2009).

Recently, nanoparticles formulated from L-tyrosine polyphosphate (LTP) have shown promise as a potential intracellular gene delivery device (Ditto et al., 2009). LTP nanoparticles are formulated by coupling multiple phosphate groups to the terminal hydroxyl group of the essential amino acid, L-tyrosine (Ditto et al., 2009). LTP nanoparticles have been shown to degrade *in vitro* over a period of seven days, which is well within the life span of most cells (Zweers et al., 2004), due to a series of unstable, hydrolytically degradable, phosphoester linkages throughout the polymer matrix (Sen Gupta and Lopina, 2005).

LTP nanoparticles have been shown to effectively deliver plasmid DNA to human dermal fibroblasts *in vitro* (Ditto et al., 2009) and have been used *in vivo* through nebulization of a silver carbene complex encapsulated within the LTP nanoparticles for treatment of an animal model of cystic fibrosis (Hindi et al., 2009). However, the effectiveness of *in vivo* transfection does not fully follow *in vitro* successes (Gharwan et al., 2003). Therefore, the efficacy of LTP nanoparticles in transfecting tissue with nucleic acid *in vivo*, however, stills remains to be determined. The objective of the current study is test the utility of LTP nanoparticles in transfecting tissue with plasmid DNA. Our hypothesis to be tested is that LTP nanoparticles with the reporter gene β-
galactosidase plasmid DNA will transfect a rat uterus model resulting in expression of the plasmid DNA product.

Materials and Methods

The objective of this preliminary study was to establish the parameters and efficacy of L-tyrosine polyphosphate (LTP) nanoparticles encapsulated with plasmid DNA (encoding for β-galactosidase) for potential \textit{in vivo} utility. LTP-pDNA nanoparticles were made and characterized for morphology, size, loading characteristics, and \textit{in vitro} cellular efficacy.

\textit{LTP-pDNA Nanoparticles}

LTP-pDNA nanoparticles were formulated as previously described in detail (Ditto et al., 2009). Briefly, LTP-pDNA nanoparticles were formed via an emulsion of water and oil through a sonication and solvent evaporation technique. A common set of LTP-pDNA nanoparticles consisted of LTP in chloroform (900mg), polyethylene glycol grafted to chitosan (PEG-g-CHN) in acetic acid (3.0mg), pDNA-LPEI complex (6mg), 10% polyvinylpyrrolidone (100mL). Chloroform was evaporated with stirring for a period of 5 hours and the nanoparticles were collected by centrifugation at 15,000 x g. Nanoparticles were then lyophilized in sterile water (Labconco Freezone 4.5, Kansas City, MO) for 72 hours and stored in a dessicator (Ditto \textit{et al.}, 2009). Blank LTP nanoparticles were also formulated with the same procedure excluding the pDNA-LPEI complex as a control.
**Scanning Electron Microscopy**

Nanoparticle samples (LTP-pDNA and Blank LTP) were examined with scanning electron microscopy (SEM, Hitachi S2150, Japan). Nanoparticle size, shape, and polymer morphology were analyzed. A nanoparticle solution was made for examination by dissolving 1mg of nanoparticles in 1mL of sterile autoclaved water. 200 μL of the nanoparticle solution were placed onto an SEM stub, allowed to dry completely at room temperature, and the sputter coated with silver/palladium for SEM examination (Ditto et al., 2009).

**Dynamic Laser Light Scattering**

Quantification of the size of the LTP-pDNA and Blank LTP nanoparticles was performed using dynamic laser light scattering (DLS, Brookhaven Instruments BI-200SM, Holtsville, NY). A nanoparticle solution of 1mg of nanoparticles into 10mL of sterile water in a glass scintillation vial was made. Nanoparticle diameters were determined by non-linear least squares (NLLS) and regularized non-negatively constrained least squares (CONTIN). Differential distribution values were reported for the nanoparticle size where the values ranged from 0 to 100 and highest value was assigned a value of 100 and all other sizes were expressed as relative amounts compared to the highest modal value (Ditto et al., 2009).

**LTP-pDNA Nanoparticle Loading**

The loading efficiency of LTP-pDNA nanoparticles was assessed to determine the total amount of DNA encapsulated in LTP-pDNA nanoparticles. A measure of 2mg of
LTP-pDNA nanoparticles was dissolved in 200 μL of chloroform and incubated at 37°C for 30 minutes. An equal volume of sterile 1X TE buffer was added to the nanoparticle solution for gently shaken for a period of 2 minutes to form an emulsion. Phase separation was allowed to take place for 30 minutes at room temperature. The nanoparticle solution was then centrifuged at 10,000 x g for 5 seconds. Then, 200 μL of the TE supernatant was assayed using PicoGreen fluorescent dye (Quant-iT) and compared with a standard curve of pDNA-LPEI complex in TE buffer. Blank LTP nanoparticles were assessed as a control (Ditto et al., 2009).

*LTP-pDNA Nanoparticle Transfection Efficacy In Vitro with Human Dermal Fibroblasts*

The efficacy of LTP-pDNA nanoparticles for in vitro transfection of cells was assessed with human dermal fibroblasts. Passage 8 human dermal fibroblasts (primary human dermal fibroblasts were originally obtained as a gift from Judy Fulton, Akron General Medical Center) were seeded as a density of 25,000 cells/well and were maintained in cell culture incubator (37°C, 5% CO₂) in a fibroblast feeding medium consisting of 90% Dulbecco’s Eagle medium, 10% fetal calf serum, and 1% antibiotic-antimycotic (Ditto et al., 2009). LTP-pDNA and Blank LTP nanoparticles were added to the fibroblast feeding media and placed onto the human dermal fibroblasts at a concentration of 0.67μg/μl. After three and seven days of transfection, the fibroblasts were fixed with 1% formaldehyde and subjected to X-gal staining for the presence of β-galactosidase according to the manufacturer’s instructions. Cells were viewed using an inverted microscope (Axiovert 200, Carl Zeiss, Peabody, MA) and imaged using a CCD camera (AxioCam ICc3, Carl Zeiss, Peabody, MA) (Ditto et al., 2009).
In Vivo LTP Nanoparticle Study Design

The objective of this study was to establish the efficacy of L-tyrosine polyphosphate (LTP) nanoparticles encapsulated with plasmid DNA (encoding for β-galactosidase) in transfection of the rat uterus in vivo. This study utilized two separate treatment groups with one group receiving LTP-pDNA nanoparticles (n=3) and the other group receiving Blank LTP nanoparticles (without pDNA) (n=3). The animals were injected in the left uterine horn with the respective treatment. Nine days post injection, the animals were terminated and the uterine horns were excised for histochemical and molecular analysis.

Animals

This investigation utilized virgin, Wistar Kyoto (WKY) females approximately 10-14 weeks of age at the beginning of the study. The animals were housed 3 animals per cage in standard polyethylene cages (45 x 25 x 20cm) with stainless steel tops. Cages contained heat treated wood chip bedding (PJ Murphy Products, Montville, NJ) and standard rat chow (Teklad Rodent, Madison, WI) and water were provided ad libitum. The animal room temperatures were held a constant temperature of ~25°C at ~40% humidity. Animal rooms had a 12 hour light (07:00-19:00)/dark (19:00-07:00) cycle. All animal protocols were reviewed and approved by the University of Akron’s Institutional Animal Care and Use Committee.
**LTP Nanoparticle Injections**

Animals were anesthetized under 2% Isoflurane (Viking Medical) with 100% O\textsubscript{2} under a flow rate of 1L/min. A midline incision along the animal’s longitudinal axis was made in the abdomen and the uterus was exposed. The left uterine horn was isolated and the myometrium was injected (0.5cc insulin syringe; 28 gauge) with 100 uL of the following treatments: 2.5mg of LTP-pDNA nanoparticles in sterile saline and 2.5mg of Blank LTP nanoparticles in sterile saline.

**Uterus Isolation**

Animals were terminated with an overdose of Sodium Pentothal (50 mg/kg; IP; EJ Lilly, Indianapolis, IN) followed by exsanguination 9 days post nanoparticle injection. The left uterine horn was quickly removed and cut into two. One portion of the uterine horn was flash frozen with liquid nitrogen for RNA analysis while the other portion was fixed in 1% buffered formalin for histochemical analysis.

**Cryosectioning of Uterine Tissue**

After uterine horn tissues were fixed with 1% buffered formalin, the tissues were washed overnight in sterile 1x PBS at 4C. The tissues were then transferred to a 30% sucrose solution for cryoprotection overnight at 4C. Tissue blocks was constructed with the use of Optimal Cutting Temperature (OCT; EMS, Hatfield, PA) and tissues were sectioned on a Bright Model OTF cryosection (Bright Instrument, Huntingdon. Cambs, England). Tissue slices were then stored at 4C until histochemical analysis was performed.
Detection of Bgal expression with X-gal Reagent

Cryosectioned uterine horn tissues were permeabilized with 1mM MgCl₂ and then stained with X-gal reagent, according to established protocols (Ditto et al., 2009), for a period of three days and counterstained with hematoxylin. Staining occurred within a humidity box and placed in a cell culture incubator (37°C, 5% CO₂) for the staining procedure. Uterine tissue staining was imaged with an inverted microscope as described previously in this chapter. LTP-pDNA and Blank LTP nanoparticles were stained in this investigation.

Immunohistochemistry

A separate set of uterine tissue sections were subjected to immunohistochemical analysis. Tissues were permeabilized with 0.2% Triton-X and blocked with 1% newborn calf serum. The primary antibody utilized was mouse monoclonal against the E. coli expressed β-galactosidase (Promega, Madison, WI). The antibody was placed on the tissue sections at a 1:50 dilution and stored overnight at 4°C. Uterine tissue sections were then washed with sterile PBS and incubated with a polyclonal anti-mouse IgG-TRITC secondary antibody (Sigma, St. Louis, MO) at a 1:50 dilution at 4°C. Tissues were imaged using confocal microscopy as described in detail below.

Confocal Microscopy Imaging

Uterine tissue samples subjected to immunohistochemical analysis were imaged using a Zeiss 510 META laser-scanning module with an inverted microscope (Axiovert,
Carl Zeiss, Peabody, MA). The laser excitation wavelength for this study was 543nm and a 40x oil-immersion objective was used. An emission reference spectrum was created with a non-stained tissue section and a separate section of TRITC secondary antibody. This spectrum spanned wavelengths from 548.6nm to 643.1nm with a step increment of 10.7nm. This reference spectrum was created to distinguish between tissue autofluorescence and TRITC secondary antibody fluorescence. Once this spectrum was integrated into the LSM 510 software used for the confocal microscope, the immunohistochemically stained uterine tissue sections were subjected to lambda stacking from 548.6nm to 643.1nm at a step increment of 10.7nm. Using the reference spectrum created, the fluorescence of the TRITC secondary antibody was unmixed from the uterine tissue autofluorescence and assigned separate colors for distinguishing (green for uterine tissue autofluorescence and red for the TRITC secondary antibody). Control images were taken from Blank LTP nanoparticle injected samples and stained with TRITC secondary antibody.

Hematoxylin and Eosin Staining

Cryosectioned uterine tissues as described previously in this chapter were subjected to hematoxylin and eosin staining (H&E) for tissue damage analysis. Uterine tissue sections were stained with Gills hematoxylin and counterstained using Eosin Y. Uterine tissue sections were then dehydrated and mounted for brightfield imaging as previously described in this chapter. Histological analysis of tissue morphology, inflammatory and leukocyte markers were assessed.
**Reverse Transcriptase PCR (RT-PCR)**

Uterine horn tissues were homogenized under liquid nitrogen with mortar and pestle and RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA for the uterine samples was isolated according to the manufacturer’s instructions. Plasmid DNA or other DNA contaminates were removed by treatment of the RNA with DNase I (1U/uL; Roche) and an RNA cleanup kit (Qiagen, Valencia, CA). Purified RNA was stored at -80°C until analysis.

RT-PCR was performed with a one-step RT-PCR kit (Qiagen) according to manufacturer’s instructions. The following primers were used:

Forward: 5’-ACCCGCATTGACCCTAAC-3’
Reverse: 5’-TGTATCGCTCGCCACTTC-3’

The protocol was modified to 30 cycles with a denaturing cycle of 94°C (1min), an annealing cycle of 53°C (1min), and an extension cycle of 68°C (1min). The RT-PCR reaction products were analyzed on a 1% agarose gel with an amplicon of 234 base pairs indicative of β-galactosidase origin (Yun *et al.*, 2004).
Results

Typical image through scanning electron microscopy of LTP-pDNA nanoparticles (Figure 6.1). Nanoparticles were generally spherical in shape and displayed a smooth morphology.

Dynamic Laser Light Scattering

Diameters were determined for LTP-pDNA and Blank LTP nanoparticles by use of dynamic laser light scattering. Regularized non-negatively constrained least squares (CONTIN) diameter measurement demonstrated a maximum distribution of LTP-pDNA nanoparticle diameter at 157nm with a range of 107 to 334nm in diameter (Figure 6.2). CONTIN also revealed a maximum distribution of Blank LTP nanoparticle diameter at 102nm with a range of 79 to 196nm in diameter (Figure 6.3). Mean nanoparticle diameters were determined to be 207 ± 49 nm for LTP-pDNA nanoparticles and 130 ± 15 nm for Blank LTP nanoparticles (Figure 6.4).

LTP-pDNA Nanoparticle Loading Efficiency

The amount of pDNA loaded in the nanoparticles was determined with the use of a PicoGreen quantification assay and spectrophotometer. The following equation was used to calculate nanoparticle loading efficiency:

\[
\text{Loading Efficiency (\%)} = \frac{\text{amount of pDNA measured from nanoparticle}}{\text{amount of pDNA put into nanoparticle}}
\]
LTP-pDNA nanoparticles exhibited a 24% ± 1% loading efficiency meaning that 24% of the nanoparticles were loaded with pDNA during the emulsion formulation of the nanoparticles. Blank nanoparticles served as a control and showed no pDNA encapsulation.

**Human Dermal Fibroblast Transfection with LTP-pDNA Nanoparticles**

*In vitro* cellular transfection efficacy was tested with LTP-pDNA nanoparticles on human dermal fibroblasts (Figure 6.5). Cells were transfected with LTP-pDNA nanoparticles or Blank LTP nanoparticles for a period of 3 and 7 days and then were subjected to X-gal staining for the presence of β-galactosidase. Three and seven days post-transfection revealed positive staining for β-gal in cells transfected with LTP-pDNA. Blank LTP nanoparticles served as controls and revealed no positive staining.

**X-gal Staining of Rat Uterus Tissue**

Figure 6.6 represent a typical image of the efficacy of LTP-pDNA nanoparticles for transfection of rat uterine tissue *in vivo*. Brightfield images of LTP-pDNA nanoparticles display enhanced transfection of plasmid DNA encoding for B-galactosidase as shown by the blue cell staining with X-gal reagent. Tissues were counterstained red with hematoxylin for contrast. All three animals injected with LTP-pDNA nanoparticles displayed varying degrees of transfection indicated by X-gal blue cells staining (see appendix). Blank LTP nanoparticles resulted in no positive staining by X-gal reagent.

Confocal Microscopy Imaging

As a further confirmation of successful transfection of LTP-pDNA nanoparticles, tissue sections were subjected to immunohistochemical analysis and then imaged using confocal microscopy (Figure 6.7). LTP-pDNA nanoparticles demonstrated successful and enhanced transfection of B-galactosidase as indicated by the red fluorescence. Green fluorescence indicates the inherent autofluorescence of the tissue. In contrast, Blank LTP nanoparticles did not result in any red fluorescence indicating no expression of B-galactosidase as a control.

Estrus Cycle Determination

Figure 6.8 represents typical images from estrus and diestrus stages of the rat estrus cycle. Xgal staining of transfected tissues during these particular phases demonstrated no visual differences between treatments (Figure 6.9).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as a molecular confirmation of the previous tissue staining procedures. Positive signal for B-galactosidase was found in LTP-pDNA nanoparticle injected uterine horns (Figure 6.10, Lane 4). Lane 2 and 3 served as control with no template control (Lane 2) and thoracic aorta control (Lane 3). Stock plasmid DNA for B-galactosidase served as a positive control (Lane 5). Replicates of LTP-pDNA nanoparticle injected animals can be found in the appendix.
Hematoxylin and Eosin Staining

LTP-pDNA and Blank LTP nanoparticle injected uterine tissue were subjected to hematoxylin and eosin (H&E) staining for the presence of inflammatory and leukocyte activity (Figure 6.11). H & E staining revealed comparable tissue morphology and no signs of inflammation and a low amount of leukocyte activity between LTP-pDNA nanoparticle, Blank LTP nanoparticle and uninjected control uterine horns. Accordingly, nuclear and cellular structures were determined to be normal in LTP-pDNA and Blank LTP nanoparticle injected uterine horns.
Figure 6.1 Scanning electron micrograph of LTP-pDNA nanoparticles.
Figure 6.2 Representative size distribution of LTP-pDNA nanoparticles. Diameters were determined using regularized non-negatively constrained least squares (CONTIN).
Figure 6.3 Representative size distribution of Blank LTP nanoparticles. Diameters were determined using regularized non-negatively constrained least squares (CONTIN).
Figure 6.4 Mean nanoparticle diameters for LTP-pDNA and Blank LTP nanoparticles (means, ± SEM). Diameters were determined using regularized non-negatively constrained least squares (CONTIN).
Figure 6.5 LTP-pDNA nanoparticle transfection of human dermal fibroblasts for 3 and 7 days. X-gal staining revealed positive staining (blue cells) after both 3 and 7 days of transfection. No positive staining for β-galactosidase was found in blank LTP nanoparticle transfected cells.
Figure 6.6 Brightfield images of X-gal stained rat uterus sections. Uterus injected with LTP-pDNA nanoparticles at various magnifications (A), (B), and (C) shows gene expression of β-gal with positive blue staining. Rat uterus injected with blank LTP nanoparticles at various magnifications (D), (E), and (F) does not show expression of β-gal.
Figure 6.7 Combined confocal fluorescent images of \textit{in vivo} rat uterus sections immunostained with anti-\(\beta\)-gal mouse primary Ab followed by anti-mouse IgG-TRITC secondary Ab. Uterus injected with LTP-pDNA nanoparticles at tissue section depths of 2.5 \(\mu\)m (A), 15 \(\mu\)m (B), and 27.5 \(\mu\)m (C) shows gene expression of \(\beta\)-gal. Rat uterus injected with blank LTP nanoparticles at tissue section depths of 2.5\(\mu\)m (D), 15 \(\mu\)m (E), and 27.5 \(\mu\)m (F) does not show expression of \(\beta\)-gal.
Figure 6.8 Typical images of vaginal lavages taken during estrus and diestrus phase of the uterine cycle (10x magnification).
Figure 6.9 Typical images of Xgal stained uteri injected with LTP-pDNA nanoparticles for animals in estrus and diestrus cycles (20x and 63x magnification).
Figure 6.10 RT-PCR from the rat uterus injected with LTP-pDNA nanoparticles. Uterine tissues were taken 9 days post-injection. (1) 100 base pair ladder, (2) No template RNA control, (3) Thoracic aorta control, (4) LTP-pDNA nanoparticles, (5) β-gal pDNA positive control.
Figure 6.11 Brightfield images of hematoxylin and eosin stained rat uterus sections. Uterus injected with LTP-pDNA nanoparticles at various magnifications (A-C) shows no detectable damage to tissue and comparable inflammation and leukocyte activity to rat uterus injected with blank LTP nanoparticles at various magnifications (D-F) and control rat uterus tissue (G-I). Blue represents nuclei staining and pink/purple represents cytoplasm.
Discussion

This study provides foundational evidence to the efficacy of rapidly degrading L-tyrosine polyphosphate (LTP) nanoparticles as a potential in vivo gene delivery device. Specifically, this investigation reports on the characteristics of the LTP nanoparticles such as morphology and size as well as provides evidence for the successful transfection of uterine tissue in vivo in a rat uterus model.

Using gene delivery as a therapeutic modality to treat a variety of diseases shows promise (Cristano, 1998; Baker, 2002), but has been lessened due to the use of viral vectors as a primary gene delivery device. Viral vectors are quite effective at entering host cells and utilizing the host’s cellular machinery for endpoint protein production. However, viral vectors can lead to severe immune responses which can compromise the overall effectiveness of the gene delivery thus non-viral vectors alternatives have been explored (Baker, 2002). Non-viral gene delivery vectors can make many forms such as condensing DNA with cationic lipids or polymers or encapsulation of the genetic material into a polymer capable of degradation (Itaka et al., 2004; Ditto et al., 2009). Recent studies have shown the promise of non-viral vectors as a potential avenue for gene delivery (see review: Ditto et al., 2009).

Biodegradable nanoparticles formulated from various polymers are an alternative vector for intracellular gene delivery device. Nanoparticles provide an appropriate size for cellular internalization (less than 1 micron) (Ditto et al., 2009) and provide a degradable platform for both protection of the encapsulated genetic material and a mechanism by which the release of the genetic material may be controlled. Various polymers have shown efficacy as a degradable gene delivery vehicle such as
poly[glycolic-lactic acid] (PLGA) (Csaba et al., 2006) and polyvinylpyrrolidione (PVP) (Saxena et al., 2006). However, both of these polymers have differential degradation rates that make them inappropriate as gene delivery vectors destined for a therapeutic avenue.

Recently, nanoparticles formulated from the polymer L-tyrosine polyphosphate (LTP) have shown promise as an intracellular gene delivery device (Ditto et al., 2009). LTP is a noncytotoxic and rapidly degrading polymer due to unstable and hydrolytically degradable phosphoester linkages in the polymer backbone (sen Gupta and Lopina, 2005). However, the rapidly degrading nature of this polymer has been shown to be controlled over a period of 7 days by altering peptide linkages in the polymer backbone. A degradation period of 7 days makes this device a prime candidate for intracellular gene delivery as this is within the life span of most cells (Ditto et al., 2009). LTP also has no effect on localized pH or localized inflammatory responses (Ditto, 2010) and has shown efficacy in vitro in a cell line of human dermal fibroblasts (Ditto et al., 2009). However, the efficacy of LTP nanoparticles in vivo as a gene delivery device remains unclear.

Our LTP nanoparticles as reported by scanning electron microscopy and dynamic laser light scattering are within the nanometer range and should be easily internalized by most cells. Scanning electron microscopy also reveals smooth and spherical shape which may likely make internalization of the nanoparticles easier for the cells. LTP nanoparticles loaded with plasmid DNA encoding for the reporter gene β-galactosidase (LTP-pDNA) were injected into the myometrium of a rat uterus model.

In the rat, the uterus is classified as a duplex organ due to two separate uterine horns emanating from their connecting point of the cervix. The uterus consists of an
inner layer (endometrium) of columnar epithelial cells and an outer layer (myometrium) of smooth muscle cells. We choose the rat uterus as our transfection tissue because of its rapidly cellular turnover (Charnock-Jones et al., 1997). The uterus cycles (estrus cycle) through a highly metabolic cellular state over a period of 4-5 days (Bertalanffy and Lau, 1963) and this provides a prime site to test the efficacy of our LTP-pDNA nanoparticles in vivo.

Other studies have investigated the use of non-viral gene delivery devices in the rodent uterus and oviduct and have shown nominal transfection efficiencies (Charnock-Jones et al., 1997; Relloso and Esponda, 1998). In the current investigation, LTP-pDNA nanoparticles injected into the myometrium of the rat uterus displayed significant transfection in both the glandular epithelium and myometrial smooth muscle of the uterus 9 days post injection as shown by X-gal staining. This successful transfection was confirmed with immunohistochemistry and reverse transcriptase PCR (RT-PCR). Currently it is not known the degradation profile of LTP nanoparticles in vivo. However, as the uterine tissue was harvested 9 days post injection, this suggests that the genetic message encoded in the pDNA encapsulated in LTP nanoparticles was passed over the course of the animals reproductive cycle or that the LTP nanoparticles were persisting in the cells as they divided. Additionally, the stage of the injection during the estrus cycle was not determined to be a factor for the transfection efficiency of the LTP nanoparticles. Thus this investigation proves the concept that LTP-pDNA nanoparticles can successfully transfect uterine tissue in vivo and provides a non-viral alternative for gene delivery.

The major concern of viral vectors is the inherent immune response to the viral protein coat (Anderson, 1998; Hollon, 2000). Our study also examined the immunogenic
response of the uterine tissue to LTP-pDNA nanoparticles. Hematoxylin and eosin staining of LTP-pDNA, Blank LTP and uninjected control uteruses revealed comparable tissue morphology, inflammation, and leukocyte activity further suggesting that our LTP nanoparticles do no elicit any appreciable immune response and may be considered a promising candidate as a non-viral gene delivery vector.

In conclusion, our LTP-pDNA nanoparticles are an intriguing candidate for intracellular gene delivery due to their nanoscale size, enhanced transfection ability, and non-immunogenic reactivity. This study provides proof of concept that LTP-pDNA nanoparticle can successfully, efficiently, and repeatably transfet the rat uterus in vivo with plasmid DNA encoding for the E. coli expressed β-galactosidase gene.
CHAPTER VII

DEVELOPMENT AND POTENTIAL IN VIVO EFFICACY OF LTP-VEGF pDNA NANOPARTICLES IN THE RAT UTERUS

Introduction

The previous chapter (chapter VI) demonstrated the efficacy of LTP-pDNA nanoparticles (β-galactosidase pDNA) as a potential in vivo gene delivery device. The E. coli expressed β-galactosidase DNA is not native to the rat genome thus differentiation between endogenous and exogenous sources is not applicable. However, potential mechanistic and therapeutic approaches may require overexpression or silencing of proteins that are endogenously produced and regulated. Thus the efficacy of the LTP nanoparticle gene delivery system requires further investigation using tagged DNA or proteins.

Vascular endothelial growth factor (VEGF) has emerged as a leading candidate for treatment of various cardiovascular diseases because of its potent angiogenic effects, vascular stabilization features, and its cardioprotective capabilities through vasodilation via the nitric oxide pathway (Kroll and Waltenberger, 1999). The potential therapeutic avenues of VEGF in the treatment of these diseases have been explored extensively (Maharaj, 2007). Of particular interest is the potential role of VEGF in pathological disorders of pregnancy. Free circulating VEGF is reduced in the pathological pregnancy...
disease preeclampsia along with a concomitant increase in soluble fms-like tyrosine kinase 1 (sFlt-1). Soluble Flt-1 is a natural antagonist to VEGF signaling (Belgore et al., 2000) and has been hypothesized to shift the balance of angiogenesis in pregnancy to an anti-angiogenic state in preeclampsia. Numerous therapies aimed at improving VEGF signaling through an increase in circulating VEGF have been demonstrated to reduce maternal hypertension and improve pregnancy outcomes in rats (Li et al., 2007; Gilbert et al., 2010). Recently demonstrated in clinical trials, extracorporeal removal of sFlt-1 in preeclamptic women improved their outcomes suggesting an integral role of VEGF and sFlt-1 in the pathology of preeclampsia (Thadhani et al., 2011). Therefore, utilizing the VEGF signaling pathway as a therapeutic pathway has great potential.

Our previous studies have demonstrated the effectiveness of L-tyrosine polyphosphate (LTP) nanoparticles in the delivery of Bgal pDNA to the rat uterus (Chapter VI). Accordingly, the use of this rapidly degrading biocompatible polymer for the delivery of VEGF DNA in vivo is essential to the success of the delivery system. Nanoparticles formulated from L-tyrosine polyphosphate have been demonstrated to degrade rapidly with non-cytotoxic byproducts formed (Ditto et al., 2009). Therefore, the hypothesis of this investigation is that LTP-VEGF pDNA nanoparticles will transfect the rat uterus in vivo.
Materials and Methods

LTP-VEGF pDNA nanoparticles were formulated as previously described in detail (Ditto et al., 2009). Briefly, LTP-VEGF pDNA nanoparticles were formed via an emulsion of water and oil through a sonication and solvent evaporation technique. A common set of LTP-pDNA nanoparticles consisted of LTP in chloroform (900mg), polyethylene glycol grafted to chitosan (PEG-g-CHN) in acetic acid (3.0mg), VEGF pDNA-LPEI complex (6mg), 10% polyvinylpyrrolidone (100mL). Chloroform was evaporated with stirring for a period of 5 hours and the nanoparticles were collected by centrifugation at 15,000 x g. Nanoparticles were then lyophilized in sterile water (Labconco Freezone 4.5, Kansas City, MO) for 72 hours and stored in a dessicator (Ditto et al., 2009). Blank LTP nanoparticles were also formulated with the same procedure excluding the VEGF pDNA-LPEI complex as a control.

VEGF Plasmid DNA

Plasmid DNA encoding for VEGF protein with a V5-Histidine epitope tag was provided was Dr. Yang H. Yun’s laboratory.

Scanning Electron Microscopy

Nanoparticle samples (LTP-VEGF pDNA) were examined a FEI Quanta 200 Environmental Scanning Electron Microscope (ESEM; Hillsboro, OR). Nanoparticle size, shape, and polymer morphology were qualitatively analyzed. A nanoparticle solution was made for examination by dissolving 1mg of nanoparticles in 1mL of sterile autoclaved water. 200 μL of the nanoparticle solution were placed onto an SEM stub,
allowed to dry completely at room temperature, and the sputter coated with silver/palladium for SEM examination (Ditto et al., 2009).

**Dynamic Laser Light Scattering**

Quantification of the size of the LTP-VEGF pDNA nanoparticles was performed using dynamic laser light scattering (DLS, Brookhaven Instruments BI-200SM, Holtsville, NY). A nanoparticle solution of 1mg of nanoparticles into 10mL of sterile water in a glass scintillation vial was made. Nanoparticle diameters were determined by non-linear least squares (NLLS) and regularized non-negatively constrained least squares (CONTIN). Differential distribution values were reported for the nanoparticle size where the values ranged from 0 to 100 and highest value was assigned a value of 100 and all other sizes were expressed as relative amounts compared to the highest modal value (Ditto et al., 2009).

**Animals**

This investigation utilized virgin, Wistar Kyoto (WKY) females approximately 10-14 weeks of age at the beginning of the study. The animals were housed 3 animals per cage in standard polyethylene cages (45 x 25 x 20cm) with stainless steel tops. Cages contained heat treated wood chip bedding (PJ Murphy Products, Montville, NJ) and standard rat chow (Teklad Rodent, Madison, WI) and water were provided *ad libitum*. The animal room temperatures were held a constant temperature of ~25°C at ~40% humidity. Animal rooms had a 12 hour light (07:00-19:00)/dark (19:00-07:00) cycle. All
animal protocols were reviewed and approved by the University of Akron’s Institutional Animal Care and Use Committee.

*LTP-VEGF pDNA Nanoparticle Injections*

Animals were anesthetized under 2% Isoflurane (Viking Medical) with 100% O₂ under a flow rate of 1L/min. A midline incision along the animal’s longitudinal axis was made in the abdomen and the uterus was exposed. The left uterine horn was isolated and the myometrium was injected (0.5cc insulin syringe; 30 gauge) with 200 uL of 2.5mg of LTP-VEGF pDNA nanoparticles in sterile saline. Control animals were injected with 2.5mg of Blank LTP nanoparticles in sterile saline (the Blank LTP nanoparticles used in this study were initially characterized in Chapter VI).

*Uterus Isolation*

Animals were terminated with an overdose of Sodium Pentothal (50 mg/kg; IP; EJ Lilly, Indianapolis, IN) followed by exsanguination nine days post nanoparticle injection. The left uterine horn was quickly removed and cut into two. One portion of the uterine horn was flash frozen with liquid nitrogen for RNA analysis while the other portion was fixed in 1% buffered formalin for histochemical analysis.

*Cryosectioning of Uterine Tissue*

After uterine horn tissues were fixed with 1% buffered formalin, the tissues were washed overnight in sterile 1x PBS at 4C. The tissues were then transferred to a 30% sucrose solution for cryoprotection overnight at 4C. Tissue blocks were constructed with
the use of Optimal Cutting Temperature (OCT; EMS, Hatfield, PA) and tissues were sectioned on a Bright Model OTF cryosection (Bright Instrument, Huntingdon, Cambs, England). Tissue slices were then stored at 4C until histochemical analysis was performed.

**Immunohistochemistry**

A set of uterine tissue sections were subjected to immunohistochemical analysis. Tissues were permeabilized with 0.2% Triton-X and blocked with 1% newborn calf serum. The primary antibody utilized was mouse monoclonal against the V5 epitope tag (Sigma, St Louis, MO). The antibody was placed on the tissue sections at a 1:500 dilution and stored overnight at 4C. Uterine tissue sections were then washed with sterile PBS and incubated with a polyclonal anti-mouse IgG-TRITC secondary antibody (Sigma, St. Louis, MO) at a 1:200 dilution at 4C. Tissues were imaged using confocal microscopy as described in detail below.

**Confocal Microscopy Imaging**

Uterine tissue samples subjected to immunohistochemical analysis were imaged using a Zeiss 510 META laser-scanning module with an inverted microscope (Axiovert, Carl Zeiss, Peabody, MA). The laser excitation wavelength for this study was 543nm and a 40x oil-immersion objective was used. An emission reference spectrum was created with a non-stained tissue section and a separate section of TRITC secondary antibody. This spectrum spanned wavelengths from 548.6nm to 643.1nm with a step increment of 10.7nm. This reference spectrum was created to distinguish between tissue
autofluorescence and TRITC secondary antibody fluorescence. Once this spectrum was integrated into the LSM 510 software used for the confocal microscope, the immunohistochemically stained uterine tissue sections were subjected to lambda stacking from 548.6nm to 643.1nm at a step increment of 10.7nm. Using the reference spectrum created, the fluorescence of the TRITC secondary antibody was unmixed from the uterine tissue autofluorescence and assigned separate colors for distinguishing (green for uterine tissue autofluorescence and red for the TRITC secondary antibody). Control images were taken from Blank LTP nanoparticle injected samples and stained with TRITC secondary antibody.

Hematoxylin and Eosin Staining

Cryosectioned uterine tissues as described previously in this chapter were subjected to hematoxylin and eosin staining (H&E) for analysis of tissue damage. Uterine tissue sections were stained with Gills hematoxylin and counterstained using Eosin Y. Uterine tissue sections were then dehydrated and mounted for brightfield imaging as previously described in this chapter. Histological analysis of tissue morphology, inflammatory and leukocyte markers were assessed.

Reverse Transcriptase PCR (RT-PCR)

Uterine horn tissues were homogenized under liquid nitrogen with mortar and pestle and RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA for the uterine samples was isolated according to the manufacturer’s instructions. Plasmid DNA or other DNA contaminates were removed by treatment of the RNA with
DNase I (1U/µL; Roche) and an RNA cleanup kit (Qiagen, Valencia, CA). Purified RNA was stored at -80°C until analysis.

RT-PCR was performed with a one-step RT-PCR kit (Qiagen) according to manufacturer’s instructions. The following primers were used:

Forward: 5’-AGGCCAGCACATAGGAGAGA-3’
Reverse: 5’-CGTAGAATCGAGACCGAGGA-3’

The protocol was modified to 30 cycles with a denaturing cycle of 94°C (1min), an annealing cycle of 53°C (1min), and an extension cycle of 68°C (1min). The RT-PCR reaction products were analyzed on a 1% agarose gel with an amplicon of 310 base pairs indicative of VEGF V5-His origin.

**Results**

Figure 7.1 is a typical scanning electron micrograph of LTP-VEGF pDNA nanoparticles. Qualitative analysis of the images demonstrates smooth and spherical morphologies. Encapsulation of plasmid DNA did not alter the morphology of the nanoparticles.

*Dynamic Laser Light Scattering*

Figure 7.2 quantifies the size of LTP-VEGF pDNA nanoparticles. CONTIN demonstrated that LTP-VEGF pDNA nanoparticles ranged from 163nm – 299nm with a mean nanoparticle diameter of 227 ± 39nm. Encapsulation of VEGF plasmid DNA did not alter the size of the nanoparticles.
**LTP-VEGF pDNA Nanoparticle Loading**

LTP-VEGF pDNA nanoparticles demonstrated a loading efficiency of 2% ± 0.005%.

**Immunohistochemistry**

Uterine tissue injected with LTP-VEGF pDNA nanoparticles demonstrated positive signal transfection with little to no appreciable signal found in Blank LTP control (Figure 7.3). Positive signal was primarily located near blood vessels suggesting possible localization to endothelial cells.

**Reverse-transcriptase PCR (RT-PCR)**

Figure 7.4 demonstrated the efficiency of VEGF 310 primers for the VEGF pDNA encapsulated in the nanoparticles. However, RNA transcripts for the VEGF pDNA were not able to be deciphered in the rat uterus.

**Hematoxylin and Eosin Staining**

Figure 7.5 demonstrates H&E staining of rat uterus tissue injected with LTP-VEGF pDNA nanoparticles, Blank LTP nanoparticles or uninjected control. No tissue apoptosis was found and minimal leukocyte activity was noted.
Figure 7.1 Scanning electron micrograph of LTP-VEGF pDNA nanoparticles (20,000x)
Figure 7.2 Representative size distribution of LTP-VEGF pDNA nanoparticles.

Diameters were determined using regularized non-negatively constrained least squares (CONTIN).
Figure 7.3 Combined confocal fluorescent images of *in vivo* rat uterus sections immunostained with anti-V5 mouse primary Ab followed by anti-mouse IgG-TRITC secondary Ab. Uterus injected with LTP-pDNA nanoparticles at tissue section depths of 2.5 µm, 15 µm, and 27.5 µm shows staining of VEGF pDNA. Rat uterus injected with blank LTP nanoparticles at tissue section depths of 2.5µm, 15 µm, and 27.5 µm does not show expression of VEGF pDNA. TRITC fluorescent probe – Red; Tissue autofloresence - Green
Figure 7.4 Hematoxylin and eosin staining of LTP-VEGF pDNA nanoparticles for A) 10x magnification, B) 20x magnification and C) 63x magnification. Blue represents nuclei staining and pink/purple represents cytoplasm.
Figure 7.5 Primer efficacy of VEGF 310 primers on VEGF plasmid DNA. 1) 100bp ladder, 2) pDNA with VEGF 310 primers, 3) pDNA positive control.

Forward primer: 5’ AGGCCAGCACATAGGAGAGA 3’,
Reverse primer: 5’ GGATTGGGAGAGGAGCCAGA 3’
Discussion

The results of this study provide evidence of the successful encapsulation and engineering of LTP-VEGF pDNA nanoparticles. Furthermore, immunohistochemical staining revealed positive transfection of experimental tissue with LTP-VEGF pDNA nanoparticles in the rat uterus. Additionally, hematoxylin and eosin staining revealed no inflammation, minimal leukocyte activity and no instances of cellular apoptosis indicating the potential use of LTP nanoparticles as a non-viral gene delivery device in vivo.

Our research group has previously demonstrated the efficacy of nanoparticles formulated from L-tyrosine polyphosphate as a gene delivery device in vitro (Ditto et al., 2009). Recent evidence suggests the potential of LTP nanoparticles as a delivery vehicle in vivo such as the delivery of silver carbene complexes to a mouse model of cystic fibrosis (Hindi et al., 2009). In this study, we engineered LTP nanoparticles encapsulated with VEGF pDNA. Scanning electron microscopy revealed smooth spherical nanoparticles in accordance with our previous studies (Ditto et al., 2009). Additionally, the nanoparticles are within the size that can be endocytosed by cells as shown by dynamic laser light scattering (Desai et al., 2002).

Delivery of VEGF to the systemic circulation has provided anecdotal data. Systemic delivery has not provided adequate levels of VEGF to a specific tissue due to its degradation by matrix metalloproteinases and other mechanisms associated with the actual delivery device composition (Lee et al., 2005). However, delivery of VEGF protein through minipump chronic infusion has been demonstrated to improve the maternal hypertension shown in preeclamptic rats (Gilbert et al., 2010). Recently,
nanoparticles have been formulated from polymers such as poly(lactic-co-glycolic acid) (PLGA) to delivery VEGF protein to promote vascular growth as a potential cardiovascular therapy (Golub et al., 2010). While these studies demonstrate the positive effects of VEGF protein delivery for cardiovascular related treatment, too much VEGF protein can lead to glomeruloid body formation throughout blood vessels due to the enhanced leakiness conferred to the vessels by VEGF (Sundberg et al., 2001). This observation can lead to compromised luminal diameter and blood perfusion throughout the vessel system (Sundberg et al., 2001). Therefore, delivery of VEGF protein will require stringent control in order to provide a therapeutic potential for cardiovascular related diseases.

Delivery of the precursor material in vivo to make, package, and deliver the cells own VEGF would pose a potential therapy relying on the cells’ own mechanisms to produce and regulate the end product and thus reducing diffusive, degradation, regulatory, and tissue specificity obstacles that have historically impeded delivery efficiency. However, delivery of naked VEGF plasmid DNA (pDNA) to patients with myocardial ischemia did not lead to any changes in cardiac functions due to low transfection efficiency in a phase 1 clinical trial (Losordo et al., 1998). In another phase 1 clinical trial, patients received adenoviral-mediated VEGF pDNA delivery injected directly into the ischemic myocardium. The results showed that there was minimal wall improvement via angiography and the authors suggest that this could be a possible treatment to patients with severe coronary artery disease (Rosengart et al., 1999). However, delivery of naked DNA and adenoviral based transfection have overall exhibited limited success and raised many safety concerns. Repeated exposure to
adenoviruses may lead to enhanced immune response which will attenuate the gene transfer overall (Harvey et al., 1999) through apoptosis of cells exposed to adenoviruses (Hao et al., 2008).

Nanoparticles formulated from L-tyrosine polyphosphate have been demonstrated to be non-cytotoxic *in vitro* (Ditto et al., 2009) and *in vivo* have been shown to elicit low immunogenic response as the nanoparticles do not activate antigen-presenting cells (Jawyn, 2011). Our study demonstrated low immunogenicity as well with H&E staining. LTP nanoparticles have also shown enhanced cellular transfection in human dermal fibroblasts (Ditto et al., 2009). In this study, LTP-VEGF pDNA nanoparticles successfully transfected the rat uterus as demonstrated by immunohistochemical staining. The positive transfection was located near blood vessel structures suggesting localization to the vascular endothelium. Unfortunately, reverse transcriptase PCR was unable to resolve RNA transcripts of VEGF pDNA origin after 9 days post-injection. Potential pitfalls for this failure to resolve the transcripts may involve the noted instability of VEGF mRNA under normoxic conditions (Dibbens et al., 1999) which is likely present in the rat uterus. Additional mechanisms may include the noted effect of VEGF mRNA forming complexes with RNA binding proteins (Vumbaca et al., 2008), formation of secondary structures in the 3’ untranslated region of the RNA (Wagner et al., 2001) as this has been demonstrated to impede translation of the mRNA (van Kovwenhove et al., 2011).

In summary, we have successfully encapsulated VEGF pDNA within the LTP nanoparticles as a potential *in vivo* gene delivery platform. Immunohistochemical analysis of LTP-VEGF pDNA nanoparticles injected into the rat uterus revealed positive
fluorescent staining that was localized near blood vessel structures. PCR analysis of mRNA isolated from injected uteri failed to elucidate transcripts of VEGF pDNA which may be due to the stability of VEGF mRNA \textit{in vivo}. Additionally, there was no apparent immune response of LTP-VEGF pDNA nanoparticles in the rat uterus. These data suggest a potential use of LTP nanoparticles encapsulated with plasmid DNA as a potential \textit{in vivo} gene delivery device.
Conclusions

Our studies in the pregnant rat demonstrate an integral role of reductions in uterine perfusion pressure on uterine artery function and mechanical properties (Reho et al., 2012). We also demonstrate the impact reductions in uterine perfusion pressure in the pregnant rat on the maternal response to the uterine insults and the overall outcomes of the fetuses. Additionally, chronic reductions in uterine perfusion pressure led to detrimentally altered uterine artery reactivity indicative of a constrictive phenotype which may play an integral role in both the maternal response to the pathology as well as the fetal growth response. Structural remodeling of resistance-caliber uterine arteries does not appear to occur in response to reductions in uterine perfusion pressure, however biophysical mechanical remodeling of the uterine arteries does occur leading to arteries that are less able to dilate. This result likely diminishes the arteries ability to provide adequate perfusion to the feto-placental unit.

Another facet of this dissertational research regarded the use of nanoparticles as a potential in vivo gene delivery device. Our studies with nanoparticles formulated from the non-cytotoxic, biodegradable polymer L-tyrosine polyphosphate (LTP) provide evidence for the use of LTP nanoparticles encapsulated with plasmid DNA as a potentially efficacious in vivo gene delivery device. Injection of LTP-pDNA
nanoparticles into the rat uterus resulted in transfection which was observed 9 days after injection of the uterine tissue \textit{in vivo} and LTP nanoparticles do not seem to elicit an immune response \textit{in vivo}.

Taken together, the data presented in this dissertation provide the foundational evidence for the vascular dysfunction that occurs in the uterine circulation in response to reductions in uterine perfusion pressure and the potential use of LTP nanoparticles as an \textit{in vivo} gene delivery vehicle.

**Future Directions**

The results of the studies comprising this dissertation represent two seemingly separate paths of research that ultimately are to be integrated together to form the basis of a potential therapeutic approach to the treatment of the pregnancy disease preeclampsia. The data collected from these studies on uterine arterial reactivity during a preeclampsia-like pathology and the efficacy of LTP nanoparticles as a potential \textit{in vivo} gene delivery device are in need of future research to continue the progress towards a potential vascular therapy for preeclampsia. The following are outlines of future research projects that may provide further insight into both uterine artery mechanisms involved in response to reductions in uterine perfusion pressure and the use of LTP nanoparticles as an \textit{in vivo} gene delivery device.

*Future Direction #1*

The data presented in this dissertation regarding uterine artery reactivity in RUPP animals is exciting as it is the first report to demonstrate altered vascular reactivity in the
uterine circulation in response to reductions in uterine perfusion pressure (Reho et al., 2012) which is thought to be a hallmark of preeclampsia. Furthermore this research has demonstrated an integral of nitric oxide in the mediation of myogenic reactivity and vasodilation of uterine arteries in response to reductions in uterine perfusion pressure and the resulting maternal hypertension. Additional research is warranted into mechanisms associated with the increased myogenic reactivity and decreased vasodilation demonstrated in this dissertation. Mechanisms of interest would be angiogenic, inflammatory and oxidative stress indices as they have been clearly demonstrated to play a role in the pathology of preeclampsia in women. A more complete understanding of how uterine arteries function in RUPP rats may provide a new avenue of research into the vascular mechanisms associated with preeclampsia.

*Future Direction #2*

The present research demonstrated no changes in structural parameters in uterine arteries from RUPP pregnant rats compared to SHAM. However, mechanical parameters of the uterine arteries associated with the extracellular matrix and the vascular wall were altered suggesting that reductions in uterine perfusion pressure during pregnancy can lead to biophysical changes in uterine arteries. Further research into the mechanisms associated with these changes is essential. Women with preeclampsia demonstrate alterations in circulating levels of matrix metalloproteinases (MMP) which may be involved in localized vascular remodeling within the uterine circulation (Shokry et al., 2009). A profile of the various MMP’s in the uterine circulation would provide insight into these mechanisms. Additionally, blocking the effects of these signals with inhibitors
or antibodies would also create a mechanistic investigation as to the effects of reductions in uterine perfusion pressure on uterine artery biophysical parameters. Measurements of collagen and elastin in the uterine arteries would also provide valuable information on mechanical parameters of the uterine arteries.

**Future Direction #3**

LTP nanoparticles encapsulated with plasmid DNA has demonstrated efficacy as an *in vivo* gene delivery device in the rat uterus. However, the mechanism by which this successful transfection occurs remains unclear. Further research is needed into the mechanisms of extracellular transport, internalization of the nanoparticle, and its subsequent release of encapsulation within the cell is warranted. How the plasmid DNA travels to the nucleus and sheds the LPEI polymer is also in need of further research. Recent studies have shown that LTP nanoparticles can be decorated with targeting molecules capable of site specific binding. Formulations of nanoparticles with targeting molecules specifically designed for uterine arteries will be essential for further research and the development of potential therapies aimed at the vascular pathology of preeclampsia.

**Future Direction #4**

Vascular endothelial growth factor (VEGF) has shown promise not only as a diagnostic tool for preeclampsia but also as a potential therapy for the maternal hypertension and vascular dysfunction associated with the disease. Recent evidence has demonstrated an integral role of the VEGF 2 receptor in blood pressure regulation and
potentially vascular reactivity (Facemire et al., 2009). This dissertation has provided evidence that the uterine circulation possesses a potent VEGF dilation response that is attenuated in response to reductions in uterine perfusion pressure. The VEGF 2 receptor has been implicated in the vasodilation response in response to VEGF. Development of LTP nanoparticles with the ability to upregulate the VEGF 2 receptor in the uterine circulation may provide a potential therapy aimed at the vascular level. Further research into this potential mechanism is warranted.
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APPENDIX A

IACUC APPROVAL LETTERS

October 7, 2008

Dr. Ramirez
Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Ramirez,

On August 29, 2008 the Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Ramirez, "Episodic Aortic occlusion; an alternative to RUPP to mimic preeclampsia pathology"

16 rats

IACUC number 08-7F

Your project has received unanimous approval.

You must notify the committee concerning modifications to the approved protocol. In addition, yearly updates regarding the status of this project are required. IACUC must also be notified of serious or adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Please be aware that approval of your protocol does not guarantee space in the animal facility.

Sincerely,

James Holda
IACUC Chair
October 27, 2008

Dr. Ramirez
Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Ramirez

On October 23, 2008 the Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Ramirez, Mr. John Reho, “Oxidative stress after reduced uterine perfusion pressure in pregnant rats.”

IACUC number 08-9D

Your project has received unanimous approval.

You must notify the committee concerning modifications to the approved protocol. In addition, yearly updates regarding the status of this project are required. IACUC must also be notified of serious or adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Please be aware that approval of your protocol does not guarantee space in the animal facility.

Sincerely,

James Holda
IACUC Chair
March 6, 2009

Dr. Ramirez  
Department of Biology  
The University of Akron  
Akron, OH 44325  

Dear Dr. Ramirez,

On March 4, 2009 the Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Ramirez, Mr. Reho "VEGFR-2 Gene Delivery to Pregnant Rats with Reduced Uterine Perfusion Pressure"

IACUC number 09-3A

Your project has received unanimous approval.

You must notify the committee concerning modifications to the approved protocol. In addition, yearly updates regarding the status of this project are required. IACUC must also be notified of serious or adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Sincerely,

[Signature]

James Horda  
IACUC Chair
February 11, 2011

Dr. Ramirez
Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Ramirez:

On February 9, 2011 the Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Ramirez, Mr. Reho, "LTP Nanoparticles as a Gene Delivery Device to the Uterus".
IACUC number 11-1A

Your project has received unanimous approval.

You must notify the committee concerning modifications to the approved protocol. In addition, yearly updates regarding the status of this project are required. IACUC must be notified of serious or adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Please be aware that approval of your protocol does not guarantee space in the animal facility.

Sincerely,

James Holdia
IACUC Chair
APPENDIX B

SUPPLEMENTAL PASSIVE STRUCTURAL, MECHANICAL, AND AREA MEASUREMENTS OF UTERINE AND MESENTERIC ARTERIES FROM SHAM AND RUPP RATS (PRESSURES 0-10 MMHG)

Table A.1 Structural parameters of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM)

<table>
<thead>
<tr>
<th>Intraluminal Pressure (mmHg)</th>
<th>Uterine Arteries</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive Diameter (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>152.2 ± 7.9</td>
<td>165.6 ± 9.1</td>
<td>174.2 ± 9.3</td>
<td>192.3 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>RUPP</td>
<td>197.3 ± 19.1</td>
<td>216.0 ± 20.0</td>
<td>228.8 ± 20.8</td>
<td>245.5 ± 20.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wall Thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>37.4 ± 2.4</td>
<td>35.7 ± 1.9</td>
<td>34.0 ± 1.9</td>
<td>32.4 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>RUPP</td>
<td>38.9 ± 3.9</td>
<td>36.3 ± 4.0</td>
<td>34.1 ± 4.0</td>
<td>31.4 ± 3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wall:Lumen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>0.25 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>RUPP</td>
<td>0.22 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.14 ± 0.02</td>
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</tr>
</tbody>
</table>
Table A.2 Mechanical parameters of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM).

<table>
<thead>
<tr>
<th>Uterine Arteries</th>
<th>Intraluminal Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Tension (dynes/cm)</td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>NA</td>
</tr>
<tr>
<td>RUPP</td>
<td>NA</td>
</tr>
<tr>
<td>Wall Stress (dynes/cm²)</td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>NA</td>
</tr>
<tr>
<td>RUPP</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table A.3 Area parameters of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM).

<table>
<thead>
<tr>
<th>Intraluminal Pressure (mmHg)</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross Sectional Area (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>22878 ± 2388</td>
<td>22802 ± 2104</td>
<td>21864 ± 1978</td>
<td>22938 ± 1948</td>
</tr>
<tr>
<td>RUPP</td>
<td>29370 ± 4376</td>
<td>29612 ± 4599</td>
<td>29135 ± 4632</td>
<td>27960 ± 4786</td>
</tr>
<tr>
<td>Lumen Area (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>18718 ± 2014</td>
<td>22240 ± 2618</td>
<td>24560 ± 2790</td>
<td>29591 ± 2596</td>
</tr>
<tr>
<td>RUPP</td>
<td>33717 ± 6502</td>
<td>40089 ± 7248</td>
<td>44656 ± 7638</td>
<td>51040 ± 8215</td>
</tr>
<tr>
<td>Mesenteric Arteries</td>
<td>Intraluminal Pressure (mmHg)</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Passive Diameter (μm)</td>
<td>SHAM</td>
<td>194.8 ± 10.9</td>
<td>203.0 ± 11.4</td>
<td>207.2 ± 11.9</td>
</tr>
<tr>
<td>RUPP</td>
<td>185.5 ± 5.7</td>
<td>191.9 ± 6.4</td>
<td>195.6 ± 6.8</td>
<td>204.7 ± 7.5</td>
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<tr>
<td>Wall Thickness (μm)</td>
<td>SHAM</td>
<td>35.3 ± 2.3</td>
<td>34.1 ± 2.2</td>
<td>32.4 ± 2.4</td>
</tr>
<tr>
<td>RUPP</td>
<td>39.2 ± 2.6</td>
<td>37.7 ± 2.5</td>
<td>36.2 ± 2.2</td>
<td>34.8 ± 2.1</td>
</tr>
<tr>
<td>Wall:Lumen</td>
<td>SHAM</td>
<td>0.19 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>RUPP</td>
<td>0.22 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

Table A.4 Structural parameters of resistance-caliber mesenteric arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM).
Table A.5 Mechanical parameters of resistance-caliber mesenteric arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM).

<table>
<thead>
<tr>
<th>Mesenteric Arteries</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension (dynes/cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>NA</td>
<td>40.6 ± 2.3</td>
<td>69.1 ± 4.0</td>
<td>144.3 ± 8.3</td>
</tr>
<tr>
<td>RUPP</td>
<td>NA</td>
<td>38.4 ± 1.3</td>
<td>65.2 ± 2.3</td>
<td>136.4 ± 5.0</td>
</tr>
<tr>
<td>Wall Stress (dynes/cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>NA</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.03</td>
<td>0.5 ± 0.07</td>
</tr>
<tr>
<td>RUPP</td>
<td>NA</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.02</td>
<td>0.5 ± 0.05</td>
</tr>
<tr>
<td>Mesenteric Arteries</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Cross Sectional Wall Area (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>25873 ± 1621</td>
<td>25475 ± 1600</td>
<td>24530 ± 1784</td>
<td>24248 ± 1717</td>
</tr>
<tr>
<td>RUPP</td>
<td>28047 ± 1856</td>
<td>27262 ± 1889</td>
<td>26872 ± 1878</td>
<td>26506 ± 1789</td>
</tr>
<tr>
<td>Lumen Area (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>30635 ± 3557</td>
<td>33266 ± 3909</td>
<td>34697 ± 4156</td>
<td>37898 ± 4551</td>
</tr>
<tr>
<td>RUPP</td>
<td>27240 ± 1710</td>
<td>29193 ± 1987</td>
<td>30356 ± 2140</td>
<td>33295 ± 2527</td>
</tr>
</tbody>
</table>

Table A.6 Area parameters of resistance-caliber mesenteric arteries from SHAM and RUPP pregnant rats (Pressures 0-10 mmHg) (means ± SEM).
Figure A.1 Myogenic reactivity of resistance-caliber uterine arteries with and without preincubation with N-methyl-L-arginine (L-NMA) from SHAM and RUPP pregnant rats (means ± SEM).
Figure A.2 Methacholine induced percent vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM). *p<0.05
Figure A.3 Methacholine induced percent maximum vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM). *p<0.05
APPENDIX E

SUPPLEMENTAL VEGF VASODILATION DATA OF RESISTANCE-CALIBER UTERINE ARTERIES FROM SHAM AND RUPP PREGNANT RATS

Figure A.4 VEGF induced percent vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM). *p<0.05
Figure A.5 VEGF induced percent maximum vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM). *p<0.05
APPENDIX F

SUPPLEMENTAL SODIUM NITROPRUSSIDE VASODILATION DATA OF RESISTANCE-CALIBER UTERINE ARTERIES FROM SHAM AND RUPP PREGNANT RATS

Figure A.6 Sodium nitroprusside induced percent vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM).
Figure A.7 Sodium nitroprusside induced percent maximum vasodilation of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM).
APPENDIX G

SUPPLEMENTAL PHENYLEPHRINE AND U-46619 VASOCONSTRICTION DATA
OF RESISTANCE-CALIBER UTERINE ARTERIES FROM SHAM AND RUPP
PREGNANT RATS

Figure A.8 Phenylephrine induced vasoconstriction of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM).
Figure A.9 U-46619 induced vasoconstriction of resistance-caliber uterine arteries from SHAM and RUPP pregnant rats (means ± SEM).
Figure A.10 Differential distribution values of LTP-pDNA nanoparticles using dynamic light scattering for both non-linear least squares (NLLS).
Figure A.11 Differential distribution values of Blank LTP nanoparticles using dynamic light scattering for both non-linear least squares (NLLS).
Figure A.12 Mean nanoparticle diameters (nm) of LTP-pDNA nanoparticles using dynamic light scattering for both non-linear least squares (NLLS) and regularized non-negatively constrained least squares (CONTIN). Data are expressed as mean ± SEM.
Figure A.13 Mean nanoparticle diameters (nm) of Blank LTP nanoparticles using dynamic light scattering for both non-linear least squares (NLLS) and regularized non-negatively constrained least squares (CONTIN). Data are expressed as mean ± SEM.
Figure A.14 Brightfield images of Xgal replicates of LTP-pDNA nanoparticles injected into rat uteri.
Figure A.15  RT-PCR analysis of RNA replicates from 4 different rat uteri injected with LTP-pDNA nanoparticles 9 days post injection.
Figure A.16 Differential distribution values of VEGF LTP nanoparticles using dynamic light scattering for both non-linear least squares (NLLS).
Figure A.17 Mean nanoparticle diameters (nm) of VEGF LTP nanoparticles using dynamic light scattering for both non-linear least squares (NLLS) and regularized non-negatively constrained least squares (CONTIN). Data are expressed as mean ± SEM.
Figure A.18 Brightfield images of LTP-VEGF pDNA and Blank LTP nanoparticles injected into the rat uterus.  A) Blank LTP 20x magnification, B) LTP-VEGF 20x magnification, C) Blank LTP 63x magnification, D) LTP-VEGF 63x magnification.
Figure A.19 Agarose gel 1) 1kb ladder 2) VEGFR-2/pEF-1 3) pEF-1
<table>
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<tr>
<th>Direction</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Forward – Kpn1</td>
<td>5’ – CGGGGTACCGAACCATGGGAGCAAGGGT – 3’</td>
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<tr>
<td>Reverse – Spe1</td>
<td>5’ – GGACTAGTAACAGGAGAGAGCTCAGTGGTC – 3’</td>
</tr>
</tbody>
</table>

Table A.7 Primer sequences used for cloning of the VEGF 2 receptor gene
Figure A.20 1) 1kb ladder; 2) pEF-1; 3) VEGFR-2/pEF-1; 4) Hind III digest
<table>
<thead>
<tr>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-VEGFRseq</td>
<td>5’ – AAACTGACTTGGCCTCG – 3’</td>
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<tr>
<td>RC-L1-VEGFRseq</td>
<td>5’ – CGAGGCCAAGTCAGTTT – 3’</td>
</tr>
<tr>
<td>L2-VEGFRseq</td>
<td>5’ – AGTCCGTCTCATGGAATT – 3’</td>
</tr>
<tr>
<td>RC-L2-VEGFRseq</td>
<td>5’ – AATTCCATGAGACGGACT – 3’</td>
</tr>
<tr>
<td>L3-VEGFRseq</td>
<td>5’ – TACCTTGGTTACCCACC – 3’</td>
</tr>
<tr>
<td>RC-L3-VEGFRseq</td>
<td>5’ – GGTTGGTACCCAAGGTA – 3’</td>
</tr>
<tr>
<td>L4-VEGFRseq</td>
<td>5’ – GTCTCAGTGACAAACCCA – 3’</td>
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<tr>
<td>RC-L4-VEGFRseq</td>
<td>5’ – TGGGTTTGTCACTGAGAC – 3’</td>
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<tr>
<td>L5-VEGFRseq</td>
<td>5’ – TGCCACCATGTCTCTTA – 3’</td>
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<tr>
<td>RC-L5-VEGFRseq</td>
<td>5’ – TAGAGAACATGGTGAGCA – 3’</td>
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<td>L6-VEGFRseq</td>
<td>5’ – TGTCTTGGCTGTGGA – 3’</td>
</tr>
<tr>
<td>RC-L6-VEGFRseq</td>
<td>5’ – GGTGGGTAACCAAGGTA – 3’</td>
</tr>
<tr>
<td>L7-VEGFRseq</td>
<td>5’ – GAAGGGAGCAACACACACAG – 3’</td>
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<tr>
<td>RC-L7-VEGFRseq</td>
<td>5’ – CTGTGTGTGCTCCTTC – 3’</td>
</tr>
<tr>
<td>L8-VEGFRseq</td>
<td>5’ – AAGTGGCTAAGGGCAT – 3’</td>
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<td>RC-L8-VEGFRseq</td>
<td>5’ – ATGCCCTTAGCCACTT – 3’</td>
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<tr>
<td>L9-VEGFRseq</td>
<td>5’ – ATGTAACCAGACCATTGCTG – 3’</td>
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<td>5’ – CGACATGCTCTGTTACAT – 3’</td>
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<td>L10-VEGFRseq</td>
<td>5’ – CTTTTTGTTGGAATGGTGC – 3’</td>
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<tr>
<td>RC-L10-VEGFRseq</td>
<td>5’ – GCACCATTCCACAAAAAG – 3’</td>
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</tbody>
</table>

Table A.8 Primer sequences used for sequencing of the VEGFR-2/pEF-1 insert
Figure A.21 Typical images of transfection of human dermal fibroblasts with VEGFR2-pEF1 and non-transfected controls.