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MECHANISMS OF SEIZURE DURING PREGNANCY AND PREECLAMPSIA

A Dissertation Presented

by

Abbie Chapman Johnson

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Neuroscience

May, 2015

Defense Date: February 23, 2015
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ABSTRACT

Eclampsia is defined as de novo seizure in a woman with the hypertensive complication of pregnancy known as preeclampsia (PE), and is a leading cause of maternal and fetal morbidity and mortality worldwide. The pathogenesis of eclamptic seizure remains unknown, but is considered a form of hypertensive encephalopathy where an acute rise in blood pressure causes loss of cerebral blood flow (CBF) autoregulation and hyperperfusion of the brain that results in vasogenic edema formation and subsequent seizure. However, eclamptic seizure can occur during seemingly uncomplicated pregnancies, in the absence of hypertension and PE, suggesting that normal pregnancy may predispose the brain to hypertensive encephalopathy or seizure, independently of PE. The overall goal of this dissertation was to investigate the effect of pregnancy and PE on the cerebrovasculature and neurophysiological properties that may promote brain injury and eclamptic seizure. For this dissertation project, a rat model of PE was established that combined placental ischemia, induced by restricting blood flow to the uteroplacental unit, and maternal endothelial dysfunction that was induced by a prolonged high cholesterol diet. Rats with PE developed several PE-like symptoms, including elevated blood pressure, fetal growth restriction, placental dysfunction, and were in a state of oxidative stress and endothelial dysfunction. We found that pregnancy had an overall protective effect on the maintenance of CBF that was potentially due to a nitric-oxide dependent enhancement of the vasodilation of cerebral arteries to decreased intravascular pressure. Further, maintenance of CBF during acute hypertension was similar in pregnancy and PE. Thus, it does not appear that pregnancy and PE are states during which CBF autoregulation is compromised in a manner that would promote the development of hypertensive encephalopathy. However, the brain was found to be in a hyperexcitable state during normal pregnancy that was augmented in PE, and could contribute to onset of eclamptic seizure. Under chloral hydrate anesthesia, generalized seizure was induced by timed infusion of the convulsant pentylenetetrazole (PTZ), with simultaneous electroencephalography that was stopped at the first onset of spikewave discharge indicative of electrical seizure. Seizure threshold was determined as the amount of PTZ required to elicit seizure. Compared to the nonpregnant state, seizure threshold was ~44% lower in pregnant rats and ~80% lower in rats with PE. Further, pregnant rats were more susceptible to seizure-induced vasogenic edema formation than the nonpregnant state. Mechanisms by which pregnancy and PE lowered seizure threshold appeared to be through pregnancy-associated decreases in cortical γ -aminobutyric acid type A receptor (GABA_AR) subunits and PE-induced disruption of the blood-brain barrier (BBB) and microglial activation, indicative of neuroinflammation. Magnesium sulfate (MgSO₄), the leading treatment for seizure prophylaxis in women with PE, restored seizure threshold to control levels by reversing neuroinflammation in PE rats, without affecting BBB permeability. Overall, this dissertation provides evidence that pregnancy increases susceptibility of the brain to seizure and vasogenic edema formation that likely contribute to the onset of eclampsia during seemingly uncomplicated pregnancies. Further, the pathogenesis of eclampsia during PE likely involves breakdown of the BBB and subsequent neuroinflammation, resulting in a state of greater seizure susceptibility that is ameliorated by MgSO₄ treatment.

CITATIONS

Material from this dissertation has been published in the following form:

Chapman, A.C., Cipolla, M.J., and Chan, S.L.. (2013) Effect of pregnancy and nitric oxide on the myogenic vasodilation of posterior cerebral arteries and the lower limit of cerebral blood flow autoregulation. *Repro Sci*, 20(9): 1046-54.

Johnson, A.C., Tremble, S.M., Chan, S.L., Moseley, J., LaMarca, B., Nagle, K.J., and Cipolla, M.J.. (2014) Magnesium sulfate treatment reverses seizure susceptibility and decreases neuroinflammation in a rat model of severe preeclampsia. *PLoS One*, Nov 19;9(11):e113670. doi: 10.1371/journal.pone.0113670. eCollection 2014.

Material from this dissertation has been submitted for publication to *PLoS One* on January 26, 2015 in the following form:

Johnson, A.C., Nagle, K.J., Tremble, S.M., and Cipolla, M.J.. (2015) The contribution of normal pregnancy to eclampsia.

ACKNOWLEDGEMENTS

First and foremost, I need to thank my mentor, Dr. Marilyn J. Cipolla for being, in a word, tremendous. You have provided me for the better part of a decade (really? that long?) with continuous guidance, advisement, and patience. You have taught me many things, too many to list, but most importantly to be fearless, to face things head on, and never go backwards, professionally or personally. Your high expectations of me at times felt impossible to meet, and even more impossible to maintain, yet your confidence in me that often far surpassed my own helped me get there. I will keep my eye on the forest through the trees. Thank you.

I also thank my wonderful lab family for being constant and understanding, and for all of your technical and emotional support over the years. In particular, Julie Sweet and Kelvin Chan: “We shall not separate!”

To my goofy, full-hearted husband, Travis Johnson: You, I do not know how to thank. It’s too much. Embarking on this seemingly endless long distance lifestyle has often felt like a cruel joke, yet both of our careers have benefited from it. Without your unyielding love and support I do not know where we would be, but I doubt it would be here. You have been there to celebrate the best times, albeit sometimes from 3000+ miles away, and more importantly, you have applied coat after coat of glue, sticking me back together during the worst. You haven’t let me lose those silly, carefree parts of myself that you fell in love with, even though at times over the past 5 years there has been no room for them. You are my night sky. For that I will always be thankful, and I cherish you very much.

I would like to thank my parents who, in each of their own way, have contributed an enormous amount to my motivation, determination and stubbornness. From a young age you gave me perspective, and taught me the crucial value of perseverance. I sometimes forget that I don’t just get my hard-headed qualities from you, but I get many of my best qualities from you as well (like my laugh-so-hard-my-body-doesn’t-know-if-it’s-still-laughing-or-now-crying cackle that is indistinguishable from yours, Mom). Dad, I thank you for being relentless, in all meanings of the word. Mom, I thank you for being a bottomless well of love and kindness.

To my co-inhabitants of Ph.D. Land: What a strange and unique existence. These friendships have been odd, and some have shifted like the tide. Cheers to the shared successes, years of misery, hours of early morning coffee rants and pity parties, and to learning to celebrate the most minute of successes and to capitalize on those times to let loose or lose your mind. Thank you Matt LeComte, Liana Merrill, Eric Gonzalez, Stephanie Spohn, Dave Harris, and Anthony Pappas, for we are, or have been, fortunate to have one another as leaning posts.

To all of my other friends and family, particularly from Down East, thank you for giving quieter strength and encouragement from the sidelines.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 Preeclampsia and Eclampsia

Preeclampsia, defined as the new onset of hypertension with significant proteinuria after the 20th week of gestation, is a life-threatening complication that afflicts 1-7% of all pregnancies (Roberts and Redman, 1993; Lisonkova and Joseph, 2013; Abalos et al., 2014; Rich-Edwards et al., 2014). Preeclampsia is a heterogeneous disease that exists on a spectrum from mild to severe and can affect many organs, including the kidney, liver, as well as the brain (Duley, 1992; Donaldson, 1994b; Aukes et al., 2007b; Aukes et al., 2009; Duley, 2009). Neurologic symptoms include severe and persistent headache, uncontrolled vomiting, visual disturbances, cortical blindness and seizure, or eclampsia. Eclampsia is the new appearance of unexplained seizure in a woman with preeclampsia, and is one of the most dangerous complications of pregnancy (Abalos et al., 2014). Eclampsia is a leading cause of maternal and fetal morbidity and mortality worldwide that accounts for greater than 50,000 maternal deaths each year with 1 in 50 women dying and 1 in 14 offspring (Donaldson, 1989a; Duley, 1992, 2009). Although by definition eclampsia is restricted to women with preeclampsia, there does not appear to be a progression from mild to severe preeclampsia to eclampsia (Sibai, 1990a; Douglas and Redman, 1994; Katz et al., 2000). In fact, de novo seizure has been reported to occur in 38-60% of seemingly uncomplicated pregnancies, without hypertension or the diagnosis of preeclampsia (Douglas and Redman, 1994; Katz et al., 2000). The finding that de novo seizure occurs in the absence of preeclampsia suggests that pregnancy alone may be a state of increased seizure susceptibility. In addition, women who develop

preeclampsia are by definition normotensive and asymptomatic prior to pregnancy, with no known underlying conditions contributing to seizure onset, supporting the concept that pregnancy alone may predispose the brain to seizure, independently of preeclampsia. Thus, a portion of this dissertation investigated the contribution of normal pregnancy to eclampsia.

A portion of women have de novo seizure during seemingly uncomplicated pregnancies, however, the majority of eclampsia occurs in the setting of preeclampsia. Further, by the growing use of magnesium sulfate (MgSO_4) for seizure prophylaxis in women with preeclampsia, the incidence of eclamptic seizure has decreased ~ 50 % (Duley, 1995). That the incidence of eclampsia has decreased by treating preeclamptic women with a seizure prophylactic support that preeclampsia, too, contributes to de novo seizure during pregnancy. Therefore, the research completed for this thesis further investigated the pathogenesis of preeclampsia as it relates to the involvement of the brain, the effect of preeclampsia on seizure susceptibility, and mechanisms by which MgSO_4 effectively reduces seizure.

1.2 Pathogenesis of Preeclampsia

Currently there is no medical exam or test that can be performed to determine women that will develop preeclampsia, making this disease unpredictable and unpreventable. However, general risk factors have been identified including primiparity, multi-fetal gestations, body mass index $> 34 \text{ kg/m}^2$, ethnicity, with highest risk for black women, and underlying medical conditions such as pre-existing hypertension, renal disease or diabetes mellitus (Stegers et al., 2010; Trogstad et al., 2011; Abalos et al., 2014). The only known cure for preeclampsia is delivery of the fetus, with preeclamptic

women improving nearly immediately upon discharge of the placenta. The finding that women improve after removal of the placenta has directly implicated the placenta in the etiology of preeclampsia (Myatt, 2002).

The pathophysiology of preeclampsia was considered as a two-stage model: poor placental perfusion leads to release of pro-inflammatory cytokines and anti-angiogenic factors into the maternal circulation and development of maternal endothelial dysfunction and hypertension, resulting in the hallmark preeclamptic symptoms (Redman, 1991; de Groot and Taylor, 1993; Brown, 1995). Stage 1 describes placental disease and involves improper spiral artery adaptation and trophoblast invasion during implantation (Khong et al., 1986). In the nonpregnant state, spiral arteries are high resistance arteries, however during normal pregnancy these arteries remodel, with smooth muscle cells and endothelial cells being replaced by invading trophoblasts (Robertson et al., 1967). This invasion results in widely dilated, low resistance arteries that allow for increased blood flow to the placenta necessary for proper growth of the fetus (Brosens et al., 1967; Pijnenborg et al., 1983; Lyall, 2005). In stage 1 of the two-stage model of preeclampsia, improper trophoblast invasion leads to spiral arteries remaining in a state of high vascular resistance, decreasing blood perfusion to the placenta, resulting in placental ischemia and a state of oxidative stress (Redman, 1991; Roberts and Hubel, 1999). Oxidative stress causes the placenta to release substances into the maternal circulation that have deleterious effects. Increases in pro-inflammatory cytokines lead to an increase in the maternal systemic inflammatory response and increased circulating levels of soluble receptors for angiogenic factors (Kupferminc et al., 1994; Myatt and Webster, 2009). Overall, this cascade of events leads to generalized endothelial dysfunction and reversal

of cardiovascular adaptations to normal pregnancy (Stage 2). Consequentially, hypertension develops and glomerular endotheliosis in the kidneys and impaired renal function results in protein being excreted in the urine (proteinuria) (James et al., 2010). Significant proteinuria (> 300 mg/24 hours) was, until early 2014, considered a hallmark preeclamptic symptom necessary for diagnosis. However, due to the variability and unreliability of proteinuria in women with preeclampsia, the American College of Obstetricians and Gynecologists (ACOG) has now stressed that the diagnosis of preeclampsia can be made in the absence of proteinuria if de novo hypertension occurs in association with thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or visual disturbances (Lindheimer et al., 2014).

The two-stage theory of preeclampsia has been adapted since first proposed in 1991 as it has become clear that preeclampsia is far more heterogeneous of a disease, and that all cases do not fit into the two-stage model (Roberts and Hubel, 2009). Some women are diagnosed with the maternal syndrome of preeclampsia that have a healthy placenta and no fetal involvement, suggesting that preeclampsia can occur in a setting absent of placental ischemia (von Dadelszen et al., 2003; Egbor et al., 2006; Szarka et al., 2010). Further, some women have placental involvement, indicated by fetal growth restriction that do not develop preeclamptic symptoms (Villar et al., 2006). Thus, the pathogenesis of preeclampsia does not necessarily appear to be a progression from Stage 1 to Stage 2, and there appear to be subclassifications of preeclampsia, including separation of maternal and placental disease, making identifying the underlying mechanism(s) of the pathogenesis of preeclampsia more difficult.

Classification of preeclampsia is complicated, and how to differentiate mild forms of the disease from severe is still being debated (Lindheimer et al., 2008; Magee et al., 2008; Lowe et al., 2009; Steegers et al., 2010). The current ACOG delineation of mild from severe preeclampsia is based largely upon the level of hypertension. Women with blood pressures of 140-159 mm Hg systolic and 90-109 mm Hg diastolic are considered to have a mild form of preeclampsia, whereas > 160 mm Hg systolic and > 110 mm Hg diastolic is considered severe (Lindheimer et al., 2014). The presence of neurologic symptoms also advances a woman to severe preeclampsia. While the intent of classification is to separate women at high risk of eclampsia and maternal and fetal morbidity and mortality from those at low risk, some of the classification systems exclude, or neglect to include, criteria that appear to be key variables in prediction of maternal and fetal outcome (von Dadelszen et al., 2003). It is now accepted that the gestational timing of the onset of symptoms should be considered when assessing the severity of the disease. Development of hypertension and proteinuria prior to 34 weeks of gestation is considered early-onset preeclampsia whereas symptoms occurring after 34 weeks of gestation, late-onset preeclampsia (von Dadelszen et al., 2003). The incidence of neurologic complications, including blurred vision and persistent headache, is greater in women with early-onset preeclampsia that is more often associated with maternal and fetal morbidity (Douglas and Redman, 1994; Odegard et al., 2000; Witlin et al., 2000; von Dadelszen et al., 2003; Lindheimer et al., 2008; Ogge et al., 2011). Further, early-onset preeclampsia is more often associated with placental disease, with greater incidence of abnormal vascular morphology and intrauterine growth restriction than in late-onset preeclampsia (Xiong et al., 2002; Oudejans et al., 2007; Crispi et al., 2008; Raymond and

Peterson, 2011). In contrast, late-onset preeclampsia is, for the most part, considered to be maternal disease, with little involvement of the placenta, and preeclamptic symptoms arising due to underlying cardiovascular diseases that have been unmasked by the physiological stress of pregnancy (Ness and Roberts, 1996; Egbor et al., 2006; Raymond and Peterson, 2011). It has further been proposed that the most severe form of preeclampsia is early-onset of both placental and maternal disease (Staff et al., 2013). The combination of the effect of an ischemic placenta in a maternal setting of pre-existing inflammation and/or endothelial dysfunction seems to amplify the disease process. Although eclampsia is most often nonfatal when occurring at term, maternal mortality due to eclampsia in women with severe early-onset preeclampsia is ~ 50 %, suggesting the combination of maternal and placental syndromes may adversely affect the brain to a greater degree than one of the syndromes alone (MacKay et al., 2001). Overall, there do not appear to be hard and fast rules that exist for the delineation of mild vs. severe preeclampsia; all women with preeclampsia are at some level of risk of eclampsia, and therefore increased risk for maternal and fetal morbidity and mortality. Strict demarcation of any one aspect of preeclampsia, whether it be the level of hypertension, or gestational age at which symptoms occur, could be misleading. Thus, seizure prophylaxis in the form of MgSO₄ now tends to be administered to most women with preeclampsia, regardless of “severity”, as eclampsia can occur all along the spectrum of disease, including during seemingly uncomplicated pregnancies.

1.3 Seizure during Pregnancy

Based upon the timing of eclamptic seizure onset in regards to gestational age, three types of eclampsia exist. Antepartum eclampsia (occurring prior to labor) most

often occurs preterm (before 37 weeks of gestation), whereas intrapartum (during labor) and postpartum (after delivery of the fetus and placenta) occur more often at term (Douglas and Redman, 1994). Postpartum eclampsia, however, can occur several weeks after delivery (Douglas and Redman, 1994). Antepartum eclampsia accounts for 38-45% of eclampsia and is associated with greater maternal and fetal morbidity and mortality (Douglas and Redman, 1994; Knight, 2007). Commonly, severe headache and visual disturbances immediately precede eclamptic seizure onset, followed by confusion, then progression from focal seizure, frequently over the face, to generalized tonic-clonic convulsion (Thomas, 1998; Katz et al., 2000; Kaplan, 2001; Shah et al., 2008; Cooray et al., 2011). Eclamptic seizure seems to be self-limiting, typically lasting no more than 3-4 minutes followed by a post-ictal period of confusion and agitation, and even coma (Norwitz et al., 2011). Seizure causes hypoxia and lactic acidosis due to cessation of respiration in the mother, and fetal bradycardia ensues for approximately 20 minutes (Donaldson, 1994b). Seizures can be recurrent, with the average frequency being three in 12 hours (Thomas et al., 1995). Acute memory deficit is common in women with eclampsia, with either retrograde or anterograde amnesia lasting hours to days after seizure manifestation (Shah et al., 2008). Besides hypoxia, eclampsia can lead to intracerebral hemorrhage, cerebral edema, acute renal failure and pulmonary edema, which are leading causes of maternal death (Donaldson, 1994b; MacKay et al., 2001). The most common causes of fetal mortality are prematurity, abruptio placentae, and severe fetal growth restriction (Sibai, 1990a, 2005). Thus, eclamptic seizure poses an immediate and serious health risk for both the mother as well as the fetus, although long-term effects remain unclear.

Eclampsia does not appear to be linked to the development of epilepsy and is not thought to have long-term neurological consequences (Zeeman et al., 2009). Thus, it remains, for the most part, an isolated event. However, it is difficult to assess whether the eclamptic seizure is simply benign. Studies using magnetic resonance imaging (MRI) showed that white matter lesions are present more often in women who had eclampsia than in healthy controls, however whether those white matter lesions were present prior to eclampsia remains unknown (Zeeman et al., 2004b; Aukes et al., 2009; Wiegman et al., 2014). A Cognitive Failures questionnaire that was administered to formerly eclamptic patients to assess the mental difficulty completing daily-life activities indicated impaired cognitive function later in life (Aukes et al., 2007b). Further, this self-report study revealed that women who had multiple eclamptic seizures reported greater cognitive impairment than those women who had a single seizure (Aukes et al., 2007b). However, neurocognitive function testing revealed no evidence for impaired executive functioning or sustained attention (Postma et al., 2010). Thus, it remains unclear if there are long-term neurological complications associated with eclampsia; however, the immediate risk for the mother and fetus remains high, making seizure prevention during pregnancy and preeclampsia critical.

The incidence of eclampsia has declined in developed countries with the advancement of prenatal care, and the use of seizure prophylactics such as $MgSO_4$ reduce the risk of eclampsia by ~ 50% (Duley et al., 2003). Incidence reports of eclampsia in the United States decreased by 22 % between 1987 and 2004 (Wallis et al., 2008), and in the United Kingdom declined from 4.9 cases per 10,000 pregnancies in 1992 to 2.7/10,000 pregnancies in 2005-06 (Douglas and Redman, 1994; Knight, 2007). Similarly, in

Scandinavia the incidence was 5.0/10,000 pregnancies between 1998-2000 (Andersgaard et al., 2006), and in Canada (excluding Quebec) 5.9/10,000 cases between 2009-2010 (Liu et al., 2011). However, in developing countries the incidence of eclampsia is substantially higher. For example, in 1990 in South Africa the incidence of eclampsia was 60/10,000 pregnancies (Moodley and Daya, 1994), 144/10,000 in Muheza, Tanga region, Tanzania in 2007-2008 (Cooray et al., 2011) and 200/10,000 pregnancies at Muhimbili National Hospital in Dar es Salaam, Tanzania in 1999-2000 (Urassa et al., 2006). Thus, despite rates of eclampsia declining in the developed world, eclampsia remains a worldwide problem.

The complexity of the pathogenesis of eclampsia is augmented by the lack of predictability of women who are most at risk of seizure. In fact, despite the definition of eclampsia being in the context of a woman with preeclampsia, it does not appear to always be a progression from preeclampsia to eclamptic seizure. Several studies have reported a substantial percentage of women with eclamptic seizure did not have the hallmark preeclamptic symptoms of hypertension and/or proteinuria prior to seizure (Douglas and Redman, 1994; Katz et al., 2000; Knight, 2007), suggesting that de novo seizure occurs during seemingly uncomplicated pregnancies. A retrospective study that sought to investigate the percentage of eclampsia that was preventable, that is with a diagnosis of preeclampsia before seizure onset so that prophylaxis could be started with MgSO₄, collected records of pregnancies complicated by eclampsia from The University of North Carolina Medical Center at Chapel Hill between January 1987 – December 1995 and Sacred Heart Medical Center from January 1990 – March 1999 (Katz et al., 2000). In this study, 53 pregnancies were complicated by eclampsia, and seizure occurred without a

prior diagnosis of preeclampsia in 32 of the 53 eclamptic pregnancies (60%) (Katz et al., 2000). A larger study investigated the incidence of eclampsia at all hospitals in the United Kingdom in 1992, and then again between February 2005 – February 2006 (Douglas and Redman, 1994; Knight, 2007). Douglas & Redman (1994) reported that 38% of eclampsia occurred prior to hypertension and proteinuria was documented in 1992 (Douglas and Redman, 1994). Interestingly, the 2005-6 study by Knight et al. (2007) reported that only 38% of women who developed eclampsia had hypertension and proteinuria the week prior to seizure onset (Knight, 2007). Together these studies suggest that eclampsia does not only occur in women with pregnancies complicated by preeclampsia, and that seizure onset is not a progression from preeclampsia to eclampsia. Instead, these studies suggest that de novo seizure can occur in the absence of preeclampsia, and that pregnancy alone may predispose the brain to seizure, independently of preeclampsia. Thus, the term “preeclampsia” seems to be misleading, as eclampsia can occur in its absence. Further, the progression from normal pregnancy to preeclampsia to eclampsia should not be considered linear. Overall, understanding the cerebrovascular and neurophysiological changes associated with normal pregnancy may shed light into the pathogenesis of eclampsia, as normal pregnancy may contribute to de novo seizure in the absence of preeclampsia.

1.4 The Cerebral Circulation during Pregnancy and Preeclampsia

1.4.1 Introduction

The brain is an organ of high metabolic demand that consumes ~ 20% of the body's oxygen at rest, despite comprising only 2% of body weight (Siegel, 1999). Importantly, the brain has a relatively narrow capacity to tolerate changes in ion and

water balance, and blood flow (Siegel, 1999). The brain is also unique in that it is enclosed in a rigid skull and therefore increased vascular permeability or volume could result in detrimentally increased intracranial pressure that can cause serious neurological symptoms, brain herniation, and even death (Rosenberg, 1999; Marmarou, 2007). Thus, there is a need to maintain tight control of cerebral blood flow (CBF) and water flux in the face of a 40-50% increase in plasma volume and cardiac output during pregnancy and a decline in systemic vascular resistance necessary for the maintenance of a healthy blood pressure (Clapp and Capeless, 1997). In contrast to other organs outside the central nervous system that undergo substantial increases in both perfusion and transvascular filtration during pregnancy, including the uterus, kidney and heart, the cerebral circulation must resist these adaptations to counterbalance global hemodynamic changes in order to maintain the delicate microenvironment of the brain. Thus, the adaptation of the brain and cerebral circulation to pregnancy appears to be to maintain normalcy despite substantial hormonal and cardiovascular changes in almost every other organ. Pregnancy has the potential to affect several aspects of the cerebral circulation, including the cerebral endothelium and blood-brain barrier (BBB), the structure and function of the cerebrovasculature, hemodynamics, and CBF autoregulation. Further, in the context of these cerebrovascular adaptations, there is risk for neurological complications, such as eclampsia, when the cerebral circulation is compromised, as all of the parameters listed above have the potential to lead to seizure onset if disrupted or maladapted. In fact, the cerebral circulation is thought to have a central role in the pathogenesis of eclampsia. Further, the cerebrovasculature is directly involved in ~40 % of maternal deaths due to

eclampsia (MacKay et al., 2001). Thus, understanding how pregnancy and preeclampsia affect the cerebrovasculature is of interest.

1.4.2 Vasomotor Responses to Circulating Factors

One of the most important adaptations of the cerebral circulation during pregnancy is to counteract the effects of circulating vasoactive factors. During pregnancy, large amounts of hormones are secreted from the placenta, ovaries, and brain into the maternal circulation, including pro- and anti-inflammatory cytokines, chemokines, steroids and growth factors (Aagaard-Tillery et al., 2006; Szarka et al., 2010). These factors are critical for the development and survival of the fetus and adaptation of other organ systems needed for a successful pregnancy. Cerebral arteries uniquely adapt during pregnancy to oppose an increase in circulating vasoconstrictors present late in gestation. Exposure of plasma from pregnant women causes vasoconstriction of posterior cerebral arteries from nonpregnant rats, however, this vasoconstrictive effect is absent in arteries from pregnant rats (Amburgey et al., 2010a). This lack of effect in arteries from pregnant rats suggests the cerebral circulation adapts to combat vasoconstrictors present late in gestation. This adaptation may be due to either development of resistance to vasoconstrictors circulating during pregnancy, or increased sensitivity to vasodilators also circulating in pregnancy. Interestingly, this finding was specific to the cerebral vasculature, as the effect of pregnant plasma was not seen in mesenteric arteries (Amburgey et al., 2010a). Thus, the adaptation of the cerebral circulation during pregnancy is unique compared to the adaptation of other organ systems. The exact mechanism by which the cerebral vasculature resists the vasoconstrictive effect of circulating factors in pregnancy remains unclear, but may involve receptor

downregulation or changes in the influence of the endothelium on vascular tone in response to plasma (Duckles and Krause, 2007; Chan et al., 2010). Regardless, this adaptation of the cerebral circulation likely occurs to prevent the cerebrovasculature from constricting in response to circulating factors and may help maintain physiologic levels of cerebrovascular resistance and blood flow to the brain during pregnancy.

1.4.3 The Cerebral Endothelium and BBB

Vasomotor responses are not the only feature of the cerebral circulation that adapt to pregnancy. The cerebral endothelium that forms the BBB is a complex interface between systemically circulating factors and the delicate microenvironment of the brain. The endothelial cells of the BBB contain specialized high electrical-resistance tight junctions and lack fenestrations (Ueno, 2007; Zlokovic, 2008). BBB tight junctions limit the passage of blood constituents into the brain parenchyma by preventing paracellular transport and are highly protective of the brain milieu (Wahl et al., 1988; Rubin and Staddon, 1999). Cerebral endothelial cells also have a low rate of pinocytosis, which limits the amount of transcellular transport, reinforcing the overall function of the BBB (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Fenstermacher et al., 1988). Pregnancy does not affect mRNA expression of the primary tight junction proteins of the BBB, including claudin-1, claudin-5, occludin and zona occludens-1, as these are similar to the nonpregnant state (Cipolla et al., 2011). In addition, paracellular and transcellular transport at the BBB remain unchanged during normal pregnancy, as BBB permeability to solutes does not increase (Cipolla et al., 2011). In addition, hydraulic conductivity, an important parameter that relates water movement through the vessel wall in response to hydrostatic pressure, is normally very low in cerebral endothelial cells due to the high

electrical resistance tight junctions and low pinocytotic activity (Rubin and Staddon, 1999). Similar to paracellular and transcellular permeability, hydraulic conductivity is not changed during pregnancy (Cipolla et al., 2012b), but is increased in response to preeclamptic plasma (Amburgey et al., 2010b). Increased hydraulic conductivity during preeclampsia could promote neurologic symptoms as increased BBB permeability has been linked to several pathologic states including preeclampsia and eclampsia as well as epilepsy (Oby and Janigro, 2006; Marchi et al., 2007; Marchi et al., 2011).

Pregnancy is a state marked by increased circulating permeability factors, including several that are known to promote BBB permeability (Brown et al., 1997; Evans et al., 1997), yet it is remarkable that no such changes in permeability have been measured (Cipolla et al., 2012b). For example, vascular endothelial growth factor (VEGF), a cytokine originally named vascular permeability factor, is secreted by the placenta and elevated during pregnancy in the uteroplacental unit and the maternal circulation (Brown et al., 1997; Evans et al., 1997; Charnock-Jones et al., 2004; Amburgey et al., 2010b). VEGF interacts with its receptors VEGFR1 (or FMS-like tyrosine kinase receptor 1, Flt-1) and VEGFR2 (or fetal liver kinase 1, Flk-1) located on vascular endothelium to initiate several critical physiological processes involved in angiogenesis, vascular growth, and endothelial cell survival (Dvorak, 2002; Shibuya, 2013). In addition to VEGF, placental growth factor (PlGF), a member of the VEGF family, is also elevated during pregnancy and contributes to angiogenesis in the uterus and placenta (Krauss et al., 2004). Most notably, VEGF and PlGF are potent vasodilators and increase peripheral microvascular permeability to serum proteins and macromolecules, considered a primary step in preparation for angiogenesis (Feng et al.,

1996; Dobrogowska et al., 1998; Dvorak, 2002; Oura et al., 2003), and increase BBB permeability (Schreurs et al., 2012). Interestingly, despite elevated circulating VEGF and PlGF during pregnancy, exposure of cerebral vessels to pregnant plasma or serum does not increase BBB permeability (Cipolla et al., 2012b; Schreurs et al., 2012). Further, VEGF receptor expression in cerebral arteries does not appear to change during pregnancy, suggesting the lack of effect of VEGF and PlGF is not due to downregulation of VEGFR1/II or neuropilin (Schreurs et al., 2012). In fact, plasma from late-pregnant rats prevents VEGF-induced increases in BBB permeability (Schreurs et al., 2012), likely due to increased levels of soluble Flt-1 (sFlt-1). The selective binding of VEGF and PlGF to sFlt-1 is important for regulating their bioavailability, thus limiting the permeability-promoting effects at the BBB during pregnancy (Schreurs et al., 2012). The prevention of circulating permeability factors from increasing BBB permeability is an important adaptation during pregnancy to help maintain brain homeostasis.

Although paracellular and transcellular permeability of the BBB appear to remain intact during pregnancy, efflux transporters present at the BBB are an important regulatory mechanism controlling passage of serum factors into the brain that appear to be gestationally regulated (Coles et al., 2009b; Chung et al., 2010). Specifically, P-glycoprotein (Pgp) is a main efflux transporter at the BBB that extricates steroids, cytokines and chemokines as well as many pharmacologic agents that can pass through the BBB, essentially acting a gatekeeper to the central nervous system (Begley, 2004; Ueno, 2007). Pgp is an obstacle in administration of therapeutics to the brain, making it difficult for pharmacological interventions to be delivered in patients with epilepsy, brain tumors, HIV, etc. (Begley, 2004). The role of Pgp in restricting drug delivery to the brain

during pregnancy has been investigated in pregnant mice and nonhuman primates. Pgp protein expression is elevated at the BBB mid-gestation, but returns to pre-pregnancy levels by late-gestation in mice (Coles et al., 2009b). In the same study, the protein expression of another efflux transporter, multi-drug resistance-associated protein 1 (Mrp1), was also elevated at the BBB mid-pregnancy that remained higher late in gestation (Coles et al., 2009b). A study using positron emission tomography scanning to investigate Pgp activity at the BBB across gestation in nonhuman primates reported that Pgp activity increases with gestational age (Chung et al., 2010). Thus, it appears that efflux transporters are gestationally regulated, potentially in response to the increase in circulating factors occurring during pregnancy (Bauer et al., 2007; Coles et al., 2009a). While it appears that Pgp expression increases at the BBB only in mid-gestation, its activity may increase late in pregnancy. This potential adaptation may play a key role in maintaining barrier function despite increases in circulating factors, some of which are hormones and steroids that can pass through the BBB due to their lipophilic nature. In fact, it is possible that during preeclampsia this adaptation fails or is overcome, resulting in eclamptic seizure (see below). Overall, increases in efflux transporter expression and/or activity across gestation are likely a critical adaptation of the BBB to prevent passage of circulating factors into the brain during pregnancy.

Preservation of BBB properties and adaptation of efflux transporters in the face of elevated circulating permeability factors during pregnancy appears to be highly protective, and may be central to seizure prevention. Seizure-provoking serum constituents are also present late in gestation. Serum from late-pregnant, but not nonpregnant rats causes hyperexcitability of hippocampal neuronal networks in cultured

slices, measured by evoked field potentials (Cipolla et al., 2012b). The increase in excitability is due to serum factors causing neuroinflammation via activation of microglia and secretion of tumor necrosis factor α (TNF α) (Riazi et al., 2008; Cipolla et al., 2012b). However, under normal conditions the brain is not likely to come into contact with circulating serum factors due to the protective nature of the BBB, highlighting the importance of the BBB in seizure prevention during pregnancy (Ueno, 2007; Johnson et al., 2014).

The adaptation of the BBB to normal pregnancy may play a critical role in seizure prevention, by protecting the brain from exposure to seizure-provoking constituents circulating late in gestation. However, during preeclampsia, maternal endothelial dysfunction appears to lead to BBB disruption and result in an increase in BBB permeability and edema formation (Kaplan, 2001; Demirtas et al., 2005). Under such conditions, seizure-provoking factors, or other deleterious proteins or pro-inflammatory cytokines that are increased in preeclampsia may cross into the brain and lead to seizure onset. In fact, plasma from women with preeclampsia caused increased BBB permeability when exposed to cerebral arteries of rats (Amburgey et al., 2010b). Further, when the effect of plasma from women with early-onset preeclampsia on BBB permeability was compared to that of late-onset preeclampsia, only plasma from early-onset preeclamptic patients increased BBB permeability (Schreurs et al., 2013). This was due to a > 200% increase in circulating oxidized low-density lipoprotein (oxLDL) present in early-onset preeclampsia (Schreurs et al., 2013). Through interaction of oxLDL with its receptor, lectin-like oxLDL receptor 1 (LOX1), subsequent peroxynitrite formation leads to BBB disruption and an increase in BBB permeability (Schreurs et al., 2013).

This differential effect of plasma supports that early-onset preeclampsia is more severe than late-onset, and may explain the greater propensity of neurologic involvement during early-onset preeclampsia. Overall, increased BBB permeability during preeclampsia likely contributes to edema formation and/or passage of seizure-provoking factors into the brain, and may represent one mechanism by which eclamptic seizure occurs (Donaldson, 1994b; Cipolla, 2007).

Cerebral edema formation is considered a leading cause of the neurological symptoms that occur in preeclampsia, including eclamptic seizure (Donaldson, 1994a; Hinchey et al., 1996; Zeeman et al., 2009). In fact, approximately 90 % of women with eclampsia have vasogenic cerebral edema formation, as indicated by diffusion-weighted MRI (Zeeman et al., 2004b; Brewer et al., 2013). Further, preeclampsia and eclampsia are commonly associated with hypertensive encephalopathy, and more specifically, posterior reversible encephalopathy syndrome (PRES) (Schwartz et al., 1992; Hinchey et al., 1996; Schwartz et al., 2000; Bartynski and Boardman, 2007). However, seizure itself leads to BBB disruption, making it difficult to determine if edema is the cause of or a consequence of eclamptic seizures (Oby and Janigro, 2006; Marchi et al., 2010).

Regardless, the presence of vasogenic edema formation in women with preeclampsia that have not had seizures is definitive evidence of increased vascular permeability that leads to an accumulation of edematous fluid in the extracellular space within the brain (Klatzo, 1987b, a). However, having the outcome of vasogenic edema in women with preeclampsia does not contribute to the understanding of what may be occurring at the cellular level at the BBB during preeclampsia in order to allow passage of solutes and water into the brain. Studies investigating changes in BBB permeability in the placental

ischemia rat model of preeclampsia have reported increased permeability of the BBB to albumin-bound Evans Blue (Porcello Marrone et al., 2014; Warrington et al., 2014). However, serum albumin levels change during pregnancy and preeclampsia (Honger, 1968b, a; McCartney et al., 1971; Gojnic et al., 2004), suggesting conclusions from these studies about the integrity of the BBB should be made cautiously. Additionally, these studies using Evans Blue highlight the necessity for future studies using animal models of preeclampsia to investigate BBB permeability to other fluorescently tagged solutes. Overall, there is evidence that the BBB is compromised in both women with preeclampsia as well as in animal models of preeclampsia that likely plays a role in the pathogenesis of eclampsia. Further, it has been suggested that such BBB disruption and subsequent vasogenic edema formation may be a consequence of impaired CBF autoregulation.

1.4.4 CBF Autoregulation and Hemodynamics

The cerebral circulation ultimately functions to deliver oxygen, glucose and nutrient rich blood to, and remove metabolic waste from the central nervous system that is crucial to ensure proper brain function. It is therefore not surprising that blood flow autoregulation is well developed in the brain. CBF autoregulation is the intrinsic property of the brain to maintain relatively constant blood flow in the face of changes in blood pressure (Harper, 1966; Hayman et al., 1981). In normal healthy adults, CBF autoregulation operates between ~ 60 – 160 mmHg (Lassen, 1959; McHenry et al., 1974). Although the effect of pregnancy on the lower limit of CBF autoregulation has yet to be investigated, pregnancy appears to shift the upper limit of the CBF autoregulatory curve. In normal pregnant rats, the upper limit of the CBF autoregulatory curve was

investigated by using a phenylephrine infusion to acutely raise blood pressure together with continuous CBF measurements using laser Doppler flowmetry (Cipolla et al., 2012a). This study found that compared to the nonpregnant state, the upper limit of CBF autoregulation was shifted rightward to higher pressure (Cipolla et al., 2012a). Thus, the effect of pregnancy on CBF autoregulation appears to be protective, making the maternal brain better prepared to maintain blood flow in the face of acute hypertension. However, this study used an animal model of pregnancy, as such measurements in pregnant women are challenging and potentially dangerous. Studies in humans using non-invasive techniques to measure dynamic autoregulation during normal pregnancy have found improved or no change in CBF autoregulation in pregnant compared to nonpregnant women (Bergersen et al., 2006; Janzarik et al., 2014). To our knowledge, no study has determined the limits of CBF autoregulation during pregnancy but is important to understand because of the potential for acute hypotensive and hypertensive episodes that exist, especially during parturition. For example, hemorrhage can occur during parturition causing hypotension, or parturition can also lead to increased sympathetic discharge resulting in an acute elevation in blood pressure (Pickering, 2003).

The cerebral circulation has a central role in neurologic complications associated with preeclampsia (MacKay et al., 2001). In fact, cerebrovascular events such as edema and hemorrhage account for ~ 40 % of maternal deaths (MacKay et al., 2001). An underlying feature of neurological complications, including seizure, in women with preeclampsia is the impairment of CBF autoregulation and subsequent edema formation (Engelter et al., 2000; Kaplan, 2001; Janzarik et al., 2014). Impaired CBF autoregulation is associated with decreased cerebrovascular resistance, hyperperfusion of the brain, BBB

disruption, and vasogenic edema formation (Schwartz et al., 2000; Janzarik et al., 2014). Studies that have assessed dynamic CBF autoregulation in women with preeclampsia using transcranial Doppler (TCD) to measure changes in CBF velocity in the middle cerebral artery (MCA) in response to hemodynamic fluctuations have found that CBF autoregulation appears to be intact in preeclampsia (Sherman et al., 2002; van Veen et al., 2013; Janzarik et al., 2014). However, these studies did not delineate disease severity. A case report assessing cerebral autoregulation in women with severe preeclampsia experiencing neurologic symptoms reported impaired CBF autoregulation (Oehm et al., 2006). Although small numbers of patients were assessed, this finding suggests that CBF autoregulation may be differentially affected in severe versus mild preeclampsia. Further, CBF autoregulation has been shown to be impaired in women with eclampsia (Oehm et al., 2003), however, whether loss of autoregulation is due to the convulsions themselves or whether it truly is an underlying mechanisms leading to seizure onset remains unclear, and difficult to determine. The use of animal models of preeclampsia has been employed to investigate the effect of preeclampsia on CBF autoregulation. A recent study investigating CBF autoregulation in the placental ischemia rat model of preeclampsia reported that autoregulation was impaired in the anterior brain region during stepwise increases in arterial blood pressure (Warrington et al., 2014). Interestingly, it is thought that CBF autoregulation in the posterior cerebral cortex is predominantly affected during preeclampsia, as the posterior cortex is a primary location of vasogenic edema formation (Schwartz et al., 2000). Further, most neurologic symptoms that occur in women with preeclampsia arise specifically from the posterior cerebral cortex, such as blurred vision and cortical blindness (Cunningham et al., 1995). Thus, while the effectiveness of CBF

autoregulation in preeclampsia has begun to be investigated using animal models, the need for more elaborate studies remains before a clear understanding of the role of impaired autoregulation in preeclampsia and eclampsia can be elucidated.

Numerous recent studies have also investigated potential changes in basal CBF during pregnancy and preeclampsia, employing several techniques including ultrasonography and magnetic resonance (MR) studies. Early human studies using inhalation of a gaseous mixture of nitrous oxide, oxygen and nitrogen and the Fick principle to assess CBF, oxygen delivery and metabolism in the brain reported no differences in CBF between the nonpregnant, pregnant and preeclamptic states (McCall, 1949, 1953). TCD studies have measured CBF velocity in cerebral arteries in pregnant women across gestation that revealed CBF velocity decreases during normal gestation (Williams and Wilson, 1994; Serra-Serra et al., 1997; Belfort et al., 2001), where as CBF velocity has been reported to be higher in preeclamptic than normotensive pregnant women (Williams and McLean, 1993; Williams and MacLean, 1994; Ohno et al., 1997). However, changes in vascular resistance and blood flow calculated from these measurements may not accurately reflect CBF due to the lack of information about vessel diameter (Kontos, 1989). In fact, despite reported increases in CBF velocity in women with preeclampsia, the majority of women with preeclampsia, regardless of severity, have normal CBF (Belfort et al., 2002; Belfort et al., 2006). Further, using dual-beam angle-independent digital Doppler ultrasonography, diameter and blood flow volume of the internal carotid artery were measured during pregnancy. This study found that CBF increased ~ 20% across gestation based on these measurements (Nevo et al., 2010). In contrast, a study using MR reported ~ 20% decrease in CBF during pregnancy, however,

this was in comparison to post-partum and not pre-pregnancy values (Zeeman et al., 2003). MR studies also report contradictory findings regarding CBF in women with severe preeclampsia. One study reported a significant increase in CBF in the MCAs and the posterior cerebral arteries (PCAs) of women with preeclampsia (Zeeman et al., 2004a), while another study conducted similarly reported no difference in MCA or PCA blood flow between severe preeclamptic and normal pregnancies (Morriss et al., 1997). A possible explanation for this particular discrepancy could be the administration of medications. Zeeman et al. (2004), who reported an increase in CBF, excluded women with severe preeclampsia (blood pressure > 160 mm Hg systolic or with the presence of neurologic symptoms) and only took measurements in preeclamptic women not being treated with an antihypertensive medication and/or MgSO₄ (Zeeman et al., 2004a). The study by Morriss et al. (1997) that reported no change in CBF was conducted in women with severe preeclampsia, many of which were being treated with either antihypertensive medications, MgSO₄ or both (Morriss et al., 1997). Studies have used microspheres to measure absolute CBF in late-pregnant rats and found a nonsignificant 5-10% increase in CBF compared to the nonpregnant state (Buelke-Sam et al., 1982; Cipolla et al., 2011). Regardless, there appears to be conflicting evidence regarding the effect of both normal pregnancy, as well as preeclampsia on CBF, potentially due to variability in methodology and study populations. Overall, while studies reporting the effect of pregnancy and preeclampsia on CBF in women are contradictory, the use of animals that allowed invasive measurements suggest CBF remains similar in pregnancy to the nonpregnant state. However, animal models of preeclampsia have yet to be used to investigate the

effect of preeclampsia on absolute CBF, making it difficult to interpret the incongruous results that are currently reported from women with preeclampsia.

1.4.5 Function and Structure of the Cerebrovasculature

Understanding changes occurring in the structure and function of cerebral arteries and arterioles during pregnancy may shed some light on potential changes in vascular resistance that may drive changes in CBF and autoregulation. Cerebral arteries and arterioles exist in a state of partial constriction and thus have basal tone. A major contributor to basal tone in the cerebral circulation is the myogenic response of vascular smooth muscle cells (Bayliss, 1902; MacKenzie et al., 1979). Pregnancy does not appear to affect myogenic tone in cerebral pial arteries or penetrating brain arterioles (Chan et al., 2010; Cipolla et al., 2011). While myogenic tone refers to the degree of basal constriction of a vessel relative to its passive diameter at a constant pressure, the myogenic response refers to the dynamic response of cerebral arteries and arterioles to changes in intravascular pressure (Kontos et al., 1978). The myogenic response is a main contributor to CBF autoregulation through increasing and decreasing cerebrovascular resistance in response to changes in intravascular pressure and appears to be different in the pregnant versus nonpregnant state (Kontos et al., 1978; Faraci et al., 1987a; Faraci and Heistad, 1990; Cipolla et al., 2004; Chapman et al., 2013). In isolated cerebral arteries from pregnant rats, increased intravascular pressure caused forced dilatation at lower pressures than arteries from nonpregnant rats, suggesting a lower capacity to maintain cerebrovascular resistance in the face of increased pressure during pregnancy (Cipolla et al., 2004). However, the CBF autoregulatory curve is shifted to higher, not

lower pressures during pregnancy, suggesting other contributors to CBF autoregulation may act in a compensatory way during pregnancy.

The vasculature of many organ systems, particularly within the uteroplacental circulation, changes structurally to accommodate the physiological adaptation of normal pregnancy (Osol and Mandala, 2009). There is evidence that the cerebrovasculature also structurally remodels during pregnancy in a selective manner. Structural remodeling describes changes in luminal diameter and vascular wall thickness in response to physiological or pathological stimuli (Martinez-Lemus et al., 2009). Remodeling can be directed outward or inward, depending upon whether the luminal diameter increases or decreases (Mulvany, 1999). Further, remodeling can be hypo-, hyper- or eutrophic, depending upon whether the vessel wall thickness decreases, increases or stays the same, respectively (Mulvany, 1999). During rat pregnancy, no changes in luminal diameter or wall thickness have been measured in cerebral pial arteries, suggesting pregnancy-induced remodeling does not occur in the pial vasculature (Chan et al., 2010). However, brain parenchymal arterioles, precapillary resistance vessels that branch off pial vessels and perfuse the brain tissue, undergo outward hypotrophic remodeling during pregnancy, resulting in larger vascular lumens and thinner vessel walls than in the nonpregnant state (Cipolla et al., 2011). Although there is no change in myogenic tone in these vessels during pregnancy, the intravascular pressure vs. luminal diameter curve is shifted upward due to the structural changes (Cipolla et al., 2011). This selective remodeling of parenchymal arterioles during pregnancy is through arteriogenesis and driven by peroxisome proliferator-activated receptor gamma (PPAR γ) activation by the hormone (ser)relaxin (Chan and Cipolla, 2011). Interestingly, the primary relaxin receptor is not

expressed in parenchymal arterioles (Chan and Cipolla, 2011). However, circulating relaxin appears to cross the BBB and is thought to activate PPAR γ on astrocytes and neurons that in turn exert a paracrine effect on parenchymal arterioles to drive outward remodeling (Chan and Cipolla, 2011). In contrast to parenchymal arterioles, pial arteries are not intimately associated with brain parenchymal cell types such as astrocytes and neurons, and this may explain the selective effect of pregnancy-induced remodeling on parenchymal arterioles. This process of arteriogenesis is unique compared to angiogenesis, however, angiogenesis also occurs in the brain during pregnancy. In fact, capillary density increases during pregnancy in the posterior cerebral cortex, a finding that has also been linked to increased activation of PPAR γ by relaxin (Chan and Cipolla, 2011; Cipolla et al., 2011). Thus, while pregnancy does not affect the structure of the pial vasculature, it seems to have an outward hypotrophic remodeling effect on parenchymal arterioles that may contribute to the extension of the CBF autoregulatory curve.

During pregnancy, outward remodeling of parenchymal arterioles and increased capillary density coupled with the approximate 10% hemodilution that occurs could decrease cerebrovascular resistance and increase CBF (Gordon, 2007). Despite these vascular and hemodynamic changes, pregnancy has little effect on cerebrovascular resistance when measured under normotensive conditions in the rat (Cipolla et al., 2011). The substantial contribution of large cerebral arteries to vascular resistance is unique to the cerebral circulation (Faraci and Heistad, 1990). In fact, large extracranial and intracranial cerebral arteries contribute ~50% of cerebrovascular resistance (Faraci et al., 1987b; Faraci and Heistad, 1990). Although downstream arterioles undergo structural changes that may decrease small vessel resistance, the pial vasculature does not change

structurally during pregnancy and may compensate to maintain normal vascular resistance. The structural remodeling of the cerebral circulation may be important when considering hypertensive pathologies of pregnancy such as preeclampsia. Under experimental conditions of acute hypertension, cerebrovascular resistance of cerebral arteries was decreased in pregnant rats, leading to autoregulatory breakthrough and ~40% increase in CBF (Cipolla et al., 2011). The decrease in cerebrovascular resistance with autoregulatory breakthrough was further associated with increased BBB permeability due to greater hydrostatic pressure on the microcirculation (Cipolla et al., 2011). Thus, while the BBB seems to remain intact during normal pregnancy under physiological conditions, it also appears to be at greater risk of injury during pathologic states such as acute hypertension. Under conditions of elevated intravascular pressure when large arteries become ineffective in regulating CBF due to forced dilatation of myogenic tone, outward hypotrophic remodeling of parenchymal arterioles may predispose the microcirculation to injury by transmitting high hydrostatic pressure downstream. Outward hypotrophic remodeling of brain arterioles could also contribute to the maternal brain being more sensitive to vasogenic edema formation after acute hypertension (Euser and Cipolla, 2007; Cipolla et al., 2012a). The increase in capillary density may further contribute to the susceptibility of the brain to hypertension-induced vasogenic edema during pregnancy by increasing the potential sites of BBB disruption. In addition, pregnancy both prevents and reverses remodeling of cerebral arteries that occurs in response to chronic hypertension (Cipolla et al., 2006; Aukes et al., 2007a; Cipolla et al., 2008). Chronic hypertension in the nonpregnant state leads to inward hypertrophic remodeling of cerebral arteries, resulting in smaller lumen diameters and thicker vascular walls

(Baumbach and Heistad, 1989; Cipolla et al., 2006). This is considered a protective adaptation by which cerebrovascular resistance is increased, thus protecting the microcirculation from elevated arterial blood pressure (Cipolla et al., 2006; Chan et al., 2010). The prevention and/or reversal of this remodeling during pregnancy may be related to the downregulation of angiotensin type 1 receptor (AT1R) expression in the cerebral circulation that also occurs during pregnancy (Chan et al., 2010). While the reduction in AT1R expression may be a physiological adaptation to normal pregnancy, reversal and/or prevention of hypertensive remodeling may make the cerebral microcirculation even more susceptible to injury during states associated with hypertension. Again, this may be important during preeclampsia, where acute elevations in blood pressure are thought to lead to cerebral vasogenic edema formation and subsequent neurologic complications including eclamptic seizure (Cipolla, 2007).

The adaptation of parenchymal arterioles during pregnancy may be further implicated in neurologic complications associated with preeclampsia and eclampsia. Parenchymal arterioles are the primary vessels involved in small vessel disease in the brain (Rincon and Wright, 2014). Women with eclampsia seem to be prone to white matter lesions later in life that may indicate the presence of small vessel disease in the brain (Aukes et al., 2009). It is possible albeit speculative at this time, that failure of parenchymal arterioles to outward remodel during preeclampsia underlies the potential for white matter lesions later in life. Lack of spiral artery remodeling during pregnancy is a feature of some women with preeclampsia that may indicate a type of small vessel disease (Egbor et al., 2006). We further speculate that the lack of adaptation of small vessels in the uterine circulation may be similar to the lack of adaptation of the small

vessels in the brain, both of which may be occurring in women with severe preeclampsia and eclampsia. Further, if the lack of remodeling occurs in parenchymal arterioles in the brain, as it does in the uterine circulation in some eclamptic women, this may suggest a common pathology that leads to white matter lesions and cognitive impairment associated with eclampsia (Aukes et al., 2007b; Aukes et al., 2009). However, the association of small vessel disease of the brain later in life in formerly eclamptic women with impaired spiral artery remodeling during pregnancy is speculative and further studies are needed to understand these processes.

In summary, pregnancy is associated with many adaptations of the cerebral circulation including changes in receptor and transporter activity, keeping increased permeability factors in balance in order to maintain brain homeostasis and protect against increases in BBB permeability. Further, structural and functional changes occur in certain segments of the cerebral vasculature; however, CBF and cerebrovascular resistance appear unchanged under normotensive conditions. The CBF autoregulation curve appears to extend to the right in the pregnant state, protecting the maternal brain against acute and drastic increases in blood pressure. It is remarkable that BBB permeability and CBF are affected so minimally during pregnancy, especially in the face of substantially increased factors that have direct effects on vascular filtration and flow in many other organ systems. However, this supports the principle that the adaptation of the cerebral circulation to normal pregnancy functions to maintain essential oxygen and nutrient delivery and waste removal similar to the nonpregnant state, especially in the face of tremendous systemic hemodynamic changes associated with pregnancy. There is evidence that the cerebral circulation may be compromised during preeclampsia, through

disruption of the BBB and/or effects on CBF autoregulation that may contribute to the onset of eclamptic seizure. However, a complete understanding of how preeclampsia affects the cerebrovasculature has yet to become clear and continued research efforts in this area would likely be fruitful.

1.5 Changes in Neuronal Excitability during Normal Pregnancy

1.5.1 Introduction

The majority of women do not seize during pregnancy; however, if there is greater susceptibility to seizure in the maternal brain, then under conditions where other protective mechanisms fail, such as at the BBB, seizure could ensue. Susceptibility to seizure during pregnancy has previously been investigated in the context of understanding epilepsy in pregnancy. However, the effect of pregnancy on seizure frequency in women with epilepsy is difficult to discern and appears variable (Pennell, 2002; Battino and Tomson, 2007; Meador, 2014). A review of over 2000 pregnancies in epileptic patients found that ~ 25% of patients experienced an increase, ~25% a decrease, and ~50% experienced no change in the frequency of epileptic seizures (Schmidt, 1982). This variability in seizure frequency noted may be complicated by the absence or presence of anti-epileptic medications during pregnancy. One study investigating the effect of antiepileptic drugs on seizure in pregnant mice found that electroconvulsive seizure threshold was increased on day 18 of pregnancy compared to nonpregnant mice (Nau et al., 1984). Another study using amygdaloid kindled rats also reported increased threshold to electroconvulsive seizure in pregnant compared to nonpregnant animals (Kan et al., 1985). However, the latency to seizure onset using the chemoconvulsant pentylenetetrazole revealed no change in seizure threshold between nonpregnant and

pregnant rats in a study investigating seizure threshold in the lipopolysaccharide (LPS) model of preeclampsia (Huang et al., 2014). Thus, there are discrepancies in the effect of pregnancy on seizure threshold depending upon which seizure-induction method is used. Further, these few studies that have investigated seizure threshold in pregnancy used latency to physical convulsion as the threshold; however, the use of electroencephalography (EEG) may allow for a more sensitive means to detect seizure threshold differences. Despite these incongruities, there is evidence of central changes that occur during pregnancy that may increase the potential for seizure. If the brain during pregnancy is more susceptible to seizure, that could account for the 38-60% of women that experience de novo seizure during seemingly uncomplicated pregnancies. Understanding the neurophysiological changes occurring during normal pregnancy may lead to a greater understanding of the pathogenesis of de novo seizure in the absence of preeclampsia.

1.5.2 Neuroactive Steroids

Pregnancy is a state during which there are tremendous elevations in estrogen and progesterone produced from several areas including the placenta. Estrogen and progesterone are precursors for neuroactive steroids that have been shown to affect neuronal excitability (Reddy, 2003). Neuroactive steroids, or neurosteroids, are synthesized from their precursor steroids de novo by neurons and glia throughout the brain and are capable of rapidly (within seconds) affecting neuronal excitability through non-genomic mechanisms (Baulieu and Robel, 1990; Mensah-Nyagan et al., 1999; Agis-Balboa et al., 2006). Neurosteroids affect neuronal excitability by interacting with ion channels and neurotransmitter receptors at the neuronal cell membrane (Reddy, 2003).

Specifically, estradiol exerts pro-convulsive effects by increasing neuronal excitability through increasing dendritic spine density and synapses on CA1 pyramidal cells of the hippocampus and facilitating N-methyl-D-aspartate (NMDA) receptor binding (Woolley et al., 1997; Pozzo-Miller et al., 1999). Further, estradiol increases excitatory responses of neurons by both enhancing inward sodium currents and attenuating outward potassium currents, independently of one another (Kow et al., 2006; Druzin et al., 2011). In contrast, many progesterone-derived neurosteroids have anti-convulsive effects through modulatory actions at gamma-aminobutyric acid type A receptors (GABA_ARs), the main inhibitory neurotransmitter receptors in the brain (Macdonald and Olsen, 1994; Herd et al., 2007). Specifically, the neurosteroid allopregnanolone has binding sites on the δ -subunit of GABA_ARs, at which it acts as a positive allosteric modulator, exerting an overall sedative effect (Stell et al., 2003). GABA_ARs that contain the δ -subunit (GABA_AR- δ) are located extrasynaptically and are involved in tonic inhibition throughout the brain (Stell et al., 2003). Studies investigating changes in GABA_AR- δ expression during conditions of increased progesterone such as pregnancy have shown downregulation of GABA_AR- δ (Maguire et al., 2009; Maguire and Mody, 2009). This downregulation has been associated with increased neuronal excitability of brain slices from pregnant mice that was normalized by the presence of allopregnanolone (Maguire et al., 2009). It is likely that the downregulation of GABA_AR- δ is an adaptation that functions to maintain the steady state of excitability and avoid overinhibition in the face of increased neurosteroids present during pregnancy. Although these in vitro studies suggest that the brain may be hyperexcitable during pregnancy, it remains unclear as to whether pregnancy is a state of increased seizure susceptibility under physiological

conditions when neurosteroids are naturally circulating. Understanding the overall status of brain excitability during pregnancy, and whether pregnancy is a state of increased seizure susceptibility seems important as that could contribute to the potential for eclampsia.

1.5.3 Neuroinflammation

Neuronal excitability may also be affected during pregnancy by inflammation. Pregnancy is considered a state of mild peripheral inflammation, and peripheral inflammation has been shown to cause neuroinflammation through the activation of microglia, the resident immune cells in the brain (Sacks et al., 1998; Riazi et al., 2008). Active microglia may be in a reparative state that clean up cellular debris and promote restoration of the injury or insult that lead to their activation (Hu et al., 2014). These productive activated microglia are classified as M2 microglia (Hu et al., 2014). However, another role of active microglia has emerged. For unknown reasons, M2 microglia can transform to M1 microglia that have a cytotoxic effect on brain repair (Hu et al., 2014). Instead of promoting and facilitating healing, M1 microglia secrete pro-inflammatory cytokines and reactive oxygen species that act in a feed-forward system to promote detrimental neuroinflammation (Hu et al., 2014). Further, the secretion of pro-inflammatory cytokines such as TNF α increase neuronal excitability through promoting the endocytosis of GABA_ARs and trafficking of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to the neuronal cell surface (Stellwagen et al., 2005; Riazi et al., 2008). By shifting the excitatory/inhibitory balance, M1 microglia increase neuronal excitability that may potentiate seizure (Rodgers et al., 2009). Whether neuroinflammation is present during normal pregnancy is currently unknown, but is

possible as pregnancy is considered a state of mild peripheral inflammation that has been shown to cause microglial activation and may increase seizure susceptibility during normal pregnancy.

Change in Neurosteroids

- Decreased GABA_AR subunits
- Pro-convulsive effects of estradiol
- Imbalance of progesterone and estrogen
- Others

Blood-Brain Barrier Disruption

- Increased permeability factors
- Failure of efflux transporters
- Endothelial dysfunction
- Autoregulatory breakthrough
- Edema formation

Inflammation & Infection

- Microglial activation
- Local cytokine production
- Others

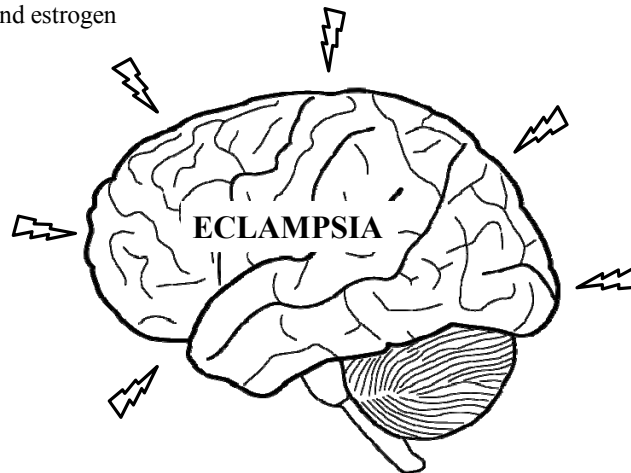


Figure 1: Summary of potential mechanisms that may contribute to eclamptic seizure onset during normal pregnancy and preeclampsia. The pathogenesis of eclampsia remains unclear and the initiation of seizure onset may differ between preeclamptic patients. Further, eclampsia can occur during seemingly uncomplicated pregnancies, suggesting that the brain during normal pregnancy may be at greater risk of seizure that is augmented during preeclampsia. However, the majority of pregnant women do not seize, and not all women with preeclampsia become eclamptic, making it likely that additional insults are required to initiate seizure onset that are multifactorial and may exist in combination:

1) BBB disruption. Seizure-provoking factors are circulating late in gestation that do not gain access to the brain under healthy conditions due to the BBB (Cipolla et al., 2012b). However, under conditions of BBB disruption, increased BBB permeability could allow such hyperexcitable factors into the brain, potentially leading to seizure onset. Disruption of the BBB during normal pregnancy that could lead to seizure onset may be due to increased permeability factors such as VEGF, PlGF, vasopressin or histamine, or failure of efflux transporters located on the luminal surface of endothelial cells to extricate serum constituents back into the vascular lumen. During preeclampsia, maternal endothelial dysfunction is present that likely affects the

cerebral endothelium and may lead to increased BBB permeability and edema formation. Further, an acute rise in blood pressure during pregnancy or preeclampsia that leads to loss of cerebrovascular resistance and CBF autoregulatory breakthrough causes BBB disruption and cerebral vasogenic edema formation. Vasogenic edema formation may be another contributor to seizure onset during pregnancy and preeclampsia.

2) Changes in neurosteroids. Neurosteroids, specifically progesterone metabolites such as allopreganolone are positive allosteric modulators of GABAARs that exert an overall sedative effect (Stell et al., 2003). For this reason, it is thought that GABAARs downregulate in response to elevated concentrations of neurosteroids to maintain a normal level of inhibition (Maguire et al., 2009). In fact, hyperexcitability of brain slices from pregnant mice is thought to be kept in balance by progesterone and progesterone metabolites; however, seizure can occur in response to changes in neurosteroids concentrations (Maguire et al., 2009). Thus, it is possible that an imbalance in neurosteroid levels that may be driven by rapid fluctuations of estrogen and progesterone concentrations could contribute to seizure onset during pregnancy and preeclampsia. It is further possible that the simultaneous and rapid decrease in progesterone and increase in GABAARs that occurs at parturition is out of balance, resulting in decreased inhibition and seizure onset (Maguire et al., 2009).

3) Inflammation and infection. Peripheral inflammation and infection lead to neuroinflammation through activation of microglia. Microglia, the resident immune cells in the brain, secrete pro-inflammatory cytokines that increase neuronal excitability and potentiate seizure (Riazi et al., 2008). Pregnancy is considered a mild form of peripheral inflammation that is thought to be exaggerated during preeclampsia. Thus, activated microglia and neuroinflammation could contribute to seizure onset during pregnancy and preeclampsia through the secretion of pro-inflammatory cytokines and subsequent neuronal hyperexcitability.

Overall, the particular insult(s) that initiate seizure in women during seemingly healthy pregnancies or women with pregnancies complicated by preeclampsia remain ambiguous. It is possible that physiological adaptations to normal pregnancy increase the potential for seizure that are exacerbated in the pathologic state of preeclampsia. This figure proposes several potential mechanisms of eclamptic seizure onset, yet seizure onset may involve a myriad of such contributors where several events such as those listed in this figure occur simultaneously. Additionally, the exact cause of seizure onset may be unique to each eclamptic patient, as convulsion can occur as a result of numerous pathologic processes, further complicating the determination of the pathogenesis of eclampsia.

1.6 Magnesium Sulfate

Eclampsia poses an immediate threat to both the mother and fetus and has been associated with white matter lesions in the brain and cognitive impairment later in life (Aukes et al., 2007b; Aukes et al., 2009; Duley, 2009). Together the immediate and potential long-term risk of morbidity highlights the importance of seizure prevention during pregnancy and preeclampsia. MgSO₄ is the leading therapeutic for seizure

prophylaxis in women with preeclampsia (Sibai, 1990b; Witlin and Sibai, 1998). MgSO₄ has a controversial history and its use as a seizure prophylactic has been scrutinized for decades (Kaplan et al., 1988). However, extensive studies have shown that MgSO₄ reduces the risk of eclampsia by > 50 %, and that it is more effective in prevention of recurrent eclamptic seizure than placebo and accepted anticonvulsant medications, including phenytoin and diazepam (Duley, 1995; Lucas et al., 1995; Altman et al., 2002; Duley et al., 2003; Duley and Henderson-Smart, 2003a, b). In fact, the incidence of recurrent eclamptic seizure in women treated with phenytoin or diazepam was 23.1% compared to 9.4% in women that received MgSO₄ (Witlin and Sibai, 1998). Further, it was suggested that MgSO₄ acted as a seizure prophylactic by lowering blood pressure through its vasodilatory properties; however, this blood pressure effect is transient, and MgSO₄ is neither considered nor administered as an antihypertensive agent (Cotton et al., 1984; Kaplan et al., 1988; Belfort et al., 2003; Lindheimer et al., 2008). Interestingly, the incidence of recurrent seizure in eclamptic women that received only antihypertensive agents was 2.8% and 0.9% in patients that received MgSO₄ (Witlin and Sibai, 1998). Similarly, in a study comparing the effectiveness of nimodipine, a calcium-channel antagonist that has antihypertensive effects and inhibits cerebral vasospasm, to the effectiveness of MgSO₄ in seizure prevention in women with severe preeclampsia reported that women who received nimodipine were more likely to become eclamptic (2.6% of patients) than those treated with MgSO₄ (0.8% of patients) (Belfort et al., 2003). Together, these studies support that simply controlling hypertension during preeclampsia does not prevent development of eclamptic seizure, and that the actions specifically of MgSO₄ are effective at preventing eclamptic seizure (Witlin and Sibai, 1998).

Eclampsia can occur antepartum, intrapartum or postpartum, with antepartum eclampsia occurring more often preterm and being associated with recurrent seizures and greater maternal and fetal morbidity and mortality (Douglas and Redman, 1994; Knight, 2007). The largest and most recent study conducted by Knight et al. in 2005 investigated the incidence of eclampsia in the United Kingdom after the introduction of MgSO₄ as a seizure prophylactic. This study reported that 45% of eclampsia occurred antepartum, 19% intrapartum, and 36% postpartum (Knight, 2007). This was in agreement with a similar study conducted by Douglas & Redman in 1992, prior to MgSO₄ being widely administered as an eclamptic seizure prophylactic (Douglas and Redman, 1994). Overall, it does not appear that the introduction of MgSO₄ as a seizure prophylactic, at least in the United Kingdom, affected the distribution of eclamptic onset in regards to the timing of labor or the type (antepartum, etc.). This suggests that while MgSO₄ effectively reduces the incidence of eclampsia, it does not suggest that one type of eclampsia is preferentially affected by MgSO₄ treatment. However, it should be noted that eclamptic seizure still occurs in some preeclamptic patients, regardless of receiving appropriate MgSO₄ treatment (Katz et al., 2000). Overall, despite being widely administered to women with preeclampsia and eclampsia, and being remarkably effective in eclamptic seizure prophylaxis and cessation, the mechanism by which MgSO₄ prevents seizure remains largely unknown, and may be multifaceted.

The normal serum concentration of Mg²⁺ in humans is 1.8-3.0 mg/dL and the target therapeutic range of treatment of women with preeclampsia is to raise serum Mg²⁺ levels to 4.2-8.4 mg/dL (Pritchard, 1979; Sibai et al., 1984a). MgSO₄ is typically dispensed intramuscularly, intravenously or a combination of the two. Despite being the

most widely administered drug for seizure prophylaxis in women with preeclampsia, its use is associated with potentially serious side effects including respiratory paralysis, cardiac arrest and death (Kelly et al., 1960; McCubbin et al., 1981; Donaldson, 1989b). Hypermagnesemia first leads to weakness of muscles, but can progress to full paralysis if severe enough (Donaldson, 1986; Fisher et al., 1988; Ramanathan et al., 1988). The effect of hypermagnesemia is due to the calcium antagonistic effects of Mg^{2+} blocking acetylcholine release at the neuromuscular junction (Ramanathan et al., 1988). Hyporeflexia begins to occur at serum Mg^{2+} levels greater than 5 mg/dL and areflexia at approximately 10 mg/dL making constant monitoring of serum concentrations of Mg^{2+} and deep patellar reflexes critical in preeclamptic and eclamptic patients receiving $MgSO_4$ treatment (Donaldson, 1986). Due to $MgSO_4$ impairing neuromuscular transmission, it was proposed in the argument against the use of $MgSO_4$ treatment in the 1980s that $MgSO_4$ did not prevent seizure, but rather prevented the physical manifestation of convulsion due to its paralytic-like effects at high serum concentrations, and that $MgSO_4$ simply masked the presence of the dangerous seizure activity that was still occurring in the brain (Donaldson, 1986; Fisher et al., 1988; Kaplan et al., 1988). In support of this theory, it was further argued that Mg^{2+} could not have central anticonvulsant effects because it could not cross the BBB, making its administration to preeclamptic women ineffective, dangerous and unnecessary (Kaplan et al., 1988). However, a study in the early 1990s investigating this controversy provided direct evidence that, not only does systemically administered $MgSO_4$ correlate with increased Mg^{2+} concentrations throughout the brain, but also that it had direct anti-convulsant effects at NMDA receptors, likely due to the Mg^{2+} -gated properties of these receptors

(Hallak et al., 1994). Since these early studies, substantial investigation has been done seeking to elucidate the mechanism by which MgSO₄ acts as an eclamptic seizure prophylactic in women with preeclampsia.

BBB disruption is a consequence of multiple pathologies and disease processes that may be central to eclamptic seizure onset, and is a potential therapeutic target of MgSO₄. Studies have shown that MgSO₄ treatment reduces BBB permeability under conditions of BBB disruption, including during dehydration of endothelial cells with hyperosmolar mannitol (Kaya et al., 2004), acute hypertension (Euser et al., 2008), hypoglycemia (Kaya et al., 2001), traumatic brain injury (Esen et al., 2003), and septic encephalopathy (Esen et al., 2005). This protective effect under pathologic conditions that increase permeability is likely due to the calcium antagonistic actions of Mg²⁺. The studies listed above measured BBB permeability to Evans Blue, a common tracer that binds to serum albumin. Serum albumin is a large protein (~ 70 kDa) and the use of Evans Blue specifically measures paracellular permeability due to disruption of tight junctions at the BBB. Tight junction proteins, including zona occludin-1, claudin-5 and occludin, are attached to the endothelial cell actin cytoskeleton. Through phosphorylation of myosin light chains by calcium-dependent myosin light chain kinase, endothelial cell contraction increases paracellular permeability, resulting in decreased barrier function (Bogatcheva and Verin, 2008). By inhibiting this calcium-dependent process, MgSO₄ seems to exert a “tightening” effect at the BBB that may be one mechanism by which MgSO₄ treatment prevents seizure onset during preeclampsia.

Treatment with MgSO₄ has been shown to decrease the activity of NMDA receptors, likely through prolongation of the presence of the Mg²⁺ gate (Hallak et al.,

1994). Removal of Mg^{2+} is necessary for the passage of cations to occur through the pore of these receptors to contribute to the depolarization of neurons, and propagation of action potentials. Thus, there seems to be a role for $MgSO_4$ as a direct anti-convulsant. Treatment with $MgSO_4$ increases Mg^{2+} concentrations within the central nervous system, having an antagonistic effect on NMDA receptor activity (Hallak et al., 1994). NMDA receptors are widely expressed in the hippocampus, a location considered central to seizure onset and propagation, as well as throughout the cerebral cortex. Interestingly, $MgSO_4$ treatment does not appear to affect EEG patterns of women with preeclampsia, which one may suspect would occur with the reduction in NMDA receptor activity shown with $MgSO_4$ treatment (Sibai et al., 1984b). The effect of $MgSO_4$ on seizure threshold has been investigated in studies seeking to understand its role as a central anticonvulsant agent. $MgSO_4$ treatment has been shown to increase the threshold for electrically-induced seizure in the hippocampus of awake rats (Cotton et al., 1992; Hallak et al., 1992), bupivacaine-induced seizure in awake pregnant rats (Okutomi et al., 2005), but shown to have no effect on lidocaine-induced seizures in rats anesthetized with nitrous oxide (Choi et al., 1991; Kim et al., 1996). It should be noted that the lack of effect of $MgSO_4$ on lidocaine-induced seizures was attributed to the $MgSO_4$ treatment regimen not increasing Mg^{2+} concentrations in the brain. More recently, $MgSO_4$ was shown to increase the latency to seizure onset induced by pentylenetetrazol (PTZ) in a LPS model of preeclampsia (Huang et al., 2014), however the mechanisms by which $MgSO_4$ affected seizure threshold in models of preeclampsia remain unclear. The pathogenesis of preeclampsia and eclampsia are likely heterogeneous and, like the actions of $MgSO_4$, likely multifaceted. Thus, the multiple sites of action of $MgSO_4$ may be why it

is more effective than a medication that functions solely as an anticonvulsant at preventing seizure during preeclampsia. Overall, understanding the mechanism(s) by which MgSO_4 acts as a seizure prophylactic in preeclampsia may allow for more targeted therapies to be developed. Further, screening processes may be established to identify women who would most readily benefit from MgSO_4 therapy, thereby targeting its use and avoiding unnecessary risk.

1.7 Methodology

1.7.1 Rat Model of Pregnancy

The rat is a useful and appropriate model of pregnancy because it has similar hemochorial implantation (Pijnenborg et al., 1981), undergoes similar cardiovascular changes (e.g. plasma volume increase) (Barron, 1987; Gilson et al., 1992), and has similar architecture of the cerebrovasculature (Edvinsson and MacKenzie, 2002) as humans. Further, the rat has a short gestation of approximately 22 days. All experiments were conducted using 12-14 week old female Sprague Dawley rats that were either virgin, nonpregnant animals or primiparous late-pregnant animals on day 20 of pregnancy, as late in pregnancy is when eclampsia occurs most often (Douglas and Redman, 1994).

1.7.2 Rat Models of Preeclampsia

In order to investigate changes in cerebrovascular and neurophysiological properties during preeclampsia, an animal model of preeclampsia was used. Preeclampsia is a disease unique to humans, with only very few incidences being reported in primates (Stout and Lemmon, 1969; Van Wageningen, 1972). Many animal models of preeclampsia exist and have been used to investigate the pathogenesis of preeclampsia, and potential

treatment options; however, there is not a single model that is able to capture the full spectrum of symptoms of the human-specific disorder. Many models target a single pathway or organ system that is afflicted in preeclampsia to mimic hypertension, proteinuria, oxidative stress and endothelial dysfunction. For example, endothelial dysfunction and vasoconstriction that is associated with preeclampsia has been investigated by inhibiting production of nitric oxide synthase (Yallampalli and Garfield, 1993; Molnar et al., 1994; Cadnapaphornchai et al., 2001). Further, the role of renal pathology associated with preeclampsia has been investigated by using the rat model of adriamycin nephropathy to induce hypertension and proteinuria in pregnant rats (Podjarny et al., 1992; Podjarny et al., 1995; Rathaus et al., 1995). Other models capitalize on the imbalance of angiogenic factors that is associated with preeclampsia by infusion of soluble receptors of the pro-angiogenic factors VEGF and PlGF, including infusion of or adenoviral administration of sFlt-1, soluble endoglin (sEng), or a combination of both (Venkatesha et al., 2006; Bridges et al., 2009; Murphy et al., 2010). There are inflammatory models of preeclampsia induced by infusion of TNF α (LaMarca et al., 2005a; LaMarca et al., 2005b), interleukin-6 (IL-6) (Gadonski et al., 2006; LaMarca et al., 2011), AT1R autoantibodies (LaMarca et al., 2009; Parrish et al., 2010), or a low-dose endotoxin such as LPS (Faas et al., 1994), as well as metabolic models of preeclampsia induced by nutritional selenium deficiency (Vanderlelie et al., 2004) and chronic insulin resistance (Podjarny et al., 2001).

One of the most common models of preeclampsia is the Reduced Uteroplacental Perfusion Pressure (RUPP) rat model of preeclampsia. Adapted from original studies in pregnant dogs in the 1940s, placental ischemia is induced by limiting blood flow to the

uteroplacental unit by placing silver clips of specific diameters on the distal abdominal aorta and uterine arcades on day 14 of pregnancy, as seen in Figure 1. This method reduces uterine perfusion pressure by ~ 40 % and raises blood pressure by ~ 25 mm Hg (Crews et al., 2000; Alexander et al., 2001). Further, rats with RUPP have proteinuria, placental ischemia and fetal growth restriction, and are in a state of oxidative stress and endothelial dysfunction similar to that of women with preeclampsia (Alexander et al., 2001; Granger et al., 2001; Sedeek et al., 2008; LaMarca et al., 2009). Rats that have RUPP have elevated circulating pro-inflammatory cytokines including TNF α and IL-6, as well as increased serum and placental concentrations of anti-angiogenic factors, including sFlt-1 (LaMarca et al., 2005a; Gadonski et al., 2006; Gilbert et al., 2007). Thus, the RUPP model of preeclampsia portrays many preeclamptic-like symptoms, as shown in Table 1. However, as with any animal model, there are limitations. The primary limitation of the RUPP model is that placental ischemia is induced. Therefore, this model does not incorporate the original disease process that causes lack of spiral artery remodeling, or any other small vessel disease pathology. Despite this, there is a broad spectrum of symptoms mimicked by this model, and the experimental model used in this dissertation was adapted from the RUPP model of preeclampsia. In addition to RUPP inducing placental disease, rats were also maintained on a high cholesterol diet (HC) days 7-20 of gestation to further induce maternal disease. This diet regimen has been previously shown to cause hypercholesterolemia as well as endothelial and cerebrovascular dysfunction during pregnancy, indicative of maternal endothelial dysfunction (Schreurs and Cipolla, 2013). As mentioned previously, although the majority of eclampsia is nonfatal and occurs at term, when eclampsia occurs in women

with severe disease, maternal mortality is high (MacKay et al., 2001). Thus, RUPP+HC rats were used to focus on a more severe model of preeclampsia that incorporated both placental and maternal disease.

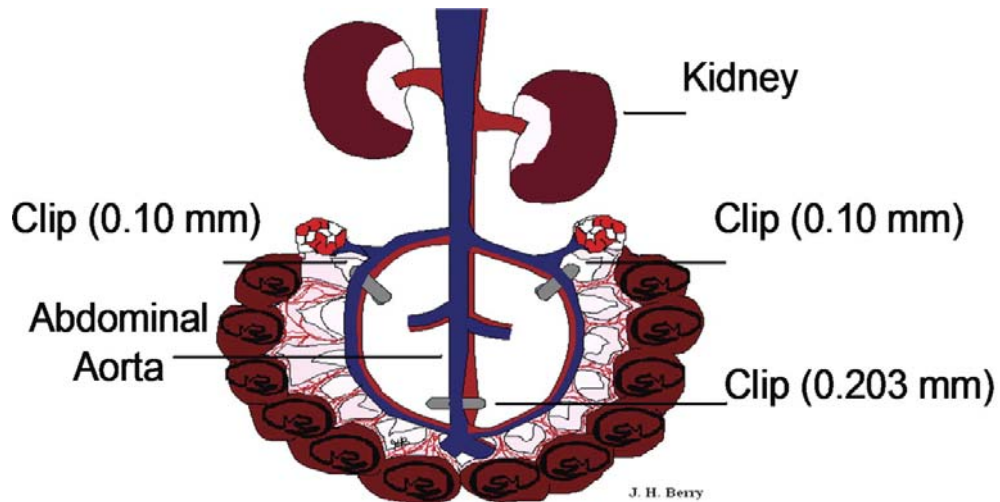


Figure 2: Illustration of the induction of placental ischemia. A silver clip 0.203 mm in diameter is placed on the abdominal aorta, distal to the renal and superior mesenteric arteries, just proximal to the iliac bifurcation, and clips 0.10 mm in diameter placed on the uterine arcade, proximal to the first segmental branches to the fetal-placental unit. Adapted from Li et al., *Am J Physiol Heart Circ Physiol* 303:H1-H8, 2012.

Table 1: Comparison of characteristics of women with preeclampsia and preeclamptic-like symptoms in the RUPP rat model. IUGR, intrauterine growth restriction; TNF α , tumor necrosis factor alpha; IL-6, interleukin-6; sFlt-1, soluble FMS-like tyrosine kinase receptor 1; sEng, soluble endoglin; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; HIF-1 α , hypoxia-inducible factor 1alpha. Adapted from Li et al., *Am J Physiol Heart CircPhysiol* 303:H1-H8, 2012.

Characteristic	Women with Preeclampsia	Rats with RUPP
New-onset hypertension	Yes	Yes
Proteinuria	Yes	Yes
Abnormal placentation	Yes	No
Fetal IUGR	Yes	Yes
Inflammation (TNF α , IL-6)	Increased	Increased
Oxidative stress	Increased	Increased
Endothelin-1	Increased	Increased
sFlt-1 and sEng	Increased	Increased
VEGF and PlGF	Decreased	Decreased
Placental HIF-1 α	Increased	Increased
AT1 autoantibodies	Increased	Increased
Glomerular endotheliosis	Yes	No
Glomerular filtration rate	Decreased	Decreased

1.7.3 Measurement of Seizure Threshold

To investigate the effect of normal pregnancy and preeclampsia on seizure susceptibility *in vivo*, seizure threshold was measured in virgin, nonpregnant and normal pregnant rats, and rats with experimental preeclampsia. Under chloral hydrate anesthesia, seizure was induced by a timed intravenous infusion of pentylenetetrazole (PTZ) while recording electroencephalography (EEG) simultaneously. EEG measures the summated activity of many neurons by detecting the extracellular current flow that occurs during

synaptic excitation of cortical neurons. PTZ infusion was stopped at the first onset of spikewave discharges, defined as a change in waveform when small amplitude, high frequency spikes that slowed and synchronized to large amplitude, rhythmic spikes, as detected by EEG (Sakamoto et al., 2008). Seizure threshold was then calculated as the amount of PTZ (mg/kg) required to elicit electrical seizure: $T_{\text{infusion}} * R_{\text{infusion}} * [\text{PTZ}] / \text{bw}$ where T_{infusion} is the time of infusion in min, R_{infusion} is the rate of infusion in mL/min, $[\text{PTZ}]$ is the concentration of PTZ in mg/mL, and bw is the body weight in kg (Riazi et al., 2008). Seizure susceptibility scores were also calculated: $\text{bw} * 10/v$ where bw is body weight in grams and v is volume of PTZ infused in μL (Riazi et al., 2008). PTZ is a chemoconvulsant that quickly and reliably elicits seizure through antagonistic actions at GABA_A receptors (Squires et al., 1984; Bough and Eagles, 2001). The use of this specific convulsant was chosen to directly investigate the role of pregnancy-induced changes in GABA_A receptor subunit expression in whole-brain excitability during pregnancy and preeclampsia. EEG was recorded unipolarly using silver subdermal corkscrew electrodes placed over the parieto-occipital cortex to monitor generalized seizure. It has previously been determined that subcutaneous electrodes are a suitable alternative for cortical electrodes for less invasive EEG recordings (Ke-jian et al., 2001). Although some studies use a single intraperitoneal injection of PTZ and measure the latency to physical manifestation of seizure to avoid the use of an anesthetic, chloral hydrate was used because it is thought to not depress neural function, and is the preferred anesthetic for studies measuring EEG (Thoresen et al., 1997; Olson et al., 2001). Further, the use of EEG allowed for a more sensitive measure of electrical seizure activity that may be more precise than monitoring for physical convulsions only.

1.7.4 Blood-brain Barrier Permeability

In order to investigate the effect of experimental preeclampsia on basal BBB permeability *in vivo*, the integrity of the BBB was assessed in normal pregnant and preeclamptic rats using two different sized fluorescent tracers. Under chloral hydrate anesthesia, tracers were infused intravenously into the femoral vein and allowed to circulate for ten minutes, a sufficient amount of time to detect changes in BBB permeability (Euser et al., 2008; Cipolla et al., 2011). After ten minutes, a cardiac perfusion of lactated Ringer's solution was performed through a thoracotomy to flush the circulation of all tracers and blood. Allowing the beating heart to flush the circulation avoided any pressure-induced artifact that can occur when the pressure at which the circulation is flushed exceeds the physiological pressure range, disrupting the BBB and pushing tracer into the brain. After the circulation was properly flushed, the brain was immediately removed, homogenized and centrifuged, and the amount of tracer that passed from the lumen of the cerebrovasculature into the brain parenchyma quantified using fluorescent spectroscopy.

Fluorescent tracers have been used to investigate the BBB for over a century, and are a common method of quantifying changes in BBB permeability (Belayev et al., 1996; Oztas et al., 2003; Esen et al., 2005; Euser et al., 2008; Cipolla et al., 2011). In fact, the presence of the BBB was originally discovered by the lack of passage of systemically administered water soluble dyes into the brain (Ehrlich, 1885; Goldmann, 1913). The smaller 470 Da sodium fluorescein is thought to pass both paracellularly and transcellularly, whereas the larger 70 kDa Texas Red dextran moves only via paracellular transport. Thus, the use of two fluorescent tracers of differing sizes allowed the

investigation of potential changes in size-selectivity of the BBB, as well as differential effects on the type of permeability (paracellular vs. transcellular) under basal conditions during experimental preeclampsia.

1.7.5 Quantification of Cerebral Vasogenic Edema

Cerebral edema has been defined as “an abnormal accumulation of fluid within the brain parenchyma, producing a volumetric enlargement of the tissue” (Klatzo, 1987b). Specifically, vasogenic edema is a type of cerebral edema that occurs in response to increased cerebrovascular permeability to proteins and solutes that disrupts the osmotic gradient and leads to passage and retention of water in the extracellular space in the brain (Klatzo, 1987b, a). Vasogenic edema is a consequence of many pathological states, including seizure, traumatic brain injury and stroke and has been shown to contribute to poor neurological outcome (Terry et al., 1990; Lin et al., 1993; Yang et al., 1994; Feldman et al., 1996; Unterberg et al., 2004). In fact, if extensive enough, vasogenic edema formation can lead to a drastic increase in intracranial pressure that exceeds the compensatory venous and cerebrospinal fluid space, and result in brain or brainstem herniation that can be fatal (Rosenberg, 1999; Marmarou, 2007).

A common and accepted measure of vasogenic edema formation is through comparison of the wet and dry brain weights (Schwab et al., 1997). Studies in this dissertation investigated the susceptibility of the posterior cerebral cortex to seizure-induced vasogenic edema during normal pregnancy and in experimental preeclampsia. To do so, after seizure threshold was measured, brains were removed and the posterior cerebral cortex isolated and immediately weighed ($\text{weight}_{\text{wet}}$). The cortices were then dried for 24 hours in a laboratory oven at 90 °C then re-weighed ($\text{weight}_{\text{dry}}$) and percent

water content calculated by the following formula: $(\text{weight}_{\text{wet}} - \text{weight}_{\text{dry}}/\text{weight}_{\text{wet}}) * 100$.

The posterior brain region was chosen as it is a primary location of edema in women with eclampsia (Sanders et al., 1991).

1.7.6 MgSO₄ Treatment

To investigate potential mechanisms by which MgSO₄ acts as a seizure prophylactic during preeclampsia, including effects on seizure threshold and susceptibility, BBB permeability and neuroinflammation, RUPP+HC rats were treated for 24 hours with MgSO₄ prior to experimentation. A previous study from our laboratory investigated the effect of MgSO₄ on BBB permeability during acute hypertension in pregnant rats using a dosing regimen of MgSO₄ shown to raise serum Mg²⁺ concentrations into the therapeutic range given to preeclamptic women for seizure prophylaxis (e.g. 4.2 – 8.4 mg/dL) (Pritchard, 1979; Hallak et al., 1994; Euser et al., 2008). This dosing regimen consisted of an intraperitoneal injection of 270 mg/kg MgSO₄ every four hours for 24 hours (day 19 – 20 of pregnancy) (Euser et al., 2008). However, rats with experimental preeclampsia (RUPP+HC) had recently undergone an invasive abdominal surgery for induction of placental ischemia on day 14 of pregnancy, making repeated intraperitoneal injections unfavorable for this dissertation project. Another study used a subcutaneous osmotic minipump for continuous delivery of 60 mg/kg/day of MgSO₄, based on the solubility of MgSO₄, however, this dose was not sufficient to raise serum Mg²⁺ above ~ 2 mg/dL (Standley et al., 2006). Thus, the dosing regimen of MgSO₄ employed in this dissertation consisted of a combination of these methodologies to effectively raise serum Mg²⁺ into the therapeutic range in a less invasive manner to avoid additional stress on the animals. RUPP+HC rats received a

loading dose of 270 mg/kg of MgSO₄ subcutaneously on the morning of the 19th day of gestation. Four hours later, under isoflurane anesthesia, three osmotic minipumps were implanted subcutaneously between the scapulae to continuously deliver ~ 180 mg/kg/day of MgSO₄. Prior to implantation, minipumps were primed overnight at 37 °C in sterile saline to allow for immediate drug delivery once placed. On day 20 of pregnancy, RUPP+HC rats received a second bolus of 270 mg/kg MgSO₄ subcutaneously approximately 1 hour prior to beginning experimentation. This course of treatment assured RUPP+HC rats received MgSO₄ continuously for approximately 24 hours. Further, after experimentation, serum was collected and Mg²⁺ concentrations determined using a colorimetric assay that uses the magnesium-dependent enzyme glycerol kinase to generate a kinetic red reaction that is proportional to the concentration of Mg²⁺ in serum (Wimmer et al., 1986). The dosing regimen of MgSO₄ used in this dissertation was clinically relevant, raising serum Mg²⁺ concentrations to ~ 5.2 mg/dL that is within the target therapeutic range administered to women with preeclampsia for eclamptic seizure prophylaxis.

1.7.7 Assessment of Microglial Activation

To investigate the potential role of neuroinflammation in pregnancy- and preeclampsia-induced changes in seizure susceptibility, a method of quantifying the activation state of microglia was used. There are many established methods to quantify the activation state of microglia, the resident immune cells in the brain indicative of neuroinflammation, nearly all of which use the morphological changes associated microglial cell activation. Discovered and characterized by Pio del Rio-Hortega in the early 1900s, inactive microglia are highly ramified cells that typically have two to six

branches radiating from the soma, often with thin, finger-like protrusions extending from primary branches (Del Rio-Hortega, 1919). These inactive, or resting microglia are continuously surveying the brain parenchyma, monitoring for any pathological stimuli (Kreutzberg, 1996). As microglia respond to injury or disruption of the brain milieu, they transform morphologically as they activate and migrate to the site of injury, as illustrated in Figure 2. The primary branches retract, shortening and becoming thicker, and the cell bodies enlarge in preparation to phagocytose damaged cells, becoming amoeboid-like (Del Rio-Hortega, 1919; Kettenmann et al., 2011). Microglia are from mesodermal/mesenchymal origin and originate from bone marrow. In rodents, monocyte progenitor cells of future microglia migrate through the vasculature and immigrate into the brain during postnatal development, specifically until postnatal day 10 (Chan et al., 2007). Once these cells have migrated into the brain parenchyma, they mature and transform into the ramified phenotype.

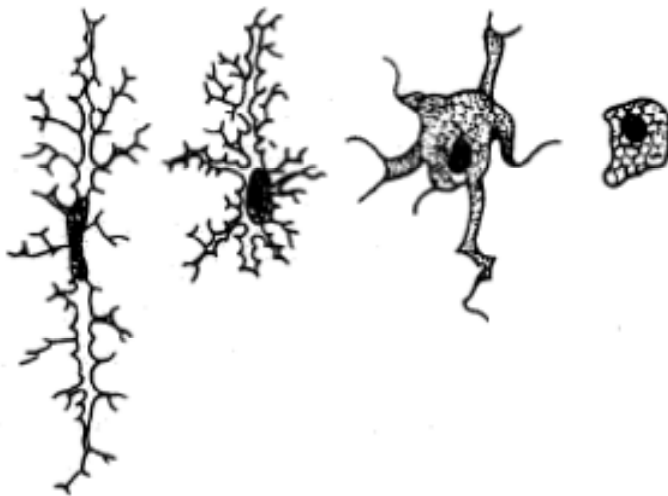


Figure 3: Illustration of the progression from left to right of inactive, resting microglia to active, phagocytotic microglia. Adapted from Kreutzberg, *Trends Neurosci* 19:312-318, 1996.

There are several antibodies that can be used to visualize microglial cells in the brain. One of the most commonly used antibodies to specifically visualize microglia with particular detail of their processes is ionized calcium-binding adaptor molecule 1

(Iba1), a protein involved in calcium homeostasis (Imai et al., 1996; Imai and Kohsaka, 2002). The morphological changes that occur when microglia become activated are well established and stereotypical, and commonly used to accurately determine the activation state of microglia (Kreutzberg, 1996; Stence et al., 2001). Thus, in this dissertation immunostaining with an antibody against Iba1 was used to assess the morphology of microglia in the cerebral cortex. A graded scale was established from 1 (relatively inactive) to 4 (relatively active) to allow each Iba1⁺ microglial cell to be ranked upon its morphology, similar to Figure 2 (Kreutzberg, 1996). Cells with highly ramified, long processes with a scattered, irregularly shaped cell body were ranked in state 1. Cells with an asymmetrical cell body and many long, defined processes were ranked in state 2. Cell bodies that were more rounded with several shorter, thicker processes were ranked 3, and large, round amoeboid-like cell bodies with few to no processes were ranked in state 4. By quantifying the activation state of microglia, the level of neuroinflammation present in the cerebral cortex during normal pregnancy and experimental preeclampsia was assessed as an underlying mechanism by which seizure susceptibility may be affected.

1.7.8 CBF Measurement

To investigate the effect of pregnancy on the lower limit of CBF autoregulation, as well as the effect of experimental preeclampsia on CBF autoregulation, laser Doppler flowmetry was used to measure changes in relative CBF in response to manipulation of arterial pressure. Laser Doppler flowmetry measures CBF by detecting the disruption of light by moving red blood cells, producing a relative measurement of cerebral perfusion in arbitrary units (Stern, 1975). The use of laser Doppler is advantageous over more invasive and absolute measures such as with the use of radio-labeled microspheres, due to

the ability of laser Doppler to provide continuous and instantaneous measurements, allowing autoregulatory curves to be obtained (Tonnesen et al., 2005). Methods measuring absolute CBF typically require immediate euthanization of the animal, and are therefore limited in use when investigating CBF autoregulation across a wide pressure range. Further, laser Doppler flowmetry has been shown to accurately detect the lower limit of CBF autoregulation in rats when compared to absolute CBF measurements via the ¹³³xenon injection technique (Tonnesen et al., 2005). As portions of this dissertation were focused on assessing CBF autoregulation across the physiological pressure range during normal pregnancy and experimental preeclampsia, laser Doppler flowmetry was an appropriate and ideal method.

1.7.9 Isolated Vessel & Arteriograph Studies

A major contributor to CBF autoregulation is the myogenic function of cerebral arteries. In order to investigate the effect of pregnancy on the myogenic vasodilation of cerebral arteries in response to decreased intravascular pressure, pial arteries were isolated and studied using arteriograph. Arteries were dissected out of the brain, cleared of connective tissue and mounted and secured onto glass cannulas in an arteriograph chamber, shown in Figure 3. The proximal cannula is connected to an in-line pressure transducer and a servo-null pressure control system that allows for the controlled manipulation of intraluminal pressure. The distal cannula remained closed throughout the experiment to avoid flow-mediated responses. Luminal diameters and wall thicknesses were measured via video microscopy: the optical window in the bottom of the arteriograph allows for visualization of vessels mounted within the arteriograph chamber by an inverted microscope. The inverted microscope is attached to a video camera and

monitor that is connected to a video dimension analyzer. The pressure transducer and video dimension analyzer signals are recorded with a data acquisition system on a computer that allows for continuous recordings to be made of the pressure-diameter relationship of cerebral vessels. By using this arteriograph system to study isolated cerebral vessels *in vitro*, it was possible to investigate the myogenic response to fluctuations in intraluminal pressure independently of other factors that contribute to CBF autoregulation that are present *in vivo*, such as neuronal or metabolic influences.

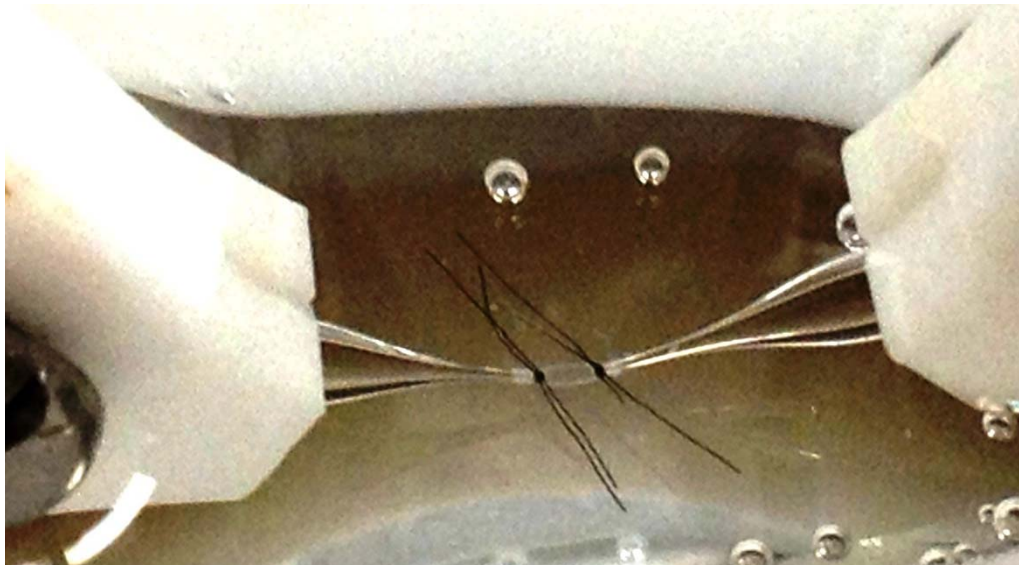


Figure 4: A cerebral artery secured on glass cannulas in an arteriograph chamber.
Image courtesy of Dr. Marilyn J. Cipolla

1.8 Project Goals and Hypotheses

The pathogenesis of the eclamptic seizure remains unclear, but is considered a form of hypertensive encephalopathy where an acute rise in blood pressure causes loss of cerebral blood flow autoregulation and hyperperfusion of the brain that result in

vasogenic edema formation and subsequent seizure (Schwartz et al., 2000). However, eclamptic seizure can occur during seemingly uncomplicated pregnancies in the absence of hypertension and preeclampsia (Douglas and Redman, 1994; Katz et al., 2000), suggesting that normal pregnancy may predispose the brain to hypertensive encephalopathy or seizure, independently of preeclampsia. The overarching goal of this dissertation was to investigate the effect of normal pregnancy and preeclampsia on the cerebrovasculature and neuronal excitability that may promote brain injury and eclamptic seizure. In addition, the pathologic processes by which preeclampsia may potentiate seizure onset that may be ameliorated by $MgSO_4$ treatment were investigated. We hypothesized that normal pregnancy affects the function of the cerebrovasculature and CBF autoregulation by shifting the limits of CBF autoregulation, making the brain more susceptible to injury. In Chapter 2 of this dissertation the effect of pregnancy on the lower limit of CBF autoregulation and the vasodilatory response of cerebral arteries to decreased intravascular pressure was investigated. We further hypothesized that normal pregnancy increases seizure susceptibility that may potentiate eclamptic seizure, and this was investigated in Chapter 3 of this dissertation. Additionally, we hypothesized that preeclampsia is a state of greater seizure susceptibility due to compromised integrity of the BBB and neuroinflammation, and that $MgSO_4$ treatment restores seizure susceptibility by protecting the BBB and reducing neuroinflammation. These hypotheses were investigated in Chapter 4 of this dissertation.

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**CHAPTER 2: EFFECT OF PREGNANCY AND NITRIC OXIDE ON
THE MYOGENIC VASODILATION OF POSTERIOR CEREBRAL
ARTERIES AND THE LOWER LIMIT OF CEREBRAL BLOOD
FLOW AUTOREGULATION**

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Repro Sci 2013; 20:1046-1054

Abstract

Hemorrhage during parturition can lower blood pressure beyond the lower limit of cerebral blood flow (CBF) autoregulation that can cause ischemic brain injury. However, the impact of pregnancy on the lower limit of CBF autoregulation is unknown. We measured myogenic vasodilation, a major contributor of CBF autoregulation, in isolated posterior cerebral arteries (PCA) from nonpregnant and late-pregnant rats (n=10/group) while the effect of pregnancy on the lower limit of CBF autoregulation was studied in the posterior cerebral cortex during controlled hemorrhage (n=8). Pregnancy enhanced myogenic vasodilation in PCA and shifted the lower limit of CBF autoregulation to lower pressures. Inhibition of nitric oxide synthase (NOS) prevented the enhanced myogenic vasodilation during pregnancy but did not affect the lower limit of CBF autoregulation. The shift in the autoregulatory curve to lower pressures during pregnancy is likely protective of ischemic injury during hemorrhage and appears to be independent of NOS.

Key words: CBF autoregulation, hypotension, myogenic vasodilation, nitric oxide, pregnancy

Introduction

Cerebral blood flow (CBF) autoregulation is an intrinsic property of the brain that maintains relatively constant blood flow despite fluctuations in blood pressure (BP).^{1,2} In normotensive adults, CBF autoregulation operates within the arterial pressure range of ~60 to 160 mmHg, outside of which autoregulation is lost and CBF becomes dependent on pressure in a linear fashion.²⁻⁵ A drop in BP within this autoregulatory range results in insignificant clinical symptoms as brain perfusion is maintained by autoregulatory mechanisms.² However, when BP falls below the lower limit of CBF autoregulation, CBF decreases with pressure, potentially causing loss of consciousness and hypoxic-ischemic brain injury.⁶⁻⁸ During pregnancy, hemorrhage occurs with parturition. In some pregnancies, hemorrhage may be severe (> 1500 mL blood loss) and cause an acute drop in maternal BP potentially below the lower limit of CBF autoregulation.⁹⁻¹¹ However, whether pregnancy alters the lower limit of CBF autoregulation is not known, but is important to understand. For example, a shift in CBF autoregulation to lower pressures during pregnancy may be protective of the brain, allowing maintenance of blood flow in the face of acute hypotension. Alternatively, a shift of the lower limit of CBF autoregulation to higher pressures could increase the susceptibility of the brain to injury during parturition.

The myogenic response of cerebral arteries and arterioles is a major contributor to CBF autoregulation.¹² Myogenic vasodilation occurs as BP decreases, contributing to the maintenance of blood flow to the brain.⁵ If BP decreases below the lower limit of CBF autoregulation, maximal dilation of cerebral vessels occurs and this vascular contributor to CBF autoregulation becomes insufficient to maintain brain perfusion.⁸ Several

mechanisms may be involved in the relaxation of vascular smooth muscle (VSM) to decreased intravascular pressure, including endothelial vasodilators such as nitric oxide (NO).^{13, 14} Pregnancy has been shown to increase expression of endothelial-NO synthase (eNOS) in several vascular beds.^{15, 16} However, the involvement of NO in the myogenic vasodilatory response of cerebral arteries and CBF autoregulation during pregnancy has yet to be investigated.

In the present study, *in-vitro* methodology was used to investigate the myogenic vasodilatory response to decreased intravascular pressure of posterior cerebral arteries (PCA) from nonpregnant (NP) and late-pregnant (LP) rats. The contribution of NO to myogenic vasodilation during pregnancy was also assessed. We found that pregnancy enhanced myogenic vasodilation in response to decreased pressure that was NO-dependent. To test if this enhanced myogenic vasodilation translates to a shift of CBF autoregulation, the effect of pregnancy on the lower limit of CBF autoregulation was measured using an *in-vivo* model of hemorrhagic hypotension and measuring changes of CBF in the posterior cerebral cortex. We further investigated the role of NO on CBF autoregulation during acute hypotension by infusing a NOS inhibitor.

Materials and Methods

Animal model. All experiments were conducted using virgin NP female (14-16 weeks) or timed-pregnant Sprague Dawley rats (Charles River, Canada). Rats were housed individually in the University of Vermont Animal Care Facility. NP females were chosen randomly and timed-pregnant rats were studied during LP on days 19 - 21 of a 22 day gestation. All procedures were approved by the Institutional Animal Care and Use

Committee and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Isolated Vessel Preparation and Pressurized Arteriograph System. NP and LP animals were anesthetized with isoflurane (3 % in oxygen) and decapitated. Brains were promptly removed and placed in cold, oxygenated physiological saline solution (PSS). PCA were carefully dissected and cleared of connective tissue. Third-order PCA were mounted and secured onto glass cannulas in an arteriograph chamber as previously described.¹⁷ Briefly, the proximal cannula was connected to an in-line pressure transducer and a servo-null pressure control system (Living Systems Instrumentation, Inc., Burlington, VT). The distal cannula remained closed throughout the experiment to avoid flow-mediated responses. Vessel diameters were measured via video microscopy. PSS was aerated with 5 % CO₂, 10 % O₂, and 85 % N₂ to maintain pH at 7.40 ± 0.05 . Temperature within the arteriograph chamber was maintained at 37.0 ± 0.1 °C throughout the experiments.

Determination of myogenic vasodilation in isolated PCA. Vessels were equilibrated at 50 mmHg for 1 hour, after which pressure was increased to 125 mmHg in 25 mmHg increments to allow for tone development. Myogenic vasodilation was measured by decreasing pressure from 125 to 5 mmHg in a step-wise manner and recording active luminal diameter at each pressure once stable. Myogenic vasodilation was measured in PCA from NP and LP rats in PSS alone (n=10/group) and in the presence of the NOS inhibitor N^o-nitro-L-arginine (L-NNA, 0.1 mM, n=7/group). Pressure steps were then repeated in zero calcium PSS to obtain passive diameter measurements.

In-vivo measurement of CBF during hemorrhagic hypotension in the posterior cerebral cortex. A separate set of NP and LP rats (n=8/group) were anesthetized initially with isoflurane (3% in O₂), which was then lowered to 1.5 – 2.0 % in O₂ for instrumentation and tracheostomy. Anesthesia was then shifted to intravenous injections of chloral hydrate (200 mg/kg, left femoral vein). Animals were mechanically ventilated to maintain blood gases and pH within normal physiological ranges (Table 1). Body temperature was monitored and maintained with a heating pad at 37 °C throughout the experiment. CBF was measured and recorded transcranially using laser Doppler flowmetry as previously described.¹⁸ The left side of the medioposterior skull was exposed and a laser Doppler probe was affixed over a thinned area 2 mm lateral to the sagittal suture and 1 mm anterior to the lambdoid suture to measure CBF in the PCA territory. Both femoral arteries were cannulated to measure arterial blood pressure via a pressure transducer (Living Systems Instrumentation, Inc., Burlington, VT), and to obtain blood samples for blood gas measurements and controlled blood withdrawal. Blood was slowly withdrawn through the femoral catheter at a steady rate (0.67 - 0.69 ml/min) to gradually decrease arterial blood pressure from 100 mmHg to 30 mmHg. The lower limit of CBF autoregulation was defined as the arterial pressure at which CBF decreased by 20 % from baseline.^{19, 20}

A separate set of LP animals (n=7) underwent the same instrumentation as described above, with the addition of cannulation of the right femoral vein for the infusion of the NOS inhibitor N^o-nitro-L-arginine methyl ester hydrochloride (L-NAME, 10 mg/kg/min, 3 min). After drug infusion, controlled blood withdrawal was

performed in the same manner as described above and the lower limit of CBF autoregulation was determined as stated above.

Real-time quantitative PCR of eNOS in pial arteries. A separate group of NP (n=5) and LP (n=4) rats were used for the isolation of arteries for real-time qPCR for eNOS. PCA segments from both right and left sides of the brain were pooled from each animal. RNA was extracted using Trizol reagent (Life Technologies) followed by purification using an RNeasy Micro Kit (Qiagen) per manufacturers' protocols. RNA concentrations and quality were determined using an Agilent Bioanalyzer (Agilent). Real time qPCR was performed in a two step process. Total RNA was reverse transcribed using a mix of oligo dTs and random primers using the iScript cDNA Synthesis Kit (Biorad). For each sample, cDNA was used to amplify the target gene eNOS and two housekeeping genes: Hprt1 and Ywhaz. One microliter of cDNA was used per reaction with 150 nM of the forward and reverse primers (eNOS: forward CCTGAGCAGCACAAAGAGTTACAA, reverse GGAGCCCAGCCCAAACACA; Hprt1: forward CTCATGGACTGATTATGGACAGGAC, reverse GCAGGTCAGCAAAGAACTTATAGCC; and ywhaz: forward GATGAAGCCATTGCTGAACTTG, reverse GTCTCCTTGGGTATCCGATGTC) and 12.5 µl of Power Sybreen Master mix (Life Technologies) in a 25 µl reaction. Primers were designed by the Obstetrics and Gynecology Departmental Molecular Core Facility at the University of Vermont using PrimerSelect (DNASTAR). The reactions were performed using an initial denaturation of 3 minutes at 95 ° C, 40 cycles of 15 seconds at 95 ° C and 60 seconds at 60 ° C followed by a melt curve analysis to ensure only the correct product was amplified. One set of PCR products for each gene were checked for

correct size on a 2 % agarose gel. Each sample was run in triplicate on the ABI 7000 Sequence Detection System (ABI). Negative water controls were run for each primer set in the real time PCR reaction to ensure no contamination in the reagents as well as no secondary primer structures were amplified. Primers were designed over an exon-exon junction or the amplicon was designed to span an exon-exon junction to ensure genomic DNA was not amplified. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.²¹

Drugs and Solutions. All experiments were conducted using a bicarbonate-based PSS containing (mmol/L): NaCl 119.0, NaHCO₃ 24.0, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ · 7H₂O 1.17, CaCl₂ 1.6, and EDTA 0.026. PSS was made and stored without glucose at 4 °C; glucose (5.5 mmol/L) was added to the PSS prior to each experiment. Zero calcium PSS was made similarly, omitting the addition of CaCl₂. L-NNA and L-NAME were purchased from Sigma-Aldrich (St. Louis, MO). L-NNA was made weekly in a 0.01 mM stock solution and stored at 4 °C. L-NAME was made fresh daily at 40 mg/ml in sterile lactated ringers solution.

Data Calculations and Statistical Analysis. Results are presented as mean ± SEM. Percent tone of isolated arteries was calculated at 100 mmHg and after addition of L-NNA as the percent decrease in active luminal diameter from the passive diameter by the equation: $[1 - (\text{diameter}_{\text{active}} / \text{diameter}_{\text{passive}})] \times 100 \%$, where $\text{diameter}_{\text{active}}$ is luminal diameter in PSS with or without L-NNA and $\text{diameter}_{\text{passive}}$ is maximum luminal diameter in zero calcium PSS. To determine the pressure at which diameters of PCA from NP and LP rats differed from baseline of 125 mmHg, repeated measures ANOVA with a *post-hoc* Bonferroni test was used. Differences in diameters between the presence and absence of L-NNA were determined using Student's unpaired t-test. The lower limit of CBF

autoregulation, defined as when CBF decreased 20 % from baseline, was determined from the laser Doppler traces for each animal. Differences in the percent change in CBF during hemorrhagic hypotension and between the pressure at which the lower limit of CBF autoregulation was reached between NP and LP, and LP and LP+L-NAME animals were determined using Student's unpaired t-test. Differences were considered significant at $p < 0.05$.

Results

Myogenic vasodilation in response to decreased intravascular pressure in PCA from NP and LP rats. We sought to determine the effect of pregnancy on the myogenic vasodilatory response of PCA to decreased intraluminal pressure. We used PCA because they are the main blood supply to the posterior cortex.²² PCA from NP and LP animals developed similar myogenic tone at 100 mmHg (33.8 ± 2.3 % and 33.7 ± 1.5 %; ns). When intravascular pressure was decreased, luminal diameter of PCA from NP and LP rats remained relatively unchanged until ~ 60 mmHg (Figure 1A). As intravascular pressure was lowered below 60 mmHg, myogenic vasodilation occurred in PCA from both NP and LP animals. However, PCA from LP rats had significantly greater dilation compared to NP rats when pressure was lowered between 50 – 30 mmHg. The diameter of PCA from LP rats was significantly greater than baseline diameter (183 ± 8 μm at 50 mmHg vs. 147 ± 5 μm at 125 mmHg; $p < 0.05$). In contrast, arteries from NP rats dilated less in response to decreased intravascular pressure, with luminal diameter never becoming statistically significantly different compared to baseline at any pressure (Figure 1A). Below 30 mmHg, the diameter of PCA from both NP and LP animals passively

decreased with pressure. Figure 1B shows that there was no difference in passive diameters of PCA from either group at any pressure studied, suggesting the difference in the magnitude of myogenic vasodilation between the groups was due to a difference in active vasodilation and not structural remodeling. Thus, the magnitude of the myogenic vasodilation in response to decreased pressure was greater in PCA from LP compared to NP rats.

Effect of NOS inhibition on myogenic vasodilation to decreased pressure. As greater myogenic vasodilation occurred in PCA from LP compared to NP rats, we investigated NO as an underlying mechanism by which pregnancy increases myogenic vasodilation in PCA by inhibiting NOS with L-NNA and measuring myogenic vasodilation. Addition of L-NNA caused similar constriction of PCA from both groups of animals and the percent tone with NOS inhibition at 100 mmHg was similar between PCA from NP and LP animals ($52.1 \pm 3.4\%$ and $51.8 \pm 3.2\%$; ns). In PCA from NP rats treated with L-NNA, vasodilation occurred and diameters were similar to PCA in PSS alone when pressure was decreased, becoming significantly greater than baseline at 60 mmHg ($176 \pm 20\ \mu\text{m}$ at 60 mmHg vs. $105 \pm 7\ \mu\text{m}$ at 125 mmHg; $p < 0.05$; Figure 2A). In contrast, vasodilation of PCA from LP rats was markedly reduced with NOS inhibition (Figure 2B). The diameters of L-NNA treated vessels from LP animals were smaller than those in PSS alone ($p < 0.01$; Figure 2B). Despite this, luminal diameter of L-NNA treated PCA from LP rats still became significantly greater than baseline at 50 mmHg ($140 \pm 20\ \mu\text{m}$ at 50 mmHg vs. $93 \pm 8\ \mu\text{m}$ at 125 mmHg; $p < 0.05$; Figure 2B).

Figure 2C compares vasodilation of PCA with L-NNA treatment from NP and LP rats. Vessels from both groups of animals underwent similar vasodilation with NOS

inhibition as pressure was decreased. However, the dilation was shifted to lower pressure in PCA from LP compared to NP rats. When pressure was lowered to 70 mmHg the diameter of PCA from LP rats was significantly smaller than the diameter of PCA from NP rats ($88 \pm 8 \mu\text{m}$ vs. $148 \pm 22 \mu\text{m}$, respectively; $p < 0.05$; Figure 2C). Thus, NOS inhibition prevented the greater myogenic vasodilation of PCA from LP compared to NP rats from occurring without eliminating myogenic vasodilation all together, and it shifted the dilation of PCA from LP rats leftward compared to PCA from NP rats.

The results above suggest that enhanced vasodilation of PCA from LP rats was NO-dependent. To determine if this was due to an affect of pregnancy on eNOS expression, real time qPCR was performed on PCA from both groups of animals. There were no differences in relative quantity (RQ) of mRNA expression of eNOS between PCA from NP and LP rats (0.86 ± 0.25 vs. 1.02 ± 0.27 ; ns; Figure 2D). These results suggest that greater vasodilation of PCA from LP compared to NP rats via NO was not due to changes in mRNA expression of eNOS during pregnancy.

CBF autoregulation during acute hypotension in NP and LP rats. The myogenic response of cerebral arteries is a main contributor to CBF autoregulation.¹² Since we found that enhanced myogenic vasodilation in response to decreased pressure during pregnancy was NO-dependent, we sought to determine the effect of pregnancy on the lower limit of CBF autoregulation and investigate the role of NOS in CBF autoregulation during acute hypotension. Table 1 shows the physiological parameters of all groups of animals used. Importantly, arterial pH and arterial gasses that can affect CBF were within physiological ranges and were not different between groups.

Figures 3A & B show the effect of pregnancy on CBF autoregulation during hemorrhagic hypotension in the posterior cortex. Baseline BPs were similar between LP and NP rats (100.1 ± 0.2 mmHg vs. 100.0 ± 0.3 mmHg; ns) prior to controlled blood withdrawal. The autoregulatory curve was shifted leftward to lower pressures in LP compared to NP animals during hemorrhagic hypotension (Figure 3A). The lower limit of CBF autoregulation was significantly lower in LP vs. NP rats (Figure 3B). To determine the role of NO in the pregnancy-specific leftward shift in CBF autoregulation, L-NAME was infused into LP rats and the autoregulatory curve determined. Figures 3C & D show the effect of acute NOS inhibition during pregnancy on CBF autoregulation and its lower limit during hemorrhagic hypotension, respectively. NOS inhibition caused a rise in BP, which has been previously shown,^{23, 24} with the BP of LP rats infused with L-NAME being 115.1 ± 2.0 mmHg prior to blood withdrawal. Despite this baseline increase in BP with L-NAME infusion, CBF was maintained similarly between LP rats with and without NOS inhibition during hemorrhagic hypotension (Figure 3C). The lower limit of CBF autoregulation was also unaffected by acute NOS inhibition during pregnancy (Figure 3D). Thus, it appears that pregnancy shifts the autoregulatory curve leftward to lower pressures, and that this is unaffected by acute NOS inhibition.

Discussion

In the present study, we investigated the effect of pregnancy and NOS inhibition on the myogenic vasodilatory response of PCA to decreased intravascular pressure and the lower limit of CBF autoregulation. PCA from LP rats dilated to a greater extent in response to decreased pressure compared to PCA from NP rats that was NO-dependent.

However, L-NNA did not prevent myogenic vasodilation from occurring in PCA from either NP or LP rats, but it eliminated the pregnancy-specific enhancement of vasodilation, causing the magnitude of dilation of PCA from NP and LP rats to be similar. Using an animal model of controlled hemorrhage, we found that pregnancy caused a leftward shift in the CBF autoregulatory curve during acute hypotension, with the lower limit being reached at significantly lower pressures compared to the NP state. We hypothesized this was due to the enhanced vasodilatory response to decreased pressure seen in PCA of LP rats, however, acute NOS inhibition in pregnancy did not affect the lower limit of CBF autoregulation. These results suggest that CBF autoregulation is more effective in pregnancy during hemorrhagic hypotension, but that this shift in the lower limit of the CBF autoregulatory curve to lower pressure is not due to NO.

NO appears to be responsible for the enhanced vasodilation of PCA from LP compared to NP rats. There are at least three possibilities by which NO may be affecting vasodilation during pregnancy. First, expression of eNOS could be increased in PCA during pregnancy, although this is unlikely as no difference in mRNA expression was seen in PCA from NP and LP rats. Second, pregnancy could enhance the sensitivity of VSM to NO. However, a previous study showed no differences in VSM sensitivity to the NO donor sodium nitroprusside between PCA from NP and LP rats.¹⁷ Finally, the activity of eNOS could be increased in endothelium from pregnant animals as intravascular pressure was lowered. Changes in phosphorylation of eNOS during pregnancy could change the activity of NO and increase NO production.²⁵ In addition, the role of NO in myogenic vasodilation appeared to be pressure-dependent because there was no

difference in the magnitude of constriction of PCA with NOS inhibition between NP and LP rats at a constant pressure, as has also been shown previously.²⁶ Pregnancy increases flow-mediated vasodilation in an NO-dependent manner in mesenteric arteries from rats^{27, 28} and subcutaneous arteries from humans.²⁹ Thus, it is possible that changes in response to shear stress during decreased intravascular pressure are responsible for increasing NO in PCA from LP rats.

To our knowledge, this is the first study investigating the effect of pregnancy on CBF autoregulation during acute hypotension and the involvement of NOS in the pregnant state. NOS inhibition did not alter the lower limit of CBF autoregulation although it inhibited pregnancy-specific enhancement of myogenic vasodilation in response to decreased pressure. Thus, the response of an isolated cerebral artery may not be indicative of what is occurring during hemorrhagic hypotension that encompasses the entire brain. Our previous study showed that, in pregnancy, brain parenchymal arterioles are significantly larger than in the NP state, an effect that may also contribute to more effective CBF autoregulation when upstream pial vessels are dilated.⁴⁴ Thus, it is possible that even when the NO-dependent enhancement of myogenic vasodilation of PCA in pregnancy was inhibited, CBF was better maintained in pregnancy during acute hypotension due to the vasodilation that was occurring in upstream vessels, coupled with structurally larger downstream arterioles.

Our findings of NOS inhibition having no effect on the lower limit of the autoregulatory curve are in accordance with other studies utilizing systemic NOS inhibition in the investigation of the role of NO in the lower limit of CBF autoregulation.³⁰⁻³³ In fact, the involvement of NO in CBF autoregulation is controversial,

with several studies showing contrasting findings.^{23, 24, 30-36} It is possible that differences in methodology, such as the method that hypotension is induced, may play a role in the outcome of a study. Our result agrees with others when hypotension was induced by hemorrhage, but not by ganglionic blockade or administration of a potassium channel activator.²⁴ Another previous study used similar methodology as the present study and found intravenous infusion of a NOS inhibitor shifted the lower limit of CBF autoregulation to higher pressure.²³ However, NOS inhibition raised BP in the present study to 115 mmHg, compared to over 150 mmHg in the previous study.²³ Therefore, it is possible that the difference between the two findings is due to the greater acute increase in BP upon NOS inhibition. It should be noted that the rise in BP in our study confirmed that eNOS was indeed inhibited by L-NAME infusion. However, it is possible that a greater degree of inhibition would have produced a shift in the autoregulatory curve seen in other studies.

Despite evidence for NO having no role in the low end of CBF autoregulation with i.v. infusion of a NOS inhibitor,^{24, 30-33} including our findings in the present study, other studies have identified a role of NO in the lower limit of CBF autoregulation when the NOS inhibitor was suffused over the cortex.^{35, 36} L-NAME suffusion over a cranial window both raised the lower limit of CBF autoregulation and depressed the height of the CBF autoregulatory curve.^{35, 36} In addition to eNOS, neuronal NOS (nNOS) contributes to CBF autoregulation and increases NO production during hypotension-induced hypoxia, effectively dilating cerebral vessels.^{37, 38} nNOS mRNA and protein levels are increased in the hypothalamus of pregnant rats¹⁵ suggesting that pregnancy increases nNOS in some brain regions. Although our recent study determined there were no

changes in nNOS mRNA expression in the posterior cortex between NP and LP rats, activity of nNOS was not measured and may be increased during pregnancy.³⁹ These data support the idea that changes in NO production by increased activation of nNOS during acute hypotension in pregnancy could contribute to the leftward shift of the CBF autoregulatory curve seen in LP rats. As inhibition of nNOS appears to take substantially longer time to achieve than that of eNOS,³⁵ it is possible that nNOS was not inhibited in the present study due to the acute nature of the L-NAME infusion. Therefore, it is possible that NO derived from nNOS was still maintaining CBF in the face of acute hypotension.

In this study, we investigated the myogenic component of CBF autoregulation. However, there are other contributors to CBF autoregulation, including metabolic and neuronal influences in addition to myogenic responses of VSM of cerebral arteries and arterioles.^{2, 8} Oxygen metabolism of the pregnant brain has been found to be similar to that of NP brain, thus this is unlikely to be contributing to the shift in the autoregulatory curve.⁴⁰ Pregnancy-induced changes in neuronal contributors, such as nNOS as previously discussed, may be contributing to the leftward shift of the autoregulatory curve. The baroreceptor reflex also influences CBF autoregulation during increases in BP, with its disruption extending the autoregulatory curve surpassing the pressure at which autoregulatory breakthrough would normally occur.⁴¹ The baroreflex stimulates sympathetic fibers which have been shown to affect CBF autoregulation during acute hypotension as well.⁴² Both alpha-adrenergic blockade as well as sympathectomy shifts the lower limit of CBF autoregulation leftward to lower pressures.^{6, 42, 43} A recent study by our group measured perivascular sympathetic fiber density of PCA of NP and LP rats

and found that pregnancy did not affect innervation of PCA; however, nerve activity was not measured.³⁹ Thus, pregnancy-induced attenuation of the baroreflex^{44, 45} may be partly responsible for the left-ward shift of the lower limit of CBF autoregulation seen in LP rats by decreasing activity of sympathetic nerves that innervate PCA.

In summary, investigation of the myogenic vasodilation to decreased intravascular pressure of PCA revealed greater dilation in vessels from pregnant rats that was NO-dependent. Pregnancy improved CBF autoregulation during hemorrhagic hypotension by shifting the lower limit of CBF autoregulation leftward in the posterior cortex, which remained unaffected by acute NOS inhibition. This leftward shift in CBF autoregulation in the posterior cortex during pregnancy may be a protective mechanism by which the maternal brain is better prepared to maintain CBF in the face of acute hypotension that can occur during parturition.

Funding

This work is supported by the National Institute of Neurological Disorders and Stroke (grant number RO1 NS045940 and RO1 NS045940-06S1).

Acknowledgments

We would like to thank Karen Oppenheimer of the Obstetrics, Gynecology and Reproductive Sciences Departmental Molecular Core Facility at the University of Vermont for her technical expertise.

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Table 1: Physiological parameters of nonpregnant (NP), late-pregnant (LP) and LP rats infused with L-NAME during hemorrhagic hypotension to assess the lower limit of CBF autoregulation.

	NP (n=8)	LP (n=8)	LP+L-NAME (n=7)
Weight (g)	342 ± 6	442 ± 11	389 ± 12
Arterial pH	7.40 ± 0.01	7.33 ± 0.01	7.42 ± 0.01
Arterial pCO ₂	40.1 ± 1.6	40.3 ± 1.5	40.2 ± 1.4
Arterial pO ₂	128 ± 8	139 ± 14	121 ± 6

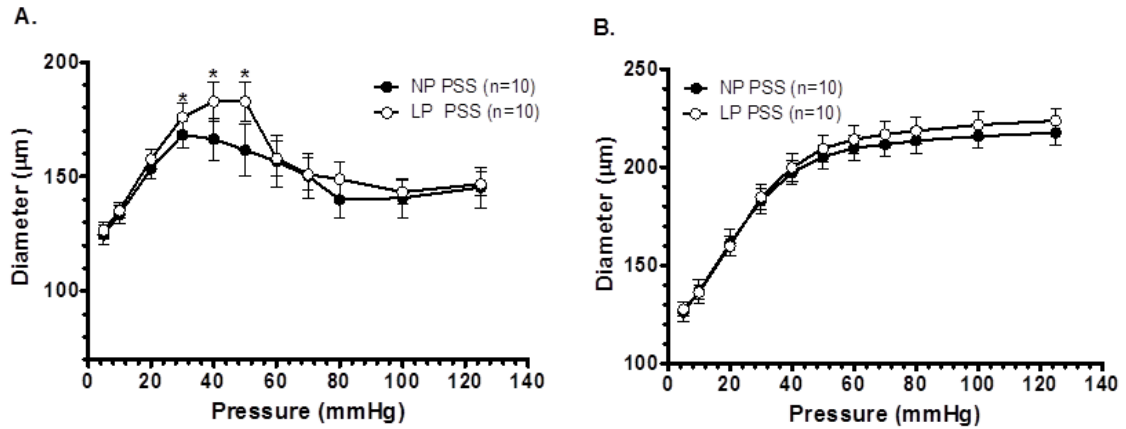


Figure 1. Impact of pregnancy on myogenic vasodilation to decreased pressure in posterior cerebral arteries (PCA). (A) Graph showing active pressure-diameter relationship in PCA from nonpregnant (NP) and late-pregnant (LP) rats. Note that greater myogenic vasodilation was seen in PCA from LP animals, with diameters becoming statistically greater than baseline at 50, 40 and 30 mmHg. (B) Graph showing passive pressure-diameter relationship in PCA from NP and LP rats. There was no difference in passive diameters between PCA from NP and LP rats at any pressure studied. * $p < 0.05$ vs. LP at 125 mmHg by repeated measures ANOVA.

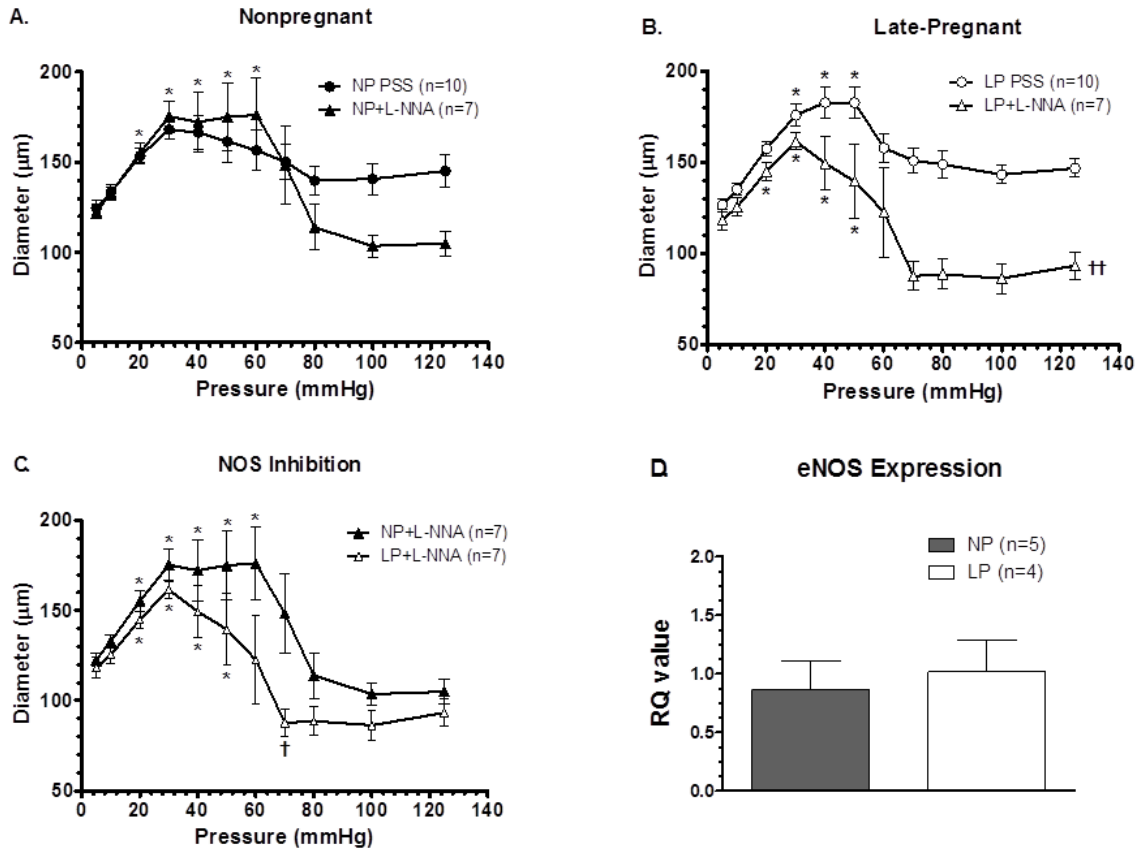


Figure 2. Role of nitric oxide synthase (NOS) inhibition on myogenic vasodilation of posterior cerebral arteries (PCA) during pregnancy. Graphs showing active pressure-diameter relationships of PCA from (A) nonpregnant (NP) and (B) late-pregnant (LP) animals in the presence or absence of the NOS inhibitor L-NNA. (C) Graph comparing the effect of NOS inhibition on myogenic vasodilation in PCA from NP and LP rats. Despite NOS inhibition, PCA from NP and LP rats had substantial myogenic vasodilation in response to decreased pressure. (D) Relative eNOS mRNA expression in PCA from NP and LP rats. There was no difference between eNOS expression in cerebral arteries from NP and LP rats. * $p < 0.05$ vs. baseline by repeated measures ANOVA; HH $p < 0.01$ vs. PSS and H $p < 0.05$ vs. NP L-NNA by t-test.

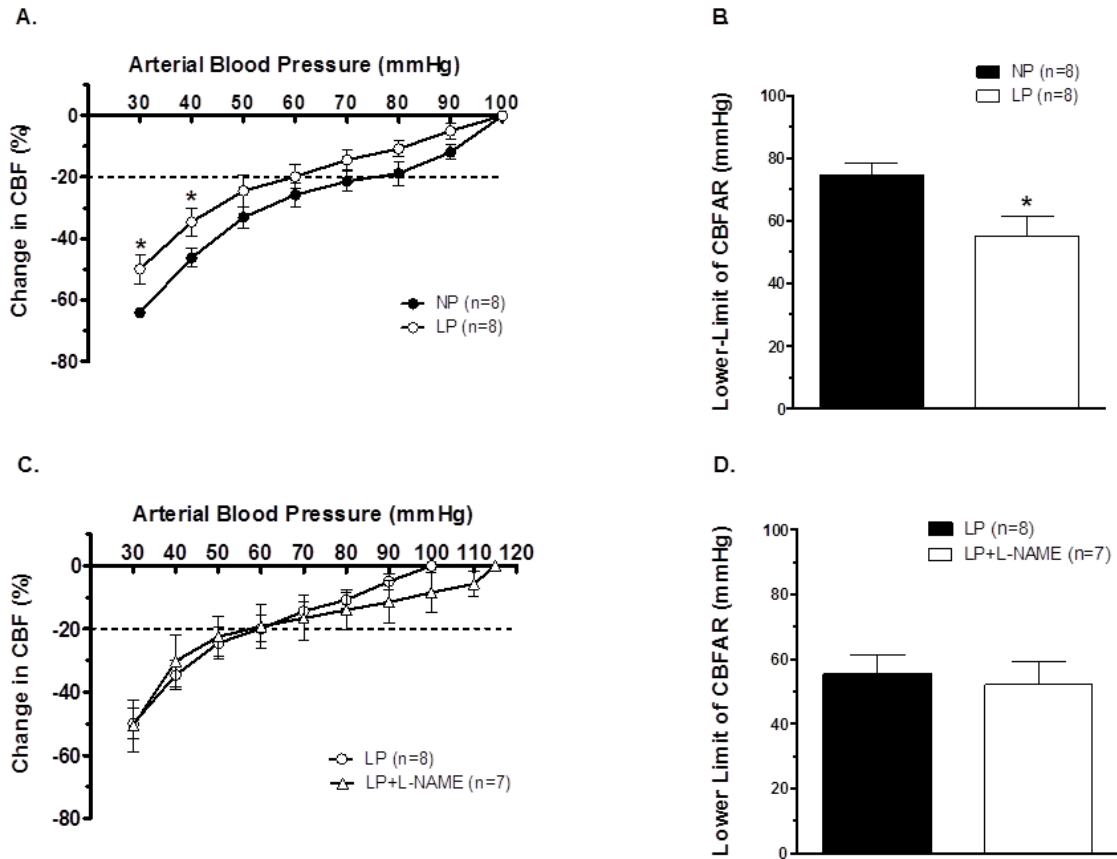


Figure 3. Effect of pregnancy on the lower limit of cerebral blood flow

autoregulation. (A) Graph showing changes of CBF during hemorrhagic hypotension in the posterior cerebral cortex of nonpregnant (NP) and late-pregnant (LP) rats, and (B) the lower limit of CBF autoregulation in NP and LP rats during hypotension. (C) Graph showing the effect of acute NOS inhibition on changes in CBF during hemorrhagic hypotension in the posterior cerebral cortex in LP rats only, and (D) the lower limit of CBF autoregulation in LP rats with and without NOS inhibition. The lower limit is indicated by the dotted line and defined as the pressure at which CBF decreased to 20% of baseline. * $p < 0.05$ vs. NP by t-test.

**CHAPTER 3: THE CONTRIBUTION OF NORMAL PREGNANCY
TO ECLAMPSIA**

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Submitted to *PLoS One* January 26, 2015

Abstract

Eclampsia, clinically defined as unexplained seizure in a woman with preeclampsia, is a life threatening complication unique to the pregnant state. However, a subpopulation of women with seemingly uncomplicated pregnancies experience de novo seizure without preeclamptic signs or symptoms, suggesting pregnancy alone may predispose the brain to seizure. Here, we hypothesized that normal pregnancy lowers seizure threshold and investigated mechanisms by which pregnancy may affect seizure susceptibility, including neuroinflammation and plasticity of gamma-aminobutyric acid type A receptor ($GABA_A$ R) subunit expression. Seizure threshold was determined by quantifying the amount of pentylenetetrazole (PTZ) required to elicit electrical seizure in Sprague Dawley rats that were either nonpregnant (Nonpreg, n=7) or pregnant (Preg; d20, n=6). Seizure-induced vasogenic edema was also measured. Further, the basal activation state of microglia, a measure of neuroinflammation (n=6-8/group), and $GABA_A$ R δ -subunit ($GABA_A$ R- δ) protein expression (n=3/group) in the cerebral cortex were determined as underlying contributors to changes in seizure threshold. Seizure threshold was lower in Preg compared to Nonpreg rats (36.7 ± 9.6 vs. 65.0 ± 14.5 mg/kg PTZ; $p < 0.01$) that was associated with greater vasogenic edema formation (78.55 ± 0.11 vs. 78.04 ± 0.19 % water; $p < 0.05$). The % of active microglia was similar between groups; however, pregnancy was associated with downregulation of $GABA_A$ R- δ in the cerebral cortex. Overall, pregnancy appears to be a state of increased seizure susceptibility that is not due to neuroinflammation, but rather is associated with reduced expression of $GABA_A$ R- δ and greater edema. Understanding neurophysiological changes occurring in normal

pregnancy could allow for better prevention and management of de novo seizure, including pathologic states such as eclampsia.

Introduction

Preeclampsia, defined as the new onset of hypertension and proteinuria after the 20th week of gestation, is a life-threatening complication of pregnancy that afflicts 1-7% of all pregnancies [1-4]. Numerous organs are affected by preeclampsia, including the brain in the form of eclampsia [5-9]. Eclampsia is the appearance of unexplained seizure in a woman with preeclampsia and is one of the most dangerous complications of pregnancy [4]. Eclampsia is a leading cause of maternal and fetal morbidity and mortality worldwide that accounts for greater than 50,000 maternal deaths each year [6,7,10]. While eclampsia by definition is restricted to women with preeclampsia, seizure during pregnancy does not appear to be a progression from severe preeclampsia to eclampsia [11-13]. In fact, de novo seizure has been reported to occur in 38-60% of seemingly uncomplicated pregnancies, without hypertension and proteinuria, or the diagnosis of preeclampsia [11]. The finding that de novo seizure occurs in the absence of preeclampsia suggests that pregnancy alone may be a state of increased seizure susceptibility. In addition, women who develop eclampsia are by definition normotensive and asymptomatic prior to pregnancy, with no known underlying conditions contributing to seizure onset, supporting the concept that pregnancy alone may predispose the brain to seizure independently of preeclampsia.

It is well-established that fluctuations in neurosteroids occur during the menstrual cycle, and to a greater extent during pregnancy, that can affect neuronal excitability [14-16]. Specifically, increases in progesterone metabolites, including allopregnanolone, act as positive allosteric modulators of gamma-aminobutyric acid type A receptors (GABA_ARs), the main inhibitory neurotransmitter receptors in the brain, and decrease neuronal excitability [16]. GABA_ARs consist of several subunits, however, the main binding site and therefore site of action of allopregnanolone is on the δ -subunit [17]. δ -subunit-containing GABA_ARs (GABA_AR- δ) are located extrasynaptically and are involved in tonic inhibition throughout the brain [16]. A delicate balance of excitatory and inhibitory inputs maintains neuronal excitability of the brain, and alterations in excitatory/inhibitory receptor expression can result in a hyperexcitable or hyperinhibited state. Neuroactive steroids such as allopregnanolone reduce neuronal excitability selectively through actions at GABA_AR- δ and cause their downregulation [16,17]. Plasticity of the δ -subunit of GABA_ARs occurs in the brain during pregnancy, and is an adaptation that likely functions to maintain a steady state of excitability in the face of increased neurosteroids [17,18]. In fact, brain slices from pregnant mice were found to be hyperexcitable due to the downregulation of the δ -subunit that was normalized by the presence of allopregnanolone [18]. While these in-vitro studies suggest that the brain is hyperexcitable during pregnancy, it is less clear if the brain is more susceptible to seizure under normal physiological conditions of pregnancy where naturally circulating neurosteroids are present.

One mechanism by which brain excitability may be affected during pregnancy is through peripheral inflammation. Pregnancy is considered a state of mild peripheral

inflammation and peripheral inflammation has been shown to cause neuroinflammation through the activation of microglia, the resident immune cells in the brain [19,20]. When microglia become active, they aid in clearance of debris and play a reparative role in the brain (M2); however, for unknown reasons they can become cytotoxic (M1) [21]. M1 microglia secrete proinflammatory cytokines such as tumor necrosis factor alpha (TNF α) and increase local neuronal excitability by simultaneously promoting the endocytosis of GABA $_A$ Rs and exocytosis of excitatory α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [20,22]. In fact, neuroinflammation has recently been shown to be present in a rat model of preeclampsia that was associated with decreased seizure threshold [23]. Whether neuroinflammation is present during normal pregnancy is unknown, however, it may be one mechanism by which the pregnant state is more susceptible to seizure. We hypothesized that pregnancy is a state of increased seizure susceptibility due to decreased expression of GABA $_A$ R- δ and/or neuroinflammation that acts to lower seizure threshold.

Onset of eclampsia may also be related to loss of cerebral blood flow autoregulation and decreased cerebrovascular resistance that increases pressure on the microcirculation and subsequent vasogenic edema formation [24]. In fact, vasogenic edema is present in ~ 90 % of women with eclampsia, as assessed by diffusion weighted MRI [25,26]. However, it is difficult to determine if edema is present prior to seizure and contributes to the occurrence of eclampsia because seizure itself causes blood-brain barrier (BBB) disruption and edema formation [27-30]. Further, vasogenic edema formation occurs to a greater extent during pregnancy under pathologic conditions such as acute hypertension, suggesting pregnancy predisposes the brain to edema [31,32].

Thus, we hypothesized that the maternal brain is more susceptible to seizure-induced vasogenic edema formation than in the nonpregnant state. Understanding if the maternal brain is more susceptible to seizure-induced edema may lead to a greater understanding of the pathophysiological process of de novo seizure during pregnancy. Further, investigating seizure susceptibility during normal pregnancy and underlying mechanisms by which pregnancy may affect seizure threshold may shed light on the contribution of pregnancy-induced neurophysiological changes to de novo seizure in the absence of preeclampsia.

Methods

Animals and ethics statement

All experiments were conducted using virgin, nonpregnant (Nonpreg) or timed-pregnant (Preg) Sprague Dawley rats that were 14-16 weeks old (Charles River, Canada). Pregnant rats were used experimentally late in gestation (day 20 of a 22 day gestation), a time point when eclampsia occurs most often [12]. All rats were housed singly in the University of Vermont Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Measurement of seizure threshold, susceptibility and severity, and brain water content

Rats that were Nonpreg (n = 9) or Preg (n = 9) were anesthetized initially with isoflurane (1 – 3 % in oxygen) for intubation, electrode placement and instrumentation. Animals were mechanically ventilated to maintain blood gases and pH within normal physiological ranges. Body temperature was monitored with a rectal thermometer and maintained with a heating pad at 37 °C throughout the experiment. The dorsal surface of the head was shaved to expose the scalp and silver subdermal corkscrew electrodes (Ambu, Glen Burnie, MD) were implanted under the scalp and secured in place with collodion glue. Electroencephalography (EEG) was recorded unipolarly using a MP150 acquisition system (BIOPAC System Inc., Goleta, CA) to monitor generalized seizure, as previously described [23]. After placement of electrodes, animals were placed in supine position for placement of venous and arterial catheters. Femoral arteries were cannulated to obtain blood samples for blood gas measurements and continuous measurement of arterial blood pressure via a pressure transducer (BIOPAC Systems Inc., Goleta, CA). Femoral veins were cannulated for administration of the anesthetic chloral hydrate and infusion of the chemoconvulsant pentylenetetrazole (PTZ). PTZ was used because it reliably elicits seizure through antagonistic actions at GABA_ARs. After instrumentation, animals were tapered off isoflurane and anesthesia maintained by continuous intravenous infusion of chloral hydrate (41.5 mg / mL in Nonpreg, 50 mg / mL in Preg; 30 µL / min) and seizure threshold measured as previously described [23]. Chloral hydrate was used because it is thought to not depress neural function, and is the preferred anesthetic for studies measuring EEG [33,34]. Seizure threshold was calculated as the amount of PTZ (mg/kg) required to elicit electrical seizure: $T_{\text{infusion}} * R_{\text{infusion}} * [\text{PTZ}] / \text{bw}$ where T_{infusion}

is the time of infusion in min, R_{infusion} is the rate of infusion in mL/min, [PTZ] is the concentration of PTZ in mg/mL, and bw is the body weight in kg. Seizure susceptibility scores were also calculated: $\text{bw} * 10/v$, where bw is body weight in grams and v is volume of PTZ infused in μL [20]. Baseline blood pressures were taken 30 seconds prior to PTZ infusion and at seizure onset. EEG was recorded for 30 minutes post-PTZ infusion and seizure severity assessed by counting the number of recurring seizures and calculating the percent of the post-infusion period spent in seizure. Two Nonpreg and three Preg rats were excluded because blood gases were outside of the physiological range. Seizure severity was not assessed in one Preg rat because EEG recordings were not available for the entire 30 minutes due to a technical issue with the ground electrode. After 30 minutes animals were euthanized under chloral hydrate anesthesia by decapitation and brains immediately removed. The posterior cerebral cortex was isolated and weighed wet ($\text{weight}_{\text{wet}}$), then dried in a laboratory oven at 90°C for 24 hours and re-weighed dry ($\text{weight}_{\text{dry}}$). The posterior brain region was chosen as it is a primary location of edema in women with eclampsia [35]. Percent water content was determined by wet:dry weights using the following formula: $(\text{weight}_{\text{wet}} - \text{weight}_{\text{dry}}/\text{weight}_{\text{wet}}) * 100$. Brain water content of one Nonpreg rat was not measured because the 24-hour drying time point was unable to be completed.

Quantification and morphological assessment of microglia

Separate groups of Nonpreg (n = 6) and Preg (n = 8) rats were euthanized under isoflurane anesthesia and brains immediately removed. A 3 mm section (4 – 7 mm posterior to bregma) was taken of the posterior cerebral cortex and fixed in 10 % buffered

formalin at 4 ° C overnight, then transferred to 0.1 M PBS and slices paraffin embedded. Immunohistochemical staining for ionized calcium binding adapter molecule 1 (Iba 1; Wako, Richmond, VA), a marker for microglia was done using standard procedure as described previously [23]. For each brain section, four micrographs of cerebral cortex were captured using an Olympus BX50 microscope at 20X magnification. Each Iba1⁺ cell was assessed by its morphology and activation state ranked using a graded scale from 1 (relatively inactive) to 4 (relatively active) as previously described [23]. To assess microglia, two analyses were performed. First, the percentage of cells in each activation state was calculated for each micrograph and averaged per group. Second, total number of Iba1⁺ cells were counted per mm² and averaged for each group. Two evaluators that were blinded to group performed all morphological assessments.

GABA_AR δ-subunit expression by Western blot

Separate groups of Nonpreg and Preg animals (n = 3 / group) were euthanized under isoflurane anesthesia and brains immediately removed. Cerebral cortex was isolated and snap frozen in liquid nitrogen and stored at – 80 ° C. Cerebral cortex from either Nonpreg or Preg rats were homogenized in COMP1 buffer and centrifuged at 16,000 g at 4 ° C for 20 min. Protein concentration was determined using the bicinchoninic acid, or BCA, protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a standard. Bromophenol blue and dithiothreitol were added to the supernatant to give a final concentration of 0.01 % and 50 mM, respectively, and heated at 95 ° C for 10 min. 40 µg was loaded on a 4 – 20 % Mini-PROTEAN TGX Precast Gel (BioRad, Hercules, CA) and ran at 35 mA. The gel was then wet transferred to a polyvinyl difluoride

membrane at 90 V for 45 min. The membrane was Ponceau-S stained to ensure that protein was properly transferred. The membrane was blocked with 5 % non-fat dried milk in TBS-T for 1.5 hrs at room temperature. The membrane was then rinsed with TBS-T and the primary antibody was applied, anti-GABA_AR- δ (1:200; Santa Cruz Biotechnology, Dallas, TX) overnight at 4 ° C with rocking. The antibody was removed and the membrane rinsed with TBS-T and secondary antibody was applied, anti-Goat (1:100,000) (Southern Biotech, Birmingham, AL) for 1 hr at room temperature. The secondary antibody was removed and subsequent TBS-T washes were done. West Pico chemiluminescent substrate (ThermoScientific, Rockford, IL) was applied to the membrane and then exposed to film. β -actin served as a loading control (1:5000, Abcam, Cambridge, MA) and all samples were normalized to a Nonpreg control.

Drugs and solutions

Chloral hydrate and PTZ were purchased from Sigma Aldrich (St Louis, MO) and made daily in sterile lactated Ringer's solution. Western blot solutions were prepared as follows: COMP1 buffer contained 60 mM TrisCl, 2 % Sodium Dodecyl Sulfate, 10 % Glycerol, pH 6.8; and TBS-T containing 15.4 mM Tris-HCl, pH 7.4, 137.0 mM NaCl, 0.1 % Tween-20.

Statistical Analysis

Data are presented as mean \pm standard error of mean. All comparisons were made between groups one-way using analysis of variance (ANOVA).

Results

The effect of pregnancy on seizure threshold, susceptibility and severity

Figure 1A shows representative EEG tracings during seizure threshold measurements from Nonpreg (top tracing) and Preg rats (bottom tracing). The black arrowheads indicate when PTZ infusion started and the time of seizure onset. The time to seizure onset was longer in Nonpreg rats compared to Preg rats. When seizure threshold was calculated, the amount of PTZ (mg/kg) required to elicit electrical seizure was significantly lower in Preg compared to Nonpreg rats (Figure 1B). Further, Preg rats had significantly higher seizure susceptibility scores compared to Nonpreg rats (Figure 1C). There were no differences in physiological parameters during seizure threshold measurements between groups, except that, as expected, body weights were significantly higher in Preg compared to Nonpreg rats (Table 1).

To determine if there were changes in seizure severity during pregnancy, the total number of recurrent seizures was counted in the 30 minutes post-infusion time period, and the percent of time spent in seizure calculated. Figure 2A shows a representative EEG tracing within the 30 minutes post-PTZ infusion recording time period. Indicated on the tracing is the rapid onset of a recurrent seizure, followed by the rapid cessation of the recurring seizure. There was no change in the number of recurrent seizures in the 30 minutes post-PTZ infusion between groups (Figure 2B). There were also no differences in the percent of time spent in seizure between Nonpreg and Preg rats (Figure 2C).

The effect of pregnancy on microglial activation and GABA_AR δ -subunit expression

To determine if normal pregnancy was a state of basal neuroinflammation, we used the microglia-specific marker Iba 1 to morphologically assess the activation state of microglia in the posterior cerebral cortex from Nonpreg and Preg rats. Figure 3A shows representative photomicrographs of Iba 1⁺ microglia in the posterior cerebral cortices of Nonpreg and Preg rats. There was no difference in the total number of microglial cells in the posterior cerebral cortex between groups (Figure 3B). Further, there were no changes in basal microglial activation during pregnancy with no differences in the percent of cells in any activation state between groups (Figure 3C).

To determine if pregnancy induced changes in protein expression of GABA_AR- δ in the cerebral cortex of Nonpreg and Preg rats, GABA_AR- δ expression was measured using Western blot and shown in Figure 4A. GABA_AR- δ expression was significantly reduced in the cerebral cortex of Preg compared to Nonpreg rats (Figure 4B).

The effect of pregnancy on seizure-induced vasogenic brain edema formation

Percent water content of the brain was calculated post-seizure in Nonpreg and Preg rats to determine if there were differences in seizure-induced vasogenic edema formation. Percent water content was significantly higher after seizure in the brains of Preg rats compared to Nonpreg rats (Figure 5).

Discussion

The major finding of this study was that normal pregnancy was a state of increased seizure susceptibility, indicated by seizure threshold being significantly lower during pregnancy compared to the nonpregnant state. This increase in seizure susceptibility did not appear to be due to basal neuroinflammation, as the percent of microglia in each activation state was similar between Preg and Nonpreg animals. However, there was decreased GABA_AR δ -subunit expression in the cerebral cortex of Preg compared to Nonpreg rats. The GABA_AR- δ are extrasynaptic neurotransmitter receptors and involved in tonic inhibition throughout the brain [17]. Thus, it is not surprising that a decrease in inhibitory input was associated with increased excitability and seizure susceptibility. However, the δ -subunit is considered the main site of action of neurosteroids elevated during pregnancy that act as positive allosteric modulators of GABA_ARs [16,17]. Thus, plasticity of the GABA_AR- δ is likely a normal adaptation to an increase in neuroactive steroids during pregnancy, necessary to maintain the steady state of excitability and avoid overinhibition of neuronal networks. Interestingly, despite the presence of naturally circulating neurosteroids, pregnant animals remained in a state of increased seizure susceptibility, suggesting this downregulation of GABA_AR- δ resulted in increased cortical excitability. Increased neuronal excitability in response to neurosteroids may contribute to the subpopulation of women who experience de novo seizure during pregnancies uncomplicated by preeclampsia. Further, the rapid and dramatic drop in progesterone and its metabolites that occur immediately after parturition may also represent one mechanism by which postpartum eclampsia occurs [36].

Susceptibility to seizure during pregnancy has previously been investigated in the context of understanding epilepsy in pregnancy. The finding of a lower seizure threshold during pregnancy in the current study would suggest that there would be an increase in seizure frequency in pregnant women with epilepsy. However, the effect of pregnancy on frequency of seizures in women with epilepsy is difficult to discern and appears variable. A review of over 2000 pregnancies in epileptic patients found that ~ 25 % of patients experienced a decrease, ~ 25 % an increase, and ~ 50 % experienced no change in seizure frequency during pregnancy [37]. This variability in seizure frequency noted may be complicated by the absence or presence of anti-epileptic medications during pregnancy. One study investigating the effect of antiepileptic drugs on seizure in pregnant mice found that electroconvulsive seizure threshold was increased on day 18 of pregnancy compared to nonpregnant mice [38]. Another study using amygdaloid kindled rats also reported increased threshold to electroconvulsive seizure in pregnant compared to nonpregnant animals [39]. The finding that pregnancy increases seizure threshold in these previous studies is in contrast to the current study in which seizure threshold was significantly lower in Preg compared to Nonpreg rats. One significant difference in the present study is that seizure threshold was defined as the onset of electrographic seizure and not based on physical manifestations as in the previous studies. Therefore, the method of measuring seizure threshold could contribute to these disparate findings. In addition, the chemoconvulsant used to induce seizure was also different. In the current study, the decrease in seizure threshold in response to PTZ is in accordance with the decrease in GABA_AR δ -subunit expression, since the mechanism by which PTZ acts as a convulsant is through antagonistic actions at GABA_ARs. Thus, the use of PTZ-induced

seizure in the current study allowed investigation into the direct involvement of GABA_AR plasticity in whole brain excitability during pregnancy. However, whether there is plasticity in excitatory receptors in the brain, such as AMPA receptors, during pregnancy is currently unknown. To our knowledge this is the first in vivo study reporting that normal pregnancy is associated with lowered seizure threshold and increased seizure susceptibility using non-kindled rats and EEG to determine seizure susceptibility during pregnancy.

The cause of spontaneous, unexplained seizure in a woman with a seemingly normal pregnancy is unknown; however, pregnancy-induced increased seizure susceptibility could be a contributing factor. It is also possible that de novo seizure in a seemingly healthy pregnant woman represents a form of status epilepticus (SE) of idiopathic nature and that eclampsia is idiopathic SE that happens to occur in a woman while she is pregnant. In addition, the incidence of eclampsia has declined in developed countries with the advancement of prenatal care and the use of seizure prophylactic agents such as magnesium sulfate that have reduced the risk of eclampsia by ~ 50% [40]. Incidence reports of eclampsia for the United Kingdom found a decrease from 4.9 cases of eclampsia per 10,000 pregnancies in 1992 to 2.7/10,000 pregnancies in 2005-06 [12,41]. Similarly, in Scandinavia the incidence of eclampsia was 5.0/10,000 pregnancies between 1998-2000 [42], and in Canada (excluding Quebec) 5.9/10,000 cases between 2009-2010 [43]. However, in developing countries the incidence of eclampsia is considerably higher. For example, in 1990 in South Africa the incidence of eclampsia was 60/10,000 pregnancies [44], and 200/10,000 pregnancies at Muhimbili National Hospital in Dar es Salaam, Tanzania in 1999-2000 [45]. Population-based epidemiologic

studies of SE report incidences of 18.3/100,000 population in Rochester, Minnesota between 1965-1984 [46], 13.1/100,000 in Bologna, Italy between 1999-2000 [47], 15.8/100,000 in Hessen, Germany between 1997-1999 [48], and 10.3/100,000 in French-speaking Switzerland between 1997-1998 [49]. Interestingly, studies reporting the incidence of SE often report that the majority of patients have no history of epilepsy [47,48]. Thus, the incidence of eclampsia seems to be magnitudes higher than SE, especially in undeveloped countries, and therefore is likely not simply an idiopathic form of SE. In fact, these comparisons likely dampen the differences in incidence since eclampsia is limited only to women who are of childbearing age. Overall, unexplained de novo seizure seems to be more prevalent during pregnancy than in the rest of the population, and supports the theory that normal pregnancy increases seizure susceptibility.

Previously we have shown that there are seizure-provoking factors present in serum that circulate late in gestation that are not present in the nonpregnant state [43]. Using a hippocampal slice culture model to measure excitability using evoked field potentials after exposure to serum from pregnant and nonpregnant rats revealed that serum from pregnant, but not nonpregnant rats increased slice excitability [50]. This was due to serum factors activating microglia and subsequent TNF α secretion increasing neuronal excitability [50]. However, under normal conditions, serum constituents do not readily gain access to the brain due to the protective nature of the BBB. In addition, the pregnant rats from which the hyperexcitable serum was taken did not appear to have spontaneous convulsions, suggesting the BBB may be central to seizure prevention during normal pregnancy. Together with the findings of the current study that the brain is

in a hyperexcitable state during pregnancy, further points to the BBB as a critical factor in preventing seizure during pregnancy. We speculate that it is the failure of this crucial function of the BBB that underlies de novo seizure during seemingly uncomplicated pregnancies, as well as during preeclampsia, by allowing the passage of circulating seizure-provoking factors into the hyperexcitable maternal brain.

The brain has been shown to be more susceptible to vasogenic edema formation under certain pathologic conditions during pregnancy, such as acute hypertension [31,32]. The current study shows that the maternal brain is also more sensitive to seizure-induced brain injury, with seizure causing greater vasogenic edema formation than in the nonpregnant state. It is important to note that previous studies have revealed no differences in brain water content basally between pregnant and nonpregnant rats [31], demonstrating that the increase in brain water content found in the present study was an effect of seizure. Although edema formation is considered a leading cause of eclampsia and is present in ~ 90 % of women with eclampsia [25,26], seizure itself can cause edema formation through disruption of the BBB [27-29]. The finding in the current study that the brain is more susceptible to seizure-induced vasogenic edema formation during pregnancy supports the concept that the brain is more susceptible to injury-induced edema formation during pregnancy. Further, this sensitivity to seizure-induced edema may help explain vasogenic edema formation that may be present in eclamptic women who experienced de novo seizure without an acute elevation in blood pressure.

There are several potential contributors to pregnancy-induced increase in susceptibility to cerebral vasogenic edema formation. Firstly, if pregnancy were associated with more severe seizure, there may be greater brain injury and edema

formation. However, there were no differences in seizure severity between Preg and Nonpreg rats, with the percent of time spent in seizure and number of recurrent seizures being similar, making this possibility unlikely. Secondly, seizure-induced acute hypertension could account for the increase in vasogenic edema formation during pregnancy. However, seizure did not cause a significant change in blood pressure in either Preg or Nonpreg rats (data not shown). The lack of effect of seizure onset on blood pressure is likely due to the sensitive measure of seizure threshold used in the current study that allowed for detection of electrical seizure, as opposed to measuring latency to tonic clonic seizure that has been associated with a rapid rise in blood pressure [51]. As there was no significant change in blood pressure with the onset of electrical seizure in either group, an acute elevation in blood pressure likely did not contribute to the increased formation of vasogenic edema in Preg rats. Thirdly, capillary density increases in the posterior cerebral cortex during pregnancy [52] that may increase the number of sites of BBB disruption during seizure. Further, plasma volume increases ~ 50 % during pregnancy resulting in a hemodiluted state that under conditions of BBB disruption such as seizure may drive water into the brain due to decreased osmolality [53]. Overall, it appears that the maternal brain is more susceptible to vasogenic edema formation during conditions that cause BBB disruption, highlighting the importance of seizure prevention during pregnancy.

In summary, this is the first study we are aware of to report that pregnancy is a state of increased susceptibility to seizure and seizure-induced cerebral vasogenic edema. While the brain appears to be hyperexcitable during pregnancy, this did not appear to be due to low-level neuroinflammation, but rather a reduction in GABA_AR- δ expression in

the cerebral cortex. Understanding pregnancy-related neurophysiological changes may clarify mechanisms by which eclamptic seizure occurs during seemingly uncomplicated pregnancies when there are failures of other protective mechanisms, such as at the BBB or neurosteroid concentrations involved in maintaining steady state excitability, as illustrated in Figure 6. Further, elucidating the contribution of normal pregnancy to seizure onset could lead to a greater understanding of pregnancy-specific pathologies such as preeclampsia and eclampsia. Our understanding of these conditions may result in development of specific screenings to identify pregnant women who are at risk of de novo seizure, aiding in seizure prevention and specific treatment during pregnancy.

Acknowledgements

We thank Nicole Bishop in the Microscopy Imaging Center at the University of Vermont for her technical expertise in performing immunohistochemistry.

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Table 1. Physiological parameters of nonpregnant (Nonpreg) and late-pregnant (Preg) rats under chloral hydrate anesthesia for seizure threshold measurements.

	Nonpreg	Preg
	(n = 7)	(n = 6)
Body Weight (grams)	287 ± 7	377 ± 10 **
Body Temp (° C)	36.3 ± 0.2	36.8 ± 0.2
Arterial P _{O2} (mm Hg)	114 ± 8	111 ± 7
Arterial P _{CO2} (mm Hg)	42.1 ± 1.9	42.8 ± 1.7

** p < 0.01 vs. Nonpreg

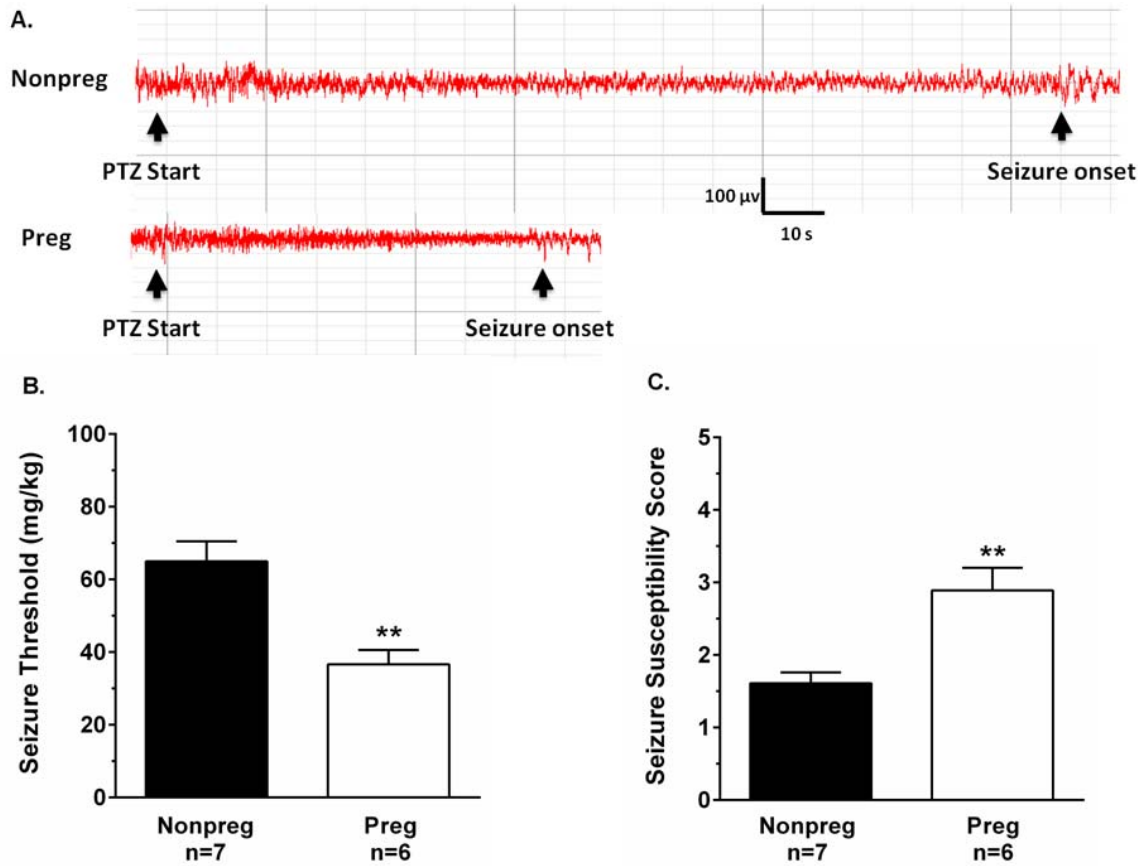


Figure 1. The effect of normal pregnancy on seizure threshold and susceptibility. (A) Representative EEG tracings from nonpregnant (Nonpreg) and late-pregnant (Preg) rats during timed-infusion of pentylenetetrazol (PTZ). Black arrows indicate when PTZ infusion started and the onset of spike-wave discharge indicative of electrical seizure. (B) Seizure threshold was significantly lower in Preg rats compared to Nonpreg rats. (C) Preg rats scored higher on the seizure susceptibility scale compared to Nonpreg rats. ** $p < 0.01$ vs. Nonpreg by one-way ANOVA.

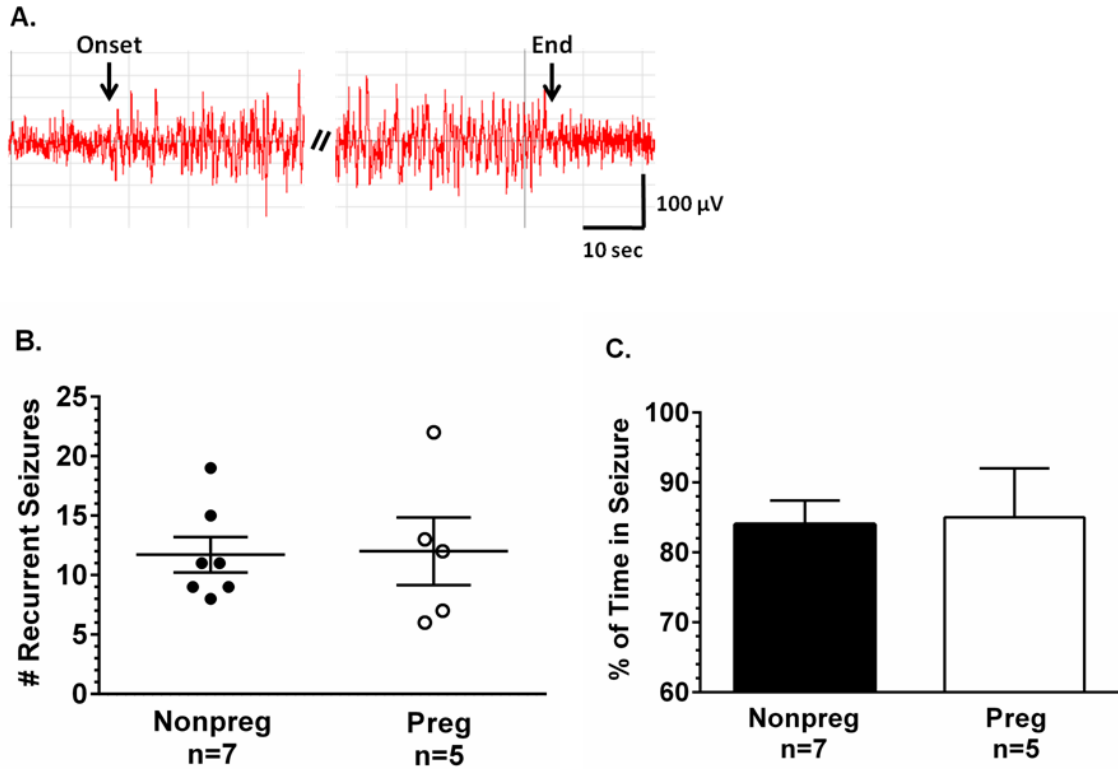


Figure 2. The effect of pregnancy on seizure severity. (A) Representative EEG tracing of a recurrent seizure during the 30-minute post-pentylenetetrazol (PTZ) infusion time period. Left black arrow indicates seizure onset and right black arrow seizure cessation. (B) Number of recurrent seizures in the 30-minute post-PTZ infusion period was similar between nonpregnant (Nonpreg) and late-pregnant (Preg) rats. (C) The percent of time spent in seizure during the 30-minute post-PTZ time period was similar between Nonpreg and Preg rats.

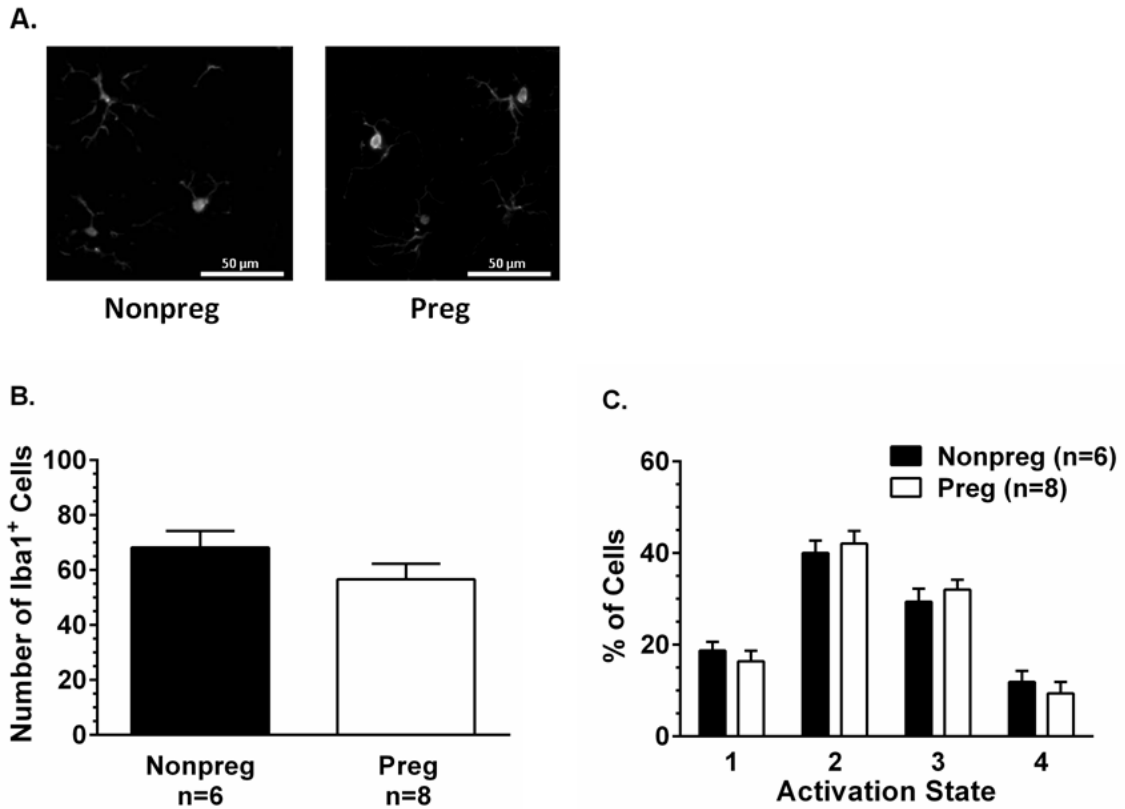


Figure 3. Basal activation state of microglia in cerebral cortex of nonpregnant (Nonpreg) and pregnant (Preg) rats. (A) Representative photomicrographs of Iba 1⁺ microglia in the cerebral cortices of Nonpreg and Preg rats. (B) There was no difference in the number of microglia in the cerebral cortices of Nonpreg and Preg rats. (C) The percent of Iba 1⁺ microglial cells in each activation state was similar in the cortices of Nonpreg and Preg rats.

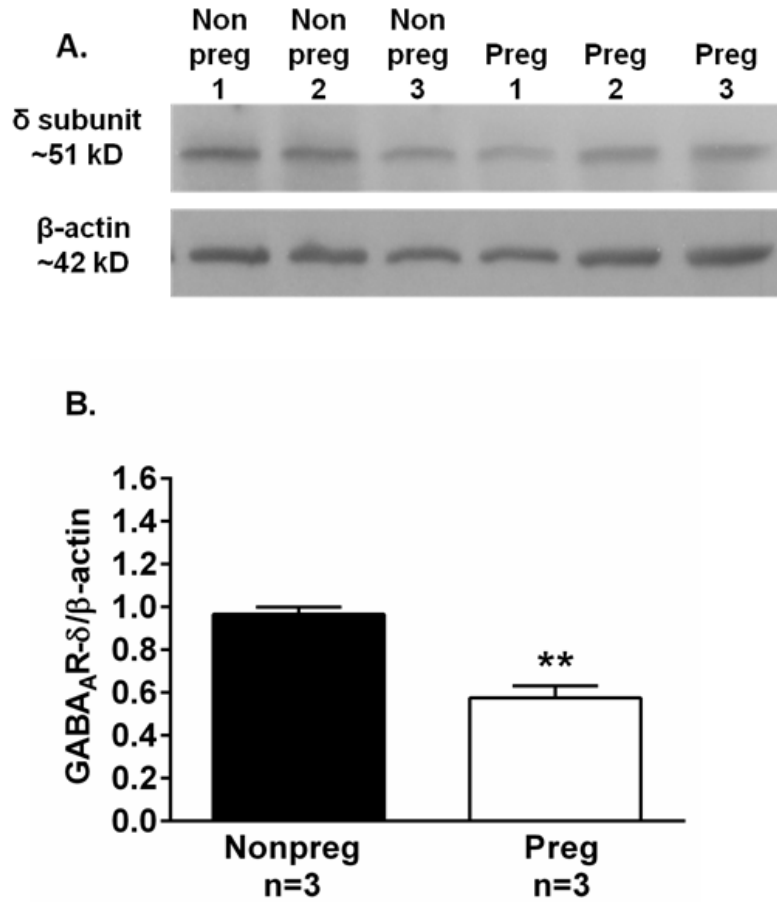


Figure 4. The effect of pregnancy on GABA_AR δ -subunit protein expression in the cerebral cortex. (A) Representative Western blot showing protein expression of the GABA_AR δ -subunit in the cerebral cortices of nonpregnant (Nonpreg) and pregnant (Preg) rats. (B) δ -subunit protein expression was significantly lower in the cerebral cortex from Preg compared to Nonpreg rats. ** $p < 0.05$ vs. Nonpreg using one-way ANOVA.

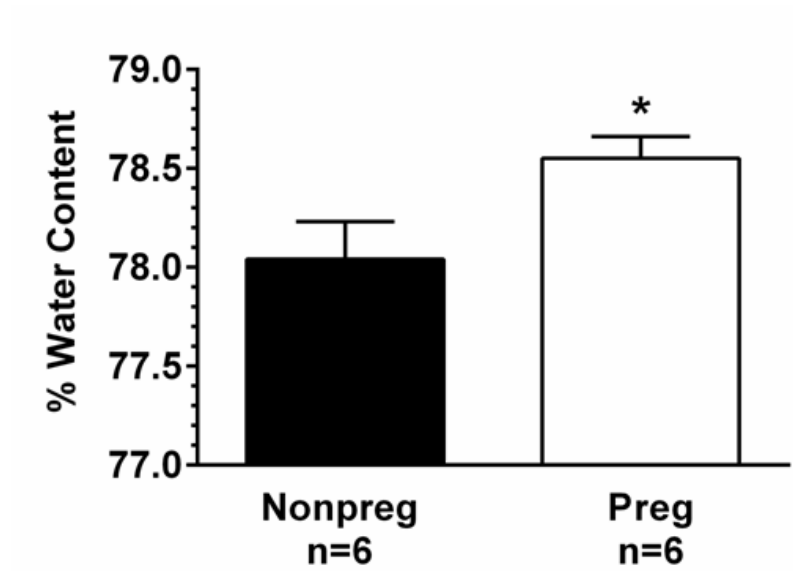


Figure 5. The effect of seizure on vasogenic edema formation in nonpregnant (Nonpreg) and late-pregnant (Preg) rats. Percent water content of the posterior cerebral cortex was significantly higher after seizure in Preg compared to Nonpreg rats. * $p < 0.05$ vs. Nonpreg using one-way ANOVA.

Change in Neurosteroids

- Decreased GABA_AR subunits
- Pro-convulsive effects of estradiol
- Others

Blood-Brain Barrier Dysfunction

- Increased vasopressin, histamine and other mediators
- Decreased osmolality and vasogenic edema
- Failure of efflux transporters

Inflammation & Infection

- Microglial activation
- Local cytokine production
- Others

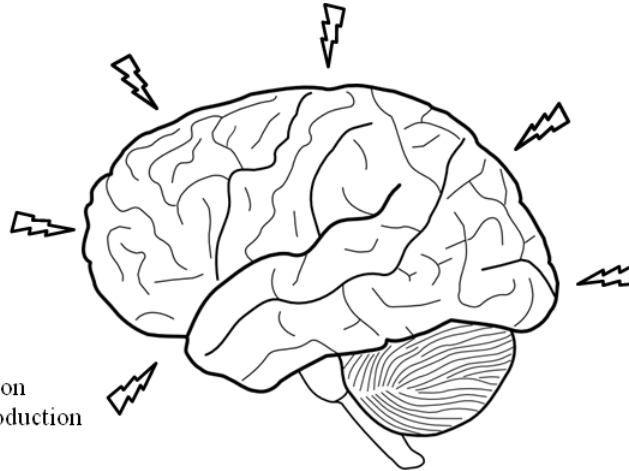


Figure 6. Schematic of the potential contribution of normal pregnancy to eclampsia.

The maternal brain appears hyperexcitable due to decreased protein expression of the GABA_AR δ -subunit that increases the potential for spontaneous seizure. However, because the majority of women do not have seizure during pregnancy, additional insults are required to cause seizure onset that are multifactorial: 1) Imbalanced neurosteroids. Hyperexcitability due to downregulation of cortical GABA_AR subunits is thought to be kept in balance by progesterone and progesterone metabolites; however, seizure can occur in response to changes in neurosteroid concentrations, such as postpartum when progesterone levels decrease to pre-pregnancy levels. Further, estradiol exerts pro-convulsive effects by increasing neuronal excitability through activity at excitatory ionotropic neurotransmitter receptors and neuronal sodium and potassium channels [54-56]. Thus, the switch from a progesterone- to an estrogen-dominated state at parturition

could facilitate seizure onset during pregnancy. 2) Increased inflammation and/or infection. During pregnancy, neuroinflammation and activation of microglia can occur to increase neuronal excitability through local production of inflammatory cytokines (e.g., $\text{TNF}\alpha$). 3) Failure or dysregulation of the BBB. Increased permeability of the BBB can occur through elevated mediators such as vasopressin and histamine that are elevated during pregnancy and preeclampsia [57,58]. An increase in BBB permeability has several effects including passage of circulating seizure-provoking factors into an already hyperexcitable maternal brain and movement of water or solutes from blood to brain that can disrupt the delicate microenvironment of the brain and cause a charge screening effect that depolarizes neurons. Decreased osmolality during pregnancy may result in greater vasogenic edema formation under conditions of disrupted BBB. In addition, failure of efflux transporters to regulate the entry of steroids, cytokines and chemokines into the brain may also lead to de novo seizure during pregnancy.

**CHAPTER 4: MAGNESIUM SULFATE TREATMENT REVERSES
SEIZURE SUSCEPTIBILITY AND DECREASES
NEUROINFLAMMATION IN A RAT MODEL OF SEVERE
PREECLAMPSIA**

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***PLoS One.* 2014 Nov 19;9(11):e113670.doi: 10.1371/journal.pone.0113670.**

Abstract

Eclampsia, defined as unexplained seizure in a woman with preeclampsia, is a life-threatening complication of pregnancy with unclear etiology. Magnesium sulfate (MgSO_4) is the leading eclamptic seizure prophylactic, yet its mechanism of action remains unclear. Here, we hypothesized severe preeclampsia is a state of increased seizure susceptibility due to blood-brain barrier (BBB) disruption and neuroinflammation that lowers seizure threshold. Further, MgSO_4 decreases seizure susceptibility by protecting the BBB and preventing neuroinflammation. To model severe preeclampsia, placental ischemia (reduced uteroplacental perfusion pressure; RUPP) was combined with a high cholesterol diet (HC) to cause maternal endothelial dysfunction. RUPP+HC rats developed symptoms associated with severe preeclampsia, including hypertension, oxidative stress, endothelial dysfunction and fetal and placental growth restriction. Seizure threshold was determined by quantifying the amount of pentylentetrazole (PTZ; mg/kg) required to elicit seizure in RUPP+HC \pm MgSO_4 and compared to normal pregnant controls (n=6/group; gestational day 20). RUPP+HC rats were more sensitive to PTZ with seizure threshold being ~65% lower vs. control (12.4 \pm 1.7 vs. 36.7 \pm 3.9 mg/kg PTZ; p<0.05) that was reversed by MgSO_4 (45.7 \pm 8.7 mg/kg PTZ; p<0.05 vs. RUPP+HC). BBB permeability to sodium fluorescein, measured in-vivo (n=5-7/group), was increased in RUPP+HC vs. control rats, with more tracer passing into the brain (15.9 \pm 1.0 vs. 12.2 \pm 0.3 counts/gram x1000; p<0.05) and was unaffected by MgSO_4 (15.6 \pm 1.0 counts/gram x1000; p<0.05 vs. controls). In addition, RUPP+HC rats were in a state of neuroinflammation, indicated by 35 \pm 2% of microglia being active compared to 9 \pm 2% in normal pregnancy (p<0.01; n=3-8/group). MgSO_4 treatment reversed neuroinflammation,

reducing microglial activation to $6\pm 2\%$ ($p < 0.01$ vs. RUPP+HC). Overall, RUPP+HC rats were in a state of augmented seizure susceptibility potentially due to increased BBB permeability and neuroinflammation. $MgSO_4$ treatment reversed this, increasing seizure threshold and decreasing neuroinflammation, without affecting BBB permeability. Thus, reducing neuroinflammation may be one mechanism by which $MgSO_4$ prevents eclampsia during severe preeclampsia.

Introduction

Preeclampsia (PE) is a hypertensive complication of pregnancy that involves many organ systems, including the kidney, liver and brain [1]. Some of the most serious complications of PE involve neurologic symptoms and include uncontrolled vomiting, severe and persistent headache, visual disturbances, unexplained seizure (eclampsia), coma and death [1]. Eclampsia is a life-threatening condition with high maternal and fetal morbidity and mortality [2,3]. The mechanism by which de novo seizure occurs in women with PE is not known, however, studies have shown that the brain is more excitable during pregnancy and PE, suggesting a lower seizure threshold that may contribute to de novo seizure. For example, network excitability in brain slices of pregnant mice was increased compared to virgin animals [4]. Further, a lower seizure threshold was reported in a lipopolysaccharide (LPS)-induced rat model of PE compared to normal pregnancy [5], suggesting pregnancy and PE may predispose the brain to seizure through increased neuronal excitability. However, the mechanism by which neuronal excitability is augmented in PE is unknown.

One of the primary mechanisms by which neuronal excitability can increase is through activation of microglia and neuroinflammation [6,7]. Microglial activation under conditions of peripheral inflammation has been shown to decrease seizure threshold via a promotional effect on neuronal excitability [8]. In addition, increased blood-brain barrier (BBB) permeability can result in neuroinflammation by allowing passage of serum constituents into the brain that activate microglia [9-12]. A previous study showed that circulating factors present during normal pregnancy are hyperexcitable to the brain through activation of microglia, and increase network excitability in a hippocampal slice

culture model [13]. In addition, circulating factors during PE have been shown to increase BBB permeability that could potentially pass into the maternal brain to promote neuroinflammation and decrease seizure threshold [14,15]. In the present study, we hypothesized that PE produces a state of neuroinflammation that lowers seizure threshold. We further hypothesized that BBB disruption during PE leads to microglia activation and is a mechanism by which seizure threshold is lowered during PE.

Magnesium sulfate (MgSO_4) is currently the most effective and commonly administered drug for eclamptic seizure prophylaxis and reduces the incidence of eclampsia by ~ 50 % [16-20]. MgSO_4 is administered to women at relatively high doses to raise serum levels to between 4.2 – 8.4 mg/dL over 12 - 24 hours [16]. However, despite its apparent effectiveness, dangerous side effects are associated with MgSO_4 use, including the potential for areflexia, respiratory paralysis and cardiac arrest [16]. Further, although it is the preferred treatment strategy in women with PE, the exact mechanism by which MgSO_4 prevents eclampsia is not clear and may be multifaceted. Animal studies have provided evidence that MgSO_4 has protective actions at the BBB, lowering permeability during acute hypertension, and reducing hyperosmolar-induced disruption of the BBB [21,22]. We hypothesized that MgSO_4 treatment during PE increases seizure threshold by preserving the integrity of the BBB and preventing microglial activation, thereby decreasing neuroinflammation.

In the present study, we developed a rat model of severe PE that incorporated placental ischemia and maternal endothelial dysfunction that are thought to contribute to the pathogenesis of PE [23]. In particular, we sought to model severe PE that encompasses both fetal and maternal symptoms and has the greatest risk of life-

threatening complications [23,24]. This model used the reduced uteroplacental perfusion pressure (RUPP) model of placental ischemia [25] combined with a high cholesterol diet previously shown to cause maternal endothelial dysfunction [15,26]. Using this model, we investigated the effect of severe PE on seizure threshold, BBB permeability in vivo and neuroinflammation. Further, rats with severe PE were treated with a clinically relevant dose of MgSO₄ for 24 hours, and the effect of MgSO₄ on these parameters was also determined.

Methods

Animals and ethics statement

All experiments were conducted using timed-pregnant Sprague Dawley rats that were 14-16 weeks old (Charles River, Canada). All rats were used experimentally and euthanized late in gestation (day 20 of a 22 day gestation), as this is when eclampsia occurs most often [1]. Rats of the same gestational age were housed in pairs in the University of Vermont Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Rat model of severe PE and assessment of pregnancy outcome

To model severe PE, we combined the RUPP model of placental ischemia with a high cholesterol diet (RUPP+HC) previously shown to cause hyperlipidemia by increasing total plasma cholesterol, maternal endothelial dysfunction and increased blood pressure [15,26]. Forty-eight pregnant rats were fed a high cholesterol diet (Prolab 3000 rat chow with 2 % cholesterol and 0.5 % sodium cholate; Scotts Distributing Inc., Hudson, NH) on days 7-20 of gestation. To induce placental ischemia, on day 14 of pregnancy while maintained on a high cholesterol diet, a midline abdominal incision was made under isoflurane anesthesia and a silver clip (diameter 0.203 mm) placed on the distal aorta, just proximal to the iliac bifurcation and distal to the renal and mesenteric arteries. Silver clips (diameter 0.10 mm) were placed on the arteries of the uterine arcade before the first segmental branch to the uteroplacental unit. This is an established method of reducing uteroplacental perfusion pressure by ~ 40 % [25]. Pregnant rats that had a sham surgery (n = 4) underwent the same surgical procedure, excluding the placement of silver clips. Animals were weighed prior to use and euthanized by decapitation after experimentation under anesthesia. Trunk blood was collected and serum stored at – 80 °C until use. Uterine horns were examined for total number of pups and any reabsorbed fetuses. To assess growth restriction and placental disease, pups and placenta from some RUPP+HC rats (n = 5) were removed, weighed individually and compared to control rats (n = 3).

Measurement of conscious, unrestrained arterial blood pressure

Separate groups of normal late-pregnant (Late-Preg; n = 6) and RUPP+HC rats (n = 6) were implanted with indwelling carotid catheters on day 18 of gestation. Under isoflurane

anesthesia, the left common carotid artery was exposed and cannulated with saline filled V-3 tubing (SCI) which was tunneled to the back of the neck and externalized. On day 19 of pregnancy, rats were lightly anesthetized with isoflurane (1 - 2 % for 3 - 4 minutes) and a pressure transducer (BIOPAC, Inc., Goleta, CA, USA) connected to the indwelling carotid catheter. Rats were placed in a rectangular, clear plastic modular chamber (10" x 7" x 5") with a metal grid floor, large enough to move freely. Average conscious, unrestrained systolic blood pressures were recorded using *AcqKnowledge* software (BIOPAC, Inc., Goleta, CA, USA). One RUPP+HC rat died due to surgical complications during catheter implantation and was excluded.

Measurement of circulating markers of endothelial dysfunction and oxidative stress

Commercially available rat ELISA kits for endothelin-1 (ET-1; R&D Systems, Minneapolis, MN, USA) and free 8-isoprostane (Caymen Chemicals, Ann Arbor, MI, USA) were used to measure circulating factors in the serum of Late-Preg (n = 6) and RUPP+HC (n = 6 – 8) rats. Serum samples were diluted 1:3 for measurements of free 8-isoprostane otherwise samples were measured undiluted. All samples were measured in duplicate.

MgSO₄ treatment in RUPP+HC rats

Eighteen RUPP+HC rats were injected s.c. the morning of day 19 of pregnancy with 270 mg/kg 1.0 M MgSO₄ (RUPP+HC+MgSO₄). Four hours later, rats were briefly anesthetized with 2 % isoflurane and three 2 mL osmotic minipumps (Alzet, Cupertino, CA, USA) primed with 1.0 M MgSO₄ were implanted s.c. between the shoulder blades.

On day 20 of pregnancy, 1.5 hours prior to surgery and experimentation rats were injected with a second bolus of 270 mg/kg 1.0 M MgSO₄ s.c. After experimentation, rats were euthanized by decapitation under anesthesia and serum collected and stored at – 80 °C until use. Serum [Mg²⁺] was measured using a colorimetric assay (BioVision Inc., San Francisco, CA, USA) according to the manufactures' instructions and compared to RUPP+HC rats that did not receive treatment with MgSO₄ (n = 4 / group).

Measurement of seizure threshold, susceptibility and severity, and brain water content

Separate groups of Late-Preg (n = 10), RUPP+HC (n = 6) and RUPP+HC+MgSO₄ (n = 6) were anesthetized initially with isoflurane (1 – 3 % in oxygen) for intubation, electrode placement and instrumentation. Animals were mechanically ventilated to maintain blood gases and pH within normal physiological ranges. Body temperature was monitored with a rectal thermometer and maintained with a heating pad at 37 °C throughout the experiment. The dorsal surface of the head was shaved to expose the scalp and silver subdermal corkscrew electrodes (Ambu, Glen Burnie, MD, USA) were implanted under the scalp and secured in place with collodion glue. Electroencephalography (EEG) was recorded unipolarly using a MP150 acquisition system (BIOPAC System Inc., Goleta, CA, USA) to monitor generalized seizure. The recording electrode was placed over the right parieto-occipital cortex (5 ± 0.16 mm lateral and 7 ± 0.16 mm posterior to bregma) [27], a reference electrode was placed in the soft tissue of the snout and a ground electrode placed posterior to the right ear. Signals were amplified and filtered (low frequency filter, 0.1 Hz; high frequency filter 35.0 Hz) and sampled at 1.0 kHz. After placement of electrodes, the animal was placed in supine position for placement of

venous and arterial catheters. Femoral arteries were cannulated to obtain blood samples for blood gas measurements and continuous measurement of arterial blood pressure via a pressure transducer (BIOPAC Systems Inc., Goleta, CA, USA). As placement of a silver clip on the distal aorta in rats with RUPP made monitoring blood pressure in the femoral artery inaccurate, blood pressures were measured in the axillary artery, as done previously [28]. Femoral veins were cannulated for administration of the anesthetic chloral hydrate and infusion of the chemoconvulsant pentylenetetrazole (PTZ). PTZ was chosen because it reliably elicits seizure by its antagonistic action at the main inhibitory receptors in the brain, gamma-aminobutyric acid (GABA) type A receptors [29]. After instrumentation, animals were tapered off isoflurane and anesthesia maintained by continuous intravenous infusion of chloral hydrate (50 mg/mL; 30 μ L/min). Chloral hydrate was used because it is thought to not depress neural function, and is the preferred anesthetic for studies measuring EEG [30,31]. Seizure was induced by a timed infusion of PTZ (10 mg/mL; 1 mL / min) that was stopped at the first onset of spikewave discharges. Seizure threshold was calculated as the amount of PTZ (mg/kg) required to elicit electrical seizure: $T_{\text{infusion}} * R_{\text{infusion}} * [\text{PTZ}] / \text{BW}$ where T_{infusion} is the time of infusion in min, R_{infusion} is the rate of infusion in mL/min, $[\text{PTZ}]$ is the concentration of PTZ in mg/mL, and BW is the body weight in kg. Seizure susceptibility scores were also calculated: $\text{bw} * 10/v$ where bw is body weight in grams and v is volume of PTZ infused in μ L.[8] Baseline blood pressures were taken 30 seconds prior to PTZ infusion and at seizure onset. EEG was recorded for 30 minutes post-PTZ infusion and seizure severity assessed by counting the number of recurring seizures and calculating the percent of the post-infusion period spent in seizure. After 30 minutes animals were euthanized under

chloral hydrate anesthesia by decapitation and brains immediately removed. The posterior cerebral cortex was isolated and weighed wet ($\text{weight}_{\text{wet}}$), then dried in a laboratory oven at 90 °C for 24 hours and re-weighed dry ($\text{weight}_{\text{dry}}$). Percent water content was determined by wet:dry weights using the following formula: $(\text{weight}_{\text{wet}} - \text{weight}_{\text{dry}}/\text{weight}_{\text{wet}}) * 100$. The posterior cortex was chosen for measurements as this is a primary brain region affected in women with PE and eclampsia [32]. Four Late-Preg rats were excluded because blood gases were outside of the physiological range.

Measurement of in vivo BBB permeability

Permeability of the BBB was measured in Late-Preg (n = 6), RUPP+HC (n = 5) and RUPP+HC+MgSO₄ (n = 8) using previously described methods with modifications [33]. Briefly, animals were anesthetized and instrumented similarly as during seizure threshold measurements, excluding EEG electrode placement and substituting the catheter for PTZ with fluorescent tracers. While under chloral hydrate anesthesia, fluorescent tracers were infused into the femoral vein (0.5 mL/min for 2.25 min) and allowed to circulate for 10 minutes. Sodium fluorescein (0.1 %; mol wt 476 Da; Stokes-Einstein radius ~ 0.45 nm) and 70-kDa Texas red dextran (0.5 mg/mL; Stokes-Einstein radius ~ 7.0 nm) in lactated Ringer's solution and heparin were used to distinguish size selectivity to small and large solutes, respectively. A thoracotomy was performed, a needle inserted into the left ventricle of the heart and the right atrium cut to allow for drainage of blood and tracers. Using an infusion pump, the circulation was flushed with 60 mL lactated Ringer's solution (5.0 mL/min) until the circulation was clear of blood. Animals were decapitated, the brain removed and the posterior cerebral cortex isolated and weighed. Each brain

section was homogenized in 5.0 mL 0.1 M PBS, 5.0 mL 50 % trichloroacetic acid (TCA) added, vortexed for 1 min, and centrifuged at 4 °C for 10 min at 4500 rpm (Sorvall Legend X1R, Thermo Scientific, Waltham, MA, USA). The supernatant was removed and re-centrifuged. The fluorescence of the supernatant was determined at excitation and emission wavelengths of 460 and 515 nm for sodium fluorescein and 595 and 615 nm for Texas red dextran. Background emissions of 50:50 lactated Ringer's and 50 % TCA were subtracted and samples normalized to brain weight to compare fluorescence as counts/gram of brain tissue. One Late-Preg rat and one RUPP+HC+MgSO₄ rat were excluded due to surgical complications.

Quantification and morphological assessment of microglia

Separate groups of Late-Preg (n = 8), RUPP+HC (n = 3) and RUPP+HC+MgSO₄ (n = 4) rats were euthanized under isoflurane anesthesia and brains immediately removed. A 3 mm coronal section (4 – 7 mm posterior to bregma) of the posterior cerebral cortex was taken and fixed in 10 % buffered formalin at 4 °C overnight, then transferred to 0.1 M PBS and paraffin embedded. Immunohistochemical staining for ionized calcium-binding adapter molecule 1 (Iba1; Wako, Richmond, VA), a marker for microglia, was done using standard procedures. Briefly, tissue was sectioned at 4 µm on a Leica 2030 paraffin microtome. Slides were allowed to air dry overnight at room temperature and then baked for one hour at 60 °C. Following deparaffinization and rehydration, the sections underwent antigen retrieval with DAKO Target Retrieval Solution, pH 6.0 in 50 % glycerol at 95 °C for 20 min. Sections were treated with 1 % bovine serum albumin, 10 % normal goat serum and 0.1 % Triton X-100. The tissue was incubated overnight at

room temperature with Iba1 antibody (1 µg/mL) and one hour in Cyanine 3 dye (1:100). For each brain section, four micrographs of cerebral cortex were captured using an Olympus BX50 microscope at 20X magnification. Each Iba1⁺ cell was assessed by its morphology and activation state ranked using a graded scale from 1 (relatively inactive) to 4 (relatively active). Cells with highly ramified, long processes with a scattered, irregularly shaped cell body were ranked in state 1. Cells with an asymmetrical cell body and many long, defined processes were ranked in state 2. Cell bodies that were more rounded with several shorter, thicker processes were ranked 3, and large, round amoeboid-like cell bodies with few to no processes were ranked in state 4 [34]. To assess microglia, two analyses were performed. First, the percentage of cells in each activation state was calculated for each micrograph and averaged per group. Second, total number of Iba1⁺ cells were counted per mm² and averaged for each group. A group of sham-operated rats were assessed for microglial activation to confirm the surgery alone did not activate microglia. Two evaluators that were blinded to group performed all morphological assessments.

Measurement of cerebral blood flow autoregulation and brain water content

Separate groups of Preg (n = 6) and RUPP+HC (n = 10) rats were anesthetized initially with isoflurane (1 - 3 % in oxygen) for intubation and instrumentation, after which anesthesia was maintained with a bolus intravenous injection of chloral hydrate (200 mg/kg into femoral vein). Animals were mechanically ventilated to maintain blood gases and pH within normal physiological ranges. Body temperature was monitored with a rectal thermometer and maintained with a heating pad at 37 °C throughout the

experiment. Relative cerebral blood flow (rCBF) was measured transcranially in the posterior cerebral cortex using laser Doppler flowmetry. The left side of the medioposterior skull was exposed and the bone thinned. A laser Doppler probe (Perimed, Ardmore, PA, USA) was affixed 2 mm lateral to the sagittal suture and 1 mm anterior to the lambdoid suture to measure rCBF in the posterior cerebral artery territory. Femoral arteries were cannulated to obtain blood samples for blood gas measurements and measurement of arterial blood pressure via a pressure transducer (Living Systems Instrumentation, Inc., Burlington, VT, USA). Femoral veins were cannulated for administration of chloral hydrate and acute infusion of phenylephrine. To measure CBF autoregulation, phenylephrine was infused intravenously at an increasing rate of 4 - 48 $\mu\text{g}/\text{min}$, and blood pressure and rCBF measured simultaneously, as previously described [35]. After experimentation, while under anesthesia, rats were euthanized by decapitation, brains immediately removed and percent water content measured by wet:dry weights. One RUPP+HC rat was excluded because of technical issues with placement of the laser Doppler probe. The percent increase in CBF of one RUPP+HC rat was greater than two standard deviations from the mean, and therefore excluded as a statistical outlier. Animals were not randomized due to the use of timed-pregnant rats.

Drugs and solutions

MgSO_4 , phenylephrine, chloral hydrate, PTZ, sodium fluorescein and TCA were purchased from Sigma Aldrich (St Louis, MO, USA) and all were made daily in sterile lactated Ringer's solution except MgSO_4 , which was ready-to-use. Texas red dextran was

purchased from Invitrogen (Life Technologies, Grand Island, NY, USA) and made daily in sterile lactated Ringer's solution.

Statistical analyses

Data are presented as mean \pm standard error of mean. Physiological parameters were compared between Late-Preg and RUPP+HC rats using analysis of variance (ANOVA). Pup and placental weights were compared using ANOVA with the n-value being total pups and placentas in each group. Differences in circulating levels of ET-1 and free 8-isoprostane were also compared using ANOVA. Percent change in rCBF was compared between Late-Preg and RUPP+HC rats at pressures between 100 and 180 mmHg using ANOVA. Comparisons of physiological parameters, seizure threshold, susceptibility, severity, BBB permeability, % water content and microglial activation between Late-Preg, RUPP+HC and RUPP+HC+MgSO₄ rats were done using a one-way ANOVA with a Bonferroni's *post-hoc* test to correct for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

Severe PE caused hypertension, oxidative stress, endothelial dysfunction, and fetal and placental growth restriction

Table 1 shows physiological parameters of Late-Preg and RUPP+HC rats. RUPP+HC rats weighed significantly less on day 20 of gestation compared to Late-Preg rats. This decrease in maternal body weight was not due to significant pup loss or fetal

reabsorptions, as the number of pups and reabsorbed fetuses were not statistically different between groups. However, RUPP+HC caused both fetal and placental growth restriction, as shown by significant reductions in pup and placental weights. Further, rats with severe PE had increased systolic blood pressure compared to Late-Preg rats. When serum markers of oxidative stress and endothelial dysfunction were compared, severe PE was associated with significantly higher levels of circulating free 8-isoprostane compared to control rats: 369 ± 25 pg/mL for RUPP+HC vs. 281 ± 8 pg/mL for Late-Preg; $p < 0.01$ and ET-1: 0.99 ± 0.25 pg/mL for RUPP+HC vs. 0.46 ± 0.06 pg/mL for Late-Preg; $p < 0.05$.

Severe PE was associated with decreased seizure threshold and increased seizure susceptibility

To determine if severe PE was a state associated with increased neuronal excitability, seizure threshold was measured in vivo in RUPP+HC and Late-Preg rats by timed infusion of PTZ and simultaneous EEG recording. Figure 1A shows a representative EEG tracing in a Late-Preg rat during PTZ infusion that had spike wave discharges indicative of seizure onset after 2.4 min of infusion. Seizure threshold was significantly lower in RUPP+HC rats, requiring less PTZ to elicit electrical seizure than in Late-Preg controls (Figure 1B). Further, severe PE was associated with a higher seizure susceptibility score than in Late-Preg controls, suggesting that RUPP+HC rats are in a state of increased neuronal excitability (Figure 1C). Comparison of recurring seizures and percentage of time spent seizing revealed no differences between RUPP+HC and Late-Preg rats (Table

2), indicating that although rats with severe PE were more susceptible to seizure than controls, seizure severity was similar between these groups.

MgSO₄ treatment reversed seizure susceptibility in rats with severe PE

Treatment of RUPP+HC rats with MgSO₄ raised serum Mg²⁺ levels into the target therapeutic range that was significantly higher than RUPP+HC rats that did not receive treatment (5.2 ± 0.5 mg/dL for treated vs. 1.2 ± 0.1 mg/dL for untreated; $p < 0.01$).

MgSO₄ treatment did not affect physiological parameters or pregnancy outcome, as maternal body weight (389.2 ± 8.9 grams; $p > 0.05$ vs. untreated), number of pups (11.5 ± 1.2 ; $p > 0.05$) and fetal reabsorptions (1.8 ± 0.9 ; $p > 0.05$) were similar to RUPP+HC rats (see Table 1). Severe PE rats that received MgSO₄ had significantly higher seizure threshold compared to rats with severe PE that did not receive treatment (Figure 1B).

Further, MgSO₄ reversed the increase in seizure susceptibility, lowering susceptibility scores back to control levels (Figure 1C). Despite these apparent protective effects, MgSO₄ did not affect seizure severity, as there were no changes in either number of recurrent seizures or percent of time spent seizing with MgSO₄ treatment (Table 2). There were no differences in any physiological parameters under anesthesia during seizure threshold measurements between groups (Table 3).

Severe PE rats with and without MgSO₄ had decreased seizure-induced cerebral vasogenic edema formation

Cerebral vasogenic edema is present in ~ 90 % of women with eclampsia [36]. Further, the maternal brain has been shown to be more susceptible to vasogenic edema formation

than the nonpregnant state under pathologic conditions including acute hypertension and in response to seizure [37,38]. However, whether the brain during severe PE is more susceptible to seizure-induced vasogenic edema has yet to be investigated, and the effect of MgSO₄ treatment under such conditions remains unknown. In the current study, seizure-induced vasogenic edema formation was significantly lower in RUPP+HC rats compared to Late-Preg controls, and remained unaffected by MgSO₄ treatment (Figure 2).

Severe PE increased BBB permeability in vivo that was unaffected by MgSO₄ treatment

BBB permeability to sodium fluorescein, the small ~ 470 Da solute, was increased in RUPP+HC rats compared to Late-Preg rats, with significantly more tracer passing from the cerebral circulation into the brain parenchyma in rats with severe PE (Figure 3A). There was no difference in permeability of the BBB to Texas red dextran, a larger 70 kDa tracer, between Late-Preg and RUPP+HC rats (Figure 3B). MgSO₄ treatment administered to rats with severe PE did not affect BBB permeability, as permeability to sodium fluorescein remained higher in RUPP+HC+MgSO₄ rats than Late-Preg controls (Figure 3A), with no change in permeability to Texas red (Figure 3B).

Severe PE increased neuroinflammation that was reversed by MgSO₄ treatment

Figure 4A illustrates the dynamic morphological changes that occur as quiescent microglia transition to an activated state and was used as a graded scale to assess microglial activation. Figure 4B shows representative photomicrographs of Iba1⁺ microglia from the posterior cerebral cortex of Late-Preg, RUPP+HC and

RUPP+HC+MgSO₄ rats. The total number of microglia was modestly increased in rats with severe PE regardless of MgSO₄ treatment, but was not statistically different than Late-Preg controls (Figure 4C). There was a significantly higher percent of cells in fully activated state 4 in rats with severe PE compared to controls, that was coupled by a reduction in the percent of cells in activation state 2 (Figure 4D). These findings indicate the presence of neuroinflammation in rats with severe PE. Treatment of RUPP+HC rats with MgSO₄ decreased the percent of activated microglia, shifting back to a relatively quiescent and inactive pattern similar to Late-Preg controls, with the majority of microglial cells residing in a relatively inactive state. Sham operated rats had similar microglial activation as Late-Preg control rats (data not shown).

CBF autoregulation and brain water content in Severe PE

An underlying feature of neurological complications during severe PE is thought to be impairment of CBF autoregulation [39]. To compare CBF autoregulation between Late-Preg and RUPP+HC rats, rCBF vs. pressure curves were generated. Figure 5A shows that as arterial blood pressure increased, the change in rCBF was similar between Late-Preg rats and rats with severe PE and stayed below 20 % until 150 mmHg (Figure 5A). To investigate if RUPP+HC rats were more susceptible than Late-Preg rats to hypertension-induced cerebral edema, percent water content of the brain was measured after measurement of CBF autoregulation. There was no difference in brain water content after phenylephrine-induced hypertension between Late-Preg and RUPP+HC rats (Figure 5B).

Discussion

When eclamptic seizure occurs in women with early-onset severe PE there is approximately a 50 % maternal mortality rate [3]. This suggests that the maternal brain is adversely affected in severe PE and at a greater risk of damage. The mechanism(s) by which eclampsia occurs is not clearly understood, but may involve a pathologic process involving BBB dysfunction, neuroinflammation and hyperexcitability of the brain. Here, we modeled severe PE by introducing placental ischemia in a setting of maternal endothelial dysfunction due to hyperlipidemia. RUPP+HC rats had increased blood pressure and were in a state of oxidative stress and endothelial dysfunction, as indicated by elevated circulating levels of free 8-isoprostane and ET-1. Further, rats with severe PE had fetal and placental growth restriction that was not associated with a change in number of pups or fetal reabsorptions. This is similar to women with early-onset severe PE that have intrauterine growth restriction and placental disease [40]. Importantly, rats with severe PE were in a state of increased seizure susceptibility, with seizure threshold being significantly lower than normal pregnant controls. Seizure susceptibility in rats with severe PE was associated with increased BBB permeability to small solutes and microglial activation. However, there was no change in seizure severity between Late-Preg and RUPP+HC rats, indicating that regulatory mechanisms limiting prolonged seizure activity were similar to the control state, despite neuroinflammation in rats with severe PE. Thus, the brain seems to be at a greater risk of seizure due to breakdown of the BBB and associated neuroinflammation in this rat model of severe PE.

Neuroinflammation, demonstrated in the current study by activated microglia, can increase neuronal excitability through microglial secretion of pro-inflammatory cytokines

such as tumor necrosis factor alpha (TNF α) [8,13]. TNF α causes trafficking of excitatory receptors to the neuronal cell surface and simultaneous internalization of inhibitory receptors, resulting in a net increase in neuronal excitability [8,41,42]. Thus, it is possible that in this model of severe PE, lower seizure threshold and increased seizure susceptibility were a consequence of neuroinflammation because they also had marked microglial activation. In the current study, severe PE rats treated with MgSO₄ had significantly less microglial activation that correlated with increased seizure threshold. In fact, MgSO₄ treatment returned neuroinflammation and seizure susceptibility to the level of normal pregnant controls. However, the effect of MgSO₄ appeared to be a direct effect on microglial activation, as opposed to limiting BBB permeability. MgSO₄ has been shown to limit LPS-induced microglial secretion of pro-inflammatory cytokines in cell culture through inhibition of L-type calcium channels and subsequent reduction in downstream signaling of nuclear factor kappa B (NF- κ B), a transcription factor involved in inflammatory pathways [43-45]. However, to our knowledge, this is the first study showing that MgSO₄ treatment increases seizure threshold in severe PE via a quiescent effect on activated microglia in vivo. This is in agreement with a recent study showing MgSO₄ treatment increased seizure threshold in a LPS model of PE, however, the mechanism by which this occurred was not investigated [5].

Overall the effect of MgSO₄ treatment on seizure threshold appeared to be due to reducing neuroinflammation, and not a protective effect at the BBB. In the present study, rats with severe PE had selectively increased BBB permeability to small, but not large solutes. Increased permeability to sodium fluorescein but not 70 kDa Texas red dextran suggests size-selectivity of the increased permeability and indicates modest tight junction

disruption [46,47]. However, as $MgSO_4$ is thought to decrease BBB permeability through a calcium-antagonistic action on tight junction permeability [16,21,22,48], it was surprising that there was no effect of $MgSO_4$ on BBB permeability in rats with severe PE. Instead, the increase in BBB permeability to sodium fluorescein may indicate an increase in transcellular permeability that may be calcium-independent, which could further explain the lack of effect of $MgSO_4$ treatment on severe PE-associated BBB permeability.

The lack of increase in permeability to large solutes is in contrast to two recent studies using the RUPP model without high cholesterol treatment. Both studies report increased BBB permeability to Evan's Blue in RUPP compared to pregnant control rats [49,50]. One explanation of these contrasting results is the different methodology used. Evan's Blue binds to albumin that changes during pregnancy and PE [51-54]. Thus, the use of 70 kDa Texas red dextran that does not bind to albumin likely provides more reliable results. Further, the two studies reporting increased BBB permeability to Evan's Blue in RUPP rats allowed the tracer to circulate for a longer duration of time: 3 – 24 hours versus 10 minutes in the current study [49,50]. These longer time frames likely provide more variable results due to differences in clearance by cerebrospinal fluid or other tissues, including placenta [55-57]. Regardless, in the present study, RUPP+HC rats had selectively increased BBB permeability that may contribute to lowering seizure threshold by allowing passage of serum constituents into the brain that activated microglia and created a neuroinflammatory state.

Cerebral edema formation is considered a leading cause of the neurological symptoms that occur in severe PE, including eclamptic seizure [58-60]. In fact,

approximately 90 % of women with eclampsia have vasogenic cerebral edema formation as indicated by diffusion-weighted MRI [36]. Seizure itself leads to BBB disruption, making it difficult to determine if edema is the cause of or a consequence of eclamptic seizure [10,61]. A recent study using PTZ to induce seizure in normal pregnant rats found that the maternal brain was more susceptible to seizure-induced vasogenic edema formation than the nonpregnant state [38]. This may be an effect of increased plasma volume during pregnancy that, under conditions of BBB disruption such as seizure, may drive water into the brain. In the current study, however, brain water content after seizure was significantly lower in RUPP+HC rats compared to Late-Preg rats, with no effect of MgSO₄ treatment. Plasma volume contraction is known to occur in women with PE that may underlie the decreased vasogenic edema formation in severe PE rats [62]. This would also support the concept that increased plasma volume contributes to increased seizure-induced edema. Further, plasma volume contraction could also explain the significant reduction in maternal body weight that occurred in rats with severe PE. Although the brain does not seem to be more susceptible to seizure-induced vasogenic edema formation in severe PE, the cerebral circulation appears to be compromised, indicated by increased BBB permeability and neuroinflammation that could increase the risk of brain injury during eclampsia.

The cerebral circulation is thought to have a central role in neurologic complications associated with PE [3]. In fact, cerebrovascular events such as edema and hemorrhage account for ~ 40 % of maternal deaths [3]. Development of neurologic symptoms in women with PE is thought to involve the impairment of CBF autoregulation that is associated with decreased cerebrovascular resistance, hyperperfusion of the brain

and vasogenic edema formation [32,39]. Studies that have assessed dynamic CBF autoregulation in women with PE using transcranial Doppler (TCD) to measure changes in CBF velocity in the middle cerebral artery in response to hemodynamic fluctuations have found that CBF autoregulation appears to be intact in PE [39,63,64]. The findings in the current study showing intact CBF autoregulation in rats with severe PE supports these previous findings in humans. Further, as autoregulation remained intact in RUPP+HC rats, it was not surprising that there was no difference in brain water content after autoregulation measurements between groups. However, a recent study investigating CBF autoregulation in rats with RUPP without a high cholesterol diet report that autoregulation was impaired [50]. While it is unlikely that the addition of a high cholesterol diet in the current study restored autoregulation, it is more likely that methodological differences account for this discrepancy. Specifically, the current study assessed CBF autoregulation in the posterior cerebral cortex where as Warrington et al. investigated autoregulation in the anterior brain region [50]. Thus, it is possible that regional differences exist due to the primary blood supply differing between regions that may explain the conflicting findings between these two studies. Further, in the current study all catheters for continuous arterial blood pressure measurement and drug delivery were placed in distal systemic arteries such as the femoral arteries/veins to avoid disrupting blood flow to the brain. Warrington et al. used a carotid catheter to continuously monitor blood pressure while simultaneously recording CBF and cannulated one jugular vein for drug infusion [50]. As the carotid arteries and jugular veins are important for hemodynamics and CBF, their cannulation may account for the discrepancies in the effectiveness of CBF autoregulation between these studies.

Regardless, the current study suggests that CBF autoregulation in the posterior cerebral cortex in rats with severe PE is similar to Late-Preg control rats. Thus it is not likely that impaired CBF autoregulation contributes to decreased seizure threshold in rats with severe PE.

In summary, PE is a heterogeneous disorder that seems to manifest along a spectrum of severity and symptoms [1,65]. It remains unclear why some women with PE develop neurologic symptoms such as seizure, and others do not. That eclamptic seizure is more often fatal in women with severe PE stresses the importance of gaining a clear understanding of the disease processes leading to seizure onset. This study provides insight into the etiology of eclamptic seizure in severe PE, as it seems to involve a pathologic process of compromised BBB function and subsequent neuroinflammation, resulting in increased seizure susceptibility. By reducing neuroinflammation, MgSO₄ effectively increased seizure threshold, without affecting BBB permeability. Overall, preventing eclampsia, particularly during severe PE, is important in preventing maternal and fetal morbidity and mortality worldwide. Further, understanding the mechanism by which MgSO₄ functions as a seizure prophylactic in PE may lead to more targeted therapies and avoid unnecessary risks associated with MgSO₄ treatment.

Acknowledgments

We thank Nicole Bishop in the Microscopy Imaging Center at the University of Vermont for her technical expertise in performing immunohistochemistry.

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Table 1. Physiological parameters in models of normal pregnancy (Late-Preg) and severe preeclampsia (RUPP+HC).

	Late-Preg (n)	RUPP+HC (n)
Body Weight (grams)	460.0 ± 10.0 (3)	371.0 ± 21.2 (5) *
# Pups	14.7 ± 1.2 (3)	11.2 ± 3.4 (5)
# Reabsorptions	1.7 ± 0.9 (3)	3.0 ± 2.5 (5)
Pup Weight (grams)	2.5 ± 0.03 (44)	2.2 ± 0.02 (56) **
Placental Weight (grams)	0.46 ± 0.01 (44)	0.42 ± 0.01 (56) **
Blood Pressure (mmHg)	114 ± 1 (6)	138 ± 3 (4) **

* p < 0.05; ** p < 0.01 vs. Late-Preg by ANOVA

Table 2. Assessment of seizure severity in late-pregnant (Late-Preg) rats, rats with severe preeclampsia (RUPP+HC), and severe preeclamptic rats treated with magnesium sulfate (RUPP+HC+MgSO₄).

	# Recurrent Seizures	% of Time in Seizure
Late-Preg (n = 6)	12 ± 3	85 ± 7
RUPP+HC (n = 6)	13 ± 4	86 ± 5
RUPP+HC+MgSO ₄ (n = 6)	6 ± 1	88 ± 5

Table 3. Physiological parameters of late-pregnant (Late-Preg) rats, rats with severe preeclampsia (RUPP+HC), and severe preeclamptic rats treated with magnesium sulfate (RUPP+HC+MgSO₄) under chloral hydrate anesthesia for seizure threshold measurements.

	Late-Preg (n = 6)	RUPP+HC (n = 6)	RUPP+HC+MgSO ₄ (n = 6)
Body Temp (° C)	36.8 ± 0.2	36.4 ± 0.1	36.5 ± 0.1
Arterial P _{O₂} (mmHg)	111 ± 7	121 ± 6	116 ± 10
Arterial P _{CO₂} (mmHg)	42.8 ± 1.7	41.5 ± 1.5	44.8 ± 3.2

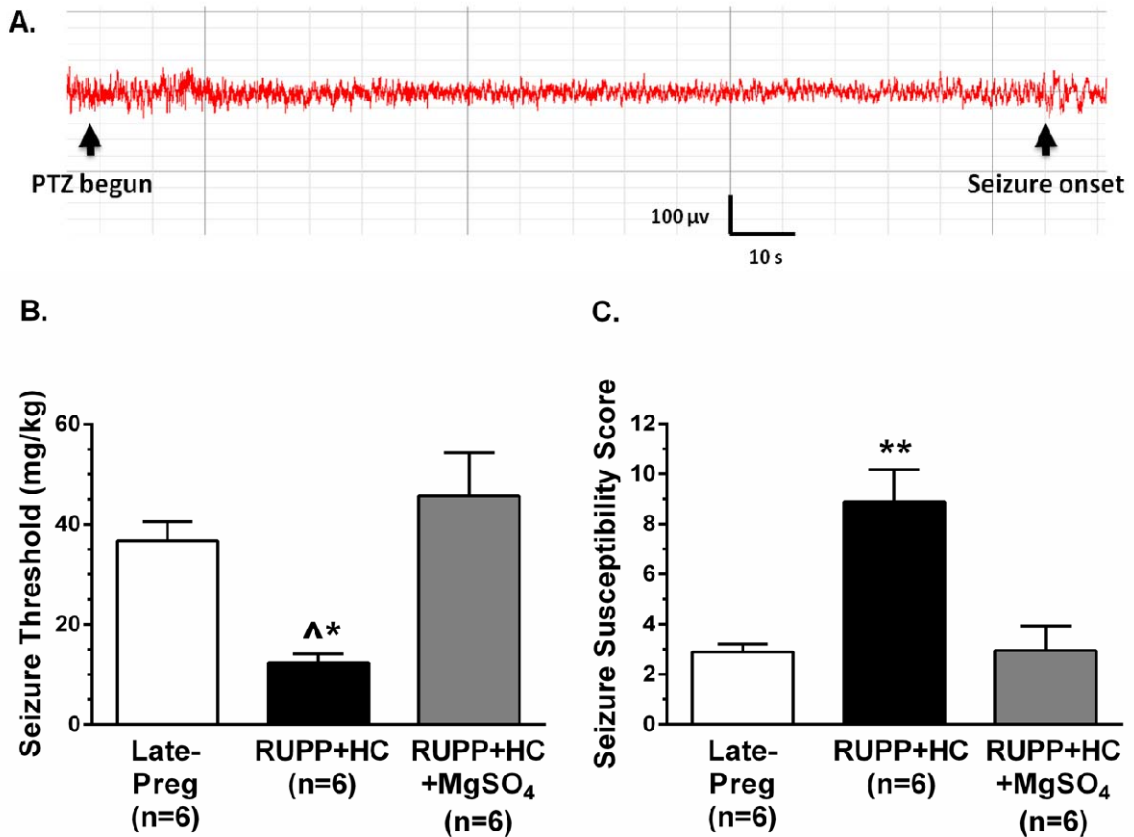


Figure 1. Effect of severe preeclampsia and magnesium sulfate (MgSO₄) treatment on seizure threshold and susceptibility. (A) Representative EEG tracing during timed-infusion of pentylenetetrazole (PTZ). Black arrows indicate when PTZ infusion begun and the onset of spike-wave discharges, or seizure onset. (B) Seizure threshold was significantly lower in rats with severe preeclampsia (RUPP+HC), and treatment with MgSO₄ (RUPP+HC+MgSO₄) significantly increased seizure threshold back to late-pregnant (Late-Preg) control levels. (C) Rats with severe PE had significantly higher seizure susceptibility scores compared to Late-Preg controls that were reversed in RUPP+HC+MgSO₄ rats. * $p < 0.05$ vs. Late-Preg; Δ $p < 0.01$ vs. RUPP+HC+MgSO₄; ** $p < 0.01$ vs. all groups by one-way ANOVA with *post-hoc* Bonferroni test.

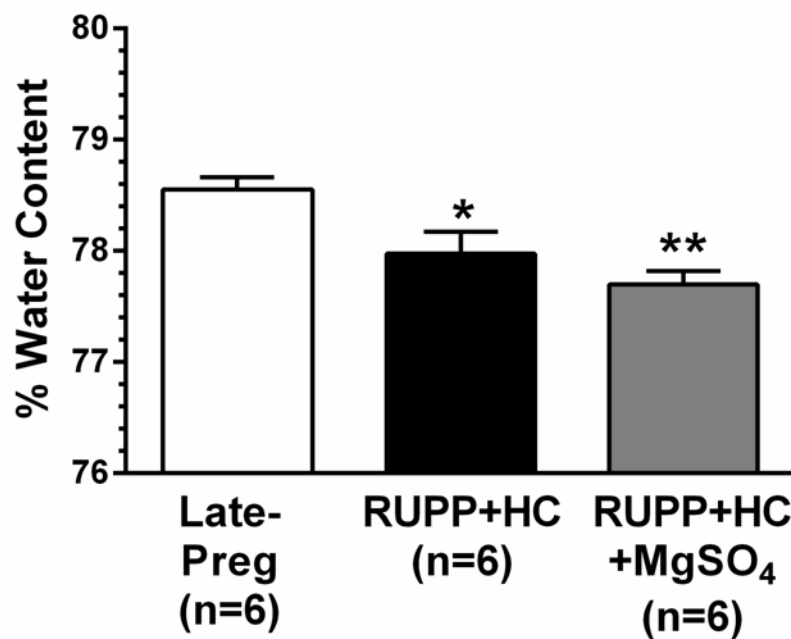


Figure 2. Effect of severe preeclampsia and magnesium sulfate (MgSO₄) on seizure-induced vasogenic edema formation. Percent water content of the posterior cerebral cortex was significantly lower after seizure in rats with severe preeclampsia (RUPP+HC) compared to late-pregnant (Late-Preg) control rats. Treatment of severe preeclamptic rats with MgSO₄ (RUPP+HC+MgSO₄) did not affect brain water content after seizure. * p < 0.05; ** p < 0.01 vs. Late-Preg by one-way ANOVA with *post-hoc* Bonferroni test.

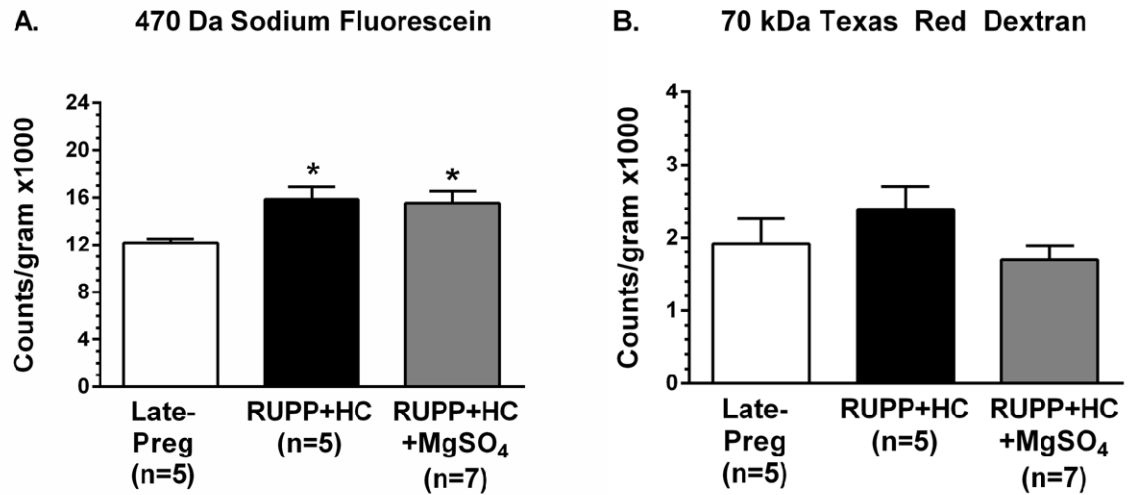


Figure 3. Effect of severe preeclampsia and magnesium sulfate (MgSO₄) on in vivo blood-brain barrier (BBB) permeability to different sized solutes. (A) BBB permeability to sodium fluorescein was increased in rats with severe preeclampsia (RUPP+HC) in the posterior cerebral cortex compared to late-pregnant (Late-Preg) control rats. MgSO₄ treatment in rats with severe preeclampsia (RUPP+HC+MgSO₄) had no effect on BBB permeability to sodium fluorescein. (B) Permeability of the BBB to 70 kDa Texas red dextran was similar between Late-Preg and RUPP+HC rats with and without MgSO₄ treatment. * p < 0.05 vs. Late-Preg by one-way ANOVA with *post-hoc* Bonferroni test.

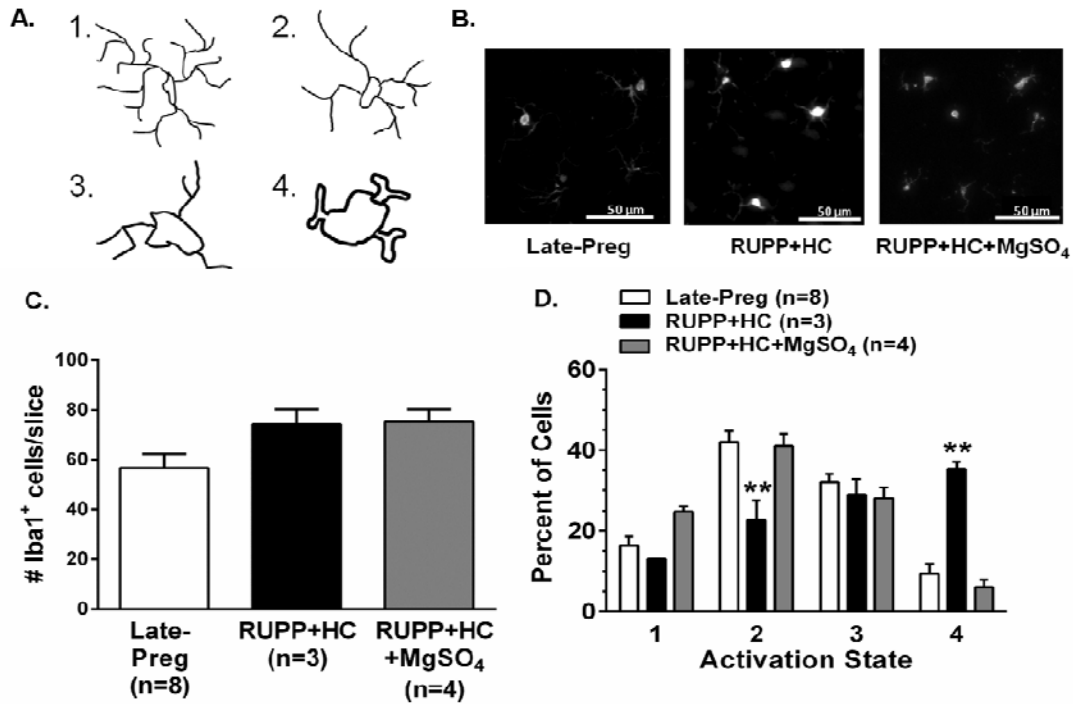


Figure 4. Effect of severe preeclampsia and magnesium sulfate (MgSO₄) treatment on microglial activation. (A) Illustration of morphological changes occurring as microglia progress from their inactive state 1, marked by long ramified processes to their active state 4, indicated by a large, amoeboid-like shape. (B) Representative photomicrographs of microglial cells stained for ionized calcium-binding adapter molecule 1 (Iba1) in the posterior cerebral cortices of late-pregnant (Late-Preg) rats, rats with severe preeclampsia (RUPP+HC) and rats with severe preeclampsia treated with MgSO₄ (RUPP+HC+MgSO₄). (C) The number of Iba1⁺ microglia was similar between groups. (D) The percentage of microglia in active state 4 was significantly higher in RUPP+HC rats compared to Late-Preg controls. Treatment of RUPP+HC rats with MgSO₄ decreased the percentage of cells that were active and was similar to Late-Preg controls. ** p < 0.01 vs. Late-Preg and RUPP+HC+MgSO₄ by one-way ANOVA and *post-hoc* Bonferroni test.

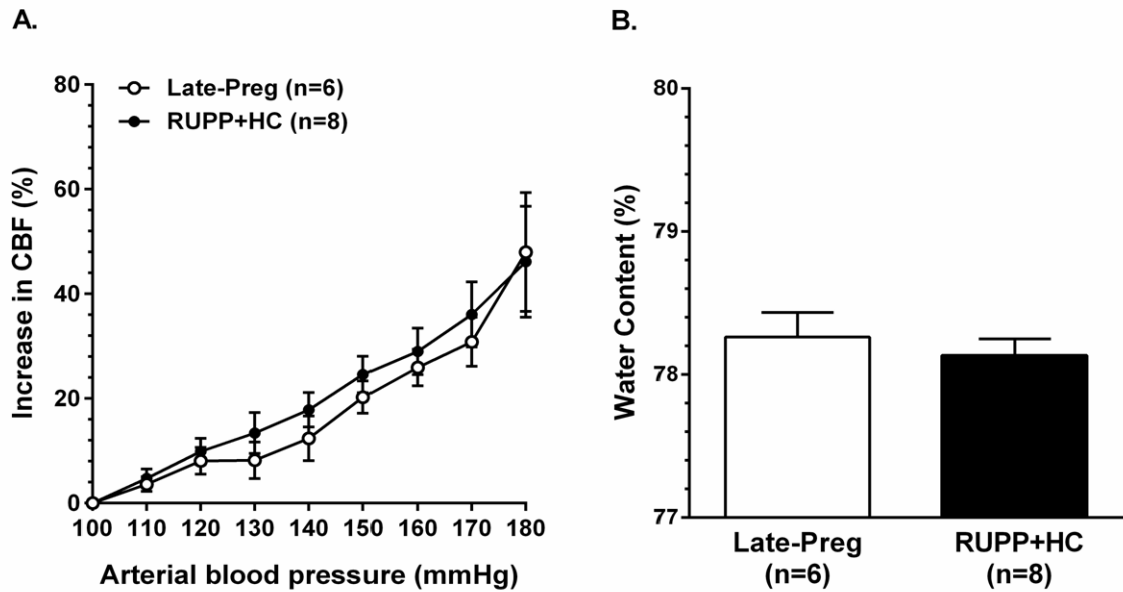


Figure 5. Effect of severe preeclampsia on cerebral blood flow (CBF) autoregulation and vasogenic edema formation. (A) Relative CBF (rCBF) increased similarly between late-pregnant (Late-Preg) and rats with severe preeclampsia (RUPP+HC) as arterial blood pressure was increased between 100 mmHg to 180 mmHg demonstrating intact autoregulation of CBF that was not different between groups. (B) Percent water content of the posterior cerebral cortex after CBF autoregulation measurements was similar between Late-Preg and RUPP+HC rats.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Eclampsia is considered a form of hypertensive encephalopathy during which an acute hypertensive episode leads to loss of vascular resistance of cerebral arteries, autoregulatory breakthrough, and increased hydrostatic pressure on the microcirculation, BBB disruption, and vasogenic edema formation (Lassen and Agnoli, 1972; Easton, 1998). However, women have eclamptic seizure during seemingly uncomplicated pregnancies at normal blood pressures and in the absence of preeclampsia (Douglas and Redman, 1994; Katz et al., 2000). Further, preeclamptic and eclamptic women are, by definition, normotensive prior to pregnancy. This may suggest that the pressure at which autoregulatory breakthrough occurs is shifted to lower pressure during pregnancy. However, previous studies in pregnant rats and findings in this dissertation suggest that the CBF autoregulatory curve is actually extended in the pregnant state, making the maternal brain better prepared to maintain CBF in the face of both acute hypotensive and hypertensive episodes (Cipolla et al., 2012a). Further, CBF autoregulation appears to be similar during preeclampsia and normal pregnancy. The findings that edema is present in over 90% of eclamptic women has supported that eclampsia is a form of hypertensive encephalopathy (Zeeman et al., 2004; Brewer et al., 2013); however, the maternal brain is more susceptible to vasogenic edema formation in response to acute hypertension with and without autoregulatory breakthrough (Euser and Cipolla, 2007; Cipolla et al., 2012a). Further, the brain appears to be hyperexcitable during normal pregnancy, and to a greater degree in preeclampsia. Thus, eclamptic seizure may occur due to vasogenic edema formation in the hyperexcitable maternal brain, potentially due to mechanisms involving

disrupted BBB function and increased permeability, rather than impairment of CBF autoregulation. In some women, eclampsia most likely occurs due to autoregulatory breakthrough similarly to hypertensive encephalopathy, however, the onset of eclamptic seizure seems to be more convoluted, and perhaps should not be considered a form for hypertensive encephalopathy.

The role of vasogenic edema formation in eclamptic seizure onset remains difficult to determine, as seizure itself leads to BBB disruption and edema formation (Oztas et al., 2003; Oby and Janigro, 2006). It is further complicated by the finding that the maternal brain is more susceptible to seizure-induced vasogenic edema, suggesting that vasogenic edema during eclampsia may be a result of the convulsions, rather than a cause, or, perhaps, a combination of both. However, this increase in seizure-induced edema was only present in normal pregnant rats. Vasogenic edema formation was significantly reduced in response to seizure in rats with preeclampsia compared to normal pregnancy, suggesting that preeclampsia may either prevent or reverse the increase in capillary density or plasma volume that appear to contribute to the susceptibility of the brain to vasogenic edema during pregnancy under conditions that promote BBB disruption. Regardless, the role of vasogenic edema formation in seizure onset during pregnancy needs to be further investigated. Further, the effect of preeclampsia on cerebrovascular adaptations to normal pregnancy needs to be investigated, as that may further elucidate roles of the cerebral circulation in eclamptic seizure onset.

The protective extension of the CBF autoregulatory curve during pregnancy appears to involve changes in nitric oxide synthase (NOS). The nitric oxide (NO)-dependent enhancement of vasodilation of cerebral pial arteries reported in this

dissertation and the pregnancy-induced outward remodeling of penetrating arterioles likely contribute to the leftward shift by increasing dilation of cerebral arteries in response to decreased intravascular pressure, thereby maintaining CBF at lower pressures (Cipolla et al., 2011). However, the systemic administration of a NOS inhibitor did not affect the lower limit, suggesting that perhaps there are compensatory mechanisms to maintain CBF, such as neuronal sources of NOS. Although in this study there was no change in mRNA expression of endothelial NOS in the cerebral arteries investigated, another study reported that endothelial NOS mRNA expression was decreased in the posterior cerebral cortex compared to the nonpregnant state (Cipolla et al., 2012a). The leftward shift of the lower limit may be due to increased activity of endothelial NOS in response to decreased intraluminal pressure, despite the decrease in expression. Further, NO production has been shown to contribute to the loss of vascular resistance that occurs in response to extreme increases in pressure, leading to forced dilatation of cerebral arteries and autoregulatory breakthrough (Talman and Nitschke Dragon, 2007). In fact, NOS inhibition shifts the upper limit of the CBF autoregulatory curve to higher pressures (Euser and Cipolla, 2007). Thus, the decrease in endothelial NOS expression in the posterior cerebral cortex during pregnancy could contribute to the rightward shift of the upper limit measured in pregnancy (Cipolla et al., 2012a). Overall, it appears that fluctuations in arterial pressure may have a differential effect on NOS and NO production in order to have protective effects at both the upper and lower limits of the CBF autoregulatory curve during pregnancy; however, whether the same mechanism exists in preeclampsia needs to be further investigated.

CBF autoregulation being similar between rats with preeclampsia and normal pregnant rats is particularly interesting, given the prominent role of NOS that has emerged in the extension of the autoregulatory curve, and that preeclampsia is a state marked by decreased bioavailability of NO. Systemically, oxidative stress and increased reactive oxygen species decrease the bioavailability of NO during preeclampsia (Lowe, 2000). However, what may be occurring in the brain during preeclampsia regarding changes in endothelial, neuronal or inducible NOS expression and NO production, or cerebrovascular function in general, remains relatively unknown. That NOS inhibition shifts the upper limit of the autoregulatory curve to higher pressure would suggest that if preeclampsia were a state of decreased NOS expression and NO bioavailability, than the limit may be shifted even further. However, this does not appear to be the case. Importantly, pregnancy prevents and reverses the protective cerebrovascular remodeling that occurs in response to chronic hypertension that has been shown to shift the upper limit of CBF autoregulation to higher pressure (Paulson et al., 1990; Cipolla et al., 2006; Aukes et al., 2007; Cipolla et al., 2008). Thus, that autoregulation was similar between pregnant and chronically hypertensive preeclamptic rats may actually indicate less effective autoregulation in rats with preeclampsia. Further investigation into the function of cerebral arteries during experimental preeclampsia would be useful to gain a better understanding of the role the cerebrovasculature may play in the potential for brain injury and eclampsia.

Preeclampsia appears to be a state of greater seizure susceptibility than normal pregnancy, which makes sense in the context that the majority of women with eclampsia are preeclamptic (Douglas and Redman, 1994). Regardless, normal pregnancy contributes

to the potential for eclampsia, not only by increasing the excitability of the brain, but also by the seizure-provoking factors that circulate late in gestation (Cipolla et al., 2012b). Under pathologic states when the BBB may be compromised due to infection, failure of efflux transporters, or acute hypertension, such hyperexcitable factors would likely gain access to the hyperexcitable maternal brain, and lead to seizure onset. Similarly, during preeclampsia where a level of basal BBB disruption appears to be present, the secondary insult necessary to initiate seizure onset is likely minor compared to the normal pregnant state. Thus, a therapeutic medication that acts as a neuroprotectant through targeting either the compromised BBB, hyperexcitable neurons, or activated microglia would likely be necessary and sufficient for seizure prevention during preeclampsia.

There is *in vivo* evidence that MgSO₄ exerts protective effects at the BBB (Kaya et al., 2001; Esen et al., 2003; Kaya et al., 2004; Esen et al., 2005; Euser et al., 2008) and an anticonvulsant effect on NMDA receptor-containing cortical neurons (Hallak et al., 1992; Hallak et al., 1994). Further, findings from this dissertation provide evidence that MgSO₄ acts as a seizure prophylactic through quiescent effects on activated microglia, thereby reducing neuroinflammation and raising seizure threshold. This is likely due to the calcium-antagonistic effects of MgSO₄, preventing the secretion of pro-inflammatory cytokines through inhibition of L-type calcium channels and subsequent activation of the NF-κB signaling cascade (Gao et al., 2013). By inhibiting the secretion of pro-inflammatory cytokines that have been shown to increase neuronal excitability (Stellwagen et al., 2005; Riazi et al., 2008), MgSO₄ restores seizure threshold to the level of normal pregnancy. Whether there are further effects of MgSO₄ on the excitability of NMDA receptor-containing neurons that also contributes to the restoration of seizure

threshold is unclear at this time. However, through the use of a GABA_AR-specific chemoconvulsant, there does not appear to be an effect at NMDA receptors. If there was an effect at NMDA receptors, one might expect seizure threshold to be even higher with MgSO₄ treatment than pregnant controls. That seizure threshold is similar to pregnant control levels suggests that it is mainly changes in the GABA_AR system that is affecting seizure threshold during pregnancy and preeclampsia. Current literature on the effects of microglial-secreted pro-inflammatory cytokines such as TNF α indicate increased excitability is due to the simultaneous increase in trafficking of AMPA receptors to neuronal cell membranes coupled with increased endocytosis of GABA_AR away from the cell membrane (Stellwagen et al., 2005). This mechanism of increased excitability does not seem to involve a role for NMDA receptors. However, direct investigation of seizure threshold using a specific NMDA receptor antagonist in preeclamptic rats with and without MgSO₄ treatment could lead to a better understanding of potential central anticonvulsant effects of MgSO₄ treatment in seizure prevention during preeclampsia.

Normal pregnancy and preeclampsia appear to be the setting of a perfect storm, with hyperexcitable factors circulating and the brain in a hyperexcitable state. There is evidence that actions of MgSO₄ have protective effects at several sites that may be involved in the pathogenesis of eclampsia, including a “tightening” effect at the BBB, a quiescent effect on microglial, and an anticonvulsant effect at NMDA receptors. This multifaceted action likely contributes to the effectiveness of MgSO₄ as a seizure prophylactic, especially given the heterogeneity of disease processes involved in the pathogenesis preeclampsia and eclampsia. It is unlikely that every woman with preeclampsia that develops eclampsia has the same insult that results in seizure onset.

Thus, by targeting several areas of potential disruption through which seizure may be initiated, MgSO₄ effectively prevents seizure in women all along the spectrum of disease. This may be the reason why MgSO₄ is more effective in seizure prevention during preeclampsia than any anticonvulsant medication that only manipulates neuronal activity.

This dissertation had several limitations that should be taken into account. Specifically, seizure threshold was determined as the amount of PTZ required to induce generalized seizure. Generalized seizure can accurately be monitored via subcutaneous EEG electrodes and, as eclampsia is generalized tonic-clonic seizure, is an appropriate measure to begin to understand the excitability of the maternal brain that may contribute to eclampsia. However, it may have also been useful to measure focal seizure using more sensitive electrode placement such as cortical or implanted hippocampal electrodes. Further, PTZ was used as the chemoconvulsant in this study due to its antagonistic actions at GABA_ARs that allowed direct investigation of pregnancy-induced changes in the inhibitory system within the brain; however, the use of a chemoconvulsant that targets excitatory glutamatergic receptors, such as AMPA or kainic acid, may provide insight into pregnancy-induced plasticity of the excitatory system. It is possible that the apparent decrease in tonic inhibition due to downregulation of the GABA_AR- δ is compensated for by a decrease in excitatory receptor expression that would only be unmasked by the use of a convulsant specific to the excitatory system. In a similar regard, only cortical protein expression of one subunit, GABA_AR- δ , was investigated via Western blot in this project. It is possible that pregnancy-induced plasticity of the γ -subunit (GABA_AR- γ) that is synaptically located and involved in phasic inhibition may compensate for the decrease in expression of GABA_AR- δ . However, studies report that GABA_AR- γ expression also

decreases over the course of gestation (Maguire and Mody, 2009), making it more likely that this pregnancy-induced decrease in GABA_AR- γ augments hyperexcitability as opposed to compensating for the decrease in GABA_AR- δ to maintain appropriate inhibition. Finally, the seizure model used in this dissertation was not a model of spontaneous seizure, and therefore, not a model of eclampsia. However, this dissertation sought to understand changes in brain excitability during pregnancy and preeclampsia that could potentiate seizure and contribute to eclampsia. Thus, seizure was induced to detect changes in seizure threshold and susceptibility. The findings in this dissertation shed new light on excitability changes during normal pregnancy and preeclampsia and potential underlying mechanisms by which that hyperexcitability may be occurring; however, we still do not have a clear understanding of eclamptic seizure onset.

This dissertation provided insight into mechanisms of seizure during pregnancy and preeclampsia; however, it also opened the door for further investigation into the pathogenesis of eclampsia during both seemingly healthy pregnancies and pregnancies complicated by preeclampsia. Future studies investigating the role of the BBB in seizure prevention during normal pregnancy would be useful in gaining a better understanding of not only the adaptation of the BBB to normal pregnancy, but potential mechanisms of disrupted barrier function that may become central to seizure onset. Specifically, studies bypassing the BBB and delivering seizure-provoking serum directly to the brain and monitoring for spontaneous seizure in late-pregnant rats would be critical proof-of-principle experiments that the BBB definitively contributes to seizure prevention during pregnancy. Further, investigation into gestational regulation of both expression and activity of efflux transporters including Pgp and Mrp1 in cerebral microvessels would

allow a greater understanding of the adaptation of the BBB to increased circulating factors occurring during pregnancy. In addition, *in vivo* studies manipulating the activity of efflux transporters at the BBB through pharmacological inhibition of Pgp and Mrp1 with simultaneous EEG recording during normal pregnancy would provide insight into the role of these specific transporters in seizure prevention during normal pregnancy. The BBB during preeclampsia should also be investigated to determine the pathologic process leading to increased BBB permeability that occurs in rats with experimental preeclampsia. Additional size-selectivity studies would be useful to determine the extent of BBB disruption, including further investigation into the type of BBB disruption that is occurring (e.g. increased paracellular vs. transcellular transport) in rats with experimental preeclampsia. Further studies investigating whether there is an effect of circulating factors during preeclampsia on efflux transporters at the BBB that may decrease their activity and result in decreased barrier function remains unclear, but could also be investigated using the experimental model of preeclampsia developed in this dissertation. These future studies could potentially lead to enhanced screening procedures to identify women who may be at risk of de novo seizure during pregnancy and preeclampsia.

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APPENDIX A: OTHER PUBLISHED WORK

Other primary research articles have been published in the following form:

Amburgey, O.A., **Chapman, A.C.**, May, V., Bernstein, I.M., Cipolla, M.J. Plasma from preeclamptic women increases blood-brain barrier permeability: role of vascular endothelial growth factor signaling. *Hypertension*. 2010; 56(5):1003-08.

Chan, S.L., **Chapman, A.C.**, Sweet, J.G., Gokina, N.I., Cipolla, M.J. Effect of PPAR γ inhibition during pregnancy on posterior cerebral artery function and structure. *Front Physiol*. 2010; 1:130. doi:10.3389/fphys.2010.00130.

Cipolla, M.J., Pusic, A.D., Grinberg, Y.Y., **Chapman, A.C.**, Poynter, M.Y., Kraig, R.P. Pregnant serum induces neuroinflammation and seizure activity *via* TNF α . *Exp Neurol*. 2012; 234(2): 398-404.

Gokina, N.I., Chan, S.L., **Chapman, A.C.**, Oppenheimer, K., Jetton, T.L., Cipolla, M.J. Inhibition of PPAR γ during rat pregnancy causes intrauterine growth restriction and attenuation of uterine vasodilation. *Front Physiol*. 2013; 23(4):134. doi: 10.3389/fphys.2013.00184.

A review article has been accepted for publication in *Physiology* on November 14, 2014 in the following form:

Johnson, A.C. and Cipolla, M.J. The Cerebral Circulation during Pregnancy: Adapting to Preserve Normalcy. *Physiology* (in press).