Placental Ischemia-induced Increases in Brain Water Content and Cerebrovascular Permeability:

Role of TNFα

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Cerebrovascular complications and increased risk of encephalopathies are characteristic of preeclampsia and contribute to 40% of preeclampsia/eclampsia related deaths. Circulating tumor necrosis factor α (TNFα) is elevated in preeclamptic women and infusion of TNFα into pregnant rats mimics characteristics of preeclampsia. While this suggests that TNFα has a mechanistic role to promote preeclampsia, the impact of TNFα on the cerebral vasculature during pregnancy remains unclear. We tested the hypothesis that TNFα contributes to cerebrovascular abnormalities during placental ischemia by first infusing TNFα in pregnant rats (200 ng/day i.p, from gestational day 14 to 19), at levels to mimic those reported in preeclamptic women. TNFα increased mean arterial pressure (MAP, p<0.05) and brain water content in the anterior cerebrum (p<0.05), however, TNFα infusion had no effect on blood-brain barrier (BBB) permeability in the anterior cerebrum or posterior cerebrum. We then assessed the role of endogenous TNFα in mediating these abnormalities in a model of placental ischemia induced by reducing uterine perfusion pressure followed by treatment with the soluble TNFα receptor (etanercept, 0.8 mg/kg, sc.) on gestational day 18. Etanercept reduced placental ischemia-mediated increases in MAP, anterior brain water content (p<0.05), and BBB permeability (202±44% in placental ischemic rats to 101±28% of normal pregnant rats). Our results indicate that TNFα mechanistically contributes to cerebral edema by increasing BBB permeability and is an underlying factor in the development of cerebrovascular abnormalities associated with preeclampsia complicated by placental ischemia.

Keywords: pregnancy, preeclampsia, cerebrovascular abnormalities, edema, BBB permeability
INTRODUCTION

Cerebrovascular events contribute to ~40% of all preeclampsia/eclampsia related deaths (32) and preeclamptic patients often present with neurological symptoms such as headaches, blurred vision, nausea, drowsiness, and seizures (in the case of eclampsia) (5). Furthermore, women with preeclampsia have increased risk for developing cerebrovascular events such as stroke during pregnancy as well as during the postpartum year (44). While studies suggest that cerebral edema is a common complication of preeclampsia, the factors contributing to the edema and increased blood brain barrier (BBB) permeability have not been identified.

Our lab previously demonstrated that placental ischemia leads to cerebral edema, impaired myogenic reactivity in middle cerebral arteries (38), impaired whole-brain cerebral blood flow autoregulation, and increased BBB permeability (51). The factors involved in placental ischemia-induced cerebrovascular abnormalities remain unknown. One candidate that may link placental ischemia to cerebrovascular abnormalities is tumor necrosis factor alpha (TNFα). TNFα levels are increased both in the circulation (27) and placentas (25) of placental ischemic rats and blockade of TNFα prevents the preeclampsia phenotype associated with this model (25). Furthermore, when TNFα is infused at a level to mimic those reported in preeclamptic women, mean arterial pressure (MAP) is elevated in normal pregnant rats (27). While these studies provide compelling evidence for the role of TNFα in the regulation of blood pressure, whether placental ischemia-induced increases in TNFα contribute to cerebral edema and increases in blood brain barrier permeability has not been established. Therefore, in this study, we determined whether infusion of TNFα into normal pregnant rats contributes to cerebral edema and increased blood-brain barrier permeability and whether blockade of TNFα in placental ischemic rats would reverse these cerebrovascular changes.
MATERIALS & METHODS

Animals:

Timed pregnant Sprague-Dawley rats were obtained from Charles Rivers Laboratories and arrived at the Lab Animal Facilities at the University of Mississippi Medical Center on gestational day 11. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Rats were housed individually, maintained on a 12 hour light / 12 hour dark cycle, and fed standard rodent chow and water ad libitum.

TNF infusion:

On gestational day 14, rats were anesthetized using 3% isoflurane, and osmotic mini-pumps were surgically implanted intraperitoneally for the constant infusion of recombinant human TNFα at a dose of 200 ng/day. This dose led to an increase in circulating human TNFα levels from 0.00 pg/mL to 0.78±0.26 pg/mL on gestational day 19. Non-pregnant rats were not included because previous studies have shown that TNF infusion increases blood pressure in pregnant rats but has no effect on blood pressure in non-pregnant rats. Tissues were collected on gestational day 19.

Reduced Uterine Perfusion Pressure (RUPP):

To induce placental ischemia, silver clips were surgically inserted around the abdominal aorta above the iliac bifurcation (0.203 mm) and each of the ovarian arteries (0.100 mm) on day 14 of gestation as described previously (17). This method has been shown to reduce blood flow to the utero-placental unit by about 40 percent (17).
TNFα Blockade:

Normal pregnant and placental ischemic rats were randomly assigned to treatment groups (etanercept or untreated group) on gestational day 18. Rats in the treated group were then given a subcutaneous injection of etanercept (0.8 mg/kg), the soluble TNFα receptor. This dose had a greater blood-pressure lowering effect in the placental ischemic group in this cohort of pregnant rats compared to 0.4 mg/kg as was previously used (25).

Blood Pressure Measurements:

Arterial catheters were implanted in the right carotid artery of rats on gestational day 18 for the measurement of arterial pressure on day 19 of gestation. Following 45-60 minutes of acclimation to the restrainer cages, blood pressure measurements were recorded using LabChart software. Blood pressure was recorded continuously for a minimum of 30 minutes.

Determination of Brain Water Content:

On gestational day 19, rats were euthanized under isoflurane anesthesia and brains were removed and weighed. Cerebellum, anterior cerebrum (rostral to the middle cerebral artery), and posterior cerebrum were weighed individually. Brain regions were then dried in an oven at 60°C for 72 hours, after which they were re-weighed to obtain the dry weight. Brain water content was then calculated as a percentage ((wet weight – dry weight)/ wet weight). To calculate cerebrum water content, the sum of the anterior and posterior brain region weights were used.

Assessment of Cerebrovascular Permeability:

A separate group of pregnant rats were utilized for determination of cerebrovascular permeability. Rats were subjected to either TNFα infusion or blockade, and jugular catheters
were implanted on gestational day 18. Evans blue dye (1% solution in saline) was then infused via jugular catheter at a final concentration of 2mg/kg. The dye was allowed to circulate overnight. The following day, a sample of blood was obtained, and PBS was then infused via the jugular vein until the blood ran clear. Brains were then removed and divided into anterior and posterior cerebrum by cutting along the middle cerebral artery. For the TNFα infusion study, hippocampi were also dissected. Brain regions were weighed and snap frozen in liquid nitrogen. Tissue and plasma samples were processed as previously described (49, 54). Briefly, 1 mL TCA (50%) was added to samples, homogenized, and sonicated followed by centrifugation at 10000 rpm for 30 minutes. Supernatants were then diluted 1:2 in ethanol, vortexed and loaded onto 96-well plates. Fluorescence was measured using a microplate reader at excitation 620nm and emission 680nm. Data are represented as concentration of Evans blue dye (ng/mL)/ tissue weight (g)/ plasma concentration (ng/mL).

**Assessment of plasma albumin concentration:**

Because of the Evans blue has been shown to bind to albumin, we measured plasma albumin to determine whether there were any differences in the amount of circulating albumin with TNFα infusion. A commercially available Rat Albumin ELISA kit (GenWay Biotech Inc.) was used and run following manufacturer’s directions.

**Statistical Analysis:**

Statistically significant differences between the normal pregnant and TNFα-treated groups were determined using unpaired one-tailed t-test while Two-way analysis of variance was used for the TNFα blockade study. All data were considered statistically different if p < 0.05. GraphPad Prism was used for statistical analysis and generation of graphs.
RESULTS

General Pregnancy Characteristics:

Infusion of recombinant human TNFα in pregnant rats resulted in a 10 mmHg increase (99±1 to 109±3 mmHg) in mean arterial pressure (MAP) (Table 1) and a modest decrease in body mass (338±5 in normal pregnant rats to 325±6g in pregnant rats infused with TNFα) at day 19 of gestation. There was no significant difference in pup weight, placenta weight, or pup number in response to TNFα infusion (Table 1).

TNFα infusion increases brain water content and blood-brain barrier permeability in pregnant rats.

Pregnant rats infused with TNFα had higher brain water content, a measure of cerebral edema, in the cerebrum (79.00±0.09 compared to 78.6±0.13%) (Figure 1A) when compared to normal pregnant rats (p=0.0098). This increase was mainly due to increases in the anterior cerebrum (79.59±0.34 versus 78.92±0.11%; p=0.032) (Figure 1B) since brain water content was unchanged in the posterior cerebrum (Figure 1C, p=0.055) or cerebellum (data not shown) of TNFα infused rats.

Blood-brain barrier permeability, measured using the Evans blue extravasation technique, was unchanged in the cerebrum of TNFα infused rats (Figure 2A). Further analysis of brain regions revealed that there was no difference in BBB permeability in the anterior cerebrum (Figure 2B) or posterior cerebrum following TNFα infusion (data not shown). TNFα infusion into pregnant rats had no effect on plasma albumin concentration (Figure 2C).

TNFα blockade reduces placental ischemia mediated hypertension.
Placental ischemia induced a significant increase in MAP compared to normal pregnant rats (121±3 vs. 102±2 mmHg; p<0.001) (Figure 3). Etanercept treatment had no effect on MAP in normal pregnant rats (104±2 mmHg) but reduced MAP in the RUPP model of placental ischemia (115±2 mmHg; p=0.03 compared to untreated RUPP rats). However, consistent with previously published work from our laboratory (25), MAP in etanercept-treated placental ischemic rats was not normalized (p<0.001).

TNFα blockade reduces brain water content and blood-brain barrier permeability in placental ischemic rats

Although brain water content was not significantly greater in the whole cerebrum from placental ischemic rats (Figure 4A), regional differences were detected with water content increased specifically in the anterior cerebrum (80.11 in RUPP group vs 79.23% in NP group) (Figure 4B). Etanercept treatment significantly reduced brain water content in the anterior cerebrum of placental ischemic rats (reduced to 78.99%; p=0.01). Neither placental ischemia nor etanercept-treatment had an effect on brain water content in the posterior cerebrum of rats (Figure 4C).

Consistent with the brain water content data, a significant difference in BBB permeability was not reported in the whole cerebrum (Figure 5A). However, when regional differences were assessed, BBB permeability increased from 0.04±0.01 in the normal pregnant group to 0.08±0.02 ng/g tissue/plasma concentration in the placental ischemic group in the anterior cerebrum (Figure 5B). Etanercept treatment prevented placental ischemia-induced increases in BBB permeability (0.05±0.01 in placental ischemic group treated with etanercept compared to 0.04±0.01 ng/g tissue/plasma concentration; p>0.05).
DISCUSSION

The American College of Obstetricians and Gynecologists recommends that in addition to blood pressure greater than 140/90 mmHg after the 20\textsuperscript{th} week of gestation, a patient can be diagnosed with preeclampsia if the increased pressure is accompanied by proteinuria, thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral or visual symptoms (19). Thus, cerebral complications are recognized as an important pathological consequence of preeclampsia. The mechanisms for preeclampsia-induced cerebral and visual symptoms remain elusive but circulating factors released by the ischemic placenta may be potential contributors. Both patients and our placental ischemic model of preeclampsia have increases in several circulating placental-derived factors. For example, increased inflammatory cytokines such as TNF\textsubscript{a} (27) and interleukin-6 (14), increased anti-angiogenic factors such as soluble endoglin (16) and soluble Fms-like tyrosine kinase -1 (sFlt) (15), and activating angiotensin II type 1 receptor auto-antibodies (AT1-AA) (26) have been reported. While these factors increase in response to placental ischemia, it is not known which, if any, mechanistically contribute to cerebrovascular abnormalities. Based on our previous work showing a role for TNF\textsubscript{a} in the pathogenesis of preeclampsia, we tested whether TNF\textsubscript{a} contributes to cerebrovascular abnormalities reported during pregnancy. The major new findings of this study are (1) Infusion of TNF\textsubscript{a} into pregnant rats results in increased mean arterial pressure and brain water content compared to the untreated controls. (2) Blockade of TNF\textsubscript{a} in the RUPP model of placental ischemia using etanercept, reduced mean arterial pressure, prevented the increase in brain water content and BBB permeability. These results demonstrate that TNF\textsubscript{a} is an important factor released in response to placental ischemia that mechanistically contributes to cerebral edema formation and increases in BBB permeability.
Several clinical studies have shown that inflammatory cytokines, including TNFα, contribute to the pathogenesis of preeclampsia. For example, polymorphisms in the TNFα gene have been reported in preeclamptic patients (34, 35) and TNFα is significantly increased in the circulation (21, 29, 55), umbilical vessels (43), and placental tissue (55) from preeclamptic women. Animal studies have also provided evidence for the pathophysiological role of TNFα in preeclampsia. Infusion of TNFα into pregnant rats (27, 28) or baboons (42), at levels to mimic those reported in human preeclampsia, produces characteristics similar to preeclampsia. Furthermore, blocking the actions of TNFα using anti-TNFα antibodies or the soluble receptor to TNFα, prevents the development of hypertension (13, 27), and cardiomyocyte fibrosis (18). TNFα-induced increases in blood pressure were not associated with a change in placental or pup weight, suggesting that the effects of TNFα occurred independent of placental ischemia. Taken together, these studies support the idea that TNFα mechanistically contributes to the pathogenesis of preeclampsia. The present study reaffirms this concept as infusing TNFα into pregnant rats increased mean arterial pressure while TNFα blockade in placental ischemic rats reduced mean arterial pressure.

In addition to the role of TNFα in regulating blood pressure, TNFα has been implicated in cerebrovascular pathologies from various disease models. For example, in acute liver failure, increased BBB permeability is correlated with elevated serum TNFα levels and can be prevented using anti-TNFα IgG treatment (31, 50). Moreover, increased circulating TNFα reportedly promotes matrix metalloproteinase-9 expression in the brain tissue and cerebrospinal fluid and may contribute to increases in BBB permeability (46). A seminal study demonstrated that serum from pregnant rats induces hyperexcitability in hippocampal neurons, a response that can be
abolished by inhibiting TNFα signaling (8). Other studies, in which status epilepticus (a characteristic symptom of eclamptic patients) has been induced, demonstrated that TNFα release is increased (23) especially in activated microglia (22) resulting in vasogenic edema. Thus, numerous lines of evidence suggest an important role for TNFα in cerebral pathologies and the present study demonstrates that TNFα is an important factor that contributes to the cerebral complications associated with preeclampsia.

While clinical studies assessing the utility of etanercept or similar TNFα inhibitors in the treatment of cerebrovascular complications have not been reported, several basic studies have shown positive cerebrovascular outcomes with TNFα blockade. For example, etanercept treatment reduced traumatic brain injury-induced TNFα expression, edema, and axonal swelling (11). In a model of intracerebral hemorrhage, anti-TNFα antibody treatment reduced microglial activation, cerebral edema, and functional deficit (30) and TNFα receptor antagonists reduced BBB opening and attenuated edema development (24). Moreover, reduction in TNFα signaling following 10% hypertonic saline treatment in a model of middle cerebral artery occlusion resulted in decreased brain water content and infarct size (20). These studies demonstrate that reducing TNFα levels following cerebrovascular insult may present a potential therapeutic. The current study supports this since treatment of placental ischemic rats with etanercept prevents placental ischemia-induced edema and BBB permeability increases.

Consistent with our previous findings in placental ischemic rats, TNFα infusion and placental ischemia increased water content in the anterior cerebrum while the posterior cerebrum was unaffected (51). While the increase in brain water content was small, the brain lies within the enclosed space of the skull, thus limiting the extent of edema formation. Therefore, small increases in brain water content are physiologically relevant. Although several clinical studies
report abnormalities in the parietal-occipital lobe of preeclampsia patients (2, 33, 45), there is evidence that the anterior cerebrum is also affected. Indeed, increased cerebral perfusion pressure has been reported in both the anterior cerebral arteries of preeclamptic patients (37) and in the anterior and middle cerebral arteries of severe preeclamptic patients (6). Other studies show that while cortical/sub-cortical lesions were detected predominantly in the occipital lobe, the frontal lobe was also affected (9). Importantly, a recent study demonstrated that white matter lesions were more common among women with previous pregnancies complicated by preeclampsia or eclampsia and that these lesions were most frequently observed in the frontal lobe (53). Additionally, in animal models of acute hypertension during pregnancy, cerebral edema occurs in both the anterior and posterior cerebrum (7). Taken together, these studies demonstrate that cerebrovascular abnormalities are not confined to the posterior cerebral circulation but also affect the anterior cerebrum.

Edema can occur through disruption of the BBB and increases in extracellular fluid as a result of increased hydrostatic pressure (vasogenic edema) or can occur through ionic imbalance and cell swelling (cytotoxic edema) (3). Because preeclamptic patients have been reported to have increased cerebral perfusion pressures (37) often with increased blood pressure, we hypothesize that vasogenic edema may be the main form of edema that occurs following placental ischemia and TNF$\alpha$ infusion. However, it is possible that ionic imbalance may also contribute to edema formation in response to placental ischemia or TNF$\alpha$ infusion since we have not directly addressed this question in any of our studies. We recently reported that expression of aquaporin 4 channel protein, the major water transport channel in the brain, is elevated in the posterior cerebrum where edema was not detected, and was unchanged in the anterior cerebrum where edema was evident (51). We therefore hypothesized that placental ischemia failed to
induce the compensatory increase in aquaporin 4 expression in the anterior cerebrum that has
been shown to be involved in edema resolution (12, 36, 51). The observation that aquaporin 4 is
differentially expressed in response to placental ischemia suggests that cytotoxic edema may
contribute to the increased brain water content observed in response to placental ischemia and
TNFα infusion. Further studies are warranted to address this question.

Increased BBB permeability is a potential underlying mechanism for cerebral edema
formation. Patients with posterior reversible encephalopathy syndrome, a condition common
among preeclampsia patients, have acute disruption of the BBB in response to abrupt increases
in blood pressure (2). Importantly, several studies have shown that plasma from preeclamptic
women is capable of inducing BBB disruption in isolated cerebral veins from non-pregnant rats
(1, 39, 40). While this study showed no change in BBB permeability in response to TNFα
infusion, it is possible that there are changes in tight junction protein expression and localization.
Additionally, the use of Evans blue dye may limit the sensitivity to detect small changes in BBB
permeability and provides no information about transcellular permeability both of which may be
altered in response to increases in TNFα. Future studies will therefore determine whether TNFα
infusion contributes to changes in tight junction and adherens junction protein expression and
will utilize other methods of assessing BBB integrity such as using varying molecular weight
dextrans that are fluorescently labeled. It should also be noted that TNFα is one of many factors
increased in response to placental ischemia and may act synergistically with other factors to
stimulate increases in BBB permeability. It is also possible that increases in BBB permeability in
response to placental ischemia may result from increased hydrostatic pressure in addition to the
effects of circulating factors.
Several studies have assessed the safety of anti-TNFα drugs during pregnancy. A majority of the studies reported no adverse effects on pregnancy outcomes (10, 41, 47, 48); however, a recent study reported increased risk of birth defects along with increased risk of preterm births and lower birthweight (52) and increased congenital anomalies with use of anti-TNFα medication (4). While some adverse effects were reported in the studies mentioned above, it is difficult to isolate the effects of the disease from the effect of the treatment. More studies are required to assess the long-term effect of TNFα antagonists during pregnancy and on the offspring. However, the work cited above, along with data from this study, suggest that anti-TNFα treatment, should not yet be ruled out as a potential option for preeclamptic patients to reduce the cerebrovascular abnormalities associated with preeclampsia.

Significance & Perspectives:

Cerebrovascular complications in preeclampsia and eclamptic patients pose serious clinical risks and contribute to a large percentage of preeclampsia/eclampsia-related deaths. To date, only magnesium sulfate is used in the clinic as a prophylactic for the prevention of seizures. At present, no treatments are available to reverse edema formation, blood-brain barrier permeability, or abnormal cerebral blood flow autoregulation in these patients. This study provides compelling evidence that the inflammatory cytokine, TNFα, may be an important clinical target for not only preeclampsia, but also for the prevention of cerebrovascular abnormalities that complicate preeclampsia. Our data demonstrate that blockade of TNFα signaling can prevent placental ischemia-induced cerebral edema and increased BBB permeability, two major symptoms observed in brain scans of preeclamptic patients.
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Disclosures: None


Figure Legends:

Figure 1: TNFα infusion in pregnant rats results in cerebral edema. Brain water content was increased in the (A) cerebrum of pregnant rats infused with TNFα. The increased brain water content is observed in the (B) anterior cerebrum but not the (C) posterior cerebrum of pregnant rats infused with TNFα. Data represents Mean ± SEM; *p<0.05 compared to pregnant untreated rats; N = 11-12 per group. NP - normal pregnant.

Figure 2: TNFα infusion in pregnant rats has no effect on blood-brain barrier permeability. Evans blue extravasation was unchanged in the cerebrum (A) and anterior cerebrum (B) of TNFα-infused pregnant rats. (C) TNFα infusion does not change plasma albumin concentration. Data represents Mean ± SEM; *p<0.05 compared to pregnant untreated rats.

Figure 3: Blockade of TNFα in placental ischemic rats attenuates blood pressure. Mean arterial pressure is reduced but not normalized by TNFα blockade in placental ischemic rats. Data represents Mean ± SEM; *p<0.05.

Figure 4: TNFα blockade reduces brain water content. Etanercept treatment did not alter brain water content in the whole cerebrum (A) but prevented an increased brain water content in the anterior cerebrum (B) with no effect in the posterior cerebrum (C). Data represents Mean ± SEM; *p<0.05.

Figure 5: Etanercept treatment reduces blood-brain barrier permeability. Blood brain-barrier permeability to Evans blue dye was unchanged in the cerebrum as a whole (A). Anterior cerebral extravasation of Evans blue dye was increased in placental ischemic rats and prevented by etanercept treatment (B). Data represents Mean ± SEM; *p<0.05.
Table 1. General Animal Characteristics at gestational day 19

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<td>N=12</td>
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<td>MAP (mmHg)</td>
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<td>Body Weight (g)</td>
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<td>Placenta Weight (g)</td>
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*p<0.05, **p<0.01 vs. Pregnant group. Data represent Mean ± SEM.