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Biphasic Effects of Vanadium Sulfate on Cerebrovascular Endothelial Cell Barrier Integrity

Yoselin Ordonez Suarez

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**Biphasic Effects of Vanadium Sulfate on
Cerebrovascular Endothelial Cell Barrier Integrity**

by

YOSELIN ORDONEZ SUAREZ

B.S. Biochemistry, UNM 2015

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Master of Science
Pharmaceutical Sciences**

The University of New Mexico
Albuquerque, New Mexico

May 2019

DEDICATION

This thesis is dedicated to my family.

Thank you for all your unconditional support and love.

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I would like to thank Matthew Campen and Barry Bleske for giving me the opportunity to do research while in pharmacy school. Thank you for all the support, I have grown as a person for the past 4 years from all the gain work experiences. I would also like to thank Alicia Bolt for all her experimental recommendations and for always being available when needed, thank you. I also want to thank everyone in Dr. Campen's lab for all their help and support for the last four years.

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ABSTRACT

The objective of this study was to investigate the biphasic effects of vanadyl sulfate in cerebral vascular endothelial cells with a focus on understanding the mechanisms underlying vanadyl sulfate benefits. To first address the effects of vanadyl sulfate on endothelial permeability, mouse cerebrovascular endothelial cells were grown to confluence in 96-well electrode ECIS plates and treated with three different vanadium containing compounds: vanadyl sulfate, vanadium pentoxide, and ammonium metavanadate. Resistance of the endothelial cell monolayer was measured at 4kHz for 24 hours for different concentrations of vanadium containing compounds. The mouse cerebrovascular endothelial cells were also treated with different concentrations of vanadyl sulfate plus a superoxide dismutase mimetic, tempol, or a rho kinase inhibitor, fasudil, for 48

hours to assess oxidative stress and rho kinase activation by vanadium. All three vanadium-containing compounds exhibited a dose dependent biphasic effect in resistance. The addition of tempol and fasudil did not increase resistance of vanadyl sulfate. Additionally, an in vivo mouse study was conducted with two concentrations of vanadyl sulfate 0.025 mg and 0.25 mg, dosed twice daily for 24 hours. Brain tissue was collected and mRNA transcription levels of intracellular adhesion molecule-1 (ICAM-1), tumor necrosis factor- α (TNF- α), zona occludens-1 (ZO-1), occludin and claudin-5 were measured by qPCR. The transcription levels for the genes of TNF- α and ICAM-1 did not change with vanadyl sulfate treatment. Blood brain barrier endothelial tight junction protein occludin transcription levels significantly increased with 0.025 mg vanadyl sulfate treatment. Levels remained unchanged compared to control for blood brain barrier tight junction proteins ZO-1 and claudin-5. These studies suggest that vanadium-containing compounds at certain concentrations may improve the blood brain barrier. Further investigation of the mechanisms of this effect may reveal novel therapeutic pathways/targets.

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CHAPTER 1. INTRODUCTION

Neurotoxicity of Vanadium

Vanadium is a transition metal that is ubiquitous in the environment. Vanadium is a contaminant of fossil fuels and people can be environmentally exposed to the metal via crude oil spills and automobile exhaust (Plunkett et al, 1976). Moreover, vanadium is used industrially in the making of pesticides, steel, and many other materials (Wenning et al, 1988). As such, chronic vanadium exposure often occurs through environmental or occupational contact (Fatola et al, 2019). Investigations of vanadium toxicity as well as the potential therapeutic use of vanadium have linked the heavy metal to tissue remodeling in several organs including the lungs, testes and liver of rats (Fatola et al, 2019). Experimental models have also showed vanadium to have neuroinflammatory effects along with physiologic biochemical changes (Thompson et al, 2009). The potential therapeutic effects of vanadium have been most substantially studied in diabetes mellitus, as vanadium has been showed to decrease glucose levels in diabetic humans (Thompson et al, 2009). A possible mechanism by which vanadyl sulfate leads to decreased glucose levels is through the up-regulation of PI3-kinase, which phosphorylates IRS-1 leading to increased inhibition of glucagon (Goldfine et al, 2000). Additional diseases where vanadium has been studied for therapeutic benefits include syphilis, hyperlipidemia, tuberculosis, anemia and malnutrition (Fatola et al, 2019). When thinking about the toxicity or therapeutic benefits of vanadium it is essential to understand that the downstream effects of vanadium exposure are dependent on dose, route of exposure and valance state (Madejon et al, 2013). As industrial applications of

vanadium continue to increase and the extent of environmental exposure becomes more evident, there is an emerging need for deeper investigations into the physiological role and mechanisms of action of vanadium to better understand its possible therapeutic effect and levels that lead to toxicity.

Several studies have been conducted in rodent models to understand the toxicity of vanadium in the brain (Fatola et al, 2019). In a chronic mouse model of exposure, metavanadate vanadium injected intraperitoneally (IP) (3mg/kg/day three times a week for 3-18 months) was found to cross the blood-brain barrier, as assessed by laser ablation inductively coupled plasma-mass spectrometry (Folarin et al, 2017). Vanadium accumulation was observed throughout the brain, with higher concentrations found in the olfactory bulb, cerebellum and brain stem. Moreover, mice exposed to vanadium had histological lesions in the pre-frontal cortex and a significant decrease of neurons in the hippocampus (Folarin et al, 2017). In another study, the effect of chronic (90 day) vanadium exposure in early life was assessed using a mouse model (Azeez et al, 2016). In this model, pups were exposed to sodium metavanadate via lactating dams injected IP with 3mg/kg of vanadium for the first 21 days. For the remaining exposure time frame, the weaned mice received 3mg/kg IP vanadium three times per week via IP injections. Early life exposure to vanadium resulted in deteriorating myelin tracts in the thalamus, hippocampus, and different layers of the cortex. Moreover, exposure animals exhibited increased levels of astrocyte-expressed tumor necrosis factor alpha (TNF α) (Azeez et al, 2016).

The most common form of human environmental exposure to vanadium is via inhalation. In an inhalation study, mice were exposed to 0.02 M vanadium pentoxide for 1 hour two times a week for 4 weeks total using an acrylic chamber (Colin et al, 2015). Mice had a decrease in olfactory bulb size, swollen organelles, and mitochondrial dysregulation (Colin et al, 2015). In a similar inhalation study with vanadium pentoxide, mice were exposed to 0.02M vanadium pentoxide for 1 hour twice a week via a nebulizer for 8 weeks resulting in loss of cilia of ependymal cells in circumventricular organs of the brain and oxidative damage in the choroid plexus (Avila-Costa et al, 2005).

Various mechanisms for vanadium neurotoxicity have been reported. One of the major proposed mechanisms for vanadium neurotoxicity is the production of reactive oxygen species (Fatola et al, 2019). When vanadium is in the blood is typically in +5 oxidation state in a pH range of 4-8 pH (Nechay et al, 1984). Vanadium can enter cells in the form of vanadate through anion channels and it can also enter cells as vanadyl ions through the process of passive diffusion. When vanadate is in the cytoplasm it is converted to vanadyl, a reduction leading to the production of reactive oxygen species (Ding et al, 1994). Vanadyl can then be converted to vanadate via hydrogen peroxide by Fenton-like reactions. This oxidation reaction can also lead to the production of free radicals (Figure 1) (Capella et al, 2002). In another study, rats exposed intratracheally to 0.5 mL of 1.0 mM vanadyl sulfate over a 24-hour period had increased levels of free radicals in the lung as detected by electron spin resonance (Kadiiska et al, 1997).

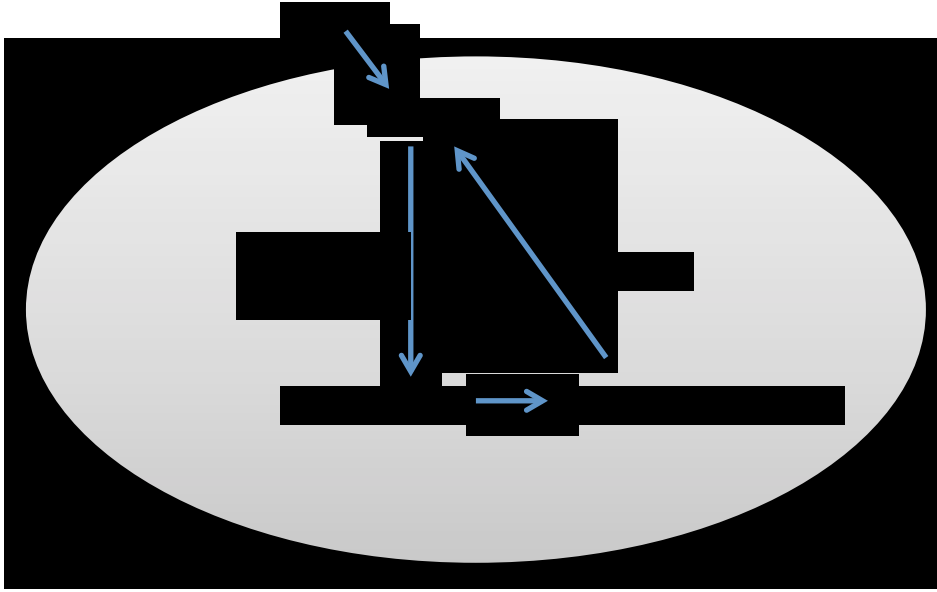


Figure 1. Mechanism of vanadium produced intra-cellular toxicity. Vanadium enters the cell typically in the +5 valence state and is converted to the +4 state by NADPH oxidation inside the cell. The conversion of vanadium +4 to +5 can produce oxygen radicals and hydrogen peroxide. Vanadium +4 can form a reaction with hydrogen peroxide to make hydroxyl particles. (Figure based on Capella, L. et al., 2002).

At high intracellular doses, vanadium can promote oxidative stress (Figure 1) and, owing to the high lipid content in the brain, such reactions can lead to lipid peroxidation affecting the myelin of neurons (Todorich et al, 2011).

Another proposed mechanism of vanadium neurotoxicity is through binding to protein phosphorylation enzymes. For example, vanadate has the capacity to bind to cysteine residues in proteins and, as a consequence, the complex can react with hydrogen peroxide to form pervanadate ions that can consequently oxidize the cysteine residues (Fatola et al, 2019). Cysteine

residues are located in active sites of several proteins such as protein tyrophosphatase, which can be inactivated by vanadate. Inhibition of protein phosphorylation results in sustained activation of specific intracellular pathways, including, for example, the MAPK cascade which plays a major role in inflammatory cytokine activation (Chien et al, 2006).

Vanadium may also exhibit possible therapeutic effects in different disease states. There does seem to be a threshold for toxicity and at low enough doses vanadium does not appear to cause overt toxicity. Bis(ethyl maltolato) oxido vanadium (BEOV) is an antidiabetic agent that is clinically being tested (Domingo and Gomez et al, 2016). In addition, the use of vanadium has been proposed to treat various forms of cancer as well as viral and bacterial diseases (Rehder et al, 2016). Low doses of vanadium have the potential to promote positive therapeutic effects, however the administration of such doses over an extended period of time should be avoided as it can lead to the development of tumors (Korbecki et al, 2012). Interestingly, vanadium has been reported to have a biphasic effect in osteoblast cells. Depending on the dose of vanadium, it can have cell proliferation and differentiation effects or it can induce inhibition of cell proliferation (Cortizo et al, 1995). This is likely related to a hormetic effect that switches from low doses that target disease pathways to higher doses that are potentially toxic due to oxidative effects. Recent work by (Dyer et al, 2015) suggests that vanadium has a beneficial effect in cognition in rats exposed to vanadium through their food for four weeks. However, while several studies explore the effects of vanadium at a high dose, there is a significant gap in

knowledge regarding the possible effects and mechanisms of action of low dose vanadium exposure on the brain (Fatola et al).

Blood-brain Barrier Cell-Cell Junctions

The blood-brain barrier (BBB) protects the major regions of the brain from the environment by providing a more controlled transport of molecules to and from the brain parenchyma. The BBB is found in the arteries and veins throughout the brain excluding circumventricular organs and is composed of specialized endothelial cells. Cerebral vascular endothelial cells are distinctive from endothelial cells outside of the nervous system. Brain endothelial cells have tight junctions that resemble epithelium cells that tightly regulate the solutes allowed to reach the brain by passive diffusion. Additionally, cerebral endothelial cells have specialized transporters that also regulate what nutrients can reach brain tissue. Similar to the peripheral endothelial cells, brain endothelial cells are also charged and have specific transporters that are able to inactivate toxic substances and efflux the toxic chemicals away from the brain. As a result of endothelial tight junctions and specialized transporters, plasma contents are closely regulated, thereby protecting brain cells from possible harmful plasma solutes. Other specialized characteristic of brain endothelial cells is that they contain a high number of mitochondria to account for the high metabolic work that endothelial cells perform. In addition, endothelial cells lack fenestrations and have limited pinocytosis thereby decreasing transcellular transport (Cipolla et al, 2009).

The BBB is composed of adherens and tight junctions that connect cells paracellularly. The tight and adherent proteins connect to adapter proteins and these proteins connect to actin and the cytoskeleton of the cells. Several neurological disorders can cause tight junction dysregulation and BBB impairment, including: Alzheimer's disease, ischemic stroke, Parkinson's diseases, multiple sclerosis, hypertension, and seizures (Zlokovic et al, 2008). Strategies to improve BBB function in such diseases may be beneficial to reduce neuroinflammation and better support cognitive function.

Tight junctions are made up of transmembrane proteins that include: claudin, occludin, junction adhesion molecules (JAM), and zona occludens (Cipolla et al, 2009). Claudins account for the majority of the BBB junction proteins with more than 20 identified claudin proteins (Morita et al, 1999). Claudin tight junction proteins connect with zona occludens (ZO) proteins ZO-1, ZO-2, and ZO-3 on their carboxyl terminus in the cytoplasm. ZO-1 and ZO-2 connect with the cytoskeleton via actin proteins in their carboxy-terminus (Furuse et al, 1999). Claudin proteins, in conjunction with different zona occludens, cingulin proteins and several other proteins, provide support to tight junctions (Citi et al, 1988). Occludin proteins also make up tight junctions in cerebral endothelial cells. Occludin proteins are transmembrane proteins that have amino and carboxyl end terminus in the cytoplasm. Occludins and claudins both have two extracellular proteins that form bonds with their neighboring cells occludins and claudin proteins making paracellular connections and forming part of the BBB (Cipolla et al, 2009). The occludin proteins form a direct link with zona occludens proteins

that form a link with actin and the cytoskeleton. Therefore, occludin proteins are able to regulate the passage of solutes in close coordination with the cytoskeleton (Mitic et al, 2000). Junction adhesion molecules (JAM) are also membrane proteins that form tight junctions with ZO-1. There have been a total of three identified junction adhesion molecules (JAM): JAM-1, JAM-2 and JAM-3. Of these three proteins only JAM-1 and JAM-3 are found in the endothelium tissue of the brain. JAM-1 is found with actin and is subsequently involved in paracellular adhesion (Aurrand-Lions et al, 2001). Besides playing a structural role in connecting claudins, occludins and junction adhesion molecules to the cytoskeleton, ZO proteins are also involved in signaling molecules by PSD-95/Discs large/**ZO**-1 (PDZ) and SRC homology 3 domains (Yuan, Rigor et al, 2010).

Adherent junctions also form the ultrastructure of the BBB. Adherent junctions in the cerebral endothelium are made from vascular endothelial cadherin proteins that form hemotypic bonds with other vascular endothelial cadherin proteins from adjacent cells. Vascular endothelial cadherin proteins are connected to beta or gamma catenin proteins that are connected to alpha catenin that, in turn, is connected to actin filaments and the cytoskeleton (Figure 2; Yuan, Rigor et al, 2010).

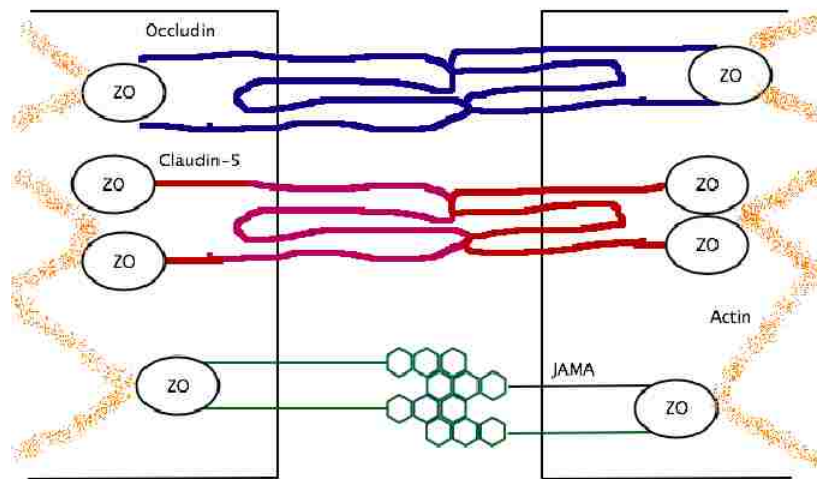


Figure 2. Tight junction protein structure of endothelial cells in the blood-brain barrier. Occludin, claudin and JAMA proteins form bonds intracellular with ZO proteins and extracellular with each other forming a selective barrier. Figure based on (Yuan SY, Rigor RR, 2010).

Blood-Brain Barrier Dysfunction

BBB dysfunction typically leads to an increase in the traffic of molecules paracellularly or transcellularly, in a way that exposes the brain to factors normally absent. For example, BBB dysfunction occurs if there is an increase in pinocytotic activity or if a large flux of molecules opens the tight junction proteins. BBB integrity is compromised in several neurological conditions including stroke, stress, multiple sclerosis. What all the above conditions have in common is the production of reactive oxygen species (ROS), has been implicated in BBB impairment (Pun et al, 2009). Experiments in frogs have showed a relationship

between amplified levels of reactive oxygen species and BBB permeability (Olesen et al, 1987). Several findings indicating the pathways by which reactive oxygen species lead to BBB disruption have been reported. One of those pathways is through the bradykinin system (Pun et al, 2009). Bradykinin levels increase in the brain when there is ischemia and reperfusion (Kamiya et al, 1993). Bradykinin has been linked to the activation of phospholipase A2 which is able to cut membrane phospholipids and subsequently releases arachidonic acid. Arachidonic acid is metabolized and produces reactive oxygen species that affect the integrity of the BBB (Pun et al, 2009). Neutrophils are white blood cells that get activated in the presence of inflammation. When there is ischemia, neutrophils are recruited to the injured site and start to increase the generation of superoxide molecules (Pun et al, 2009). Macrophage accumulation has also been implicated in the generation of reactive oxygen species when there is insult to the brain. Macrophages recruit pro-inflammatory factors such as TNF- α and early growth response protein (Egr-1), which have been implicated in blood-brain barrier disruption (Lynch et al, 2004). The depletion of glutathione levels has been correlated to increased levels of oxidative stress. Oxidative stress caused by low levels of glutathione is produced when proteins like sulfhydryls are left vulnerable to oxidative attack (Hall et al, 1997). Changes in tight junctions, as mentioned earlier, have been linked to changes in barrier integrity. Similarly, exposure of xanthine/xanthine oxidase reduces protein levels of the tight junction protein occludin. In addition, hydrogen peroxide leads to increased levels of occludin and, interestingly, occludin is found at the cell membrane and not

necessarily at the tight junctions (Pun et al, 2009). Similar to tight junctions, the cytoskeleton plays a vital role in blood-brain barrier integrity. The production of reactive oxygen species can cause changes in the cytoskeleton. For example, reactive oxygen species can cause the activation of Rho phosphorylates which, in turn, activate proteins such as rho-associated protein kinase (ROCK), focal adhesion kinase (FAK) and mDIA (Pun et al, 2009). When proteins such as (ROCK) get activated, myosin light chain proteins are phosphorylated causing structural changes to the cytoskeleton, particularly by actin proteins. Increased levels of ROS/RNS including peroxide, peroxynitrite (ONOO-) and nitric oxide (NO), also phosphorylate tight junction proteins such as occludin and cause changes in cytoskeletal organization (Haorah et al, 2005). The phosphorylation of various proteins in the cytoskeletal and tight junctions leads to rearrangement of the cytoskeleton and an increase in blood-brain barrier permeability (Pun et al, 2009).

Matrix metalloproteinases (MMP) are capable of breaking down proteins from the extracellular matrix and basement membrane into amino acids .The activation of MMPs leads to an increase in blood-brain barrier permeability. One possible mechanism by which MMPs disrupt blood-brain barrier integrity is by decreasing levels of the tight junction protein occludin and preventing the development of an endothelial gap (Reijerkerk et al, 2006). The specific activation of MMP's is not known but studies have shown that protein tyrosine kinase inhibitors can decrease levels of MMP's (Haorah et al, 2007). The activation of protein tyrosine kinase is believed to increase under oxidative stress

leading to increase barrier integrity dysfunction (Pun et al, 2009). Inflammatory mediators have also been well studied in the disruption of the BBB. Reactive oxygen species can activate the transcription factor NF- κ B and this transcription factor promotes the activation of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) (Kim et al, 2008). ICAM-1 activates calcium dependent signaling pathways and makes modifications to the blood-brain barrier. The adhesion molecules are known to promote the recruitment of white blood cells such as neutrophils and leukocytes. Recruitment is thought to be rho dependent and the white blood cells can release inflammatory mediators such as TNF- α and IL-1 beta that can damage the BBB. Not only are the mediators causing damage to the BBB, but also the movement of cells across the membrane can cause further endothelial barrier damage by decreasing occludin levels. Additionally, neutrophils and macrophages cause an increase in reactive oxygen species further contributing to endothelial barrier dysfunction (Pun et al, 2009). All together, reactive oxygen species can create blood-brain barrier disruption by activating different proteins and pathways (Zlokovic et al, 2008).

Cerebrovascular Disease, Stroke, and the Blood-brain Barrier

BBB structure and dysfunction is a prominent pathologic factor in many diseases such as stroke. Stroke attributed to 16.9% of all cardiovascular deaths in 2016. Hospitalizations due to stroke have also increased by approximately 40% from 2003 to 2012. Patients with a higher risk of stroke include those who suffer from high blood pressure, obesity, high blood glucose levels,

hyperlipidemia and kidney dysfunction. Other risk factors contributing to stroke include life-style choices such as smoking, living a sedentary lifestyle and not consuming healthy foods. Also, a global risk contributing to stroke is air pollution, adding a 29% increased risk (Benjamin et al, 2019). Stroke can occur through several different mechanisms. Two of the most common types of stroke are ischemic and hemorrhagic. In ischemic stroke there is a blockage in the blood vessels that prevents normal blood flow typically caused by a blood clot. In a hemorrhagic stroke, blood vessels become weak and rupture leading to decreased blood flow to the brain. During an ischemic stroke the BBB is disrupted and starts to leak due to an increase in MMP's, inflammatory factors, oxidation pathways, and disruption in junction-junction and junction-cytoskeleton interactions (Sifat et al, 2017). The available treatments for an ischemic stroke are limited and include tissue type plasminogen activator (tPA) and thrombectomy in certain patient populations, which function to remove clots. The Food and Drug Administration has not approved any neuroprotective or neurorestorative treatments for acute ischemic stroke. New treatments are warranted to decrease neuronal death (Sifat et al, 2017).

The phosphorylation of tight junction proteins such as occludin, claudin-5 and ZO-1 by factors such as cyclic AMP, Rho/ROCK kinases and vascular endothelial growth factor have been reviewed over the years (Jiang et al, 2018). In stroke models, phosphorylation of tight junctions and an increase in BBB permeability is typically induced and results from several inflammatory factors. In cultured brain cells, biomolecules such as cytokines including TNF α , interleukin-

6, and monocyte chemoattractant protein 1 can promote phosphorylation of the tight junction protein ZO-1 (Jiang et al, 2018). When endothelial cells are cultured with monocytes, Rho kinases become activated and subsequently phosphorylate occludins and claudin-5 leading to increased ability of white blood cells to cross the BBB (Persidsky et al, 2006). Controlling the changes in tight junction protein integrity in stroke disease is important. Many studies have focused on the phosphorylation changes of tight junction proteins, but the proteins can also undergo other structural modifications such as methylation, acetylation, or ubiquitination, which have not been studied as extensively (Jiang et al, 2018).

Tight junction proteins can translocate depending on the regulatory biochemical environment. Studies have demonstrated the translocation of tight junction proteins occludin and claudin-5 when endothelial brain cells are exposed to C-C Motif Chemokine Ligand 2 (CCL-2) resulting in a decreased TEER (Stamatovic et al, 2009). Another approach that can result in the translocation of tight junction proteins after an ischemic stroke event is within the cytoskeleton. The cytoskeleton in ischemia can undergo changes resulting in actin polymerization and alterations that can lead to tight junction proteins moving to the cytoplasm. The cell-to-cell adhesion is reduced and the junction proteins can more easily become degraded. Preventing negative cytoskeletal changes can prevent tight junction protein translocation and changes leading to negative barrier integrity (jiang et al, 2018). Several studies have tried to improve the BBB by different pathways. In one of the pathways, steroids are given to try and decrease inflammatory factors (Jiang et al, 2018). In another study, to decrease

the phosphorylation of tight junction proteins, rho kinase inhibitor fasudil was given to mice after transient middle cerebral artery occlusion and fasudil inhibited BBB disruption (Jiang et al, 2018). While other studies have used lentivirus to over express tight junction protein claudin-5 in retinal endothelial cells leading to an enhance in barrier integrity (Jiang et al, 2018).

Tight junction proteins are not only affected by phosphorylation and translocation, but they can also be degraded by (MMP's). Such proteins are activated by zinc, which is increased in events such as ischemic stroke (Rempe et al, 2016). Zinc is able to activate MMP-9 and MMP-2, which consequently decrease the levels of occludin and claudin-5. After a stroke, MMP-9 and MMP-2 levels remain high, leading to decreased function of tight junction proteins and an increase in blood-brain barrier integrity (Jiang et al, 2018). In a stroke, endothelial cells have to react to ischemia and hypoxic conditions. As stated above, the cytoskeleton starts to rearrange and tight junction proteins start to degrade. In addition, during a stroke, endothelial cells have to deal with oxidative stress and inflammation leading to an increase in neutrophil infiltration, MMP's, inflammatory cytokines leading to autophagy (Jiang et al, 2018). There are many regulators of the BBB, some of which are activated by stressors such as stroke and lead to further damage and other chemicals that are activated help to restore the BBB integrity. Chemicals that have been reported to negatively influence the BBB include inflammatory cytokines TNF- α , interleukin-6 (IL-6), interleukin-1 (IL-1), bradykinin, histamine, thrombin, nitric oxide, glutamate, and free radicals (Johann et al, 2013).

As mentioned earlier, there are many risk factors for the development of stroke, including but not limited to hypertension, hyperglycemia and hyperlipidemia (Benjamin et al, 2019). These diseases can cause functional and structural changes in the blood-brain barrier (Benjamin et al, 2019). Animal studies that include the use of spontaneous hypertension rat models have shown BBB dysfunction in the cerebral cortex at five months and hippocampus BBB dysfunction at 3 months (Jiang et al, 2018). Spontaneous hypertensive rat models are similar to essential hypertension in humans (Jiang et al, 2018). In renal hypertensive rats, 8-weeks of hypertension results in changes to endothelial tight junctions (Fan et al, 2015). Tight junctions such as occludin and ZO-1 levels were significantly lower at 8-weeks and also gradually decreased over time (Fan et al, 2015). When hypertension in rats is induced by constricting the aorta, the gene expression of the tight junction proteins claudin-3 and claudin-5 is decreased (Mohammadi et al, 2014). In addition, in spontaneously hypertensive rats, pro-inflammatory factors such as IL-1 β and TNF- α levels are increased (Tayebati et al, 2016). TNF- α expression levels were high in the frontal cortex of the hypertensive rats (Tayebati et al, 2016). Furthermore, acutely-induced hypertensive rat models have an increase in oxidative stress (Poulet et al, 2006). Transverse aortic coarctation done between the two carotid arteries in rats leads to hypertension and an increase in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme activity 1 day after hypertensive surgery. One and seven days after the surgery, albumin extravasation increased in the surgery rats compared to the sham-operated rats

in the hippocampus and the cortex indicating BBB damage (Poulet et al, 2006). These studies highlight the importance of controlling high blood pressure in patients to prevent BBB damage.

Diabetes is another disease that has the potential to damage the endothelial BBB. Studies indicate that hyperglycemia leads to a decrease in the tight junction proteins occludin, claudin and ZO-1. Hyperglycemia increases the oxidative stress that leads to a decrease in tight junction proteins. Hyperglycemia in numerous studies has been related to an increase in BBB permeability and increase rate of mortality (Jiang et al, 2018). Diabetic mice brain periventricular regions exhibit increased BBB permeability measured by albumin leakage (Fujihara et al, 2016). In other studies conducted in 40-weeks old diabetic rats exposed the damage of untreated diabetes in the BBB. Rats displayed a decrease in the protein levels of occludin and claudin-5 in the hippocampus (Yoo et al, 2006). In other rat model studies of induced hyperglycemia and brain ischemia, enhanced BBB disruption is evident. The hyperglycemia rats demonstrated greater barrier disruption early after artery occlusion compared to a longer time frame of ninety minutes. This evidence suggests that patients with high blood glucose levels should be treated immediately when having a stroke (Ennis et al, 2007).

Thus, impaired BBB is intimately related to the severity of outcomes in stroke and other related diseases. Therapeutic strategies to improve BBB integrity in stroke models have led to limitations of cerebral necrotic regions and overall improvements in neurocognition (Sifat et al, 2017). Conversely,

environmental stressors may have an important role in exacerbating BBB impairment therefore compounding the effects of stroke.

Summary and Research Objectives

The purpose of this study is to investigate dose-dependent toxicologic and therapeutic effects of vanadium sulfate in the permeability of the BBB with a focus on the therapeutic benefit in murine cerebral vascular endothelial cells and animal studies. As exposure studies have noted a seeming threshold of toxicity from systemic vanadium, we posit that there may be a hermetic effect of vanadium on endothelial cell function, permitting a potential therapeutic window for improvement in cell-cell interactions and overall barrier integrity.

Thesis objectives:

1. Investigate vanadium sulfate dose dependent BBB permeability.
2. Evaluate vanadium sulfate mechanism of oxidative stress and inflammatory factors in the BBB.
3. Characterize the effect of vanadium sulfate in BBB tight junctions.

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Chapter 2. Biphasic effects of vanadium sulfate on cerebrovascular endothelial cell barrier integrity.

Introduction

Vanadium is a transition metal that can readily be found in the environment and reflects a potential environmental and occupational exposure risk for many. While it has not been extensively studied, there are numerous reports of systemic vascular toxicity of vanadium. Vanadium has been shown to cross the BBB and such exposure leads to lesions in different parts of the brain including layers of the cortex (Folarin et al, 2017) In combination with structural changes in the brain, vanadium can also lead to an increase in pro-inflammatory factors such as TNF- α (Azeez et al, 2016) and oxidative stress (Avila-Costa et al, 2005). In addition to having a toxicological effect, vanadium has been reported to have therapeutic effects, albeit at lower concentrations. Vanadium-containing compounds have been used as a diabetic treatment compound leading to decrease levels of glucose (Domingo and Gomez et al, 2016). Additionally, Vanadium (IV) and vanadium (V) have been shown to counteract reactive oxygen species through the generation of a superoxovanadium complex, which transforms ROS to oxygen molecules by superoxide oxidation (Kelm et al, 2001). In a recent study, rats exposed to vanadium via food mash containing vanadium 0.05mg/1g showed cognitive improvement compared to control rats who received no vanadium in their diet (Dyer et al, 2015). A gap in knowledge exists of the possible beneficial or harmful effects of vanadium in the brain, in terms of dosage, valence states, and mechanism of action (Fatola et al, 2019).

The BBB is composed of specialized cell-cell junctions. In the brain, endothelial cells have tight junctions similar to epithelial cells. The tight junctions play a critical role in regulating the solutes that are able to reach the cells of the brain. The tight connection between brain endothelial cells confers an electrical resistance of the BBB approximately $1500\text{-}2000\ \Omega\text{-cm}^2$ (Cipolla et al, 2009). The endothelial barrier is composed of transmembrane tight junction proteins. Tight junction proteins include but are not limited to claudin, occludin and zona occludens (Cipolla et al, 2009). Claudin proteins connect with zona occludens proteins in the cytoplasm and the zona occludens proteins connect to the cytoskeleton via connection to actin proteins (Furuse et al, 1999). Occludin proteins also connect with zona occludens proteins that connect to the cytoskeleton by binding to actin; in this way occludin tight junctions are able to regulate the passage of solutes in close relationship with the cytoskeleton (Mitic et al, 2000). Tight junction proteins occludin and claudin have two extracellular proteins that form bonds with the extracellular occludin and claudin proteins of neighboring cells (Cipolla et al, 2009). Such proteins connect brain endothelial cells paracellular and regulate passage of solutes arising from the circulatory system.

The BBB can be disrupted when there is an increase in paracellular or transcellular traffic, which can cause the tight junctions to open. Cerebrovascular events such as stroke cause disruption of the BBB and one of the mechanisms of barrier disruption is by the generation of reactive oxygen species (Pun et al, 2009). During ischemia, neutrophils get activated by inflammation processes.

The recruitment of neutrophils leads to an increase in the generation of reactive oxygen species (Pun et al, 2009). Macrophages can also be activated when there has been an insult to the brain. These white cells can recruit pro-inflammatory factors such as TNF- α , which can negatively affect BBB (Lynch et al, 2004). Changes in endothelial brain barrier tight junctions can also lead to barrier disruption. Studies have demonstrated that when claudin-5 junction proteins are exposed to ONOO⁻, the tight junction protein levels decrease leading to brain permeability similar to when occludin proteins are exposed to hydrogen peroxide. Similar to tight junction dysfunction, reactive oxygen species can also disrupt the cytoskeleton. ROS activates rho phosphorylates that in turn activate proteins like ROCK (Pun et al, 2009). When the protein ROCK gets activated, myosin light chains get phosphorylated provoking structural changes in the cytoskeleton (Haorah et al, 2005). Reactive oxygen species can also activate transcription factors that activate ICAM-1 and VCAM-1 (Kim et al, 2008). These adhesion molecules recruit white blood cells, which can damage the BBB by activation of inflammatory mediators (Pun et al, 2009).

In this study, we propose that vanadium sulfate may be a possible therapeutic option when used in low doses. We show that low vanadium doses are not toxic to cerebral vascular endothelial cells and such doses have a potential benefit in increasing electrical BBB permeability. We also demonstrate by animal studies that vanadium does not alter transcription levels of tight junction proteins but increases occludin tight junction protein translation. Our results indicate that vanadium, at optimal doses, can have therapeutic effects.

Identification of the mechanisms of action could potentially be applied to diseases such as stroke when the integrity of the BBB is disrupted.

Materials and Methods

Electric Cell-substrate Impedance Sensor (ECIS)

Mouse cerebral vascular endothelial cells were used to assess electrical resistance. The electric cell impedance sensing system was used to determine the changes in BBB integrity. A monolayer of cerebral vascular endothelial cells was placed in each well. Approximately 5×10^5 cells were placed in each well and the cells were grown to confluence demonstrated by a plateau line in resistance. After the cells reached an electric plateau state, the media of the endothelial cells was replaced with media containing different experimental treatments. As soon as the different treatments were added, the resistance of the cells was measured at 4kHz for 24 hours. All treatment groups were run in triplicates. All treatment groups were then compared to the media-only control groups or to dose-equivalent groups in the case of pharmacological treatments. ECIS at 4kHz measures transcellular electrical current flow of endothelial cells.

In Vivo Vanadium Exposure

A total of three groups of C57BL6/J male mice (Taconic Laboratories) were utilized for this study (n=5/gp). Upon arrival to our laboratory at 6 weeks old mice acclimated for one week under AAALAC-approved housing conditions (12h light:12 hr dark cycle, 21-25°C, food and water available *ad libitum*). After the acclimation, mice were divided into the following groups: saline 0.9%, vanadyl

sulfate 1 mg/kg (low dose), and vanadyl sulfate 10mg/kg (high dose). Vanadyl sulfate was dissolved in 0.9% isotonic saline solution. The injection volume was 100 μ L with a vanadyl sulfate high concentration of 0.25 mg vanadyl sulfate/100 μ L and a vanadyl sulfate low concentration of 0.025mg vanadyl sulfate/100 μ L. The mice were exposed to one dose of vanadyl sulfate and another dose 12 hours after the initial dose. Vanadyl sulfate was administered via intraperitoneal injections under isoflurane anesthesia. After 24 hours the mice were anesthetized with isoflurane and euthanized. Brain tissue was harvested by doing a vertical midline cut starting at the nape of the neck to the tip of the nose using sterile microscissors and forceps. The brains were then instantly frozen in liquid nitrogen. All procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee.

RNA Extraction from Brain Tissue

RNA was extracted from the frontal cortex of mice exposed to both concentrations of vanadyl sulfate as well as control animals. Lysis buffer was added to tubes containing the isolated brain cortex and the tissue was homogenized using beads for 1 minute. Shredders and RNeasy columns and collection tubes were labeled and used for each sample. The homogenized tissue was then added to the shredder tubes and centrifuged for 2 minutes at 10,000 RPM. Tissue was then transferred to RNeasy columns and centrifuged for 30 seconds at 10,000 RPM. Different buffers including buffer RW1 and buffer RPE were used to extract the RNA. RNase free water was then added directly to the columns and centrifuged for 1 minute at 10,000 RPM. After centrifuge

samples were stored.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR was utilized to determine gene expression of the following genes: ZO-1, claudin-5, occludin, ICAM-1 and TNF- α . All primers were acquired from Thermo Fisher. A master mix was created with taqman (Life Technologies). A total volume of 8 μ L of master mix was added first to the plate followed by 2 μ L of cDNA from the brain tissue. After addition of master mix and cDNA, the plate was centrifuge for 2 minutes at 2000 rpm. The plates were run for three hours in the light cycler. To calculate a possible change in gene transcription, the delta-delta Ct method was used compared to housekeeping gene.

Statistical Methods

ECIS data were analyzed by a two-way ANOVA with tukey's posthoc testing to identify temporal regions of difference among groups. For qPCR statistic analysis were measured using two-tailed Student's t-test or one-way ANOVA. The p-values <0.05 are considered to be statistically significant. GraphPad Prism 7.0 software was utilized for all statistical analyses.

RESULTS

Vanadium containing compounds increase cerebral vascular endothelial cell resistance

We exposed confluent primary murine cerebrovascular endothelial cells to different vanadium containing compounds: vanadyl sulfate, vanadium pentoxide and ammonium metavanadate. We first exposed the cells to vanadyl sulfate and noticed an increase in electrical resistance at specific doses. The endothelial

cells treated with vanadyl sulfate at 0.01mM and 0.03mM doses constantly held a greater resistance compared to control cells treated with media for 24 hours. At a higher concentration of vanadyl sulfate 0.3mM, the electrical resistance was higher compared to control for 5 hours and for the remaining of the 24 hours the resistance decreased in cells treated with vanadyl sulfate (Figure 1). To verify that vanadium in other forms would have the same effect, cerebrovascular endothelial cells were also exposed to vanadium pentoxide and ammonium metavanadate. Endothelial cells treated with 0.01mM and 0.003mM of vanadium pentoxide demonstrated to have a constant increase in resistance for 24 hours compared to control cells. Vanadium pentoxide at a dose of 0.1mM and 0.3mM consistently decreased resistance over 24 hours (Figure 2). Endothelial cells were also exposed to ammonium metavanadate and at a dose of 0.03mM, treated cells show constantly higher resistance for 24 hours compared to control endothelial cells. Ammonium metavanadate at 0.3mM decreased the electrical resistance over 24 hours (Figure 3). All three different forms of vanadium were able to consistently increase the electrical resistance over time at lower doses compared to the control endothelial cells. At higher doses all three vanadium-containing compounds decreased the electrical resistance of cerebral vascular endothelial cells

Vanadyl sulfate does not cause oxidative stress

Vanadium compounds have been associated with an increase in reactive oxygen species. Tempol is an antioxidant that is used in studies to decreased reactive oxygen species. In human vein umbilical endothelial cells the addition of tempol

to arsenite exposed cells leads to a decrease in permeability (Bao et al, 2010). To test whether different doses of vanadyl sulfate increase reactive oxygen species, we decided to add tempol, an antioxidant, to vanadium treated endothelial cells. Cerebral vascular endothelial cells exposed to 1mM of tempol had a constant decreased in resistance compared to control. The combined addition of tempol 1mM and vanadyl sulfate 0.03mM lead to a decrease in resistance 5 hours after the addition of the treatment. The addition of tempol 1mM and vanadyl sulfate 0.1mM also lead to a decrease in resistance 10 hours after the treatment. When tempol 1mM and vanadyl sulfate 0.3mM are combined the cells resistance consistently decreases over 24 hours. We observed that the addition of tempol did not increase BBB permeability at our low and high doses of vanadium (figure 4). When the different concentrations of vanadium sulfate 0.03mM, 0.1mM, and 0.3mM are combined with tempol 1mM the decreased in resistance is dose dependent over 24 hours.

Vanadyl sulfate does not inhibit rho kinases

Vanadium containing compounds can have contractile effects that involve the activation of rho kinases (Ito et al 2015, Mori et al 2004). Rho kinases play a vital role in the organization of the actin cytoskeleton. RhoA effects on the cytoskeleton also control cell-cell adhesion (Shi et al, 2013). Rho kinases can not only phosphorylate the cytoskeleton but also tight junction proteins leading to an increase in endothelial cell permeability (Jiang et al, 2018). To evaluate if vanadyl sulfate activate rho kinases and decreased BBB permeability, fasudil, a well-documented and known rho kinase inhibitor, was added to vanadium treated

endothelial cells and electrical resistance was measured. The addition of fasudil (20 μ M) to cerebral vascular endothelial cells consistently decreased the cells resistance over 24 hours. The combination of vanadyl sulfate 0.03mM and fasudil at 20 μ M decreased endothelial cell electrical resistance over 24 hours compared to media control endothelial cells. The addition of vanadyl sulfate 0.1mM plus fasudil also consistently decreased the resistance over 24 hours as did the combination of vanadyl sulfate 0.3mM and fasudil. A decrease in resistance is dependent on the concentration of vanadyl sulfate with 0.3mM concentration having the largest decrease and 0.003mM of vanadyl sulfate resulting in the lowest decrease in resistance. The addition of fasudil did not increase endothelial cell resistance (figure 5).

Vanadyl sulfate does not increase pro-inflammatory factors

Pro-inflammatory factors have been linked to BBB dysfunction and vanadium has been shown to increase inflammatory factors in specific settings. To test whether low doses of systemically-administered vanadium could alter pro-inflammatory factors in the brain, mice were injected IP with vanadium and qPCR was conducted to measure inflammatory gene transcription levels in the frontal cortex. No significant difference was observed in the gene transcription levels of TNF- α (Figure 8) and ICAM-1 (Figure 7) in mice exposed to 0.025mg vanadium or 0.25mg vanadium as compared to control mice treated with saline.

Vanadyl sulfate increases tight junction protein occludin

We demonstrated that three different vanadium-containing compounds increased the electrical resistance across a monolayer of murine cerebrovascular

endothelial cells. Tight junction proteins and the cytoskeleton of brain endothelial cells are involved in the regulation of barrier integrity. Any changes to tight junction proteins can have a structural effect on the cytoskeleton as these proteins form bonds with proteins of the cytoskeleton such as actin. Studies have demonstrated that an increase in tight junctions such as claudin-5 leads to enhanced barrier function (Jiang et al, 2018) To investigate if vanadium has an effect on tight junction protein gene expression, mRNA levels were measured for the tight junction proteins ZO-1, claudin-5 and occludin. Mice treated with 0.025mg of vanadyl sulfate had significantly increased transcription levels of occludin (Figure 9). While the levels of ZO-1 (Figure 10) and claudin-5 (Figure 11) remained unchanged compared to the control group with 0.025mg and 0.25 mg of vanadyl sulfate

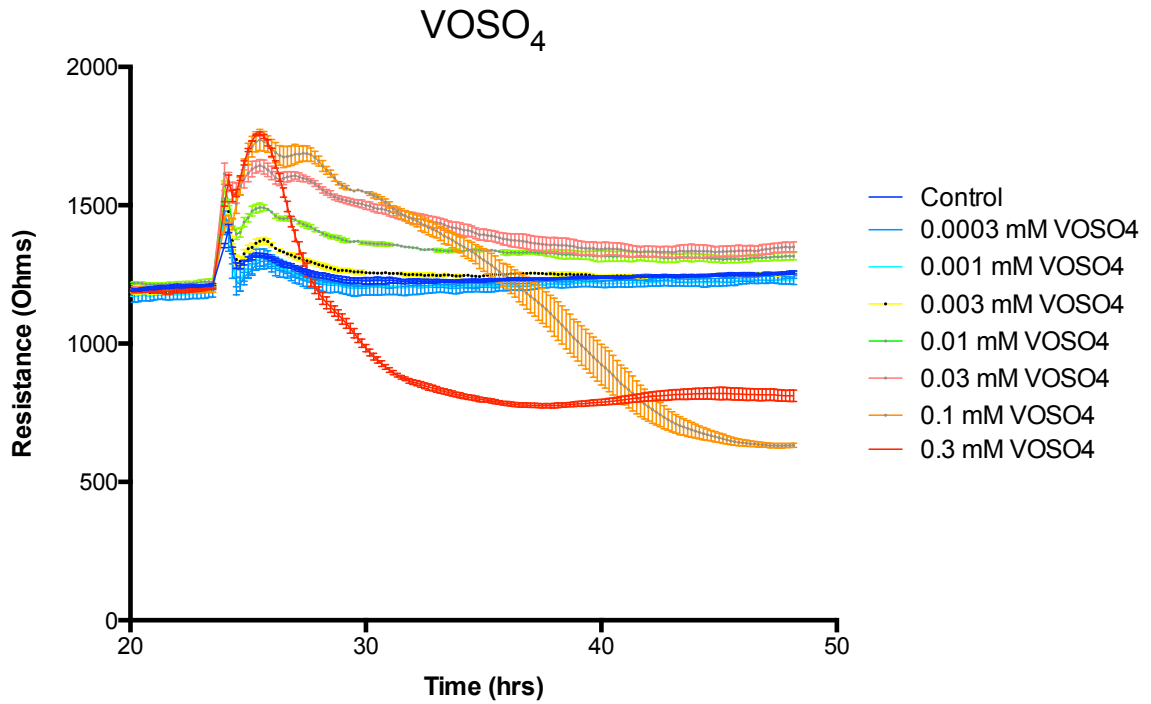


Figure 1. Murine cerebral vascular endothelial cells resistance with vanadyl sulfate using ECIS. Endothelial cells were grown to confluence and treated with different concentrations of vanadyl sulfate. Each concentration of vanadyl sulfate including control groups treated with media were run in triplicates. Vanadyl sulfate 0.01mM and 0.03mM consistently increased resistance for 24 hours.

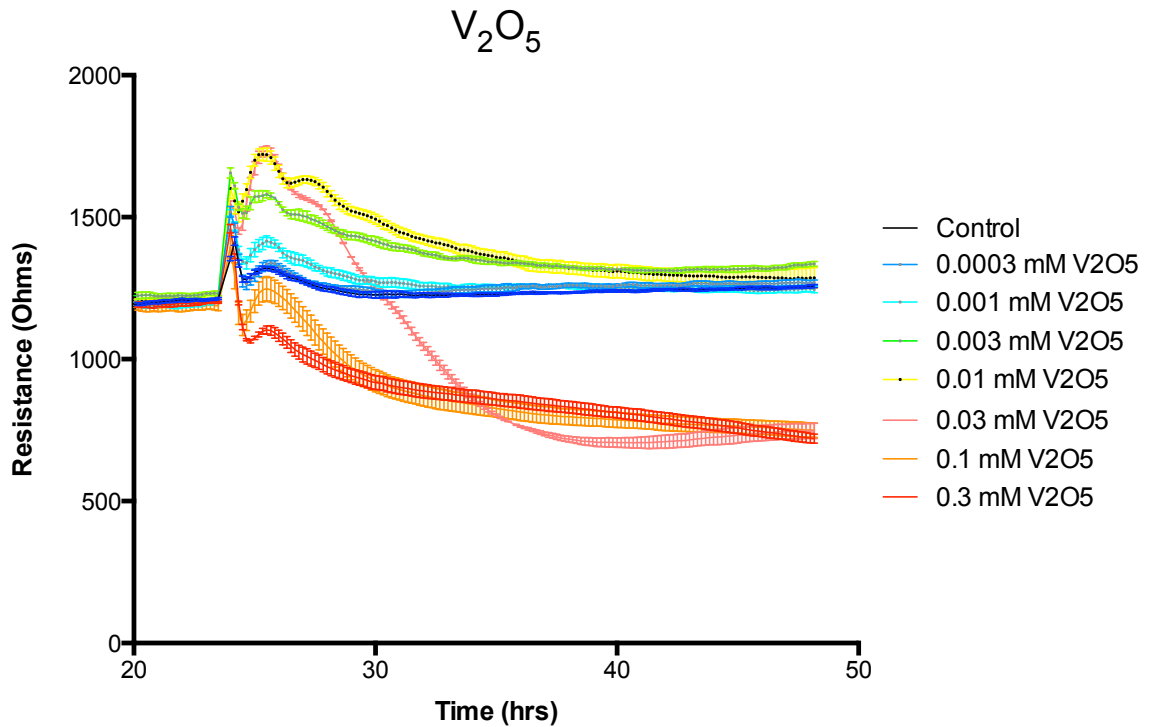


Figure 2. Murine cerebral vascular endothelial cells resistance with vanadium pentoxide using ECIS. Endothelial cells were grown to confluence and treated with different concentrations of vanadyl sulfate. Each concentration of vanadium pentoxide including control groups treated with media were run in triplicates. Vanadium pentoxide 0.01mM and 0.003mM consistently increased resistance for 24 hours.

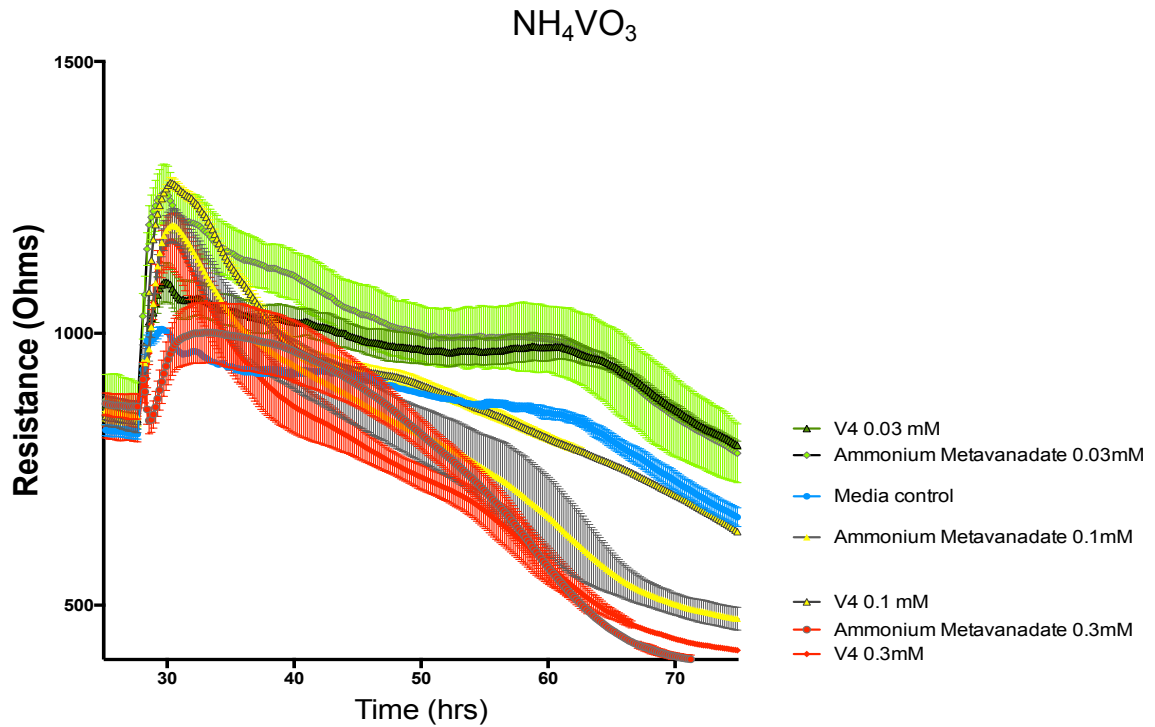


Figure 3. Murine cerebral vascular endothelial cells electrical resistance with ammonium metavanadate using ECIS. Endothelial cells were grown to confluence and treated with different concentrations of ammonium metavanadate and vanadyl sulfate. Each concentration including control groups treated with media were run in triplicates. Vanadium metavanadate 0.03mM consistently increased resistance for 24 hours.

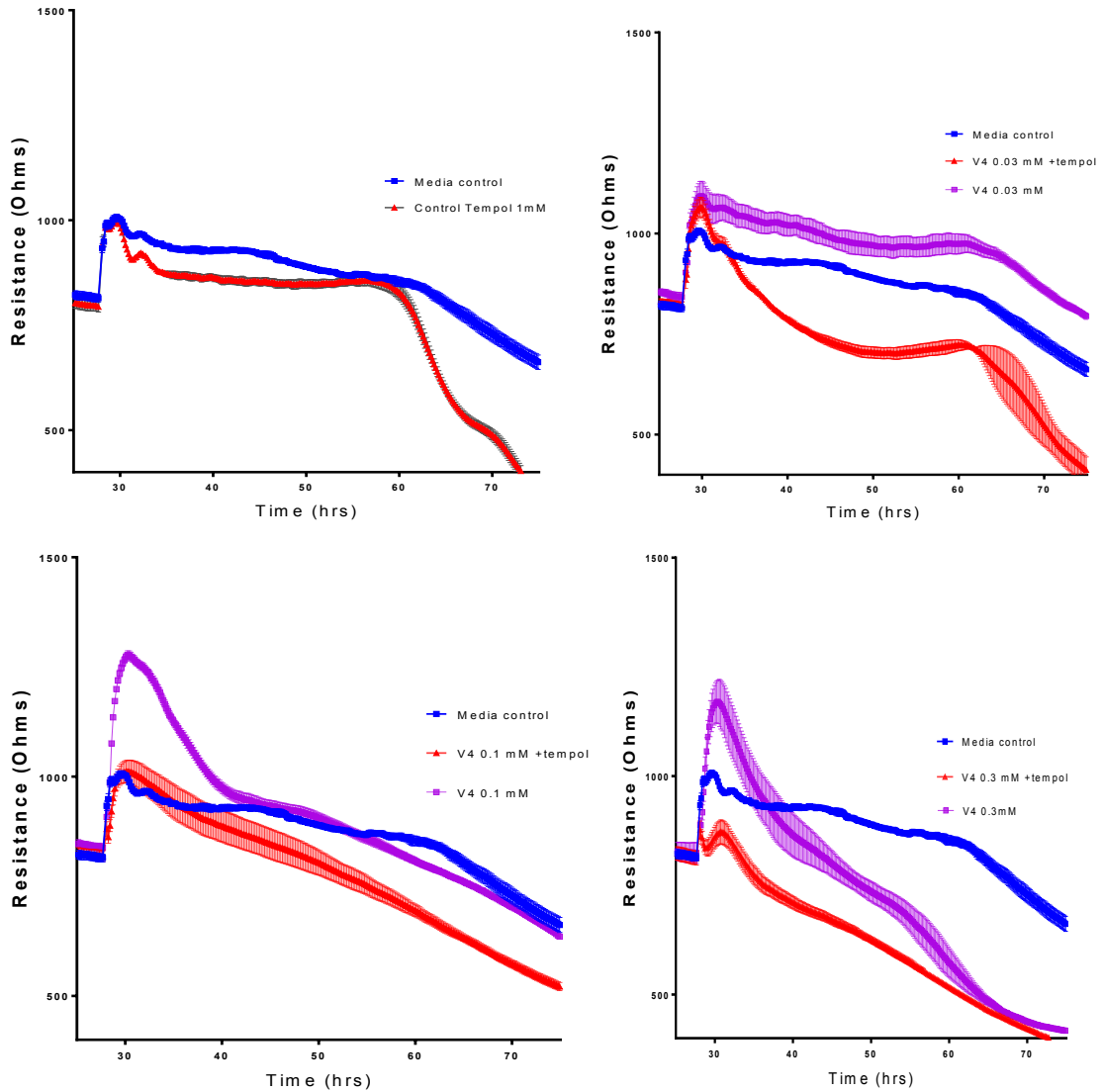


Figure 4. Murine cerebral vascular endothelial cells electrical resistance with tempol and vanadyl sulfate using ECIS. Endothelial cells were grown to confluence and treated with tempol 1mM and different concentrations of vanadyl sulfate. Each concentration including control groups treated with media were run in triplicates. The addition of tempol to vanadyl sulfate 0.03mM, 0.1mM and 0.3mM decreased the resistance of the endothelial cells in a dose dependent matter with higher doses of vanadyl sulfate leading to an increase in resistance over 24 hours.

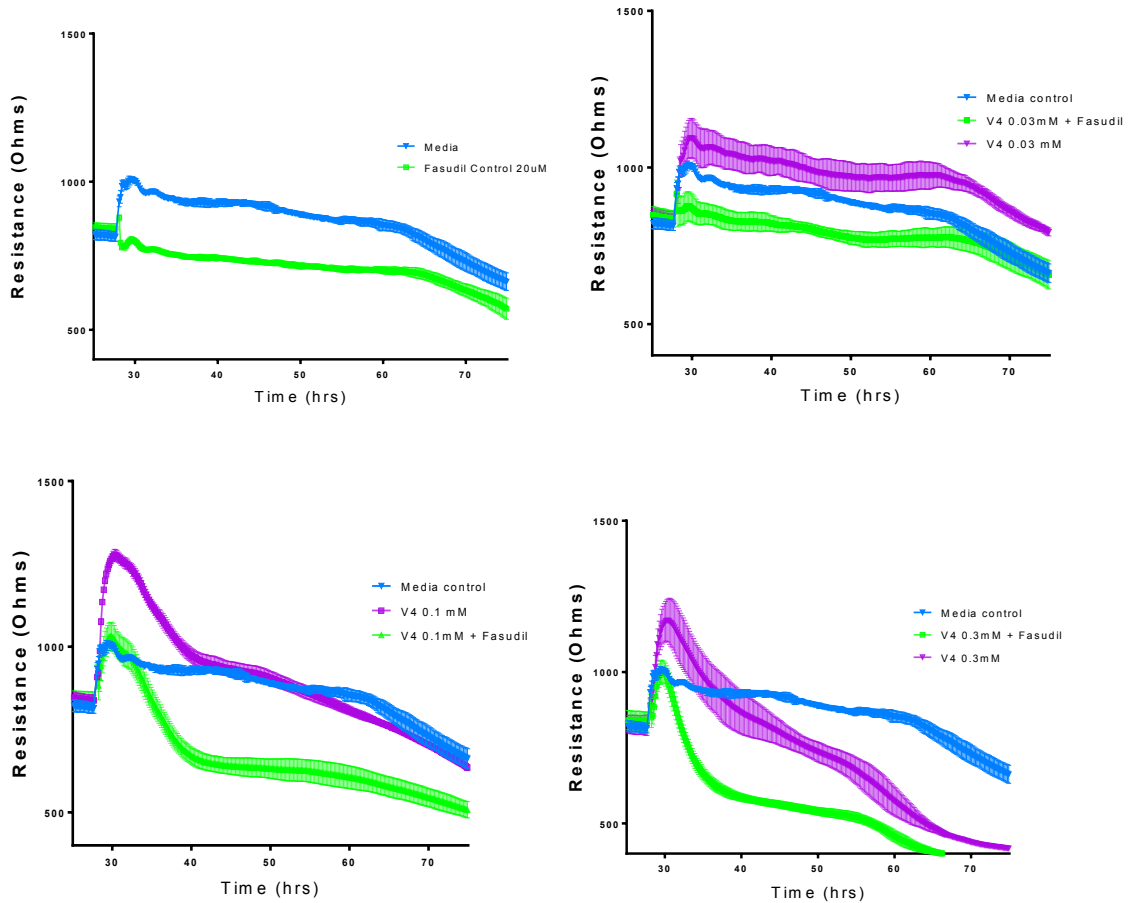


Figure 5. Murine cerebral vascular endothelial cells electrical resistance with fasudil and vanadyl sulfate using ECIS. Endothelial cells were grown to confluence and treated with fasudil 20 μ M and different concentrations of vanadyl sulfate. Each concentration including control groups treated with media were run in triplicates. The addition of fasudil to vanadyl sulfate 0.03mM, 0.1mM and 0.3mM decreased the resistance of the endothelial cells in a dose dependent matter with higher doses of vanadyl sulfate leading to an increase in resistance over 24 hours.

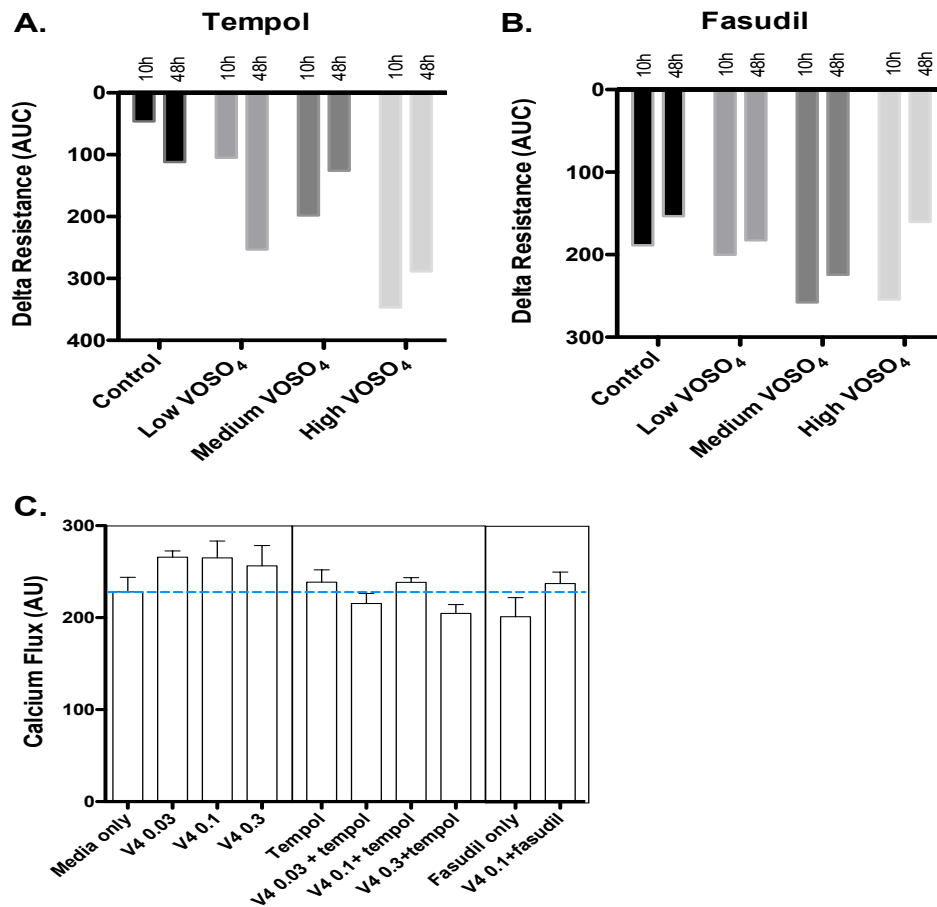


Figure 6 a-c. Area under the curve (AUC) summations for the tempol (A) and fasudil (B) permutations. AUC values were determined based on the differences between the VOSO₄ responses at each dose compared to each dose with tempol or fasudil present. Each AUC plot presents the response from 0-10h and 0-48h, as we suspect different cellular mechanisms may be at play. Increasing doses of VOSO₄ appear to create a greater tempol-sensitive response, especially in the 10h timeframe. Fasudil did not seem to have such an effect. (C) The role of VOSO₄ in stimulating calcium release was also assessed (n=3/group), in the presence and absence of tempol and fasudil, but no permutation made a significant impact on intracellular calcium flux.

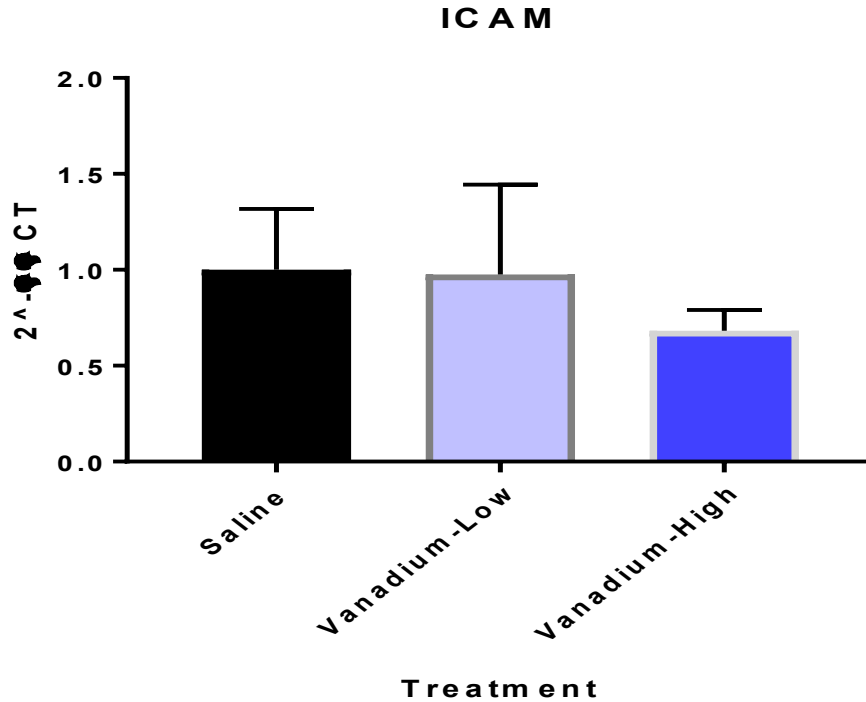


Figure 7. In vivo ICAM frontal brain cortex qPCR gene transcription levels of vanadium exposed mice. Mice were exposed to vanadyl sulfate (low dose: 0.025mg, 0.25mg high dose) via IP injection twice a day. Animals were sacrificed after 24 hours of vanadyl sulfate exposure. Control mice received saline IP injections twice a day. The control group contained an n=4, low vanadium group n=5, and high vanadium group n=5. No significant difference was observed ($p>0.05$) using one-way ANOVA between saline and vanadium treatment groups for ICAM mRNA levels.

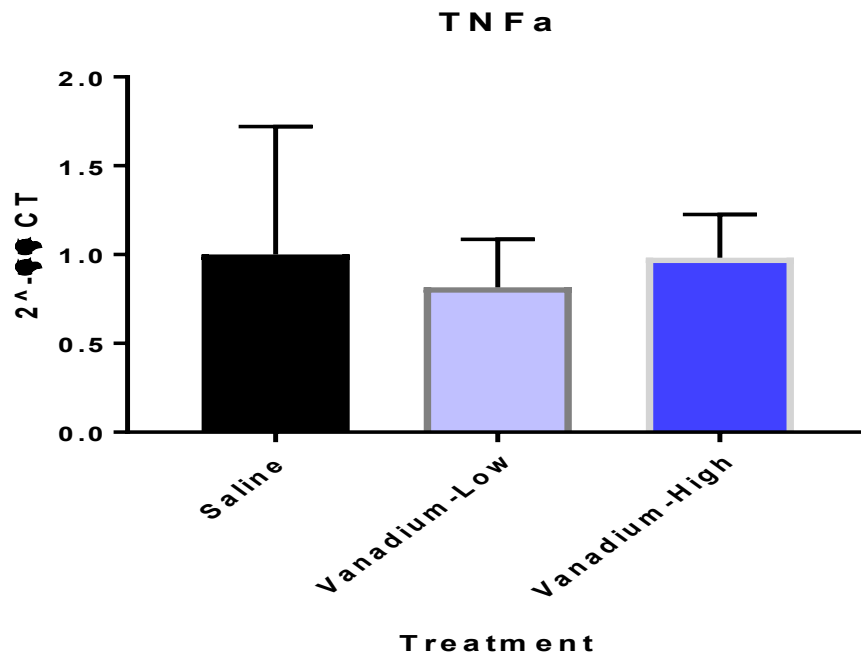


Figure 8. In vivo TNF- α frontal brain cortex qPCR gene transcription levels of vanadium exposed mice. Mice were exposed to vanadyl sulfate (low dose: 0.025mg, 0.25mg high dose) via IP injection twice a day. Animals were sacrificed after 24 hours of vanadyl sulfate exposure. Control mice received saline IP injections twice a day. The control group contained an n=4, low vanadium group n=5, and high vanadium group n=5. No significant difference was observed ($p>0.05$) using one-way ANOVA between saline and vanadium treatment groups for TNF- α mRNA levels.

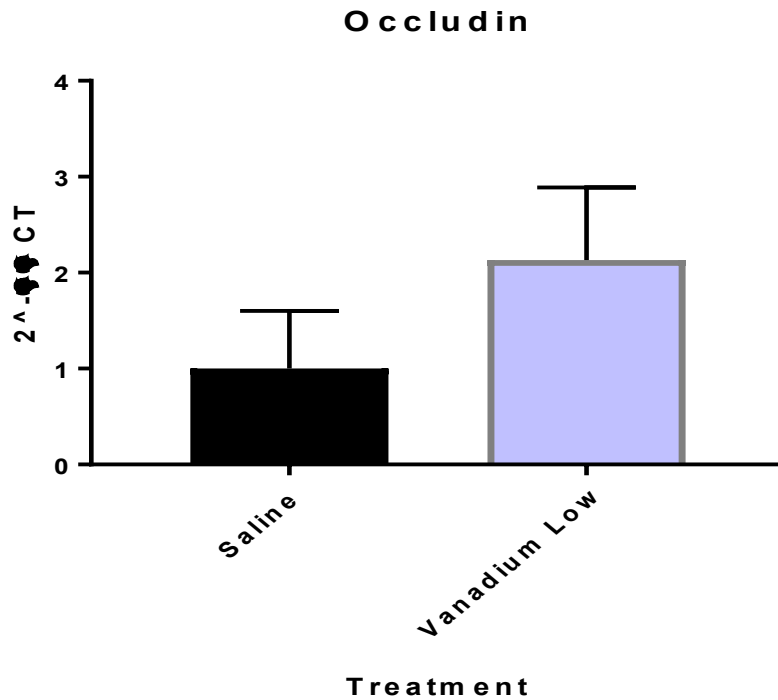


Figure 9. In vivo occludin frontal brain cortex qPCR gene transcription levels of vanadium exposed mice. Mice were exposed to vanadyl sulfate (low dose: 0.025mg, 0.25mg high dose) via IP injection twice a day. Animals were sacrificed after 24 hours of vanadyl sulfate exposure. Control mice received saline IP injections twice a day. The control group contained an n=4, low vanadium group n=5. A significant difference was observed ($p < 0.05$) using two-tailed Student's t-test between saline and low vanadium treatment groups for occludin mRNA levels.

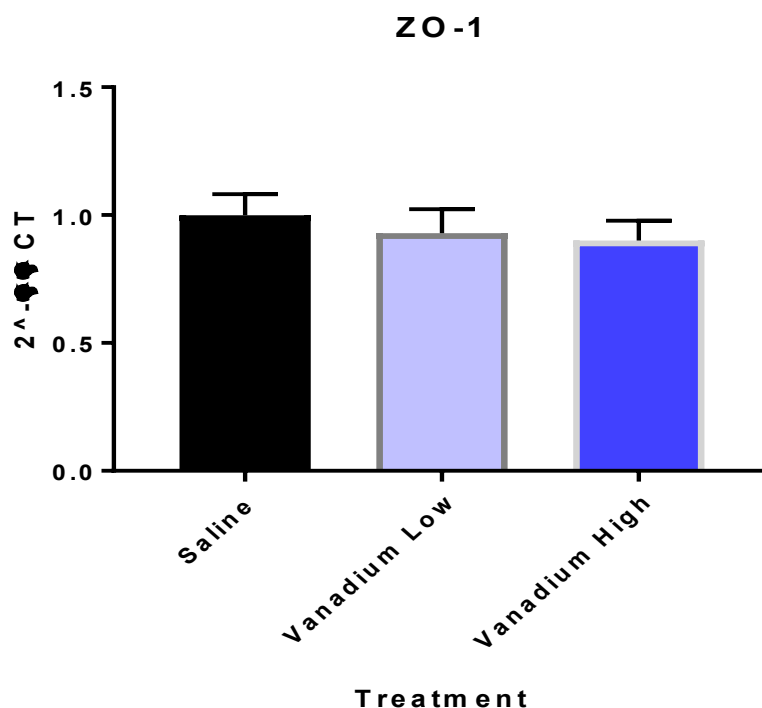


Figure 10. In vivo ZO-1 frontal brain cortex qPCR gene transcription levels of vanadium exposed mice. Mice were exposed to vanadyl sulfate (low dose: 0.025mg, 0.25mg high dose) via IP injection twice a day. Animals were sacrificed after 24 hours of vanadyl sulfate exposure. Control mice received saline IP injections twice a day. Saline group contained an n=4, low vanadium group n=5 and high vanadium group n=5. No significant difference was observed ($p>0.05$) using one-way ANOVA between saline and low and high vanadium treatment groups for ZO-1 mRNA levels.

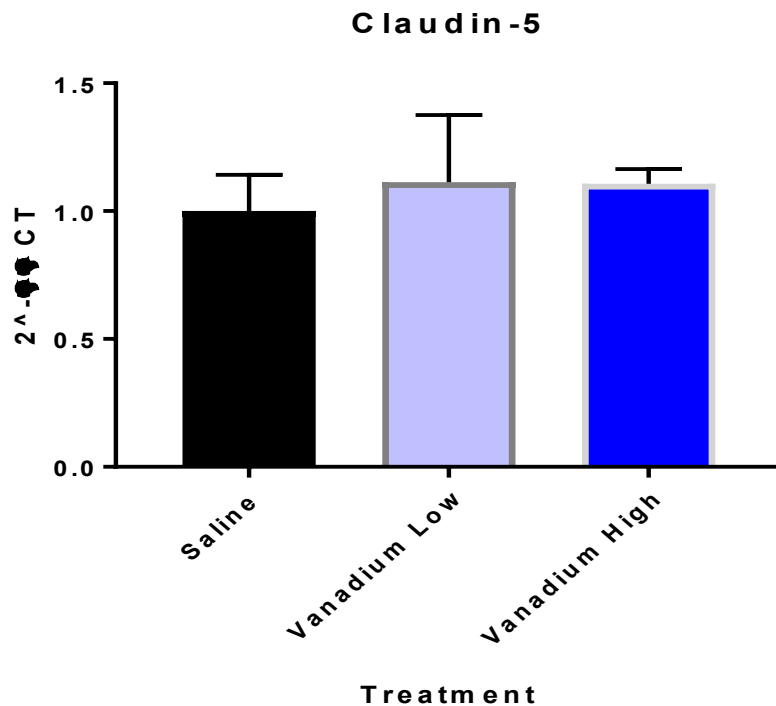


Figure 11. In vivo claudin-5 frontal brain cortex gene transcription levels of vanadium exposed mice. Mice were exposed to vanadyl sulfate (low dose: 0.025mg, 0.25mg high dose) via IP injection twice a day. Animals were sacrificed after 24 hours of vanadyl sulfate exposure. Control mice received saline IP injections twice a day. The control group contained an n=4, low vanadium group n=5 and high vanadium group n=3. No significant difference was observed ($p>0.05$) using one-way ANOVA between saline and low and high vanadium treatment groups for claudin-5 mRNA levels.

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Chapter 3. Discussion

This study identifies vanadyl sulfate as a possible therapeutic agent in BBB disruption. Or, perhaps more conservatively, these results suggest that the mechanism of action of vanadium – as yet uncertain – may provide a novel path towards BBB improvement in cerebrovascular disease. Our results show vanadyl sulfate increases electrical resistance in murine cerebrovascular endothelial cells. The addition of tempol and fasudil partially abrogated the enhanced electrical resistance from vanadyl sulfate exposure in endothelial cells. In mouse studies, intraperitoneal vanadyl sulfate injections did not alter pro-inflammatory factors TNF- α and ICAM-1. In fact, while underpowered, ICAM-1 expression may have been reduced, suggesting an anti-inflammatory effect of vanadium at this dose. A possible mechanism by which vanadyl sulfate is increasing electrical resistance is by increasing the tight junction protein occludin. Our results also show that vanadyl sulfate does not alter the mRNA for tight junction proteins claudin-5 and ZO-1.

The literature for vanadium mainly emphasizes its toxicologic effects, with less research done on investigating possible therapeutic benefits. The chemistry of vanadium has been related to the formation of intracellular reactive oxygen species (ROS). It is believed that the conversion of vanadate (V) to vanadyl (IV) inside cells can generate oxygen radicals and hydrogen peroxide (Capella et al, 2002). Other studies have associated vanadium with the generation of oxidative stress leading to lipid peroxidation that consequently affects neurons (Todorich et al, 2011). In this study, we demonstrate that the addition of tempol, a well-used

antioxidant, antagonized the vanadium-induced electrical resistance of cerebral vascular endothelial cells in our ECIS experiment. This suggests that at least a portion of the seemingly beneficial effect of vanadium does relate to the generation of ROS. Studies use tempol to combat the effects of generating ROS from agents such as arsenic, another metal that can decrease brain permeability (Bao et al, 2010). In our experiments, tempol did not increase the resistance of endothelial cells treated with different concentrations of vanadium suggesting that vanadyl sulfate was not creating reactive oxygen species (figure 4). Studies by (Matsubara et al, 1995) suggest that vanadate is an oxyradical scavenger.

Vanadium has been reported as an activator of rho kinases (Ito et al 2015, Mori et al, 2004). The activation of rho kinases in the BBB brings structural changes to the cytoskeleton and tight junction proteins (Shi et al 2013, Jiang et al). Our ECIS *in-vitro* experiments (figure 5) demonstrate that the addition of fasudil, a well-documented rho kinase inhibitor, does not have an effect on cells treated with vanadyl sulfate. This suggests that vanadyl sulfate at the specific doses used does not disrupt the BBB through phosphorylation of the cytoskeleton or tight junction proteins. Fasudil is a known rho kinase inhibitor, but it has also been reported as an MMP inhibitor (Ishiguro et al 2012, Deng et al 2010). The activation of MMPs increases the permeability of the BBB (Reijerkerk et al, 2006). This could also suggest that vanadyl sulfate is not activating MMPs, which have been link to disruption of tight junction proteins.

Vanadium has not only been associated with ROS, but also with the production of pro-inflammatory factors. The BBB can be disrupted when pro-

inflammatory factors are activated which, in turn, can activate neutrophils (Pun et al, 2009). Pro-inflammatory factors such as TNF- α and Egr-1 have been referenced as BBB disruptors. ROS can also indirectly activate ICAM-1 and VCAM-1 (Kim et al, 2008). Once ICAM-1 is activated, it can recruit WBC to the BBB and increase barrier permeability. When we exposed mice to vanadium via IP injections, the mice exposed to vanadyl sulfate did not have an increase in pro-inflammatory factors TNF- α and ICAM-1. This advocates for the idea that vanadium can be toxic depending on dose and length of exposure.

Tight junction proteins play a vital role in the regulation of electrical resistance in BBB endothelial cells. Tight junction protein disruption is associated with a decrease in BBB resistance. The data from figures 9-11 demonstrate that vanadyl sulfate does not decrease tight junction proteins. Vanadyl sulfate exposure increased the tight junction protein occludin, which is one of the possible mechanisms by which vanadium increases BBB resistance. Vanadyl sulfate at the appropriate dose has been indicated to be therapeutic and not toxic. The BBB is an important component of the brain that in many diseases such as stroke is disrupted. The BBB can increase in permeability when there is ischemia such as in the case of stroke, but the BBB can also be disrupted by the stroke treatment tPA. (Zhu et al, 2019). During a stroke, the BBB can be affected by multiple parameters including but not limited to tight junction proteins phosphorylation by rho kinases, pro inflammatory factors (TNF- α , interleukin-6) (Jiang et al 2018), cytoskeleton actin polymerization, (Jiang et al, 2018) and MMP activation (Rempe et al, 2016). Risk factors to stroke include hypertension

and diabetes. In hypertension, tight junction protein levels can decrease over time (Fan et al, 2015) and in diabetes BBB leakage has been documented (Fujihara et al, 2016). Our studies suggest a potential therapeutic effect of vanadyl sulfate that highlights novel pathways that could benefit diseases such as stroke. Moreover, treatment with vanadium may also help control blood glucose levels in patients with diabetes. In a clinical situation when a patient has a stroke, high blood glucose levels and receives tPA treatment, vanadyl sulfate could potentially restore glucose levels and BBB disruption. A more thorough elucidation of the intracellular pathways contributing to the improvements in endothelial barrier integrity following vanadium treatment will be needed to understand such therapeutic pathways.

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