THE SOLE EFFECT OF HYPOTHERMIA ON BLOOD-BRAIN BARRIER TIGHT JUNCTION PROTEIN EXPRESSION

By

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Abstract

While the use of hypothermia as an organ preservation technique has been around since the mid-20th century, its use in combination with cardiopulmonary bypass (CPB) did not gain popularity until 1975 when it was demonstrated to offer a practical and safe approach for aortic arch surgery. It has since been the cornerstone of successful cerebral protection during complex cardiovascular procedures. The use of deep hypothermia (<28°C) in deep hypothermic circulatory arrest (DHCA) procedures can prevent cerebral ischemic injury through reductions in both anaerobic glycolysis and uncontrolled excitatory neurotransmitter release, which normally cause cell damage and eventual death. Although these neuroprotective effects are well understood, neurological dysfunction is still the number one cause of morbidity and mortality following DHCA procedures, highlighting the need for improvements to increase cerebral protection.

The blood-brain barrier (BBB) provides precise regulation of CNS uptake of specific solutes required for maintenance of cerebral homeostasis while maintaining protection from harmful substrates that circulate in the blood. Tight junction (TJ) proteins, found between brain microvascular endothelial cells, significantly limit paracellular diffusion and prevent free exchange of compounds between the parenchyma and blood. Indeed, TJ protein complexes are critical in providing a “physical barrier” to solute permeation at the level of the cerebral microvasculature. Modulation of TJ protein complexes, via changes in expression or trafficking of constituent proteins, can cause increased BBB permeability (i.e., leak). The consequences of BBB leak include an increased ability of potentially toxic substances to accumulate in brain parenchyma and cause significant cellular damage to neurons. In fact, changes in BBB TJ
protein expression during deep hypothermic conditions alone has not yet been investigated. This highlights the need for a clinically translational model that can be used to study TJ protein expression during these drastic changes in body temperature.

The purpose of this study was to develop a clinically relevant rodent model for inducing deep hypothermia and to investigate changes in expression of transmembrane TJ proteins occludin and claudin-5. Animals were assigned to either control (CTRL), anesthesia (AN), or anesthesia and ice (AN+ICE) groups. Desired rectal temperature ranges were set at 33-31°C for AN group and 26-24°C for AN+ICE group for the Pilot Experiment and Experiment #1. AN group temperature range was adjusted to 37-35°C for experiment #2. Improvements to the model included: tighter control of active cooling the AN+ICE group, increased time within the assigned temperature ranges, implementation of EKG monitoring, control of respiration through ventilator use, verification of ventilator settings through arterial blood gas monitoring, and the use of ketamine/isoflurane combination as an anesthetic. Changes in expression of occludin and claudin-5 were evaluated via western blotting and subsequent densitometric analysis.

Results of western blot experiments showed significant decreases in occludin in both AN (p<0.05) and AN+ICE (P<0.01) groups when compared to CTRL following the pilot experiment. The significant decrease (p<0.05) in the AN+ICE group when compared to CTRL was observed following Experiment #1 and #2. The AN group showed no significant differences compared to CTRL following Experiment #1 and #2. Significant differences in claudin-5 expression were not detected following the Pilot Experiment and Experiment #1. A significant decrease in claudin-5 expression was observed in the AN+ICE group when compared to the AN group following Experiment #2.
In summary, we have successfully developed a rodent model of deep hypothermia that can be used for the in vivo study of brain microvascular changes that occur in response to clinically relevant changes in body temperature. Specifically, we have utilized this model to show that decreased body temperature causes measurable changes in occludin expression at the BBB. These observations form the basis for a more detailed examination of BBB dysfunction following deep hypothermia in an effort to understand how BBB injury relates to neurological complications following DHCA.

Introduction

Background-DHCA

Cardiovascular disease has been, and still is the number one cause of death worldwide, and is the primary reason as to why cardiovascular surgery is an ever-growing and ever-evolving field within healthcare\textsuperscript{1}. With modern advancements in healthcare, more people are living long enough to experience consequences of aging and poor health habits upon their own cardiovascular system, causing over 600,000 deaths in the U.S. alone\textsuperscript{1}. The advancement of surgeries performed on cardiovascular birth defects has led to an increase in the life-span of these pediatric patients as well, causing a higher demand for cardiovascular surgical procedures and an increased need to advance surgical practices to increase efficiency and safety for this subpopulation of patients. These factors together highlight the need to keep the field of cardiovascular surgery moving forward in terms of researching new techniques, as well as revamping old techniques to improve the quality of life for these patients.
The use of hypothermia in the surgical setting as an organ preservation technique has been around since the mid-20th century. In 1952, Dr. John Lewis used topical hypothermia in the first successful open-heart procedure to close a secundum-type atrial septal defect. Although the introduction of the heart-lung machine and implementation of cardiopulmonary bypass (CPB) was around this time, the combination of the two did not occur for a number of years. In 1955, Dr. Cooley performed the first successful aortic arch replacement in which they relied on CPB with an ascending-to-descending aortic shunt for abdominal and lower-extremity perfusion, as well as carotid artery side arms for cerebral perfusion. This technique however did not provide adequate cerebral protection, and the patient died 6 days postoperative due to neurological complications. This highlighted the need for a new technique that provided the necessary cerebral protection for recovery after complex operations of the aorta and aortic arch. The use of hypothermia and CPB together to facilitate aortic arch surgery began to appear in the 1960’s, however it wasn’t until 1975 that Griep demonstrated and published that this combination of techniques offered a practical and safe approach for aortic arch surgery. Since then, the induction of systemic hypothermia to preserve organ function while stopping circulation, termed deep hypothermic circulatory arrest (DHCA), has been the cornerstone technique used for cerebral protection during complex cardiovascular procedures.

The core process of deep hypothermic circulatory arrest is straightforward. Once CPB has been initiated, the core temperature of the patient is dropped to below 28°C (usually between 26-18°C) using the heart-machine, and the circulation is then completely arrested. This cooling of the tissue leads to a decrease in cellular metabolism, especially in the brain, which is critically important to reducing ischemic injury during the time that the circulation is
stopped. This lowering of cellular metabolism preserves high-energy phosphate stores, which protects organs from these periods of ischemia. The brain, while only accounting for two percent of body weight, accounts for twenty percent of total oxygen consumption and without the ability to store glucose, a shortage in delivery of these nutrients immediately impairs neuronal function. This is why at normothermia, significant cerebral injury occurs after only four minutes of circulatory arrest. There are two cellular pathways that lead to ischemic injury within the brain. The first is related to the production of lactate, caused by the formation of ATP through anaerobic glycolysis. This pathway is not only insufficient in maintaining adequate energy, but also lowers the intracellular pH due to lactic acid buildup, the combination of which quickly leads to permanent cell damage. The second relates to calcium ion imbalance, caused by uncontrolled release of excitatory neurotransmitters, which occurs in hypoxic conditions. Excitatory neurotransmitters can directly activate neuronal N-methyl-D-aspartate (NMDA) receptors allowing positively charged ions, mainly calcium, to enter the cell. Intracellular accumulation of calcium ions leads to activation of intracellular proteases and mitochondrial dysfunction, ultimately resulting in neuronal cell death. Hypothermia has been shown to provide neuroprotection by counteracting the pathophysiological effects of both of these pathways. Decreased body temperature has been shown to decrease metabolic demand, which lowers the rate of anaerobic metabolism and lowers lactate buildup, leading to decreased cellular acidosis. Furthermore, hypothermia significantly reduces temperature-dependent release of excitatory neurotransmitters and subsequent NMDA channel activation, thereby reducing the calcium ion accumulation intracellularly.
The reasoning for why this technique is needed is apparent after identifying the types of surgeries it is most commonly used for. In adults, the most common procedures requiring DHCA are done on the ascending aorta and aortic arch, such as in an aortic aneurism or dissection. There are also cases, such as in a heavily calcified aorta, where the application of the cross clamp is at an increased risk of causing a thromboembolic event. In pediatrics it is used more frequently, as there are a number of complex congenital defect surgeries that require this technique, such as for the Norwood operation, which involves manipulation of both ventricular outflow tracts, as well as enlargement of the aorta\(^8\). Overall, the reasoning behind using DHCA in these procedures is to provide the surgeon with a clear, motionless, and bloodless field to work on\(^2,4,9\). In the case of both an aortic dissection and Norwood operation, the surgeon needs to be able to replace part or all of the aortic arch, an objective that is virtually impossible if the patient is on normal bypass based on cannulation sites and the path of blood, shown in Figure 1\(^1\). This is why the patient is cooled and circulation is stopped, so the surgeon can work on the aorta without blood being pumped through it to the rest of the body.

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\(^1\) Reprinted from Surgery of the Chest, 6th Ed, John A. Waldhausen et al, Cardiac Support Techniques, pg 285, Acquired Heart Disease, pg 561, 1996, with permission from Elsevier.
While the core process of DHCA remains constant, there are a number of techniques that have been developed to protect the brain during these circulatory arrest periods. Straight DHCA was the first to be used, and is still a popular option for some institutions today to provide cerebral protection during aortic arch and complex congenital defect surgeries. As the term implies, straight DHCA adds no adjunctive cerebral perfusion, and relies on hypothermic conditions, maintained by the heart-lung machine and topical cerebral cooling, to provide cerebral protection during the circulatory arrest period\(^2\). The most apparent benefit is the undisputed simplicity, and the ability to perform the procedure without added cannula placement\(^2\). While simple, the fact that there’s no additional cerebral support means circulatory arrest durations longer than 40 minutes are more likely to cause neurological

**Figure 1:** A diagram of normal CPB cannulation without (A) and with (B) placement of the aortic cross-clamp. The ascending aorta is cannulated so that blood is pumped through all aortic arch vessels and the descending aorta, as well as down the coronary ostia prior to and after cross-clamp application\(^{10}\).
damage, and some groups have described its use only be implemented in safe and easy cases lasting less than 30 minutes\textsuperscript{11}. Studies that have compared straight DHCA to techniques where adjunctive cerebral protection has been employed have found no differences in outcomes and mortality rates\textsuperscript{2,12,13}. Overall, DHCA has considerable utility when used carefully and appropriately in a surgical setting. That is, surgeons must be cognizant to ensure that circulatory arrest is not maintained for lengthy times in order to limit the degree of neurological damage\textsuperscript{13}.

The idea to utilize adjunctive cerebral protection during DHCA was developed due to the fact that neurological deficits following aortic arch operations were still relatively high. These continuous flow methods were developed to enhance cerebral protection by delivering oxygen and nutrients to the brain while under circulatory arrest, so as to protect those parts of the brain still functioning during hypothermia\textsuperscript{14}. The first and most popular of these approaches is termed Retrograde Cerebral Perfusion (RCP)\textsuperscript{14}. In this technique, cooled oxygenated blood is slowly pumped into the superior vena cava (SVC) and travels retrograde through the venous system back into the arterial system, supplying the brain with both oxygen and glucose while the rest of the body undergoes circulatory arrest\textsuperscript{5}. As opposed to straight DHCA where the type of cannula placement does not matter, bi-caval venous cannulation is critical for RCP as the cannula placed in the SVC is then used for cerebral flow\textsuperscript{9}. The main proponents for RCP identify the advantages to include i) uniform brain cooling; ii) de-airing of the arch vessels; iii) the capability of flushing both cerebral emboli and toxic metabolites; and iv) the provision of oxygen and nutrients\textsuperscript{15}. However, these claims are supported by multiple controversial findings, and are based on the assumption that cerebral veins lack valves that impede
retrograde flow\textsuperscript{4}. RCP has been shown to extend the safe time under circulatory arrest out to 60-80 minutes, so for cases that cannot be achieved within the 40 minutes with straight DHCA, one of the options that should be considered is retrograde cerebral protection\textsuperscript{4,15}.

The third popular cerebral protection technique, and the other continuous flow technique, is termed antegrade cerebral perfusion (ACP). In this system, cerebral protection is provided again through the slow pumping of cold oxygenated blood, but via the normal physiologic pathway through the arteries and out through the veins. While cannulation for RCP is limited to the SVC, cannulation for ACP can occur at several sites. A method that has proven simple, reproducible, and safe is through the direct cannulation of the innominate artery with a balloon-tipped arterial cannula\textsuperscript{16}. Alternatives include cannulation of either the right or both carotid arteries while clamping the left subclavian, cannulation of the axillary artery, or via the side branch of an arch graft\textsuperscript{4}. While it is agreed that bi-caval venous cannulation is important, it is used in ACP for the purpose of ensuring adequate SVC venous drainage to prevent cerebral edema and safeguard acceptable cerebral perfusion pressure\textsuperscript{9}. Use of this cerebral perfusion technique provides a more reliable global cerebral oxygenation than RCP, helps maintain cerebral cooling, and has been shown to require less hypothermia than both previously mentioned techniques\textsuperscript{4}. This technique has also been shown to increase safe circulatory arrest time by a larger margin than RCP (90 minutes compared to 60-80 minutes), and has also shown to result in a lower incidence of transient brain dysfunction (TBD) compared to RCP\textsuperscript{17}. Some of the problems identified with ACP include its increased complexity leading to longer operation times and increased risk of cannula kink or displacement, the need for an intact Circle of Willis
for even blood distribution, and an increased risk of embolization (although ACP is not associated with an increased risk of stroke)⁴.

Although DHCA has allowed for countless lives to be saved, it subjects the body to a highly unnatural state, leading to a number of physiologic changes and reactions to both the hypothermia, and the circulatory stasis, shown in Figure 2, for which the solutions have not been found

**Figure 2:** A schematic showing potential cellular and systemic mechanisms of injury during DHCA

While all physiological sequelae from DHCA are important to understanding injuries received following these procedures and bettering outcomes, the predominant cause of morbidity and mortality post-DHCA remains cerebral complications. It has also been demonstrated to increase risks of neurocognitive abnormalities in children with complex congenital heart abnormalities. There are two distinct types of neurological injury that are caused by surgeries requiring circulatory arrest: localized strokes caused by embolic events, and
a complex of symptoms collectively known as TBD\textsuperscript{17}. While not associated with any structural abnormality in the brain, they are associated with confusion, agitation, delirium, prolonged obtundation, and parkinsonism without localizing neurological signs\textsuperscript{19}. These TBD’s, although temporary, have been found to be suggestive of long-term neurological deficits, and increased frequency of occurrence has a direct correlation to the length of circulatory arrest, as well as the patient’s age\textsuperscript{20}. While the neuroprotective effects of hypothermia are well understood, the fact that nearly ten percent of DHCA patients develop some neurological deficit highlights the remaining need for advancements in cerebral protection during circulatory arrest procedures\textsuperscript{20}.

**Background-Blood-Brain Barrier**

The blood-brain barrier (BBB) is both a biochemical and physical barrier that exists at the level of the cerebral microvasculature, and separates the CNS from the systemic circulation\textsuperscript{21,22}. A number of efflux transporters, including P-gp and Multidrug Resistance Proteins, that are highly expressed at the luminal membrane of these endothelial cells transport a range of structurally diverse compounds out of the CNS, and contribute significantly to the BBB biochemical properties\textsuperscript{21}. In contrast, SLC transporters allow the selective uptake of endogenous and exogenous substances across the BBB via carrier-mediated transport\textsuperscript{21}. This selectivity, in combination with the diversity of substrates transported by efflux transporters, significantly limit the number of compounds in circulation that can accumulate in the brain parenchyma through transcellular means.
Brain microvessels exhibit very low permeability when compared to microvessels outside of the CNS\textsuperscript{22}. This can be attributed partially to the lack of fenestrations in the microvasculature, and low rates of pinocytosis; however, it is mostly due to the tight junction (TJ) protein complexes that are found between endothelial cells\textsuperscript{22}. These multiprotein complexes form a seal between endothelial cells that contributes to a high transendothelial electrical resistance, which greatly restricts the paracellular permeability of the BBB\textsuperscript{23}. Proteins that comprise these TJ complexes include occludin and claudins, junctional adhesion molecules (JAMs), and transmembrane proteins attached to accessory proteins (zonula occludens [ZO]) that link to the cytoskeleton, depicted in Figure 3\textsuperscript{2}.

\textbf{Figure 3}: Basic molecular organization of tight-junction protein complexes at the blood–brain barrier. ZO=Zona Occludens\textsuperscript{24}.

\textsuperscript{2} Reproduced from Therapeutic Delivery. (2011) 2(8), 1015-1041 with permission of Future Science Group.
Monomeric occludin is a 65 kDa transmembrane protein that is consistently found along endothelial cell margins in the cerebral microvasculature\textsuperscript{24}. The c-terminal domain, found in the cytoplasm, is likely involved in the association of occludin with the cytoskeleton via ZO (-1, 2, and 3) proteins and likely intracellular communication\textsuperscript{24}. This region is also capable of disulfide bonding, allowing the formation of dimers and other oligomeric structures that are essential in TJ permeability limitation and intracellular signaling\textsuperscript{24}. Occludin also contains a number of phosphorylation and ubiquitination sites that allow for intricate regulation in response to stresses, such as oxidative stress caused by ischemic stroke\textsuperscript{22,25}. Occludin null mice are viable and have no apparent barrier dysfunction; however, the neuropathological finding of progressive mineral deposition in the basal ganglia and cerebellum as well as along small cerebral vessels (i.e., arterioles, venules) is characteristic of the occludin knockout phenotype\textsuperscript{44}. This seminal study emphasizes the critical role of occludin in maintaining functional integrity at the BBB.

While there are 27 different claudin genes in, all in the range of 20-24 kDa proteins, it is likely that at the BBB claudin-1, 3, 5, and 12 proteins are expressed, with claudin-5 thought to be the predominant isoform\textsuperscript{22}. Since claudin expression in fibroblast cells can reconstitute TJ strands, in comparison to occludin which will only localize to TJs in fibroblasts after claudin expression is established, it is hypothesized that claudins form the primary seal of the TJ\textsuperscript{22,24}. Claudin-5 knockout mice show normal development and morphology of cerebral microvessels; however these animals do show significant increases in BBB leak to small molecules (i.e., <800 Da) suggesting a critical role for claudin-5 in conferring barrier properties to the cerebral microvasculature\textsuperscript{22}. More recently, antibodies directed against claudin-5 were shown to
enhance paracellular delivery of small molecules, further demonstrating the paramount role for claudin-5 in forming the physical seal of the TJ\textsuperscript{45}.

It is noteworthy that the integrity of the BBB is directly linked to CNS protection from toxins in systemic circulation, and BBB disruption, especially TJ disturbances, can lead to significant damage to the brain parenchyma. One example of this is observed in diseases with a hypoxia/reperfusion injury (H/RI) component, such as ischemic stroke. These pathophysiological changes include increased cerebrovascular permeability and leak, activation of cell death mechanisms, autoimmune system responses, activation of the complement system, infiltration of inflammatory cells, and an increase in the number of reactive oxygen species (ROS)\textsuperscript{26}. Oxidative stress causes changes in TJ protein organization and localization, contributing to endothelial dysfunction and increased BBB permeability\textsuperscript{26}. Studies have shown oxidative stress caused by H/RI can significantly alter the structure and localization of oligomeric occludin TJ assemblies, which showed increased BBB permeability\textsuperscript{26}. It has also been observed that acute (60 minute) hypoxia, followed by acute (10 minute) reoxygenation leads to increases in phosphorylated occludin, with no changes in claudin-3 or ZO-1\textsuperscript{22}. Vasogenic edema following ischemia/reperfusion, in part caused by these TJ disruptions, leads to extravasation of plasma proteins and eventual fluid accumulation in the brain parenchyma, causing increased brain volume and intracranial pressure\textsuperscript{26}. This, in combination with the loss of protection from potentially neurotoxic endogenous and exogenous substances normally restricted from the parenchyma, lead to increased CNS damage. In conclusion, injury models where TJ complexes are altered, such as H/RI seen in ischemic stroke, show BBB permeabilization which leads to exacerbation of brain tissue damage\textsuperscript{26}. In pathologies where neurological deficits are observed,
BBB integrity should be investigated as a potential cause of injury, especially in models where ischemia/reperfusion injury could be a component.

**Significance of Study**

Although the neuroprotective effects of hypothermia are well understood, the reaction of the BBB to significant hypothermic conditions, as typically occur during DHCA, has yet to be investigated. While there are literature reports highlighting experiments that have investigated BBB protein expression in hypothermic conditions, these experiments utilized decreased body temperature to protect against CNS damage in injury models. For example, in a rodent intracerebral hemorrhage injury model, anesthesia-induced mild hypothermia (33°C) attenuated the decrease in occludin and claudin-5 mRNA and protein expression seen in normothermic injury animals. These changes coincided with an attenuation of BBB permeability seen in normothermic injury animals. Similarly, mild hypothermia (33°C) in the setting of cardiopulmonary resuscitation (CPR) in swine was shown to attenuate significant decreases in occludin, claudin-5, and ZO-1 mRNA and protein expression, that were observed in normothermic CPR animals. This same study demonstrated that attenuation of decreases in TJ proteins was associated with a reduced level of cerebral edema, a disease marker associated with BBB disruption. In a rat model of global cerebral ischemia, mild hypothermia (32°C) was shown to attenuate the loss of vascular basement membrane proteins including agrin and SPARC in both mRNA and protein expression that was observed in normothermic injury animals, which also led to a decrease in permeability. It is important to note that in these experiments: a) hypothermia was induced either at the time of injury, or after the injury and b) only mild hypothermia was used. These are important distinctions because, first and foremost,
DHCA involves cooling the body to temperatures below 28°C. It is also essential to consider that DHCA requires cooling of the body prior to initiation of the ischemia/reperfusion injury caused by the circulatory arrest. In terms of identifying hypothermia’s effect on the BBB from these studies, it is not known whether these changes are the result of a direct effect on the BBB, or if the observation was a decreased severity of the injury due to lowered metabolism and blood flow.

Few studies have investigated effects of hypothermia alone on BBB function. Anesthesia-induced mild hypothermia (32°C) was shown to decrease the BBB disruption seen following hyperosmolar challenge in rats when compared to normothermic animals. In contrast, another study found that mild hypothermia (33-32°C) caused an increase in albumin positive cells in the cortex, indicating an increase in BBB permeability. Experiments investigating changes in protein expression at the BBB during deep hypothermic conditions in a non-injury model are lacking. This, coupled with the fact that the leading cause of morbidity and mortality following DHCA procedures remains neurocognitive deficit, highlights the need for an understanding of BBB integrity during this extreme change in body temperature.

The purpose of this project was two-fold. The main purpose was to design a clinically translational model of hypothermia in rats that allowed for investigation into protein expression changes at the BBB during deep hypothermic conditions. Animals were to be cooled to deep hypothermic (<28°C) temperatures without additional circulatory support. The model was designed so that hypothermia’s individual effect on the BBB could be investigated prior to use in conjunction with an injury model. The duration and depth of hypothermia was to be tightly regulated, and it was to be simple enough to eventually use in combination with an
MCAO injury model to induce a DHCA simulation. The model was developed in stages, first addressing cooling and maintenance of core temperatures within designated ranges for a given time while ensuring the animal’s survival. It was then updated to improve upon its clinical relevance, using physiological monitoring and control to confirm hypothermia as the only variable between groups.

The second purpose of the project was to investigate changes in transmembrane TJ protein expression at each stage in the development of the model. Occludin and claudin-5 were identified as the critical TJ proteins of interest, due to their connection with increased BBB permeability upon changes in expression.

**Methodology**

*Animals and Procedures - Pilot Experiment*

Animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC) and were designed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (NIH). Female Sprague-Dawley rats (230-300 g; three-four months old; Envigo, Denver, CO) were used for all experiments. Rats were randomly assigned to one of three experimental groups: control (CTRL), anesthesia (AN), or anesthesia and ice (AN+ICE). For the Pilot Experiment and Experiment #1, all animals were anesthetized (100 mg/kg ketamine, 20 mg/kg xylazine, i.p.) and CTRL animals were immediately prepared for brain microvessel isolation. For the Pilot Experiment, animals in the remaining two experimental groups were sedated for 60 minutes with two maintenance doses (50 mg/kg ketamine, 10mg/kg xylazine i.p.) administered every 20 minutes. AN animals
were placed on blue laboratory pads and their rectal temperature allowed to drift to 33-31°C. AN+ICE animals were placed on ice pack covered with paper towel to prevent skin damage. Animals remained on the ice pack until a rectal temperature of 26°C at which point they were placed on blue lab pads to remain within 26-24°C. Rectal temperatures were to be recorded every 10 minutes. The goal was to reach an average rectal temperature within the designated range over the last 30 minutes. After the 60-minute time course, animals were prepared for brain microvessel isolation according to our published method\textsuperscript{33}.

**Procedures-Experiment #1**

For Experiment #1, animals in the two experimental groups were sedated for 90 minutes with three similar maintenance doses every 20 minutes for the first 60 minutes. Animals in each group followed a similar experimental protocol to the Pilot Experiment, with the exception that AN+ICE animals were removed from the ice pack at 28°C rectal temperature, and allowed to drift to 26-24°C. Rectal temperatures were to be recorded every 15 minutes. The goal was to reach an average rectal temperature within the designated range over the last 60 minutes.

**Procedures-Experiment #2**

All animals were given a 50 mg/kg ketamine, 10mg/kg xylazine i.p. injection followed by 5% inhaled isoflurane to induce anesthesia. Anesthesia was maintained by inhaled isoflurane at 1.5%. Following sedation, animals were placed supine on the Indus Instruments (Houston, TX) MouseMonitor™ S surgical platform with electrode gel applied to all paws and taped to the platform leads. The rectal temperature probe was then inserted and taped in place. Nair was
used to remove the hair from the neck and chest area prior to surgery. Animals then underwent a tracheostomy procedure as previously described with few modifications. Briefly, a midline neck incision was made with scissors and the skin was removed. The pretracheal muscles were pulled apart with the use of forceps and a mosquito forceps. The trachea was then dissected from the tissue underneath to increase visibility and mobility. A #0 surgical silk suture was passed underneath the trachea and kept distal to the anticipated incision site. A small cut was made in the trachea, and the 2.3mm O.D. tracheal cannula (Harvard Apparatus) was inserted, with the suture used to secure the cannula in place. The surgery was performed on the MouseMonitor surgical pad with the heater set at 40.7°C to maintain normothermia throughout the surgery. Ventilator settings were determined using the equations in Figure 4.

### Ventilator settings:

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{T}=6.2 \times M^{1.01}$</td>
<td>Tidal volume (mL)</td>
</tr>
<tr>
<td>$M=mass \ (kg)$</td>
<td></td>
</tr>
<tr>
<td>$RR=53.5 \times M^{-0.26}$</td>
<td>Respiratory rate (min$^{-1}$)</td>
</tr>
</tbody>
</table>

**Figure 4:** Equations used for ventilator parameters used in Experiment #2, with abbreviations defined.

PEEP was maintained at a low setting (2 mmHg). After initiation of the Harvard Apparatus Inspira asv ventilator, baseline heart rate and rectal temperature were recorded. CTRL animals then immediately underwent arterial blood gas sampling, as previously described. Samples were read using an ABL77 blood gas analyzer (Radiometer). Following the blood draw, animals underwent the same MVI protocol. AN animals were kept on the surgical pad for continuous EKG monitoring for 75 minutes. The goal was to keep this group normothermic (37-35°C), so the heating pad was kept at 40.7°C. AN+ICE animals were cooled as in previous experiments to a temperature of 26°C. The animal was then moved to the surgical for continuous EKG monitoring. The heating pad was also turned off to maintain the hypothermic temperatures.
Heart rate and rectal temperature were recorded every 15 minutes, with the goal that the average temperature be within the designated ranges for the last 60 minutes.

**Microvessel Isolation**

Microvessel isolation was performed as previously described\textsuperscript{33}. Following euthanasia by decapitation, the brain was harvested and the meninges and choroid plexus were removed. The isolated cerebral cortex was homogenized in 5 mL of microvessel buffer containing protease inhibitor cocktail (Sigma-Aldrich). Homogenates were mixed with 8 mL of 26% dextran and centrifuged at 5000 \( g \) for 15 minutes at 4°C. The supernatant was aspirated and the capillary pellet was resuspended in 5 mL of microvessel buffer. After resuspension, 8 mL of 26% dextran was again added and underwent the same centrifugation step. After aspiration of this supernatant, the capillary pellet was resuspended in 5 mL of microvessel buffer and centrifuged at 150,000 \( g \) for 60 min at 4°C. Pellets now containing total cellular membranes were resuspended in 400 \( \mu \)L of storage buffer (50% microvessel isolation buffer: 50% diH\( \text{H}_2\text{O} \), v/v) with protease inhibitor cocktail, and stored at -80°C.

**Western Blot Analysis**

Western blotting was performed as previously described\textsuperscript{34}. The microvessel fraction samples were quantified for total protein using Bradford reagent (Sigma-Aldrich) and heated at 37°C for 30 min under non-reducing conditions in 1 × Laemmli sample buffer (Bio-Rad, Hercules, CA). Following SDS-PAGE and transfer, PVDF membranes were incubated overnight at 4°C with primary antibodies against occludin (Rabbit IgG polyclonal, Thermo Fisher, 1:4000), claudin-5 (Mouse IgG1 monoclonal, Thermo Fisher, 1:3000), GAPDH (Rabbit IgG polyclonal, Abcam,
1:2500), α-tubulin (Mouse IgG1 monoclonal, Abcam, 1:20,000), and Na+/K+-ATPase (Mouse IgG1 monoclonal, Abcam, 1:40,000). Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, 1:40,000 dilution) or anti-mouse IgG (Jackson ImmunoResearch, 1:40,000 dilution) for 60 min at room temperature. Membranes were developed using enhanced chemiluminescence (Super Signal West Pico, Thermo-Fisher). Bands were quantitated using ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD) and normalized to either the strongest control band (Pilot and Experiment #1) or normalized to Na+/K+-ATPase (Experiment #2).

Statistical Analysis

Western blot data are reported as mean ± SEM, where each group consisted of 4 individual animals (n=4) per experiment. Statistical significance was determined using one-way ANOVA followed by post hoc multiple-comparison Bonferroni t-test. A value of p < 0.05 was accepted as statistically significant.

Results

Pilot Experiment

Temperature data for this experiment is shown in Table 1. Animals in both experimental groups maintained average rectal temperatures within the designated range for the last 30 minutes of the experiment. It should be noted that the AN+ICE temperature average was below the designated range at the last timepoint, and markedly decreased heart rate and respiratory rate were observed in all four animals; however, all animals survived through the time course.
<table>
<thead>
<tr>
<th>AN Average Rectal Temp (°C)</th>
<th>AN+ICE Average Rectal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline 37.0</td>
<td>Baseline 35.2</td>
</tr>
<tr>
<td>10’ 35.4</td>
<td>10’ 31.7</td>
</tr>
<tr>
<td>20’ 34.1</td>
<td>20’ 28.3</td>
</tr>
<tr>
<td>30’ Avg Temp: 32.3</td>
<td>30’ 25.0</td>
</tr>
<tr>
<td>40’ 32.6</td>
<td>40’ -</td>
</tr>
<tr>
<td>60’ Avg Temp: 24.2</td>
<td>60’ 23.3*</td>
</tr>
</tbody>
</table>

**Table 1**: Temperature data from the Pilot Experiment. An average temperature goal for the last 30 minutes was set between 33-31°C for AN animals to represent anesthesia-induced hypothermia. The AN+ICE animal temperature range was set between 26-24°C to represent actively cooled deep hypothermic temperatures. Averages are from four animals per group (n=4) per timepoint. * indicates an out-of-range temperature.

**Figure 5**: Results of western blot analysis of (a) monomeric occludin (65 kDa) and (b) claudin-5 (20 kDa) following the Pilot Experiment. Each lane represents one animal, with four animals per group (n=4). GAPDH (37 kDa) and Tubulin (50 kDa) were used as loading controls. The results are expressed as mean ± SEM. * indicates p<0.05. ** indicates p<0.01.
Figure 5 a and b represent western blot results and analysis for occludin and claudin-5 respectfully. Occludin showed a significant decrease in expression in both the AN (p<0.05) and AN+ICE (p<0.01) groups when compared to CTRL (Fig 5a). No significant difference was seen between the two experimental groups when compared. Claudin-5 showed no changes in expression level between the three groups (Fig 5b).

Experiment #1

The temperature data for this experiment is shown in Table 2. The goal of extending the average temperature in the given range to the last 60 minutes was achieved for both experimental groups. It should be noted that AN animals were placed on heating pads at 60 minutes to increase core temperature back into the specified range. Additionally, all AN+ICE animals were taken off the ice block at the 30-minute timepoint and this time were intermittently cooled to the desired temperature range, producing a more gradual and less visually pronounced decrease in heart rate and respiratory rate.

<table>
<thead>
<tr>
<th>AN Average Rectal Temperature (°C)</th>
<th>AN + ICE Average Rectal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Baseline</td>
</tr>
<tr>
<td>37.0</td>
<td>37.1</td>
</tr>
<tr>
<td>15’</td>
<td>35.2</td>
</tr>
<tr>
<td>15’</td>
<td>31.6</td>
</tr>
<tr>
<td>30’</td>
<td>32.4</td>
</tr>
<tr>
<td>30’</td>
<td>28.5*</td>
</tr>
<tr>
<td>Avg Temp:</td>
<td>Avg Temp:</td>
</tr>
<tr>
<td>45’</td>
<td>31.7</td>
</tr>
<tr>
<td>45’</td>
<td>26.5*</td>
</tr>
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<td>26.0</td>
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<td>75’</td>
<td>25.0</td>
</tr>
<tr>
<td>90’</td>
<td>32.5</td>
</tr>
<tr>
<td>90’</td>
<td>24.6</td>
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</tbody>
</table>

Table 2: Temperature data from Experiment #1. Similar designated temperature ranges were used for both groups as seen in the Pilot Experiment, with the goal time extended to 60 minutes. Averages are from four animals per group (n=4) per timepoint. * indicates an out-of-range temperature.
Figure 6 a and b represent western blot results and analysis for occludin and claudin-5 respectfully. Occludin showed a significant decrease in expression in the AN+ICE (p<0.05) group when compared to CTRL (Fig 6a). No significant difference was observed between the AN group and CTRL group, nor between the two experimental groups when compared. Claudin-5 again showed no changes in expression level between the three groups (Fig 6b).

**Figure 6**: Results of western blot analysis of (a) occludin (65 kDa) and (b) claudin-5 (20 kDa) following Experiment #1. Each lane represents one animal, with four animals per group (n=4). GAPDH (37 kDa) and Tubulin (50 kDa) were used as loading controls. The results are expressed as mean ± SEM. C=claudin-5. * indicates p<0.05.

**Experiment #2**

The temperature data for this experiment is shown in Table 3. The goal of maintaining the average temperature in the given range for the last 60 minutes was achieved for both
experimental groups while decreasing the overall experimental duration to 75 minutes. A reminder the new designated temperature range for the AN group for this experiment was 37-35°C.

<table>
<thead>
<tr>
<th>AN Average Rectal Temperature (°C)</th>
<th>AN + ICE Average Rectal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>36.0</td>
</tr>
<tr>
<td>15’</td>
<td>35.7</td>
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<tr>
<td>30’</td>
<td>Avg Temp: 35.4</td>
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<tr>
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<td>35.2</td>
</tr>
<tr>
<td>60’</td>
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<tr>
<td>75’</td>
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<tr>
<td>75’</td>
<td>26.0</td>
</tr>
</tbody>
</table>

Table 3: Temperature data from Experiment #2. The designated temperature range for the AN group was set between 37-35°C to represent normothermia. AN+ICE temperature range was kept the same, and the average temperature goal maintained at 60 minutes. Averages are from four animals per group (n=4) per timepoint. * indicates an out-of-range temperature.

Heart rate data is shown in Table 4. Average weight was included to show no significant difference in ventilator settings between groups. No significant differences in baseline heart rate were observed between the three groups. AN animals maintained a steady average heart rate just above 200 BPM for the 75-minute time course. AN+ICE animals experienced a statistically significant (p<0.01) decrease in heart rate following initiation of cooling, and remained consistent once the temperature reached the designated range.

<table>
<thead>
<tr>
<th>AVG±SD</th>
<th>Weight (g)</th>
<th>Baseline HR</th>
<th>15’</th>
<th>30’</th>
<th>45’</th>
<th>60’</th>
<th>75’</th>
</tr>
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<tbody>
<tr>
<td>CTRL</td>
<td>243</td>
<td>202±32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AN</td>
<td>243</td>
<td>225±20</td>
<td>207±13</td>
<td>205±10</td>
<td>209±8</td>
<td>211±9</td>
<td>214±6</td>
</tr>
<tr>
<td>AN+ICE</td>
<td>240</td>
<td>232±29</td>
<td>137±28**</td>
<td>117±23***</td>
<td>115±26***</td>
<td>115±26***</td>
<td>119±27***</td>
</tr>
</tbody>
</table>

Table 4: Heart rate results from Experiment #2. Heart rate (HR) units used were beats per minute. Each mean ± SD is representative of four animals per group. At each timepoint, HR averages from AN and AN+ICE groups were compared for differences. ** indicates p<0.01. *** indicates p<0.001.
Arterial blood gas results are shown in Table 5. No significant differences between the three groups were found in the major blood gas measurements including: pH, pO$_2$, pCO$_2$, HCO$_3^-$, Na$^+$, and K$^+$. Un-ionized Ca$^{2+}$ was shown to be significantly increased (p<0.05) in the AN group when compared to CTRL and AN+ICE groups.

<table>
<thead>
<tr>
<th></th>
<th>AVG±SD</th>
<th>pH</th>
<th>pO$_2$</th>
<th>pCO$_2$</th>
<th>HCO$_3^-$</th>
<th>HCT</th>
<th>Na$^+$</th>
<th>K$^+$</th>
<th>Ca$^{2+}$</th>
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<tbody>
<tr>
<td></td>
<td>(mmHg)</td>
<td>(mmHg)</td>
<td>(mmol/L)</td>
<td>(%)</td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>7.52±0.08</td>
<td>257±30</td>
<td>29±6</td>
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<td>35±1.8</td>
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<tr>
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<td>33±5</td>
<td>23.4±2.1</td>
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<td>140±1.1</td>
<td>5.0±0.3</td>
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<tr>
<td>AN+ICE</td>
<td>7.50±0.07</td>
<td>252±41</td>
<td>27±7</td>
<td>20.7±1.8</td>
<td>36±2.3</td>
<td>139±2.9</td>
<td>4.9±0.1</td>
<td>1.23±0.04</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Arterial blood gas results collected after completion of the 75-minute timecourse from Experiment #2. HCT=Hematocrit. Each mean ± SD is representative of four animals per group. * indicates p<0.05.

Figure 7a and b represent western blot results and analysis for occludin and claudin-5 respectfully. Occludin showed a significant (p<0.05) decrease in expression in the AN+ICE group when compared to CTRL (Fig 7a). No significant difference was observed between the AN group and CTRL group, nor between the two experimental groups when compared. There was also no significant difference scene in occludin dimer expression between the three groups. Claudin-5 showed a significant decrease in AN+ICE group expression when compared to AN group expression but not with the CTRL group (Fig 7b).
The use of hypothermia in cardiac surgery remains widespread, and has played an important part in improving clinical outcomes for this patient population. While the use of deep hypothermia is primarily restricted to circulatory arrest cases, it allowed for complex cardiac cases to be performed with high success rates that were not previously attainable. Although this technique has greatly improved surgical success rates, deep hypothermia is not without

**Figure 7**: Results of western blot analysis of (a) occludin (65 kDa) and (b) claudin-5 (20 kDa) following Experiment #2. Each lane represents one animal, with four animals per group (n=4). Na⁺/K⁺-ATPase (95 kDa) was used as a loading control. The results are expressed as mean ± SEM. Cl=claudin-5. * indicates p<0.05.
limitations and negative sequelae on the body (Figure 2). A common occurrence following DHCA is acute kidney injury and renal dysfunction, due to hypoxic conditions combined with systemic metabolic acidosis and hyperglycemia that occur following circulatory arrest\textsuperscript{4,35}. Another prevalent problem following this procedure is perioperative bleeding associated with hypothermia-induced coagulopathies. This is a complex, multifactorial process that involves kinetic factors, kinin/kallikrein perturbations, platelet dysfunction, and imbalance between hemostatic systems in response to the hypothermia\textsuperscript{36}. Although these complications have a major impact on patient outcomes, neurological protection remains a top priority because neurocognitive deficit remains the leading cause of injury and death following such procedures\textsuperscript{20}. While adjunctive cerebral protection strategies have been implemented to address this continued problem, and both RCP and ACP have increased the duration of safe circulatory arrest time, continued efforts need to be made to improve neuroprotection during these circulatory arrest periods.

The BBB serves as the major defense mechanism for the brain parenchyma against potentially harmful substances that may be present in the systemic circulation. The ability to tightly regulate uptake of essential solutes while providing a barrier to potential neurotoxins is essential for proper CNS function. TJ proteins serve to greatly limit paracellular diffusion between cerebral microvascular endothelial cells. Changes in expression levels of these proteins during injury, such as seen in ischemic stroke, cause enhanced BBB permeabilization and increased cerebral edema and intracranial pressure, factors that are well known to exacerbate neurological injury. Occludin and claudin-5 serve as two critical transmembrane TJ proteins that form the physical seal between these endothelial cells, and changes in expression
have been seen during ischemic injury models. While the neuroprotective mechanisms of deep hypothermia are well characterized, the consequences of this drastic temperature change on the BBB are yet to be understood. This lack of information, combined with the unsatisfactory cerebral protection during DHCA highlights the need for investigation into this possible pathway of neurologic injury.

_Hypothermia Model Development_

The goal of this study was to develop a clinically relevant model of deep hypothermia in rats that would allow investigation into BBB changes at hypothermic temperatures. The first question that needed to be answered was whether these animals could survive at deep hypothermic (<28°C) temperatures without additional circulatory support. We found that animals could tolerate changes in core temperature down to a range of 26-24°C. Since these temperatures are achieved in humans undergoing DHCA, we selected this deep hypothermia for our AN+ICE group. It is important to note here that a new technique, moderate hypothermic circulatory arrest (MHCA) with ACP, has recently gained popularity. Since the body is only cooled between 28-20°C, a less pronounced hypothermia-induced coagulopathy is observed, and lower transfusion rates are seen when compared to DHCA, while maintaining similar cerebral protection\(^\text{41,42}\). Figure 8\(^3\) depicts the growing number of cases that are being done using MHCA instead of DHCA\(^\text{42}\). While we could not cool the animals to below 20°C without circulatory support, the fact that a temperature could be reached that was well within the

\(^3\) Reprinted from The Journal of Thoracic and Cardiovascular Surgery, Vol 152;6, Jeffrey E. Keenan et al, Does moderate hypothermia really carry less bleeding risk than deep hypothermia for circulatory arrest? A propensity-matched comparison in hemiarch replacement, pg 14, 2016, with permission from Elsevier.
range of that which is used during MHCA, highlights the clinical relevance of this model in terms of the degree of hypothermia achieved.

The use of an ice pack for active cooling provided an easy and reproducible way of consistently achieving core temperatures within the designated range in a non-invasive manor. Minor adjustments were made between the Pilot Experiment and Experiment #1 that allowed a higher degree of control over the cooling process, and included selectively cooling the animals once they fell below 28°C until they reached the designated range. Previous studies investigating hypothermia and the BBB used anesthesia-induced mild hypothermia (33-31°C), and so we used this as our temperature range for our AN group for the first two experiments. To better simulate the clinical application of DHCA, the time within the designated temperature ranges was lengthened from 30 to 60 minutes. As shown in Tables 1 and 2, temperature goals were met for each group in both experiments, showing consistency and accuracy of our cooling method.

Figure 8: Bar graph showing the number of cases of hemiarch replacement per year using either deep or moderate hypothermic circulatory arrest.42
After the cooling method was determined and made consistent, the next improvement between Experiment #1 and #2 was to implement physiological monitoring. AN+ICE animals were observed by visual inspection to experience a marked decrease in both heart and respiration rate. Monitoring of these parameters was essential to ensure that ischemic injury was not being induced during these hypothermic temperatures. To help prevent ischemic injury, we chose to control respiration through the use of a ventilator. Adequacy of ventilation was to be monitored with arterial blood gases to access suitable ventilator settings and function. Additionally, we incorporated our rodent electrocardiogram system to measure heart rate and heart rhythm in all experimental animals. The results in Table 4 and 5 indicate successful implementation of monitoring and control of these parameters. The data in Table 4 confirmed the observation that the hypothermic conditions were inducing a significant decrease in heart rate. Although this observed drop was about 50% from baseline, heart rate steadied after the active cooling was terminated, and no arrhythmias were observed in any of the 4 animals in this treatment group. Arterial blood gas results in Table 5 showed no significant changes in pO$_2$ or pCO$_2$ between the three groups, verifying that our ventilator was providing adequate gas exchange for the duration of the experiment. We also saw no change in pH or HCO$_3^-$ indicating neither acidosis nor alkalosis was being induced in either experimental group. We did not observe significant changes in the concentration of the major ions Na$^+$ or K$^+$, however we did see a significant increase in free Ca$^{2+}$ concentration in the AN group, although these values were all within normal range$^{50}$. It is also important to note that the percent hematocrit (HCT) was not significantly different between the three groups, indicating similar
oxygen-carrying capacities. These results help to indicate that ischemic injury was not being induced by the hypothermic conditions in this model.

For this experiment, the designated temperature range for the AN group was modified from 33-31°C to 37-35°C to represent normothermia. This was done to ensure only one experimental group experienced a change in temperature, and so the cause of changes in protein expression could be determined as either the hypothermic temperature or the anesthetic being delivered. Table 3 shows that core temperatures for both groups were successfully maintained within the specified ranges for the last 60 minutes, and the implementation of the EKG monitoring and placement on the ventilator did not affect temperature management. With the successful monitoring of heart rate and adequate control of respiratory rate, the model has been demonstrated greater translational potential, allowing for investigation into BBB protein expression during hypothermic conditions.

**Tight Junction Protein Expression**

Western blot analysis revealed changes in TJ protein expression at each stage in model development. Results following the Pilot Experiment found significant decreases in occludin expression in both the AN and AN+ICE groups when compared to CTRL, however no significant changes were seen between the experimental groups (Fig 5). This meant that at this time, the cause of the changes in expression could not be attributed to either the hypothermic condition or the anesthetic being delivered, as both experimental groups experienced some degree of hypothermia (Table 1). Following Experiment #1, the significant decrease in occludin expression in the AN+ICE group when compared to CTRL was also observed, however the decrease in the
AN group was not seen (Fig 6). This is important to note as this is the first observation that could indicate a temperature-dependent decrease in occludin expression. These same observations were repeated following Experiment #2 (Fig 7), again indicating a possible temperature-dependent decrease in occludin expression. This is an important observation, as increases in BBB paracellular permeability in H/RI models have correlated with decreases in occludin expression or increases in redistribution\(^2\). Occludin has shown increased transport away from TJ in certain pathologies, including H/RI, which correlates to an increase in the breakdown of oligomeric occludin structures vital to maintaining BBB integrity\(^4\). While the mechanism of occludin changes in hypothermia is unknown at this point, an increase in trafficking could pose a possible explanation for our observations.

Changes in claudin-5 expression were not detected through western blot analysis performed following the Pilot Experiment and Experiment #1 between the three groups. Following Experiment #2, a significant decrease in expression was seen in the AN+ICE group when compared to the AN group. This is an interesting finding as claudin-5 expression had remained unchanged in the previous two experiments. However, replication of this observation following further modifications to the model is necessary before explanations and implications will be investigated.

Clinical Impact

DHCA remains a popular technique used for the correction of aortic aneurysms and dissections in the Unites States. Deep hypothermia has been used in combination with circulatory arrest for over 40 years because of the ability to lower whole body metabolic
demand for protection against the temporary cessation of circulation, as well as its neuroprotective ability against brain ischemic injury. Although this is well understood, the effect of deep hypothermia, or hypothermia in general, on certain structures of the body such as the BBB are not well understood. This is important to highlight, as the mechanisms behind observed neurologic deficits following these procedures remains poorly defined. The development of this model, as well as investigation into TJ protein expression at the BBB, can provide possible answers for the pathogenesis of these neurocognitive deficits.

Our observed changes in expression of both occludin and claudin-5 under hypothermic conditions indicate that DHCA may cause clinically significant changes to the BBB. Changes in protein expression of both these transmembrane TJ proteins observed in reperfusion injury are associated with increases in BBB permeability to circulating solutes. Additionally, disruption of the BBB can cause increased water content in the brain, a process that can lead to clinically significant cerebral edema. Furthermore, this increase in brain water content increases intracranial pressure, leading to a reduction of cerebral blood flow and an increase in distortion and herniation of vital structures of the brain. These pathophysiological mechanisms may provide an explanation for CNS injury and neurocognitive complications that are still relatively common following DHCA procedures.

While the use of deep hypothermia is limited to circulatory arrest procedures, mild (34-32°C) to moderate (32-28°C) hypothermia is commonly used in the majority of more than 300,000 cardiac surgeries performed annually. The major benefit to using some degree of hypothermia is to reduce metabolic demand of the body, and thereby increasing tolerance to ischemia while on CPB. Similarly, the effect of either mild or moderate hypothermia on TJ
protein expression has yet to be investigated. Since it is well established that BBB disruption is correlated with an increase in adverse neurocognitive effects, it is critical to identify and characterize biological mechanisms that contribute to barrier disruption following DHCA. Such knowledge will enable development of more effective strategies to ensure neuroprotection (i.e. administration of neuroprotective drugs prior to onset of DHCA) in patients who require such procedures.

**Future Works**

While advances have been made in terms of the clinical relevancy of this hypothermia model, improvements need to be made to ensure that hypothermia is the cause of changes in BBB protein expression. Though the implementation of EKG monitoring and ventilation control, we are confidence that ischemic injury is not being introduced during these hypothermic temperatures; however, it is vital to measure cerebral blood flow in order to fully understand how deep hypothermia can affect the brain. Although this decrease in cerebral blood flow could be possible, the coinciding decrease in cellular metabolism induced by hypothermia could ameliorate the possibility of ischemic injury from occurring in these conditions. Another potential cause of ischemic injury in the model is due to the decrease in temperature causing an increase in the viscosity of blood, making perfusion of the microvasculature more difficult. Hemodilution through the CPB circuit is sometimes employed by the perfusionist to decrease the viscosity of the blood in an attempt to better perfuse the microvascular beds, however this is still controversial as studies have found no difference in outcomes with higher (30-35%) HCT when compared to diluted (20-30%) HCT\textsuperscript{37,38}. A way to address this variable is to implement the use of Laser Doppler flowmetry (LDF) for measurement of cerebral blood flow during these
hypothermic temperatures. In combination with this, the use of both arterial and venous blood gases could also be implemented for a more complete picture. In this way, the oxygenation status of the blood reaching the tissues will be measured, the rate at which the oxygen is being delivered will be monitored, and the oxygen extraction of the tissues will be determined. With the combination of both new strategies, a better understanding of the tissue's oxygenation status can be obtained, and thus a more concrete understanding as to whether an ischemic injury is being induced at this hypothermic temperature.

While occludin and claudin-5 are both critical TJ proteins in terms of maintaining barrier integrity, other TJ proteins play important roles in BBB function, and could have a large effect on resulting permeabilization of the barrier if expression is changed. Zona Occludens (ZO-s) are scaffolding proteins that are localized to TJ structures in BBB endothelial cells\(^{22}\). ZO-1 links transmembrane TJ proteins (i.e., occludin and claudin-5) to the actin cytoskeleton (Fig 3), and plays a role in maintaining TJ function\(^{43}\). It is also likely involved in signal transduction pathways which regulate gene expression and cell behavior\(^{22}\). Similar to occludin and claudin-5, changes in ZO-1 expression and localization are associated with diseases that exhibit BBB disruption, and studies have shown that ZO-1 dissociation from the TJ complex is associated with increased BBB leak\(^{22,43}\). Increases in trafficking of ZO-1 away from TJ complexes and decreases in protein expression have been observed in H/RI models, and was associated with increases in BBB permeability\(^{46}\). This highlights the ZO-1-transmembrane protein interaction as critical to tight junction stability and function, and the changes in its expression under hypothermic conditions requires investigation to gain a better understanding of BBB integrity. Furthermore, understanding TJ protein regulation and/or trafficking under hypothermic conditions will
inform future studies designed to identify and develop novel BBB protection strategies that can be translated to an OR setting.

Another important class of transmembrane proteins in maintaining TJ function is the junctional adhesion molecules (JAMs) -1, -2, and -3. JAMs have been shown to regulate transendothelial migration of neutrophils and macrophages into the CNS, and also mediate early attachment of adjacent endothelial cells during BBB development\textsuperscript{43}. Loss of JAM protein expression is directly correlated with BBB disruption and injury, and in certain injury models, such as inflammation or oxidative stress, changes in JAM localization and decreases in protein expression correlate with increases in BBB permeability\textsuperscript{43}. The fact that changes in the expression and localization of both ZOs and JAMs have been correlated with increased BBB permeability and disruption highlight the need for investigation as to whether drastic changes in temperature seen during our hypothermia model are inducing similar changes previously observed in other injury models.

If these changes in TJ proteins are continued to be detected, visualization of these changes will be necessary. It has been observed that H/RI is associated with movement of occludin away from TJs, and correlative increases in intracellular staining, indicating increased trafficking of occludin\textsuperscript{39}. Of interest, these changes were observed after 1 hour of hypoxic stress, in close relation to our experimental duration, and could provide an explanation for the observed occludin protein changes within the same timeframe. This highlights the need to investigate localization changes in these TJ proteins, and this could be accomplished through immunofluorescence microscopy. In addition to this, investigation into changes in BBB permeability is essential when a change in TJ protein expression is observed. In-situ perfusion
experiments are needed with both small (\(^{14}\text{C}\)-sucrose) and large (10, 15, 20 kDa dextran) molecule tracers to investigate size-dependent leak associated with the different TJ proteins.

The long-term goal of this model is to be used in conjunction with an ischemia/reperfusion (I/R) injury model to fully simulate the conditions seen in a DHCA procedure. The ease and relative non-invasiveness of the hypothermia model allow for this possibility, and the ideal I/R injury model is still to date the middle cerebral artery occlusion (MCAO) model. This will be eventually done in tandem so a better understanding of the BBB response to DHCA conditions can be elucidated.

**Conclusions**

The need for improved cerebral protection during DHCA is critical. The lack of knowledge regarding BBB changes following hypothermic temperatures provides an interesting avenue into exploration of possible pathologies of neurocognitive deficit following these procedures. The link between BBB disruption and increased CNS injury seen in ischemic stroke illustrates the potential for neurologic damage when this barrier is diminished.

A model of hypothermia was to be developed that allowed for investigation into hypothermia’s effect on BBB protein expression, that remained clinically translatable as to better understand potential reactions to DHCA. Once temperature management was established, advancements were made to measure and control physiologic parameters to ensure injury outside of hypothermia (i.e. ischemic) was not being induced. This resulted in a mostly non-invasive, easy to replicate model that consistently achieved ideal hypothermic temperatures while allowing for clinically relevant parameters to be controlled or measured.
At each stage in development of the model, the TJ proteins occludin and claudin-5 were investigated for changes in protein expression via western blot. TJ proteins, and these two in particular, were chosen due to their integral role in maintaining TJ integrity, which has been shown to be vital in protecting the CNS from injury due to cerebral edema and loss of protection from neurotoxic substances. After model improvements were made, occludin was observed to have a potentially temperature-dependent decrease in expression. The results of claudin-5 require further investigation into reproducibility.

Future directions should be focused on investigation of the changes in localization in these TJ proteins, as are seen in H/RI, and whether these changes correlate to an increase in BBB permeability. Finally, this model can be used in combination with MCAO to induce an I/R injury so that a better understanding of the BBB during DHCA can be investigated.

References


3. Lewis FJ, Taufic M. Closure of atrial septal defects with the aid of hypothermia; experimental accomplishments and the report of one successful case. Surgery 1953;33:52-9


