

The Blood-Brain Barrier integrity and function in ischaemic injury and multiple sclerosis with role of hypothermia

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Abstract

Background

Brain injury following transient or permanent focal cerebral ischaemia matures from a multifaceted succession of pathophysiological events that progress in time and space. The key biochemical events related with these pathologies include excessive cytoskeletal remodeling, modulation of tight junction proteins, induction of oxidative stress and excessive release of pro-inflammatory cytokines. In addition, other neuropathologies such as multiple sclerosis have been shown to account for the development of these events and the consequent disruption of human blood-brain-barrier (BBB) integrity and function. However this study emphasizes on potentially new avenues of treatment which can protect the integrity of the blood-brain barrier and decrease post ischaemic consequences such as vasogenic oedema. Hypothermia has been hypothesised as a potent neuroprotectant and its putative effects have been assessed in the present research work.

Objectives

This thesis focuses on the putative role of hypothermia during intra- and post-ischaemic stroke mimicked by oxygen-glucose deprivation and followed by reperfusion. The impact of hypothermia has been studied on various inflammatory pathways such as pro-inflammatory cytokine levels, rate of apoptosis, extent of cytoskeleton remodeling and integrity and function of the BBB. In addition, we have also focused on the neuroinflammatory effects of multiple sclerosis (MS) on the BBB integrity.

Method To mimic ischaemic insult, human brain microvascular endothelial cells, human astrocytes and human pericytes were exposed to oxygen-glucose deprivation (OGD) for 4 and 20 hours in the absence or presence of hypothermia (intra-ischaemic

episode) and 4 hours of OGD followed by 20 hours of reperfusion in the presence of hypothermia (post-ischaemic episode) and cells left untreated were served as controls. Similarly, to assess BBB properties, all three cells were cultured simultaneously to establish an *in vitro* model of human BBB before exposing them to the experimental conditions. The integrity and function of triple cultures were measured via transendothelial electrical resistance (TEER) and paracellular flux via permeability markers such as sodium fluorescein (NaF) and Evan's blue-labelled albumin (EBA). Various inflammatory inhibitors were also used during the insult to assess the distortion of the barrier. Furthermore, the triple cultures were exposed to multiple sclerosis patients' serum mainly to study the neuroinflammatory effects. Moreover, with help of specific enzyme-linked immunosorbent assay-based kits pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1 β were measured during intra- and post-ischaemic episodes in the absence or presence of moderate hypothermia. Lastly, to evaluate the status of oxidative stress during acute ischaemic insult, the levels of superoxide anion generation and NADPH oxidase activities were measured.

Results

This study shows that moderate hypothermia significantly decreases pro-inflammatory cytokine levels when introduced during intra- and post-ischaemic episodes. Additionally, moderate hypothermia also maintains the BBB integrity and function during intra- and post-ischaemic scenarios. As evidenced by significant increase in resistance across the barrier and by concurrent decrease paracellular flux values. In addition, moderate hypothermia manages to reduce the oxidative stress impact during intra- and post-ischaemic episodes as documented by a decrease in NADPH oxidase and superoxide anion levels. Furthermore, the outlined study shows

that MS patients' serum is fortified with various inflammatory components such as pro-inflammatory cytokines, increase rate of apoptosis and resulting in destruction of the BBB architecture.

Conclusions

In this study, we have found moderate hypothermia to be a robust neuroprotectant in during intra- and post-ischaemic episodes of ischaemic injury mimicked by oxygen-glucose deprivation alone or followed by reperfusion. If adequately and appropriately maintained, moderate hypothermia may be an effective therapeutic option with minimal adverse effects. Furthermore, MS associated BBB destruction shows a new conduit for future research.

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“Jai Shri Ram”

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List of Abbreviation

ABG	: Arterial Blood Gas
ANOVA	: Analysis of variance
ATP	: Adenosine triphosphate
BBB	: Blood-Brain Barrier
BP	: Blood Pressure
CA	: Cardiac Arrest
Ca²⁺	: Calcium
CBC	: Complete Blood Count
CD	: Cluster Difference
CNS	: Central Nervous System
CT	: Computer Tomography
Cu	: Copper
DMEM	: Dulbecco's Modified Eagle's medium
DNA	: Deoxyribonucleic acid
EBA	: Evan's Blue Albumin
ECF	: Extracellular Fluid
ECM	: Endothelial cell Medium
ELISA	: Enzyme-Linked Immunosorbent Assay
FBS	: Fetal Bovine Serum
H₂O	: Water Molecule
HA	: Human Astrocytes
HBMEC	: Human Brain Microvascular Endothelial Cells
HIV	: Human Immunodeficiency Virus
HP	: Human Pericytes
HT	: Hypothermia
I/R	: Ischaemic/Reperfusion Injury
ICH	: Intracerebral Hemorrhage
ICP	: Intracranial Pressure
IL-1β	: Interleukin-1beta
IV	: Intravenous
K⁺	: Potassium

MCAO	: Middle Cerebral Artery Occlusion
MI	: Myocardial Infarction
MMP	: Matrix Metalloproteinase
Mn	: Manganese
MRI	: Magnetic Resonance Imaging
MS	: Multiple Sclerosis
Na⁺	: Sodium
NADPH	: Nicotinamide adenine dinucleotide phosphate
Naf	: Sodium Fluorescence
NMDA	: <i>N</i>-methyl-D-aspartate receptor
NO	: Nitric Oxide
O₂	: Oxygen
O₂⁻	: Superoxide Anion
OGD	: Oxygen Glucose Deprivation
PBS	: Phosphate Buffer Saline
PKC	: Protein Kinase C
PPMS	: Primary Progressive Multiple Sclerosis
PRMS	: Progressive Relapsing Multiple Sclerosis
RNA	: Ribonucleic acid
ROS	: Reactive Oxygen Species
ROSC	: Return of spontaneous circulation
RRMS	: Relapsing Remitting Multiple Sclerosis
RT-PA	: Recombinant tissue plasminogen activator
SOD	: Superoxide Dismutase
SPMS	: Secondary Progressive Multiple Sclerosis
TBI	: Traumatic Brain Injury
TEER	: Transendothelial Electrical Resistance
TGF-β	: Transforming growth factor beta
TNF-α	: Tumor Necrosis Factor-alpha

Introduction

Inflammation is a term referring to an extensive and complex process of cellular and biochemical changes that can affect specific organ or the entire body. It has been documented that apoplectic brains are hallmark for inflammation. Until recently, researchers have suggested that inflammation following stroke attack develops as a secondary reaction to cerebral tissue damage and is cleared by invading leukocytes and macrophages [1]. Further, this concept has been modified with results documented from the recent research work on stroke induced neuroinflammation. Numerous studies have now been able to show the damaging effects of inflammation associated with neuropathologies such as intra- and post-ischaemic stroke and multiple sclerosis (MS) [2-6]. MS and ischaemic injury represent the most common chronic inflammatory pathologies of the central nervous system. Recent studies have documented that early attack of MS leads to disruption of BBB [7], similar to the disruption caused during an ischaemic insult as published by our group [8]. Therefore, our research has attempted to examine the effect of neuroinflammation on the blood-brain barrier (BBB) during oxygen-glucose deprivation (OGD) induced acute stroke injury in the absence or presence of moderate hypothermia (35°C). Furthermore, the effects of MS on the BBB were studied following an exposure of a triple culture model of human BBB to serum obtained from MS patients' blood.

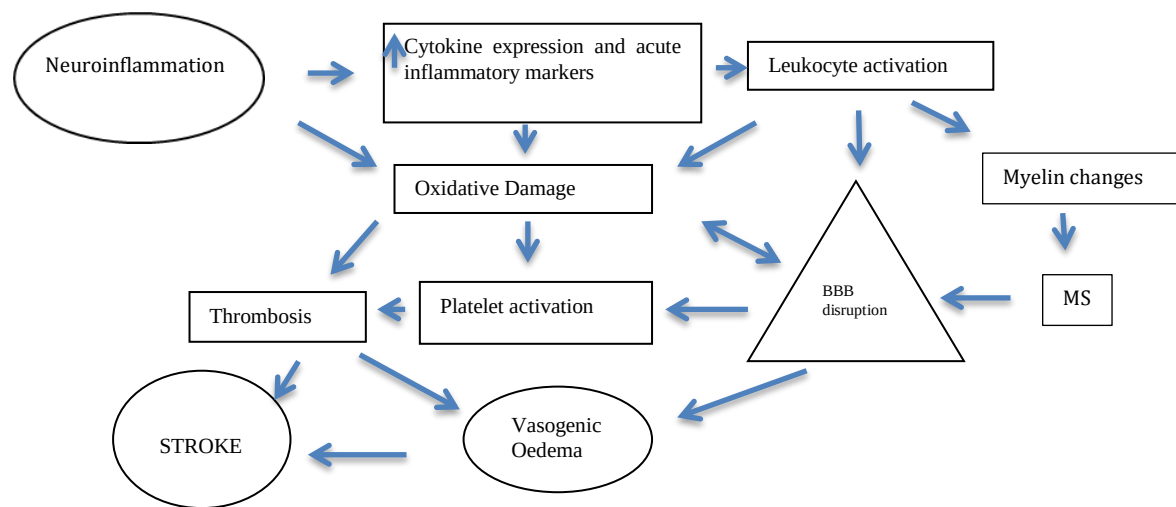


Figure 1 Schematic layout highlights the brief consequences of neuroinflammation via demonstrating increase in inflammatory counterparts and their magnitudes. Also highlights interrelation between neuropathologies ultimately resulting in BBB damage. MS: multiple sclerosis, BBB: Blood-brain barrier

Stroke Incidences

World Health Organisation defines stroke as a focal neurological impairment of sudden onset, lasting more than 24 hours or leading to death. This excludes transient ischaemic attacks, which is defined as stroke symptoms that resolve within 24 hours of onset. World Health Organisation reports that 15 million people suffer stroke worldwide each year. Of these, 5 million die, and another 5 million are left permanently disabled [9]. Regardless of the higher risk in men, the lifetime risk of stroke is higher in women. The Framingham study calculated lifetime risk of stroke between middle age men and women and found that the risk in women age 55 was 21% and in men age 55 was 17% [10]. Stroke occurs approximately 152,000 times a year in the UK. That is one stroke every 3 minutes and 27 seconds. It ranges from 115/100,000 to 150/100,00 population with doubling the risk of stroke by every decade after the age of 55 [11]. It is certain that by the age of 75, 1 in 5 women and 1

in 6 men will have stroke in the UK [12]. Black people are as twice as likely to have a stroke compared to white [13]. Likewise, South Asian people also tend to have a significant stroke attack at younger age when compared to whites [14].

Stroke according to vascular pathology is classified into haemorrhagic or ischaemic. Ischaemia triggers a series of pathological events in the brain, leading to neuronal loss by apoptosis and other mechanisms of cellular injury. Ischaemic stroke is usually characterised by the sudden loss of blood circulation to a section of the brain, resulting in a corresponding loss of neurological function. It constitutes one of the most vital public health challenges that are threatening the world in the 21st century. It is the most common type of stroke, comprising about 80 to 85% of all cerebrovascular injury and the foremost cause of long-term disability [15, 16].

Stroke Features

Acute ischaemic stroke is caused by thrombotic or embolic phenomena occluding any of a cerebral artery. Common stroke signs and symptoms are such as abrupt onset of hemiparesis (weakness of the entire left or right side of the body), monoparesis (one limb is very weak, but not completely paralysed), quadriparesis (means muscle weakness affecting all four limbs), hemi-sensory deficits, monocular or binocular visual loss, dysarthria (difficult or unclear articulation of speech that is otherwise linguistically normal), facial drop and sudden decrease in level of consciousness. Though such symptoms can happen alone, they are more liable to occur in muddle. No patient associated historical features differentiate ischaemic from haemorrhagic stroke, although nausea, vomiting, headache, and sudden change in level of consciousness are more commonly observed in haemorrhagic stroke. In younger patients, a history of recent trauma, coagulopathies, illegal drug abuse (especially

cocaine), migraines, or practice of oral contraceptives should be matter of concern. Patients with history of stroke are at higher risk of vascular events, including recurrent stroke and myocardial infarction (MI) [17]. Apparently, the risk of stroke is highest in the early period following an acute event therefore a rapid instigation of tailored prevention strategies is obligatory [18]. A meta-analysis showed that the risk of stroke was as high as 12.8% during first week following transient ischaemic attack [19]. It is estimated that 80% of such cases could have been treated if early use of comprehensive approach such as dietary modification, smoking cessation, blood-pressure (BP) lowering, anti-platelet therapy, statin therapy and robust neuroprotectant is applied [18].

Clinical Examinations

With the handiness of fibrinolytic therapy for acute ischaemic stroke, the physician must be able to complete a brief but precise neurological examination. The goals of such examination include the following, confirming the presence of a stroke syndrome, distinguishing stroke from other pathologies producing similar clinical features (differential diagnosis) and establishing a neurologic baseline. Additionally, it is crucial to establish stroke severity, using a planned neurologic exam and score (National Institutes of Health Stroke Scale) to assist in prognosis and therapeutic selection. Furthermore, brain imaging is one of the most essential parts of stroke diagnosis and treatments. Non-contrast computed tomography (CT) scan is the most frequently used form of neuroimaging in the acute evaluation of patients with apparent acute stroke syndrome, other imagining techniques are such as CT angiography and CT perfusion scanning, magnetic resonance imaging (MRI), carotid duplex scanning, digital subtraction angiography are also preferred. Additionally,

procedures such as lumbar puncture are essential in ruling out other neuro-pathologies, which do mimic stroke symptoms such as meningitis [20]. There is a wide range of laboratory investigations for ischaemic stroke such as complete blood count (CBC), basic chemistry panel, baseline study that may reveal a stroke imitator (e.g., hypoglycemia, hyponatremia), coagulation studies to uncover a coagulopathy and are useful when fibrinolytics or anticoagulants are to be used, cardiac biomarkers, pregnancy testing and arterial blood gas (ABG) analysis. The system of classifying stroke developed in the multicenter Trial of ORG 10172 in Acute Stroke Treatment (TOAST) divides ischaemic stroke into the following 3 major subtypes [21] :

- ✓ Large-artery
- ✓ Small-vessel or lacunar
- ✓ Cardio-embolic infarction

Risk Factors

There are numerous risk factors for ischaemic stroke, classified under modifiable and non-modifiable aspects. Identification of risk factors in each patient can expose the aetiology of the stroke and the most suitable treatment including secondary prevention plan. Non-modifiable risk factors include age, race, sex, and ethnicity with history of migraine and hereditary characteristics. These all issues play a dominant role in the development of ischaemic stroke. Additionally, the category for modifiable risk factors include hypertension, diabetes mellitus, cardiac disease such as atrial fibrillation, valvular disease, heart failure, mitral stenosis, structural anomalies allowing right to left shunting (e.g. patent foramen ovale), and atrial and ventricular enlargement, hypercholesterolaemia (high levels of cholesterol in blood stream), transient ischaemic attacks, carotid stenosis and lifestyle issues such as extreme alcohol intake, tobacco use, illegal drug use and physical inactivity [22]. In 2014, the

American Heart Association and the American Stroke Association delivered guidelines for the reduction of stroke risk precisely in women. These gender-specific recommendations comprised the following [23]:

- ✓ A stroke risk score must be introduced for women.
- ✓ Women with a history of high BP before pregnancy should be considered for low-dose aspirin and/or calcium (Ca^{2+}) supplement to decrease the risk of pre-eclampsia (a condition in pregnancy characterised by high BP, sometimes with fluid retention and proteinuria).
- ✓ BP medication can be considered for pregnant women with moderately high pressure (150-159/100-109mmHg), and pregnant women with severe BP (160/110 mmHg or above) must be treated.
- ✓ Women with migraine headaches with aura should be encouraged to abandon smoking.
- ✓ Women over the age of 75 years should be screened for atrial fibrillation.

Moreover, indication lingers to gather that inflammation and genetic factors have important roles in the development of atherosclerosis related to further development of stroke. According to the current model, atherosclerosis is not an insipid cholesterol storage disease, as initially thought, but on the contrary is an active, chronic, inflammatory condition caused by a response to endothelial injury [24]. Old-style risk factors, such as oxidised low-density lipoprotein cholesterol and smoking are the major factors involved in the upsurge of atherosclerotic injuries. Host genetic factors, also, may adapt the response to these challenges, although inherited risk for stroke is likely to be multigenic. A number of genes are recognised to increase vulnerability to ischaemic stroke. Mutations to the F2 (coagulation factor 2 gene) and F5 (coagulation factor 5 gene) are comparatively similar in the general population and increase the

risk of thrombosis and other genes such as NOS3 (nitric oxide synthase 3) [25, 26]. Furthermore, hyperhomocysteinaemia (abnormally high level of homocysteine in the blood) is implicated in the pathogenesis of ischaemic stroke. The most common concern is mutations in the 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene [27]. Thromboembolic events are the most common cause of death for patients with homocystinuria and may be of any type, including MI [28].

Large-artery occlusion characteristically results from embolisation of atherosclerotic debris originating from the common or internal carotid arteries or from a cardiac source. Large-vessel ischaemic strokes more commonly affect the middle cerebral artery (MCAO) territory. On the other hand, lacunar strokes represent 13-20% of all ischaemic strokes [29]. They result from occlusion of the penetrating branches of the middle cerebral artery (MCA), the lenticulostriate (anterolateral-ganglionic branches) arteries, or the penetrating branches of the circle of Willis. The great majority of lacunar strokes are related to hypertension. In addition, cardiogenic emboli may account for up to 20% of acute strokes. Emboli may be of cardiac origin or from extracranial arteries [30]. Lastly, thrombotic strokes are generally thought to originate from ruptured atherosclerotic plaques [31]. Although multiple hypotheses are currently prevalent about the complex aetiology of ischaemic stroke, the inflammatory cascade hypothesis has been attracting more and more attention. Increasing evidence from the past has proposed that the detrimental role of inflammation on the drastic changes during or after cerebral ischaemia is essential to evaluate [32]. It is commonly understood that brain cells cease to function when blood flow decreases or diminishes. Ischaemia results in cellular hypoxia and depletion of cellular adenosine triphosphate (ATP). In turn, the ATP loss leads to ions flowing down their concentration gradients, and eventual efflux of potassium (K^+)

and influx of sodium (Na^+) and Ca^{2+} . These influxes may lead to direct neurotoxicity as well as extracellular accumulation of glutamate, which are further known to augment damage at the site of injury [33]. Therefore, in the absence of ATP, there is no longer the energy preserved to uphold ionic gradients across the cell membrane and cell depolarisation. Moreover, influx of Na^+ and Ca^{2+} ions and inert inflow of water (H_2O) into the cell results in cytotoxic oedema [34].

Stroke and Cerebral Blood Flow

A severe vascular occlusion harvests varied areas of ischaemia in the affected vascular territory. Local blood flow is restricted to any residual flow in the major arterial source plus the collateral supply. Affected areas with cerebral blood flow of lesser than 10 mL/100 g of tissue/min are denoted as the core. These cells are reputed to expire within minutes of stroke attack [35]. Zones of decreased or bordering perfusion (cerebral blood flow < 25 mL/100g of tissue/min) are jointly termed as the ischaemic penumbra. This area comprises of tissue having ability to remain viable for several hours due to marginal tissue perfusion [35]. Further, the subsequent arrival of Ca^{2+} due to loss of membrane integrity leads to the release of a number of neurotransmitters, which in turn activates N -methyl-D-aspartate (NMDA) and other excitatory receptors on former neurons [36]. Also, free radicals and nitric oxide (NO) are generated by this process, which adds up to further neuronal damage. There are at least three fundamental mechanisms leading to cell death during ischaemic brain injury: excitotoxicity and ionic imbalance, oxidative/nitrosative stress, and apoptotic-like cell death [37]. These mechanisms further validate overlying features and facilitate injury within neurons, glia and further affecting the function of mitochondria [38].

Oedema and herniation are the most common reasons of early death in stroke.

Seizures are experienced in 2 to 23% of patients within the first days after ischaemic stroke [39]. Most importantly, ischaemia also directly results in dysfunction of the cerebral vasculature, with breakdown of the BBB occurring within 4 to 6 hours after infarction [40]. Following the barrier's failure, proteins and water flood into the extracellular space, leading to again vasogenic oedema. This accounts to greater stages of brain swelling and mass effect that peak at 3 to 5 days [41]. Within hours to days following initial attack of stroke there is augmentation of formation and release of cytokines and cause more damage. Ultimately, the ischaemic penumbra is consumed by these progressive insults, merging with the infarcted core. Infarction results in the death of cells and this infarcted tissue ultimately suffers liquefaction necrosis and is removed by macrophages.

In the Framingham and Rochester stroke studies, the overall mortality rate at 30 days after stroke was 28%, the mortality rate at 30 days after ischemic stroke was 19%, and the 1-year survival rate for patients with ischemic stroke was 77%. [42]. However, the prognosis following acute ischaemic injury varies greatly in individual patients, depending on the stroke severity and on the patient's premorbid condition, age, and post-stroke complications. Haemorrhagic alteration is projected to occur in 5% of uncomplicated ischaemic strokes in the absence of fibrinolytic therapy [43]. Acute ischaemic stroke has been related with acute cardiac dysfunction, which then relate with worse functional outcome and morbidity at 3 months [44]. Data also highlight that high levels of hyperglycaemia is independently related with poor outcome and reduced reperfusion in fibrinolysis, as well as extension of the infarcted territory [45, 46].

The Blood-Brain Barrier and Inflammation

In 1900, Lewandowsky documented the presence of a barrier in the central nervous

system and showed its efficacy in preventing the passage intravenously (IV) administered drug to pass into the brain parenchyma. Later in 1909, Goldmann confirmed the presence of this barrier [47] and finally in 1967 by the help of electron microscopy the presence of the BBB was unequivocally confirmed and shown to localise at the level of tight junctions between adjacent endothelial cells [48]. Currently, it is known that the BBB limits the access of plasma components, red blood cells, and leukocytes into the brain and they only cross the BBB in cases of various pathologies such as traumatic brain injuries, ischaemic insult, intracerebral hemorrhage, neurodegenerative process, inflammation, or vascular disorder. The BBB is one of the most important barriers in cerebral vasculature and is primarily formed by microvascular endothelial cells, which are surrounded by basement membranes, human pericytes (HP), and human astrocytes (HA).

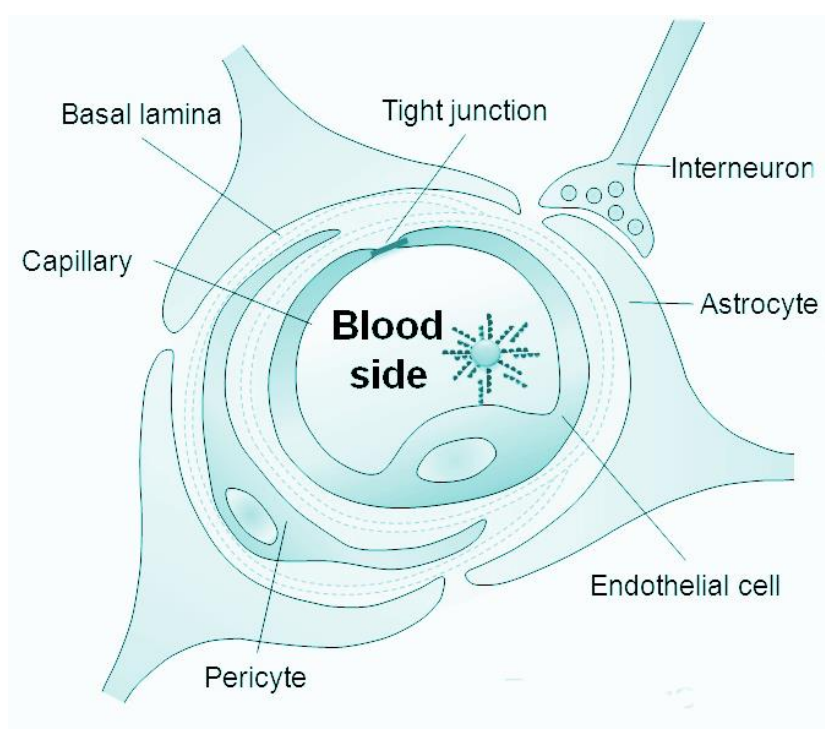


Figure 2-Schematic diagram showing the Blood-Brain Barrier

Cross-sectional representation of a cerebral capillary, where the circumference of the capillary lumen is completely surrounded by human brain microvascular endothelial

cells, the opposing membranes of which are connected by tight junctions, pericytes are attached to the abluminal surface of the endothelial cells and these two cell types are surrounded by the basal lamina, which is contiguous with the plasma membranes of human astrocytes end-feet and endothelial cells. Picture courtesy: CUHK Shun Hing institute, 2014-2016. http://www.bme.cuhk.edu.hk/jchchoi/r_SHIAE14-16.php.

Figure above demonstrates a schematic cross-sectional picture of a characteristic cerebral capillary. Anatomically, the HBMECs of the BBB are distinguished from those in the periphery by augmented mitochondrial content, absence of fenestrations and merely pinocytotic activity [49-51]. The preservation of the tight junctions is governed by three essential transmembrane proteins such as claudin, occludin, and junction adhesion molecules [52] that are self-assemble into 'zip-locked' structures, forming the physical barrier in the endothelial cell clefts [53]. There are two kinds of junctions present in the intercellular junctional complex namely; tight junctions (zona occludens) and adherens junctions [54]. The tight junction proteins and their connector molecules help in connecting the junctions to the cytoskeleton and are frequently affected during acute and chronic ischaemic episodes. Recent studies with mouse model of experimental autoimmune encephalomyelitis and MS have shown that tight junction's dephosphorylation leads to evident signs of disease and deceptive changes in the BBB permeability [55]. The claudin found at the site of the BBB are claudin-5, claudin-2 and claudin-3 and are found to be a multigenic with more than 20 members that form junctional strands [47, 56]. Observed in case of epilepsy where selective downregulation of claudin affects the BBB architecture [57]. Not only associated with neurological disorders, the reduction in claudin expression is observed in pathologies such as human immune deficiency virus (HIV) infections, where crossing of this virus results in the BBB breakdown [58]. Under normal physiological conditions, substances with a molecular weight greater than 180Da do not gain access

through the tight junction [59]. Yet, the sealing properties of tight junction can vary between brain endothelial cells of different locations. For example, as endothelial capillaries proceed to post-capillary venules a reduced sealing capacity is observed [60].

While, human brain microvascular endothelial cells form a proper barrier and their interaction with surrounding cells remains crucial for the maintenance of the BBB [61]. The BBB expresses a large number of ion channels and transporters, with a low rate of pinocytosis, and forms intercellular tight junction protein complexes that limit paracellular permeability [62]. It is a diffusion barrier, consisting of an inter-dependent network of cells designed to segregate the central nervous system from the systemic circulation. The tight junction-controlled paracellular impermeability of the brain suggests that the hydrophilic molecules must cross transcellularly to reach their neuronal targets or leave the brain as suggested by Deane et al. [63]. In overall, the two-way transport system across the BBB can be classified into five main groups' such as active efflux transport, receptor-mediated transport, caveolae-mediated transport, carrier-mediated transport and ion transport. Astrocytic endfoot processes form the glia limitans, which, along with its own basement membrane, postulate the parenchymal aspect of the perivascular space [64]

The role of cytoskeleton in maintaining the BBB has recently become evident with recent studies with mice lacking actin-binding protein dystrophin resulted in increased brain vascular permeability mainly due to muddled α -actin cytoskeleton fibers in neuronal cells [65]. Equally, ischaemic insult or viral attack has been found to be associated with increased stress fibers and finally resulting in increased BBB permeability [66]. Understandably, when the BBB is compromised in presence of

inflammation, circulating immune cells gain access into the injured brain and activate immune molecules that aggravate ischaemic cell death. A few studies focusing on astrocytes as additional brain resident immune cell which also activates in response to ischaemia [67]. Migration of inflammatory cells into the brain is mediated by cell adhesion molecules such as selectins, integrins, and immunoglobulins, and the expression of these is regulated both intracellularly and by cytokine signalling [68]. Selectins are Ca^{2+} -dependent, transmembrane glycoproteins that bind to carbohydrate residues, and mediate rolling and adhesion to vascular endothelium. Namely, E-selectin, P-selectin, and L-selectin have all been shown to be involved in leukocyte trafficking in brain ischaemia [67, 69-71]. E-selectin or P-selectin have been shown to participate in an initial leukocyte rolling and recruitment, whereas L-selectin guides un-stimulated leukocytes to areas of activated endothelium [72]. P-selectin and E-selectin expression has been documented in various experimental stroke models and their upregulation appears to be involved in promoting both ischaemic inflammatory responses and injury [72-74]. Among the immunoglobulin superfamily members, intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) have been the most investigated in brain ischemia [75]. It has been previously noted that expression of ICAM-1 upsurges soon following ischaemia which in turn is followed by pro-inflammatory cytokines [76]. ICAM-1 is also seen to increase within hours during intra-ischaemic insult, and peaks at about 12–48 hours [77]. Further, in a study, mice lacking ICAM-1 had smaller infarcts compared to wild-type mice [78].

Other adhesion molecules such as VCAM-1 have lesser contribution to stroke and also its role is unclear. Although, some groups have shown that VCAM-1 messenger ribonucleic acid (mRNA) and protein both increase after cerebral ischemia [79].

Integrins are adhesion molecules composed of heterodimeric mixture of various α and β subunits. Previous to leukocytes binding, integrins are expressed on cell exteriors and will subunits will associate in order to recognise endothelial cell adhesion molecules. Almost all leukocytes express cluster of differentiation (CD), CD11a/CD18 and CD11b/CD18, also known as Mac-1, which are integrins that contain a common beta-2 (β 2) chain (CD18) and are thus known as β 2 integrins. These integrins allow the cells to bind to endothelial ICAM-1 and migrate through the vessel. In addition to the β 2 integrins, lymphocytes and monocytes express α 4 β 1 (CD49d/CD29) and α 4 β 7 (CD49d/CD103) [80]. By now, we do understand that disruption of the BBB during intra- and post-ischaemic stroke licenses penetration of circulating immune cells into the brain, and also exacerbates oedema and hemorrhage. Moreover, experimental strategies to prevent BBB disruption have largely involved the inhibition of matrix metalloproteinases (MMPs) [81].

Human brain microvascular endothelial cells

Human brain microvascular endothelial cells directly mediate the BBB function by resting on a basement membrane, which consists mainly of collagen IV (collagen found mainly in basal lamina), fibronectin, laminin, and proteoglycans, these HBMECs are known to act as mediators between the blood and brain [82]. They have specialised transport systems, constant thickness with no transendothelial fenestrations, little pinocytic activity, unceasing intercellular tight junction, and high mitochondrial volume [83, 84]. In addition, they have a negative luminal surface charge that rejects negatively charged compounds [84]. Due to absence of fenestrations and the reduced pinocytic activity with limited paracellular flux, acceptance of essential molecules occurs through specific carrier and transport systems [85]. In addition, because of the presence of continuous tight junctions and

adherens junctions, the paracellular space between adjacent lateral endothelial membranes is almost completely sealed [86, 87]. Furthermore, brain endothelial cells are found deliver higher energy and regulate selective transport mechanism due to presence of larger mitochondria when compared to endothelial cells elsewhere in the body [88]. Endothelial cells are actively involved in processes of microvascular stasis and are the first cells which face the effect of ischaemia. When damaged by ischaemic stimuli, these cells swell or detach from the underlying basement membrane, leading to a compromise in barrier function. This leads to the increased BBB permeability instigating serum protein extravasation and interstitial oedema as well as entry of immune molecules and cells [89].

Human Astrocytes

Astocytic processes known as astrocytic end-feet enclose the brain capillaries' abluminal surface [90]. These are placed among neurons, pericytes, and capillary endothelial cells. Astrocyte-endothelial cell interactions not only play a major role in maintaining the barrier properties but also have a major role in regulating brain water and electrolyte metabolism [91]. These further contribute to the CNS via communication pathways by modulating synaptic transmission and neuronal plasticity [92]. Apart from ischemic injury, an abnormal astrocytic activity attached to vascular instability has been observed in Alzheimer's [93]. These are vital components of the BBB and their end-feet processes cover 99% of the surface of brain microvessels from which their processes are detached only by a thin basal membrane [51]. These processes form a web responsible for the progress of the BBB phenotype of the brain endothelium [94, 95]. To support this, Janzer et al observed the strong bond between HA and the endothelium in an *in vitro* study [90]. Neuhans et al signifying an

importance of HA in forming an optimal barrier further explained this [96]. Moreover, HA are basis of important regulatory factors such as transforming growth factor- β (TGF- β) [97], glial-derived neurotropic factor, and the fibroblast growth factor, and interestingly, they can provide these secreted factors to endothelial cells [98]. The current main view is that HA regulates various aspects of BBB physiology with secreted factors and effect particular BBB features such as permeability, leading to tight junction formation and expression in endothelial cells [99].

Human Pericytes

Rouget in 1870 found pericytes as perivascular cells [47] belonging to vascular smooth muscle cell family and share a common basement membrane with endothelial cells [100]. These further contribute to constancy of microvessels and cover a major part of the abluminal endothelial surface [101]. Pericytes are vital cellular constituents of capillaries and post-capillary venules. Pericytes regulate various neurovascular functions such as angiogenesis, BBB formation in embryogenesis, maintenance, vascular stability, regulation of capillary blood flow, and clearance of toxic cellular products [102]. Pericytes can control the expression of tight junction molecules in endothelial cells by secreting factors such as TGF- β , (14) and angiopoietin (23). TGF- β improves BBB function by inhibiting the immigration of leukocytes and the proliferation of endothelial cells. The release of angiopoietin can persuade remodeling and stabilization of capillaries. Platelet-derived growth factor- β (PDGF β), secreted by endothelial cells, is an important factor for recruitment and preservation of HP on vessels and vascular maturation [103-105]. Absence of HP has been testified to subordinate with endothelial hyperplasia, an augmented capillary diameter and variations in permeability of the BBB and resulting in formation of oedema [106].

Other cell types

The interaction between endothelial cells and neurons plays an essential role in the neurovascular network. The perivascular glia limitans are thought to influence the BBB properties under the presence of microglial end-foot processes [107]. However, their contribution to BBB function remains unknown.

BBB and pro-inflammatory cytokines

Amplified cytokine release has been documented in the central nervous system following various acute insults including ischaemic stroke. Microglia and infiltrating leukocytes are known to enhance as well as produce cytokines, but some reports have shown that cytokines can also be produced by resident brain cells following acute ischaemia, including glia and neurons in humans [108]. Interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) have been the most studied cytokines in brain ischaemia [109]. Of these cytokines, IL-1 β and TNF- α has been documented to facilitate ischaemic and traumatic brain injury (TBI) [110]. In both focal and global ischaemia these cytokines are reported to be elevated in animal brains under ischaemic insult and in cerebrospinal fluid [111].

IL-1 β has been nominated in the pathogenesis of ischaemic brain as a neurotoxic mediator. There are two IL-1 isoforms, IL-1 α and IL-1 β , plus an endogenous inhibitor [110]. IL-1 β , rather than IL-1 α is thought to be more engaged in the ischaemic pathogenesis [112]. IL-1 β group results from exchange from its pro-form by IL-1 β converting enzyme (ICE-1, a member of the Caspase family) to its active form [113]. It contains two receptors, IL-1R1, which is involved in signal transduction and IL-1R2, which has no known function but may serve as a lure receptor [114]. On the

other hand, TNF- α expression has been observed in neurons, astrocytes in addition to the peripheral immune system in stroke models [115]. Although TNF- α and IL-1 β often work synergistically, TNF- α appears to have both neurotoxic and neuroprotective effects, while IL-1 β seems usually neurotoxic [116]. TNF- α sparks apoptosis of endothelial cells and contributes to vasogenic oedema and penetration of circulatory immune cells is stimulated by the BBB breakdown. On the other hand, TNF- α triggers repair processes of the cerebral microvasculature and facilitates neuronal plasticity [117]. The causes for this inequality are still anonymous, but a few theories have been projected. Firstly, the activities of TNF- α may depend on the timing of inflammatory process in that it appears to reveal disadvantageous effects in the early stage of the inflammatory response, but may have more beneficial effects at a later stage [118]. Another hypothesis narrates to the receptors to which TNF- α binds, where soluble TNF- α binds to TNF receptor 1 primarily leads to unfavourable effects, whereas membrane bound TNF- α which binds to TNF receptor 2 leads to neuroprotection [119]. The exact role of IL-6 in the ischaemic stroke has not been clearly identified. IL-6 has been shown to increase its expression continuously up to 24 hours after ischemia onset [77]. Yet, there has been a new emphasis on brain derived IL-6 where it seems to add to neo-angiogenesis and neuronal survival through STAT3 activation and manganese-superoxide dismutase (MnSOD) [120].

Oxidative Stress in Ischaemic Injury

Ischaemic attack diminishes O₂ levels in the brain. Obstruction of a blood vessel in the brain leads to rapid cell death in the ischaemic core while activating a series of deleterious mechanisms which collectively further exacerbates cell death. In particular, a complex succession of biochemical cascades takes place in the neurons leading to serious damage of the brain tissue and mediated by at least in part by pro-

inflammatory cytokines that produce neuronal loss, axonal destruction, and demyelination during the secondary injury cascade. [121]. Ischaemic condition associated with hypoxia and re-oxygenation leads to an upsurge in the BBB permeability, which results in passage of neurotoxic substances from the blood into the brain and subsequently formation of vasogenic oedema. Cerebral ischaemia and re-oxygenation is vital component of several disorders or conditions affecting the CNS. These include ischaemic stroke, cardiac arrest, acute respiratory distress syndrome, obstructive sleep apnea and high-altitude cerebral oedema [62, 122-124]. Much of the cellular damage caused by hypoxic insult is believed to occur during the subsequent re-oxygenation phase. Hypoxia and re-oxygenation is associated with an increased production of ROS [125].

Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to the possible cellular damage. As the brain consumes about 20% of the body's total O₂, neuronal cells are more vulnerable than other cells to oxidative damage. Interestingly, the brain constitutes less than 2% of the total body weight [126]. The contribution of oxidative damage to the pathogenesis of many diseases affecting the central nervous system is vastly studied. One of the major contributors of oxidative stress is free radicals; superoxide anion, hydroxyl radical, lipid radicals and reactive nitrogen species such as nitric oxide (NO) [127]. The roles of oxidative stress and reactive oxygen species (ROS) in stroke injury have been firmly cemented by research and are one of the main targets for the stroke therapy research studies. The design of ROS scavengers and antioxidants, which can improve the antioxidant defenses of the brain, has become the major part of modern stroke research.

Experimental stroke studies have confirmed that oxidative stress plays an important role in cerebral damage following ischaemia [128]. Basically, oxidative stress is the

overproduction of ROS [129], which are produced at low levels in the cell via many biochemical processes such as the mitochondrial electron transport chain. ROS perform many vital cellular functions including redox signaling and defense against invading bacteria and other microorganisms. When produced in excess numbers, these radicals can injure cells by attacking energetic cellular constituents, such as proteins, fatty acids, lipids and deoxyribonucleic acid (DNA). During ischaemia, NOS activity increases by many folds in the neurons. The glutamate fluxing at synapses, activation of NMDA receptors and elevation in the intracellular Ca^{2+} level are the main contributors behind the elevated activity of NOS. In the setting of brain ischemia, the activity of NOS is broadly deleterious and their inhibition or inactivation is neuroprotective. In addition, ischaemia leads to the generation of superoxide anion, through the action of NADPH oxidase, xanthine oxidase, cyclooxygenase, and leakage from the mitochondrial electron transport chain and other mechanisms [130]. Highly reactive NO and O_2^- are themselves deleterious and they can associate to form a highly toxic anion, peroxynitrite. This anion and other ROS destroy the DNA, activating the poly (ADP-ribose) polymerase-1 (PARP-1), a DNA repair enzyme. PARP-1 catalyse the conversion of β -nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and long polymers of poly-ADP-ribose. As a consequence of PARP-1 over-activation, cellular levels of NAD^+ depletes impairing NAD^+ dependent processes such as glycolysis and mitochondrial respiration, which leads to ATP starvation, energy failure and consequently neuronal death [131]. NADPH oxidase (NOX) is a multisubunit enzyme complex found in phagocytic leucocytes and vascular tissues. This membrane bound enzyme is suspected to be the key factor behind abnormal rise of the ROS level following ischaemic reperfusion. Activated NADPH oxidase responds with increased generation of O_2^- , which can

further react with water to generate hydroxyl radical and peroxynitrite by interacting with NO [132]. Among the five NOX isoforms, NOX2 and NOX4 isoforms are highly localised in the hippocampus and cerebral cortex. The central nervous system replies to a diversity of attacks with multifaceted inflammatory processes. Inflammation takes part in the recovery after stroke and cerebral injury, but concurrently it is one of the threats which may degrade the stroke condition [133]. Intra-ischaemic attack is associated with both factors, specifically cytokines and also cellular components such as leukocytes and microglia, many of which ensure pro-inflammatory properties with promoting or critical penalties [134]. These inflammatory factors launch a platform for leukocytes to adhere the vascular endothelial cells, so enhancing their growth. As a result, infiltration of leucocytes from the circulation into the ischaemic regions soared within minutes to hours after the onset of stroke [135].

Management

So far, two likely approaches for the treatment of stroke exist: one being thrombolysis and other neuroprotection. The basic strategy involved in the management of stroke is to cover the following measures within 60 minutes of patient arrival in the emergency unit such as assessment of airway, breathing, and circulation including initial evaluation, initiation of reperfusion therapy e.g., fibrinolysis with recombinant tissue-type plasminogen activator (rt-PA) or intra-arterial approaches), ideal BP control, administration of antiplatelet agents and mechanical thrombectomy [136]. The US Food and Drug Administration have approved the use of rt-PA in patients who meet criteria set forth by the National Institute of Neurologic Disorders and Stroke. Precisely, rt-PA must be given within 3 hours of stroke onset and only after CT scanning has ruled out haemorrhagic stroke. Four trials have publicised the efficacy of

endovascular therapy, the most recent of which, SWIFT-PRIME [137]. On the basis of recent European data, the American Heart Association and American Stroke Association recommended expanding the window of treatment from 3 hours to 4.5 hours, with more stringent exclusion criteria for the later period. Subsequently, fibrinolytic therapy administered 3-4.5 hours after symptom onset was found to improve neurological outcomes in the European Cooperative Acute Stroke Study III (ECASS III), suggesting a wider time window for fibrinolysis [138]. A 10-center European study of nearly 6,900 patients found IV rt-PA to be most beneficial when given within 90 minutes of the onset of stroke symptoms [139]. Even though other agents including platelet inhibitors (aspirin), anti-coagulants (heparins) and pro-urokinase have been recommended but with poor or no confirmation that these tactics improve the consequences [140]. Whereas, neuroprotective selection antagonises, diminishes or slows the mechanisms involved in the injury. Also, these primarily focus on penumbra thus improving the overall outcome of the attack [141].

Inclusion/exclusion criteria for the use of the Fibrinolytics

If the fibrinolytic therapy is required to be administered in a patient, a detailed review of the inclusion and exclusion criteria must be carried out. The exclusion criteria largely focus on recognising risk of haemorrhagic complications linked with fibrinolytic use. The American Heart Association/American Stroke Association (AHA/ASA) inclusion guidelines for the administration of rt-PA are as follows [39]:

- ✓ Neurologic signs not clearing spontaneously to baseline
- ✓ Symptoms not suggestive of subarachnoid haemorrhage
- ✓ No head trauma or prior stroke in past 3 months

- ✓ No MI in past 3 months
- ✓ No arterial puncture in a non-compressible site during the past 7 days
- ✓ No major surgery in past 14 days
- ✓ No history of previous intracranial bleeding
- ✓ Systolic BP under 185 mmHg, diastolic under 110 mmHg
- ✓ No indication of acute trauma or bleeding
- ✓ Not consuming an oral anticoagulant, or if so, international normalized ratio (INR) under 1.7
- ✓ Platelet count of more than 100,000/ μ L
- ✓ Blood glucose greater than 50 mg/dL (2.7 mmol/L)
- ✓ No reported seizure attacks.
- ✓ CT scan does not show evidence of multi-lobar infarction or intracerebral haemorrhage

The early molecular signals triggered by cerebral ischaemia promote inflammatory signaling and aggravate brain damage, which is characterised by an orderly sequence of events involving the activation of glia, release of vasoactive mediators, activated macrophages and release of pro-inflammatory cytokines [142]. Moreover, recent preclinical studies suggest that suppression of inflammation offers multiple advantages: First, many anti-inflammatory agents have an extended therapeutic window and are effective when administrated up to 12 hours after ischaemic insult [143] Second, considering that inflammation is particularly deleterious during

ischaemia associated with reperfusion [144], overthrow of inflammation would be a complement to reperfusion therapy using thrombolytic agents. Thus, the fact that inflammation is central to the pathophysiology of stroke and MS has raised the prospect of new anti-inflammatory approaches to counteract ischaemic damage. Natural products and/or natural product structures have been playing important roles in drug discovery and development [145], and can sometimes bring surprising new treatments to the preclinical and clinical areas of research [146]. Screening of natural products isolated from traditional Chinese medicines that were used for the treatment of neurological diseases enabled identification of certain caffeoyl triterpenoids as potential neuroprotective agents [147].

Stroke Assessment Scales

To evaluate the degree of a stroke, various scales are available and these also help in assessing the usefulness of stroke interventions. The National Institutes of Health Stroke Scales records the deficit caused by stroke and is specific for cerebral infarction. An increase in this score correlates to an upsurge in stroke severity [148]. The modified Rankin scale (scale range 0-6) provides a reliance assessment based on a global disability scale drawn from motor changes in the stroke patient, while the Barthel index helps in measuring the capability of the patient. The score ranges from 0-20, higher the score better functionality [149].

Neuroprotection

As we have discussed that initial reperfusion therapy to restore blood flow to salvageable ischaemic brain can prevent cell death and enable neurological recovery. Reperfusion may be accomplished by administration of IV thrombolytic agents, intra-arterial thrombolysis, mechanical thrombo-embolism, ultrasound-enhanced

thrombolysis and various combinations of these approaches. Other or adjunct approaches comprise improved O₂ delivery, haemodilution (increasing the fluid content of the blood) and systemic central haemodynamic augmentation therapy [150]. Risks associated with reperfusion therapies, apart from failure to reperfuse, comprise intracerebral haemorrhage (ICH), ischaemia–reperfusion (I/R) injury and catheterisation complications [150]. Despite increased risks of ICH and early death, a Cochrane review of 27 controlled trials of thrombolytic agents given during 6 hours of acute ischaemic attack onset quantified better survival and neurological consequences at 3 to 6 months; benefits were greater in patients treated within 3 hours of the onset [151]. The evidences acquired in such studies have been jointly merged into the guidelines and protocols, which approve early IV thrombolysis [150]. As mentioned above restoring perfusion by selectively aiming the occluded artery through intra-arterial thrombolysis or mechanical thromboembolectomy have been beneficial in patients with ischaemic stroke symptom onset of >4.5 hours or those for whom systemic IV rt-PA is contraindicated [43, 152]. Auto-regulation also plays an important role in augmenting neuroprotection. Cerebral vascular accident (CVA) auto-regulation is the aptitude of the CNS to uphold a constant cerebral blood flow (CBF) through a range of cerebral perfusion pressures [150]. Apart from this and IV thrombolysis there is one more contender who has managed to expand the group of therapeutic measures against ischaemic attack and that is therapeutic hypothermia mainly due to multiple-stage protection capabilities and tagged with controllable complications [153] and it has been used therapeutically for centuries [154]. In the past 6 decades, quite a few experimental models and human clinical trials performed in a variety of clinical-pathologic conditions have proven that lowering body core temperature may show benefit [155]. Furthermore, certain clinical trials manage to

show association among brain temperature, initial stroke severity and infarct volume [156, 157]. Additionally, it has been documented that 25% of stroke patients have body temperature of 37.5°C or more within first 4-6 hours of attack [158]. However, it is only recently that hypothermia was actually revealed to enhance the probability of neurologically intact survival in patients with cardiac arrest [159]. Brain temperature has been shown to surpass measured core temperature by at least 1 and 2°C in the presence of injury and inflammation [160]. Numerous studies in patients with acute brain injury have revealed relations between fever and adverse outcome, irrespective of the source of fever [161]. Measured dropping of core body temperature to ease secondary injuries (including reperfusion injury) following acute ischaemic attack has been widely studied. Several animal studies have demonstrated that induced hypothermia may mitigate I/R injury and decrease brain oedema [153]. Both mechanisms may be present within the same patient, but the two courses vary and, critically, have unlike time frames necessitating vitally diverse approaches to treatment. I/R involves mechanisms that commence within minutes to hours following injury, and post-effects last up to 72 hours [162]. Hence, at least theoretically, I/R injury need speedy treatment, preferably before or in the early stages of attack and lasting up to at least 60-72 hours as an ideal time frame. In contrast, brain oedema would require treatment for as long as it persists, and thus treatment stages would be highly variable. Results have documented positive aspects for the usage of hypothermia in traumatic brain injury, acute ischaemic stroke with oedema and acute hepatic encephalopathy, as it helps in lowering intracranial pressure (ICP). Though, reduced ICP does not certainly associate with improved result, and clinical results have been variable [150]. However, a recent study on rat models have shown significant decrease in both ICP and infarct volume under influence of moderate

hypothermia (35°C) [153]. Hypothermia in acute ischaemic stroke was firstly cast-off to control malignant brain oedema in patients with large middle cerebral artery stroke [161]. Following out of hospital cardiac arrest, recent data suggest that mild to moderate hypothermia may be as protective [163]. From the past research, patients with post-traumatic cardiac arrest have a grim prognosis, where surgeons often struggle to halt bleeding to avoid irreversible damage to the brain. Also, in one of the animal model cardiac arrest study, cooling has permitted survival with good neurological result after lengthy hemorrhage leading to it [164].

Brain cooling is well known to limit injury attributable to ischaemic insults. Deep hypothermia (25°C) has long been used during high-risk surgery to protect the brain from ischaemic insults. Mild to moderate hypothermia (cooling in the range of 28 to 35°C) provides similar improvement in neurological outcome with fewer deleterious side effects. By the mid-1960s moderate hypothermia had become a treatment of choice for patients with severe traumatic brain injury in number of centers worldwide [165]. However this did not last for long due to potential infections tagged to it with prolonged uncontrolled use. By 1990s there was a renewed interest in use of hypothermia in cardiac arrest [166]. There has been some contradiction in the literature in defining the range of temperature establishing each level of hypothermia. Some classify mild hypothermia as a core body temperature of 32 to 34°C, moderate hypothermia as 28° to 31.9°C, deep as 11°C to 28°C, profound as 6°C to 10°C, and ultra-profound as 5°C or less [167]. Others consider an induced decrease of body core temperature to 32°C to 34°C as being mild-to-moderate hypothermia [168]. Either way, most authors, as well as the clinical recommendations for hypothermia, currently suggest a target temperature range of 32°C to 35°C when it is induced for post cardiac arrest therapeutic purposes [169]. Historical evidences of the use of hypothermia for

various illnesses such as cardiac surgeries, traumatic brain injuries and traumatic limb injuries are abundant. In instance, during ancient Egyptian times, several references were made regarding the use of cold as local therapy in the Edwin Smith Papyrus, the most ancient medical text known [170]. Also, Tannic acid derived from acacia seeds were used for “cooling the vessels” [168]. Furthermore, Hippocrates supported the use of cold water for treating “swellings in the joints, ulceration, those of a gouty nature, and sprains” because it “reduces the swelling, and removes the pain” [171]. Mercurialis provided a personal example for his patients by submerging himself in the cold springs of the river Arnus so as to comfort the pain of renal colic [171]. Soon after the invention of the mercurial thermometer, James Currie recognised the first records of human temperatures in health, disease, and experimental conditions. He executed a series of studies on the consequence of innumerable methods of cooling upon physiological parameters of the human body and then used cooling practices for treating numerous clinical disorders.

In November 1938, Temple Fay, a Philadelphia neurosurgeon, began inducing systemic hypothermia in patients with cancer to ease obstinate cancer pain. Using a laboratory thermometer that permitted measurements lower than normal, the first patient was cooled to 90°F (32.22°C) by introduction to the elements and was therefore maintained for 18 hours. Rewarming was achieved by applying heat to the body surface and performing hot coffee enemas. Overall, 169 patients who wanted a break from intractable cancer pain were treated by Fay with hypothermia [171]. This sequence of patients was presented at the third international cancer congress in 1939. In a late report on this series of patients, Fay described a mortality rate of 11.2% and a 95.7% rate of success in pain relief [172]. He also introduced and encouraged hypothermia program for patients with severe head injuries and reported better

outcome following the treatments. Additionally, during Napoleon's Russian campaign (1812), Larrey tried to preserve injured limbs by using hypothermia and used the numbing effect of the cold for decreasing pain during limb amputations [173]. He also noted that injured soldiers who were warmed died earlier than those who were not [174]. Shortly after, two studies of post–return of spontaneous circulation (ROSC) hypothermia were published [175, 176]. One study documented that hypothermia group had a survival rate of 50%, and the normo-thermic group had a survival rate of 14% [176]; These results are fairly suggestive of those published more than 40 years later [177, 178].

Initial laboratory studies on hypothermia hinted that the central nervous system displays a “cold narcosis” equal to hibernation that is exclusively reversible [179]. By 1960, the association between hypothermia and decreased O₂ consumption and ICP had been discovered [180]. This led to acceptance of hypothermia for not only cardiovascular surgery but for head and spinal cord injuries as well [181]. However, the number of obstacles detected throughout execution of hypothermia and rewarming led many clinicians to abandon this procedure [182]. Interest in the beneficial effects of hypothermia after cardiac arrest was renewed sometime during the 1990s when animal experiments began to clarify the difference between mild-moderate hypothermia and profound hypothermia and demonstrated that the former may improve neurological outcome [183]. As data supporting the positive effects of hypothermia continued to accumulate, both scientists and clinicians began showing interest in this treatment modality. By 2003, the American Heart Association and the European Resuscitation Council recommended therapeutic hypothermia as a treatment modality for out of hospital comatose patients with cardiac arrest with ROSC [184].

For patient comfort, monitoring and the prevention of shivering at hypothermic levels of 32–34°C generally requires sedation, mechanical ventilation, and therefore admission to an intensive care unit [185]. Due to the limited availability of intensive care beds in hospitals and care centers in most countries, treating even minute cerebral vascular attacks is therefore precluded by substantial practical and logistical problems. The fact that intensive care treatment has long been common in clinical practice for patients with severe traumatic brain injury or after CA may explain why large and definitive randomised clinical trials of moderate hypothermia have been accomplished in these patient populations, whereas such trials have not even been started in patients with acute ischaemic stroke. The only temperature-driven phase III randomised acute stroke trial currently ongoing is the Paracetamol (Acetaminophen). The present observation that cooling to only 35°C consistently reduces infarct volume in animal experiments may help in the design of future randomised clinical trials [186]. Cooling acute stroke patients to temperatures between 32 and 35°C using conventional methods (cooling blankets, ice water and alcohol rubs) is reported to take around 4 hours [187]. With use of an endovascular cooling device inserted into the inferior vena cava, achievement of a target body temperature of 33°C has been reported in a mean of 77min [188]. Although external cooling has been shown to improve outcome after CA despite a delay of 8 hours between start of treatment and attaining the target temperature [189], methods to improve the speed of cooling deserve further study. Small and uncontrolled or non-randomised studies in patients with acute ischaemic stroke have suggested that cooling to 32 to 33°C carries a high risk of adverse events, including arterial hypotension, cardiac arrhythmias, pneumonia and thrombocytopenia [187].

The finding of an inverse relation between the duration of active cooling and efficacy

is remarkable. In animal models of focal cerebral ischaemia, the diverse pathophysiological processes, which are invoked, exert their deleterious effects over different time-courses extending from the first hours to several days after vessel occlusion [190]. Such observations would suggest that hypothermia should in fact be more efficacious if prolonged. For instance, in clinical trials of cardiac arrest, hypothermia has been proven efficacious if maintained for 12 or 24 hours [178]. Furthermore, in studies that assessed both rectal and brain temperatures, the relation between both was not unequivocal. Some authors reported brain temperature about 1°C higher than that measured rectally [191], whereas others found an opposite relation [192]. Whereas, temperature in the temporal muscle is considered to reflect brain temperature well [193].

As a result of these findings, hypothermia has gained admiration as a neuroprotectant for neurological diseases and brain where it became a strategy for comatose survivors of sudden cardiac death [168, 194]. Numerous pathophysiological stages may elaborate in the neuroprotective effect of hypothermia [195]. These may include: (i) surge in denaturation and misaggregation of proteins; (ii) slow development through the cell cycle, with phase G1 classically being the most sensitive; (iii) an inhibition of transcription and translation (iv) a disruption of cytoskeletal elements and (v) changes in membrane permeability.

Hypothermia can also produce variations in the properties of the lipid bilayer, such as phase transitions or changes in the fatty acid composition of the membrane, reflecting a cellular physiological response to cold stress. It is known that a 1°C reduction in core temperature leads to a 9.6% decrease metabolic reduction, such as in the rate of cellular respiration, O₂ demand or carbon dioxide production. This altered O₂ demand

during hypothermia treatments is particularly critical for highly aerobic organs such as the brain. Based on these values, if the brain could survive an ischaemic insult for 5 mins at 37°C, hypothermia to 27°C and 17°C would afford 15-40 mins neuroprotection, respectively, based solely on decreased cerebral metabolic rate of O₂ [196].

Dissimilar experimental data exist on the consequences of hypothermia on cerebral blood flow. Systemic hypothermia of 28-33°C is followed by decreased cerebral blood flow as measured by 14C-butanol [197], whereas selective brain cooling (33°C) with normal core temperature is tailed by enlarged cortical blood flow as measured by laser-Doppler flow-metry [196]. Thus, the hypothermia-induced hemodynamic changes may hinge on diverse experimental methods of brain tissue cooling as well as the depth and duration of hypothermia. Furthermore, external brain cooling crops a centripetal temperature value resulting in temperature differences amid superficial and deep cerebral structures [196]. On the other hand, in newborn piglets, it was demonstrated that cerebral blood flow, decreased more than cerebral metabolic rate of O₂ required, if brain temperature was below 34°C during systemic hypothermia [198]. In addition, moderate hypothermia dilates cerebral arterioles *in vitro*, suggesting that this effect may *in vivo* scenario as well [199]. From this pathophysiological feature, it appears that the cerebrovascular response fluctuates, depending on the depth of hypothermia, the method of cooling, the model of ischaemia, and the animal species. These differences may signify the key factor in defining the local hemodynamic consequences of hypothermia [200].

In forebrain global ischaemia, normothermic (37°C) but not hypothermic (33°C) rats demonstrated early post-ischaemic BBB disruption to protein tracers after an

ischaemic event [201]. Same study have also demonstrated that hypothermia reduce the leakage of radiolabeled tracers across the BBB [201], suggesting that decreased formation of brain oedema as induced by hypothermia is mainly facilitated by the protection of BBB integrity. Furthermore, hypothermia also found to decreases brain oedema formation in the basal ganglia at 24 hours after induced ischaemia [202]. Moreover, reduced brain oedema formation by hypothermia is very likely, when the hypothermic reduction of glutamate surge helps to maintain brain tissue integrity and therefore reduces brain oedema formation [196, 203]. In addition, hypothermia protects the BBB and applies a neuroprotective effect by reducing the passage of potentially injurious substances across the endothelial barrier [204, 205].

A metabolic reduction has been recommended to be the main pathophysiological mechanism of hypothermia-induced neuroprotection [206]. Protein synthesis and ion-balancing ATPase are the leading energy-consuming processes of typical metabolic rate in mammals [207]. Fluctuating temperature of 27 to 35°C induced beforehand of global ischaemia in cats and maintained during the first 1.5 to 2.0 hours of re-circulation abbreviates intracellular acidosis as assessed by magnetic resonance spectroscopy [208]. Disturbances in cerebral tissue ATP during acute ischaemia can lead to brain tissue damage. Conservation of ATP during intra ischaemic hypothermia has been documented in adult rats [209]. However, at the end of short periods of complete cerebral ischaemia, ATP stores are not preserved by hypothermia [208, 210, 211], even though the rate of reduction is hindered in some studies [208] but does acknowledges that hypothermia exerts a protective effect regardless of amplified reduction of ATP.

Also, mild perioperative hypothermia suppresses mitogen-induced activation of

lymphocytes and reduces the production of IL-1 and IL-2 [212]. In animal experiments, it has been documented that hypothermia leads to a decrease in neutrophil phagocytosis and mobility as well as reduced production of reactive oxygen species [213]. Although inflammatory response in development of ischaemic infarction has attracted the attention over the last years, little work has been done to assess the effects of hypothermia on leukocyte accumulation in ischaemic damaged brain tissue. Recently, the effects of intra-ischaemic moderate hypothermia on neutrophil infiltration following 3 hours of transient middle cerebral artery occlusion demonstrated that, at 24 hours after induction of ischaemia, hypothermia treated animals exhibited significantly less myeloperoxidase activity in the peri-core region compared with normothermic controls [214]. Moreover, Maier et al. found that 1 hour of hypothermia is adequate to reduce neutrophil growth by 75% within the ischaemic core [191]. This is clear that, by reducing the cerebral metabolic rate of O_2 and depressing the rates of all enzymatic reactions, hypothermia may blunt the group of reactive oxygen species and protect the BBB.

Recently, suppression of apoptotic neuronal cell death has been proposed as a probable mechanism of the protective effect of hypothermia [215]. Experimental data confirmed that following 3 days of an ischaemic event, 1 and 2 hours of mild hypothermia are adequate to decrease the number of apoptotic cells, as determined by TUNEL-positive staining [191]. Another study, using a rat model of mild focal cerebral ischaemia, qualitative differences in Bcl-2, Bax and cytochrome c release from mitochondria immunostaining could be demonstrated after 1 hour of mild hypothermia, suggesting that mild hypothermia declines the mass and intensity of staining for Bax and cytochrome c, but upsurges Bcl-2 staining [216]. Mild hypothermia leads to a break of a necrotic process such as mitochondrial swelling,

potentially also interfering with other apoptotic pathways.

Combination therapy with other stroke remedies

A few studies have addressed whether hypothermia could be used in combination with thrombolytics [217]. Considering that hypothermia seems to protect cerebral injury by multiple mechanisms, rather than by just focusing on one mechanism and this is what most pharmaceutical agents aim for [218]. However, despite its good qualities hypothermia is still armed with limitations, and some may not be easily overwhelmed at the clinical settings. For instance, it is understandable that cooling must be commenced as soon during or after ischaemic attack to achieve better outcomes. Early reperfusion is also desirable following an insult. However, many stroke attack survivors do not receive treatments straight away following an attack and also vast majority of these do not meet standards for treatment with rt-PA or mechanical thrombectomy. Thus, it would make sense to associate therapeutic approaches to boost the probability of a favorable outcome. Previous researches have documented failure of neuroprotectants for diverse reasons. Nevertheless, it is now evident that neuroprotection may be achievable if multiple treatments are combined and applied synergistically [219, 220]. In general, authors have suggested three-way approaches to improve the changes of hypothermia-mediated neuroprotection. The first is a “kitchen sink” approach where manifold therapies are introduced at once with the hope that if each therapy is effective on its own; they should give better results when work as a team. A second is to find situations where hypothermia is less likely to be effective (delayed cooling) and when combined with another neuroprotective strategy to may increase the likelihood of a positive result. Last approach should take into consideration when pre-clinical data displays that long-standing protection by hypothermia is possible and the degree of protection is lost as

time goes by [217].

Cooling methods

Surface cooling is a broadly used technique since the trials on hypothermia have been introduced. The objects used in delivering surface cooling are such as convective air blankets, water mattresses, alcohol bathing, and ice packing, but these strategies are also equipped with limitations such as slow rate of temperature change and longer durations required to achieve the target [221]. Furthermore, conscious patients have been reported to intolerate hypothermia mainly due to body shivering and general discomfort. On the other hand, to overcome these glitches several groups planned endovascular, or internal cooling to reduce shivering [222]. To achieve this chilled saline infusions endorse quick fall in core temperatures in anaesthetised patients but also require huge infusion volumes. Additionally, experimental studies have also explored the possibility of retrograde perfusion of cooled blood through the internal jugular in pigs [147]. This method latter led to rapid reduction of ipsilateral (same side of the brain) brain temperature, which could be controlled by adjusting perfuse temperature; however, this needs to be researched in humans. Recently, three new endovascular catheter-based devices for lowering temperatures have been used in preliminary safety trials [147]. Interestingly, cooling rates of 1.5°C/hour have been achieved using these devices, with greater patient comfort provided modest sedation with meperidine is used. The rewarming phase following hypothermia treatment is also mentioned as a crucial phase, as rapid rewarming may lead to rebound phenomena [223]. Moreover, Berger et.al., compared fast versus slow rewarming and their results showed that slow rewarming significantly reduced infarct volume compared to fast rewarming [224]. In light of the above, the current study assessed the effects of moderate hypothermia during intra- and post-ischaemic attacks over

various inflammatory counterparts and the BBB.

Multiple Sclerosis and the Blood-brain barrier

As discussed above the various inflammatory counterparts and their effects on the integrity and function of the BBB, it is important to evaluate the effect of another condition associated with neuroinflammation on the integrity of the BBB. To this end, MS was chosen, as it is the most common cause of chronic neurological disability beginning in premature to middle adult life. The global burden of MS has high impact, including growing incidences and an amplified risk in both females and white populations [225]. Cerebral blood vessels form the chief interface among the blood and brain tissues, providing the root for the immunological appropriation of the brain tissues and the prevention of agitation of the neuronal microenvironment by fluctuations in the systemic circulation. They are active, highly metabolic structures that look similar to epithelial membranes in their high electrical resistance and aptitude to form fluids through the action of an ATPase pumping mechanism. Capillaries are critical for transporting the vital nutrients and maintaining a constant supply of O₂. Much of our knowledge of the physiology of the BBB was exposed much earlier to the modern era of neuroimaging as discussed above [226]. However, discoveries in the past 25 years have added new aspects to our understanding of which is now labelled as disruption of the BBB has been detected in many neurological disorders such as MS, acute ischaemic stroke, meningitis, encephalitis, traumatic brain injury, diabetes, hyperlipidaemia and peripheral pain syndromes [227].

In some circumstances, extrinsic systemic factors, such as infection and autoimmune processes, play a dominant role in affecting blood vessels and introducing the

damage, while in others, including cerebral ischaemia and MS account to secondary damage to the blood vessels following an injury by the activation of intrinsic cellular mechanisms that are referred to collectively as neuro-inflammation [228]. Though the cerebral vasculature offers a vital protective role in upholding homeostasis by formation of the BBB, which further prevents the entry of drugs, making treatment of disorders of the central nervous system more difficult in comparison to systemic circulation [227]. Preliminary *in vivo* research studies have exhibited strong electrical resistance within tight junctions and also, revealed the complex transport mechanism underwritten as the properties of the BBB. However, the recent stress in BBB studies is on the molecular biology of the blood vessels and surrounding cells and the applications to human disease [227, 229]. Discovering the tight junction proteins that form main constituents of the barrier was a major invention in understanding normal BBB function [230]. With those molecular biology tools, it was possible to untie the mechanisms behind injury due to proteases and free radicals [231].

Moreover, MS is figured as an immune-mediated inflammatory disease that attacks myelinated axons in the nervous system. Furthermore, it destroys the myelin and the axon in variable degrees, resulting in momentous physical disability within 20 to 25 years in more than 30% cases [232]. MS can further be classified on basis of clinical scenario, occurrence of clinical relapses, time to progression and lesion development on magnetic resonance features [233-235]. These are named as follows: 1. Relapsing-remitting MS (RRMS), 2. Secondary progressive MS (SPMS), 3. Primary progressive MS (PPMS), 4. Progressive-relapsing MS (PRMS). Additionally, RRMS is further sub divided in clinically isolated syndrome where a single episode is encountered and benign MS with almost complete remission. The classic clinical picture in MS consists of the following symptoms: sensory loss, spinal cord symptoms (motor and

autonomic), cerebellar symptoms such as dysarthria (difficulty in speaking), ataxia (loss of control over the moments) and tremor (involuntary movements), optic neuritis, facial myokymia (muscle contraction without any underline cause), heat intolerance, and depression.

There is an extrinsic BBB disruption pattern detected in MS, where the initial injury occurs to the blood vessels, allowing T and B cells (T and B-lymphocytes) to cross the BBB [236]. Current concepts of pathophysiology of MS consider it a heterogeneous disease [237]. Recent data propose that in a subset of MS patients, the brain lesions show deep resemblances to tissue alterations found in vascular diseases, suggesting that a hypoxia-like metabolic injury is occurring in the inflammatory brain lesions. Both, vascular pathology as well as metabolic disturbances may be responsible for apoptosis of oligodendrocytes (myelinating cells of the central nervous system) [238]. Early pathologists defended the concept of vascular lesions being a central module of the MS attack. Contraindicating this, Dawson et al in 1916 presented that inflammatory cells clustered around veins in the centre of the demyelinated plaque [227]. Furthermore, Putnam and Adler in 1937 reconstructed serial brain sections from MS patients, and exhibited that fibrin deposits fenced the inflamed vessels [239]. RRMS presents in >80% of patients with a female to male dominance of 2:1 and the age of onset differs among 10 and 60 years [240]. The bases for MS are unknown but are supposed to involve immune-inflammatory reactions against auto-antigens, particularly myelin proteins. The most extensively accepted theory proposes that dialogue mediated by T cell receptors on CD4-T (cluster of differentiation-T cells) lymphocytes, with myelin antigens, presented by class II major histocompatibility complex expressed on macrophages/microglia, human astrocytes, and endothelial cells that leads to an immune attack on the myelin-

oligodendrocyte complex [240]. These interactions between active CD-T cells and myelin antigens apparently incite an enormous unhelpful inflammatory response and authorise ongoing proliferation of T and B cells and macrophage activation, which bears secretion of inflammatory mediators, e.g., IL-1 β and TNF- α . Hence, the immune dysregulation in MS eventually represents a failure of the immune system to energise T cells to myelin sheath components, and a disparity among pro-and anti-inflammatory cytokines. TNF- α is elevated in peripheral blood mononuclear cells as well as in serum of MS patients and its level associates with disease activity and secondary progression [241-243]. BBB endothelial cells express TNF receptor-1 and TNF- α is also found to elevate other cytokine levels [244, 245]. In the periphery, TNF- α and other cytokine receptors affect the cellular distribution of tight junction proteins [99]. It has been well documented that IV administration of TNF- α to mice resulted in an increased BBB permeability and high levels of cytokine in serum downregulate occludin expression [246]. These chemokines promote both adhesion of leukocytes to endothelial cells and migration of leukocytes across BBB. Clinically, the relapsing-remitting course of MS is considered to be a disturbance in cognitive, cerebellar, motor, sensory, bladder and bowel, brainstem, optic nerve, and cognitive functions. Additionally, besides loss of the BBB solute barrier, enhanced trans-endothelial leukocyte migration into the CNS is detected early in the course of MS, and likely to contribute to disturbances in neural integrity [247]. MRI-neuropathological studies of acute MS lesions have exposed that these focally enhanced areas entail active lesions, characterised by severe inflammation, loss of barrier, perivascular cuffing, and often extensive infiltration of tissue by monocytes [248, 249]. Part of the MS-inflammation cascade may be activated by the infiltration of the BBB by activated leukocytes, which in turn recruits the production of cytokines

and chemokines within the cerebral interstitium [250]. Several cells in active lesions release Th1-cytokines, including active T-cells, macrophages/microglia, and astrocytes. The major Th1-cytokines involved in pathogenesis of relapses-MS are interferon-gamma, TNF- α , IL-1 β , and IL-6. Raised plasma levels of these pro-inflammatory cytokines in MS patients lead to a heave of disease activity. Levels of Th1-cytokines are intensely increased during periods of active disease and decrease during remission [251]. Endothelial cell activation by these cytokines can temper the BBB phenotype by induction of several inflammatory genes. Also, cytokines induce expression of endothelial cell adhesion molecules [252]. Therefore, in light of above we assessed integrity and function of the BBB through mimicking the MS-associated BBB insult by exposing an *in vitro* model of human BBB to serum samples obtained from MS patients. Also, we added certain inflammatory inhibitors such as matrix metalloproteinase-2 (MMP-2) inhibitor III [2-((Isopropoxy)-(1,1'-biphenyl-4-ylsulfonyl)-amino)-N-hydroxyacetamide], rotenone, apocynin, Rock inhibitor (Y27632) and protein kinase- β inhibitor (LY333531) to evaluate their putative effects over the MS-associated BBB injury. Furthermore, to assess other inflammatory mediators specific enzyme-linked immunosorbent assay (ELISA)-based kits were used to measure pro-inflammatory cytokine levels in serum obtained from MS patients.

AIMS

1. To investigate inflammatory cascades during intra- and post-ischaemic injury in the absence or presence of moderate hypothermia (35°C).
2. To further evaluate pro-inflammatory cytokine level during acute ischaemic insult in the absence or presence of moderate hypothermia (35°C).

Material and Methods

Human cell culture

Human brain microvascular endothelial cells (HBMEC, SC-1000; TCS Cellworks; Buckingham, UK), human astrocytes (HA, SC-1800; TCS Cellworks; Buckingham, UK) and human pericytes (HP, SC-1200; TCS Cellworks; Buckingham, UK) were cultured up to passage 7 in their respective specialised media to retain the barrier specific properties. HBMEC were grown in specific endothelial medium (ECM, Sciencell, Caltag Systems, Buckingham, UK) consisting of 500 ml of basal medium, 25 ml of 5% foetal bovine serum (FBS), 5 ml of endothelial cell growth supplement and 5 ml of 1% penicillin/streptomycin solution containing penicillin G salt 1×10^7 units/L, streptomycin 10g/L and sodium chloride 8.5g/L. HA were full grown in specific astrocyte media (Sciencell, Caltag Systems, Buckingham, UK) containing 500 ml of basal medium, 10 ml of 5% FBS, 5 ml of astrocyte growth supplement, and 5ml of 1% penicillin/streptomycin solution penicillin G salt 1×10^7 units/L, streptomycin 10g/L and sodium chloride 8.5g/L. Lastly, HP were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL) in a humidified atmosphere (95% relative humidity) under normal conditions at 37°C with 5% CO₂.

Human blood serum samples for multiple sclerosis study

Human blood samples (~10-15 ml) were collected from relapsing-remitting MS patients while they were in relapse or remission phase of MS. Samples collected from MS-free healthy volunteers to serve as controls. Following collection, blood samples were centrifuged at 2000g for 15 mins to separate serum from the cellular debris which is accumulated at the bottom of the test tube. Samples of serums were then allocated in 1mL Eppendorf tubes and stored at -80°C until use.

Induction of oxygen-glucose deprivation in absence or presence of hypothermia

To study the effects of intra- and post-ischaemic stroke injury mimicked by oxygen-glucose deprivation (OGD), the confluent cells were placed in specific OGD incubator (SANYO, MCO-18M Multi-gas cell culture incubator, 94% N₂, 1% O₂ and 5% CO₂ at 37°C) with growth media replaced with glucose-free RPMI-1640 media (Life Technologies, Paisley, UK) under hypoxic conditions. To expose cells to hypothermia, above mentioned Sanyo incubator was used. A desired temperature of 35°C was attained before putting cells inside the incubator. Cells were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C) to mimic intra-ischaemic episode. To generate post-ischaemic scenario cells were exposed to OGD for 4 hours followed by 20 hours of moderate hypothermia. Cells cultured under normal conditions without OGD were served as controls.

***In vitro* model of human blood-brain barrier**

To create an *in vitro* model of human BBB and to study precise barrier properties associated with ischaemic insult and MS, HBMEC, HA and HP were concurrently cultured to maximum confluence using transwell inserts. In brief, HA (~75,000 cells/150 µL) were seeded on the basolateral aspect of polyester membrane under specialised growing astrocyte media. Transwell inserts (Corning Lifesciences, Tewksbury, USA; 12 mm diameter, 0.4 µm pore size) directed upside down in the 6-well tissue culture plates. Following overnight adherence of astrocytes to the membranes, the inserts were inverted the correct way in sterile 12-well tissue culture plates containing fresh astrocyte growth media (1.5 mL) and were kept under normal tissue culture conditions until attainment of ~90% confluence. Thereafter, the HBMEC (~50,000 cells/150 µL) in ECM were seeded onto the inner part of the same inserts and the cells were cultured to the maximum confluence, once this is achieved,

the co-culture sandwich was then transferred to a fresh 12-well plate containing HP which was grown in normal cell growing medium DMEM. Following addition of HBMEC-HA co-culture in fresh HP containing plate, the triple culture model was then grown to maximum confluence with help of DMEM medium. Equal number of HBMEC and HA were seeded in a separate insert to monitor the cell proliferation rate and confluence simultaneously with the co-cultures. For OGD study, following attainment of full confluency the triple culture inserts were then exposed to OGD.

To investigate the effects of serum obtained from MS patients on the BBB, triple cultures were exposed to serum samples for 4 or 6 hours. Following the exposure, the assessment of the integrity and function of the BBB was carried out by measuring transendothelial electrical resistance (TEER) using an Ohm's meter and paracellular flux via measuring the flux of permeability markers sodium fluorescein (NaF; 376 Da), and Evan's blue-labelled albumin (EBA; 67 kDa) across the barrier, respectively. To evaluate leakage across the barrier, either NaF (10 µg/mL) or EBA (165 µg/mL; 0.5 mL) were gently added to the inserts and incubated for 1 hour at 37°C. Following incubation, samples were collected from the lateral and basolateral sides (400 µL). The concentration of EBA and NaF molecules in the samples were measured by using a BMG LABTECH Omega plate reader at absorbance: 610 nm and fluorescent TECAN GENios plate reader at excitation: 440 nm, emission: 525 nm. The flux of permeability markers were calculated as cleared volume by the formula: Cleared volume (µL) = $\frac{\text{concentration (abluminal reading)} \times \text{volume (abluminal)}}{\text{concentration (luminal reading)}}$.

Furthermore, to evaluate various inflammatory pathways involved in inflammation-associated BBB damage and the role of inflammatory cascade inhibitors as potential

treatment options, the inhibitors indicated below were added to the experimental conditions.

1. Apocynin (1mmol/L; Calbiochem, Nottingham, UK): inhibitor of Nox and also results in decrease production of superoxide anion. Therefore reduces oxidative stress on the BBB during the insult.
2. Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, 2.5µmol/L; Cambridge Bioscience, Cambridge, UK): anti-oxidant on TNF-α production, also prevents generation of intracellular reactive oxygen species.
3. Rotenone (2µmol/L, Sigma-Aldrich, Poole, UK): Inhibits mitochondrial electron at NADH-ubiquinone oxidoreductase resulting in a decrease in Cytochrome b.
4. Y27632 (2.5µmol/L, Calbiochem, Nottingham, UK): Highly potent, cell-permeable ROCK inhibitor.
5. PKC-β inhibitor (LY333531, 0.05µmol/L; Alexis Biochemical; Bingham, UK): Potent and ATP-competitive inhibitor of PKC-β isozyme.
6. MMP-2 inhibitor III (444288, Calbiochem, Nottingham, UK [2-((Isopropoxy)-(1,1'-biphenyl-4-ylsulfonyl)-amino)-N-hydroxyacetamide], 100µmol/L): Reversible inhibitor of MMP-2, a protein implicated in pathological breakdown of extracellular matrix proteins.

Western blotting

Western blotting was performed to resolve proteins on polyacrylamide gels. Gels were then used to compare the fold differences in their levels observed by immuno-

specific reactions on the polyvinylidene fluoride or nitrocellulose membranes. Equal quantities of protein samples and cell homogenates were reduced (2-mercaptoethanol in 2X Laemmli dye, Bio-Rad Laboratories, Hemphstead, UK) and denatured to open tertiary/quaternary absorbance at 610 nm structures (100°C, 5 minutes). The samples were resolved on SDS-polyacrylamide gels 7.5-15% and proteins were transferred on the methanol-activated PVDF membranes followed by blocking with 5% dry milk for 1 hour. The membranes were probed with primary antibodies (Claudin-5: 1:500, β -actin: 1:300) against human antigens followed by washing with the wash buffer 1X Tris-buffered saline, 0.1% Tween 20 (TBST). The membranes were subsequently probed with species-specific infrared dye conjugated appropriate secondary antibodies (goat anti-rabbit, cat no 926-32211; 1:30,000; Li-Cor Biosciences, Cambridge, UK) for 1 hour and were again washed to remove excessive antibodies to reduce the background and increase the signal intensities. The protein bands on the membrane were visualised by scanning on the Odyssey infrared imaging system and band intensities obtained by Li-Cor imaging software were used to calculate the relative band intensities between different treatment regimens after normalisation against the loading control β -actin.

Immunocytochemistry

To analyse the cellular localisation of proteins of interest, the cells were cultured on glass coverslips to subconfluence under experimental and corresponding control conditions. Following the treatments, the growth media was carefully aspirated and the cells were fixed in 4% paraformaldehyde/PBS, 15 minutes, permeabilised 0.1% Triton X-100/PBS for 15 minutes and blocked in 10% BSA/PBS for 1 hour at room temperature. The cells were either incubated with Rhodamine-labelled phalloidin dye (Invitrogen, Paisley, UK) for F-actin staining 200 U/ml, 20 minutes, room

temperature. The coverslips were washed and incubated in 4', 6-diamidino-2-phenylindole (DAPI) for 5 minutes in room temperature in dark. The excessive DAPI was removed by washing and the coverslips were mounted using glycerol/PBS (3:1), and the edges were sealed by nail polish. Cellular localisation of F-actin were observed under 20x magnification on a Zeiss Axio Observer fluorescence microscope and pictures captured by Sony cyber shot digital camera.

Cytokines

To measure the levels of pro-inflammatory cytokines TNF- α and IL-1 β in MS patients' serum and similarly in samples subjected to OGD specific ELISA-based kits were used. Assessment of cytokines in extracellular medium (ECF) obtained following OGD linked stress in HBMEC, HA and HP. The kit for TNF- α was TNF- α solid phase sandwich ELISA, Human TNF-alpha DuoSet DY210 ELISA (R&D systems, Abingdon, UK) and for IL-1 β it was IL-1 β solid phase sandwich ELISA, Human IL-1 beta/IL-1F2 DuoSet DY008 ELISA, (R&D systems, Abingdon, UK). All the components of the kits were further diluted to desired levels or working concentrations. Fresh 96-well plate was coated with 100 μ l of diluted capture antibody provided in the above mentioned ELISA kit and sealed for overnight incubation. On the following day, the plate was rinsed 3 times with wash buffer 400 μ l and blocked by 300 μ l/well reagent diluent followed by incubation for 1 hour at room temperature. Subsequently, we added 100 μ l of serum/ECF into each well and continued incubation for a further 2 hours at room temperature, followed by addition of the detection antibody (in ELISA kit), to each well and leave it for next 2 hours. Following this, working dilution of Streptavidin-HRP provided with the respective kits was added to each well and incubated for 20 minutes at room temperature. Lastly, to halt the reaction we added 50 μ l of Stop Solution and finally, to determine the

optical density we directly used microplate reader which was already set to 450 nm (FLUOstar Omega multiplate microplate reader, BMG LABTECH, Ortenberg, Germany). To measure these cytokines in MS, we replaced human blood serum from cell supernatant and conducted the experiment similar to above mentioned protocol.

Caspase-3/7

Caspase-3/7 assay was performed using Apo-ONE® homogeneous caspase-3/7 as per manufacturer's instructions (cat no. G7790; Promega; Southampton, UK). Briefly, cells were grown to 95% confluence in 96-well plates and subjected to time-dependent OGD in absence or presence of hypothermia. Following exposure, 100µl of media was removed from each well and substituted with caspase-3/7 assay buffer of similar amount to initiate the reaction. Plates were kept overnight at -80°C. On the following day, plates were thawed before reading fluorescence. The cleavage of the non-fluorescent caspase substrate by caspase-3/7 produced the fluorescent rhodamine-110, which was read at excitation wavelength of 485 nm and emission of 520 nm. Blanks were subtracted from reading prior to normalising activity against mg protein. To assess rate of cellular apoptosis during MS disease progression, caspase-3/7 activity was measured in serum samples obtained from relapse or remitting MS patients.

Terminal deoxynucleotidyl transferase biotin-dUPT nick-end labeling (TUNEL)

TUNEL staining was performed using a DeadEnd™ Colourimetric TUNEL system detection kit according to the manufacturer's instructions (cat.no.G7130, Promega, Southampton, UK). Briefly, cells cultured on coverslips were fixed and permeabilised as above before equilibrating in a buffer containing potassium cacodylate (200mmol/L), Tris-HCL (25 mmol/l), dithiotheitol (0.2 mmol/L), BSA (250 mg/L)

and cobalt chloride (2.5 mmol/L) for 10 min. The coverslips were then intubated with recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mix containing equilibrium buffer (98µl), biotinylated nucleotide mix (1µl) and rTdT enzyme (1µl) for 60 min to allow end-labeling to occur. The reaction was stopped by immersing coverslips in 20 X SSC buffer before blocking and staining them with 0.3% H₂O₂ and horseradish peroxidase-labelled streptavidin (500mg/L; 1:500), respectively. TUNEL-positive cells were visualised by diaminobenzidine, which gave dark brown staining to apoptotic cells. Cells were viewed under a light microscope and counted TUNEL-positive versus total from 3 different fields per coverslip before calculating mean values for these areas.

Measurement of superoxide anion generation

The superoxide anion production was measured by cytochrome C reduction assay as previously described [253]. In brief, cell pellets were sonicated in cold lysis buffer containing 20 mmol/L HEPES buffer (pH 7.2), 1 mmol/L EGTA, 210 mmol/L mannitol and 70 mmol/L sucrose. Equal amounts of homogenate (50 µl) were then incubated with 50 µmol/L cytochrome C for 60 minutes at 37°C. Superoxide anion generation was measured as the reduction of cytochrome C and monitored as the change in absorbance at 550 nm using a FLUOstar Omega plate reader (BMG, Aylesbury, UK).

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescent detection of superoxide anion aka lucigenin-chemiluminescence assay. HBMEC homogenates (50 µl) were incubated at 37°C with assay buffer 50 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EGTA, 150 mmol/L sucrose, and 5µmol/L

lucigenin containing the specific inhibitors of enzymes that are known to generate reactive oxygen species, namely nitric oxide synthase (L-NAME, 100 $\mu\text{mol/L}$), xanthine oxidase (allopurinol, 100 $\mu\text{mol/L}$), mitochondrial complex I (rotenone, 50 $\mu\text{mol/L}$) and cyclooxygenase (indomethacin, 50 $\mu\text{mol/L}$). After 15 min NADPH (100 $\mu\text{mol/L}$; Sigma-Aldrich, Poole, UK) was added to initiate the reaction. The reaction was monitored every minute for 4 hours and the rate of reaction calculated. Buffer blanks were also run for both assays and subtracted from the data.

Detection of superoxide dismutase activity

Superoxide dismutase activity was measured by detecting the level of variation in superoxide anion levels using a tetrazolium salt. OGD subjected cell homogenates were diluted 1:20 with radical detector, provided in the kit containing 250 μl of tetrazolium salt and assayed in triplicate in a 96-well ELISA plate (Calbiochem, Nottingham, UK). Reactions were initiated by the addition of 20 μl diluted xanthine oxidase to generate superoxide anion and the plate was incubated on a shaker for 20 min at room temperature. Absorbance was read at 450 nm and one unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide anion. MnSOD activity was detected following inhibition of Cu/Zn-SOD activity through incubation with 3 mmol/L potassium cyanide (BDH Chemicals Ltd, Dorset, UK), at room temperature for 45 min. CuZnSOD activity was subsequently calculated by the subtraction of MnSOD activity from total SOD activities.

Cell counting

To overcome inter-experimental errors HBMEC, HA or HP were seeded in accurate numbers and monitored for the cellular viability. To obtain this, small aliquots of cell suspension (10 μL) was mixed with equal volume of 0.1% trypan blue for 5 minutes

and the mixture was carefully pipetted on haemocytometer for cell counting. Cells situated on the 5 big grids were counted by the aid of a light microscope, and cells/ml was calculated by the following formula: Average number of cells in one large square x dilution factor x 10^4 , dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) cell suspensions. Calculation of cell viability: $\text{No. of Viable Cells Counted} / \text{Total Cells Counted (viable and dead)} \times 100$ = % viable cells

Protein Quantification

Cells grown in the cell culture flasks were washed twice with sterile PBS. The cells were dislodged from the flask by a sterile cell scraper and were concentrated in the cell-lysis buffer (0.1% Triton X-100/PBS) containing protease cocktail inhibitor (P8340, Sigma-Aldrich, Gillingham, UK) and phenylmethanesulfonyl fluoride (PMSF, 93482, Sigma-Aldrich, Gillingham, UK). The cells were lysed by sonication and cell homogenates were collected in pre-cooled Eppendorf tubes on ice. Half of the cell homogenates were aliquoted and stored at -80°C and cell debris in the remaining protein samples were removed by centrifugation (12,000 rpm/10 minutes) followed by storage at -80°C . A small volume of protein samples and cell homogenates were used for protein quantification by Bradford assay using detergent-sensitive Coomassie plus assay kit (23236, Life Technologies Ltd. Paisley, UK). Briefly, in a 96-well plate the bovine serum albumin (BSA) standards (10 μL) with known protein concentrations (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 $\mu\text{g/mL}$) were mixed with Coomassie plus reagent (300 μL) in duplicates and a standard curve with its equation were derived from the absorbance values (610 nm) measured on the BMG LABTECH Omega plate reader after the colorimetric analysis.

Statistical analysis

Data are presented as mean SEM. Statistical analyses were performed using GraphPad Prism 6.0 statistical software package. Data were analysed by nonparametric Mann Whitney U test or one-way ANOVA followed by Dunnett's post-hoc analyses, where appropriate. $P < 0.05$ was considered significant.

Results

Chapter 1

The effects of intra-and post-ischaemic injury on the integrity and function of the blood-brain barrier with role of hypothermia

Introduction

Neuroinflammation plays a significant role in the evolution of the ischaemic event [190], and the blockage of blood supply leads to significant ischaemic damage. Previous studies have demonstrated the key role of pro-inflammatory cytokines such as TNF- α and IL-1 β in the deleterious effects of neuroinflammation and are hence likely targets for the future stroke therapy. There are numerous studies signifying extensively complex cascade involved in an acute ischaemic injury [190, 254-256].. The progression of ischaemia is known to be time-dependent, including the release of cytokines and the BBB opening, therefore, it becomes mandatory for therapeutic interventions to be time-dependent [257]. Cytokines activate second messengers and signaling molecules, such as Ca²⁺. These substances in-turn activate many critical reactions in the central nervous system by aiding as catalysts for rate-limiting enzymes involved in neurotransmission. Cytokines may also damage the BBB by increasing its permeability without even entering the brain and by activating and destructing tight junction of microvascular endothelial cells forming the BBB. The BBB dysfunction is a hallmark of neuroinflammatory diseases such as human immune deficiency virus associated encephalitis, bacterial meningitis, and MS [258, 259]. The breakdown of the BBB is an important pathological trademark of ischaemia in the absence or presence of reperfusion, and is linked with vasogenic oedema. Hypoxia associated ischaemic conditions are known to persuade an inflammatory response in the central nervous system ultimately resulting in disruption of the BBB [260]. Furthermore, *in vitro* experiments have also shown that cytokines like TNF- α , IL-1 β , and IL-6 disturbs the BBB [261]. The BBB breakdown at high concentrations of cytokines correlates with caspase-3/7 activation via Jun amino-terminal kinases

(JNK) and protein kinase C (PKC) signaling pathways and with an increase in the number of cells undergoing apoptosis [262].

It has been reported on numerous occasions that BBB associated endothelial cells and HA are potent producers of cytokines [99, 263-265]. These locally produced cytokines are foremost responsible for enrollment and activation of immune cells not only in acute ischaemic injury but also in MS. It has been known that some cytokines can directly cross the BBB, in some cases within thirty minutes of ischaemic attack for example TNF- α [266]. Mechanisms involved in swift passage of cytokines across the barrier are usually either by saturable influx transport or by circum-ventricular organs, i.e. areas in the brain where the BBB is incomplete [267]. TNF- α is a pleiotropic inflammatory cytokine, usually demonstrates a bi-phasic release pattern with first peak in 1 to 4 hours and second being in 24 to 36 hours of acute ischaemic insult [268, 269]. Once microglia and macrophages are activated they tend to become a major sources of TNF- α within first 6 hours of the insult [270]. The BBB associated endothelial cells have reported to contain TNF receptor-1 and these also have further been adapted to play significant role in upregulation of Toll-like receptors-2 [244]. These receptors are further found to downregulate the tight junction expression inversely affecting the BBB [271]. Although IL-1 β and TNF- α are found to work synergistically, they are secreted by non-overlapping subsets of microglia/macrophages. Similarly, IL-1 β is found to be biphasic as well, first peak being in 1 hour following reperfusion and second being after 6 to 24 hours [268]. It induces vasogenic oedema and summons the endothelium for leukocyte adherence [272]. Secondly, it contributes to influx of calcium into the neurons making them more vulnerable to ischaemia.

Likewise, caspases enzymes form a large family comprising 12 members and they play an important role in the apoptotic process and also as cytokine activators in the immune system. They are the executioners of the apoptotic program and assure the irreversibility of apoptosis by proteolytical slicing targeted cellular proteins. This leads to the controlled degradation and disruption of all essential cellular pathways during an acute ischaemic phase. Moreover, these are a family of genes that are important for upholding homeostasis through regulating cell death and inflammation. These further hydrolyse peptide bonds in a reaction that hinge on catalytic cysteine residues in the caspase active site [273]. Accordingly, caspases have been broadly classified by their known roles in apoptosis (caspase-3, -6, -7, -8, and -9 in mammals), and in inflammation (caspase-1, -4, -5, -12 in humans and caspase-1, -11, and -12 in mice) [273]. Caspases involved in apoptosis have been sub-classified by their mechanism of action and are either initiator caspase such as caspase-8 and caspase-9 or executioner caspases (caspase-3, caspase-6, and caspase-7). Unfortunate activation of the executioner caspases is prohibited by their production as inactive procaspase dimers that must be cleaved by initiator caspases. This cleavage among the large and small subunits permits a conformational alteration that conveys the two active sites of the executioner caspase dimer together and makes a functional mature protease [274]. Once activated, a single executioner caspase can cleave and trigger other executioner caspases, leading to an accelerated response circle of caspase activation.

Therefore, there is a pressing need to discover a neuroprotectant that can decrease the concentrations of the above mentioned cytokines during and after an ischaemic phase and manages to decrease the activities of caspase-3/7. While, speedily re-establishing the blood supply is vital as it possibly will allow salvation of neurons in the penumbra

from irreversible damage, as energy metabolites and cellular membrane ionic gradients would be restored to normal levels [275].

Aims

1. To investigate the effects OGD on the release of pro-inflammatory cytokines in the absence or presence of moderate hypothermia (35°C).
2. To investigate the effects OGD on the integrity and function of the blood-brain barrier during intra- and post-ischaemic injury in the absence or presence of moderate hypothermia (35°C).

Results

Effects of oxygen-glucose deprivation on IL-1 β secretion in human astrocytes, human brain microvascular endothelial cells and human pericytes in the absence or presence of moderate hypothermia (35°C)

Using a specific ELISA-based kit, IL-1 β concentrations were measured in HBMEC, HP and HA. To mimic ischaemic insult in *in vitro* settings, the cells were subjected to OGD for 4 and 20 hours. Additionally, to mimic intra-ischaemic hypothermic treatments cells were subjected to 4 and 20 hours of OGD in the presence of hypothermia. To mimic post-ischaemic setting the cells were subjected to OGD for 4 hours prior to induction of hypothermia for 20 hours. In HA and HBMEC, exposure to 4 hours of OGD in the absence of hypothermia led to a reductions in the amount of IL-1 β secretion compared to controls. HBMECs subjected to 4 hours of OGD in the presence of hypothermia demonstrated slight but insignificant increase in IL-1 β secretion. This signifies that IL-1 β is secreted in lesser extent at the time of injury. Although, HP subjected to 4 hours of OGD in the absence of hypothermia showed an

increase in IL-1 β levels when compared to controls, addition of hypothermia resulted in a significant decrease in IL-1 β concentrations compared to OGD alone groups. Furthermore, all three cells when subjected to 20 hours of OGD in the absence of hypothermia led to a massive upsurge in IL-1 β concentrations. On the contrary, cells exposed to 20 hours of OGD in the presence of hypothermia led to a significant decrease in IL-1 β levels. High concentrations of IL-1 β at 20 hours of ischaemic insult signify the late release property associated with the particular cytokine. In the post-ischaemic scenario, hypothermia caused a decrease in IL-1 β levels in all three cell lines when compared to intra-ischaemic episodes or controls (n=6, p<0.05). Hence, these results demonstrate the beneficial effects of hypothermia in declining IL-1 β levels during intra- and post-ischaemic injury in HA and HP.

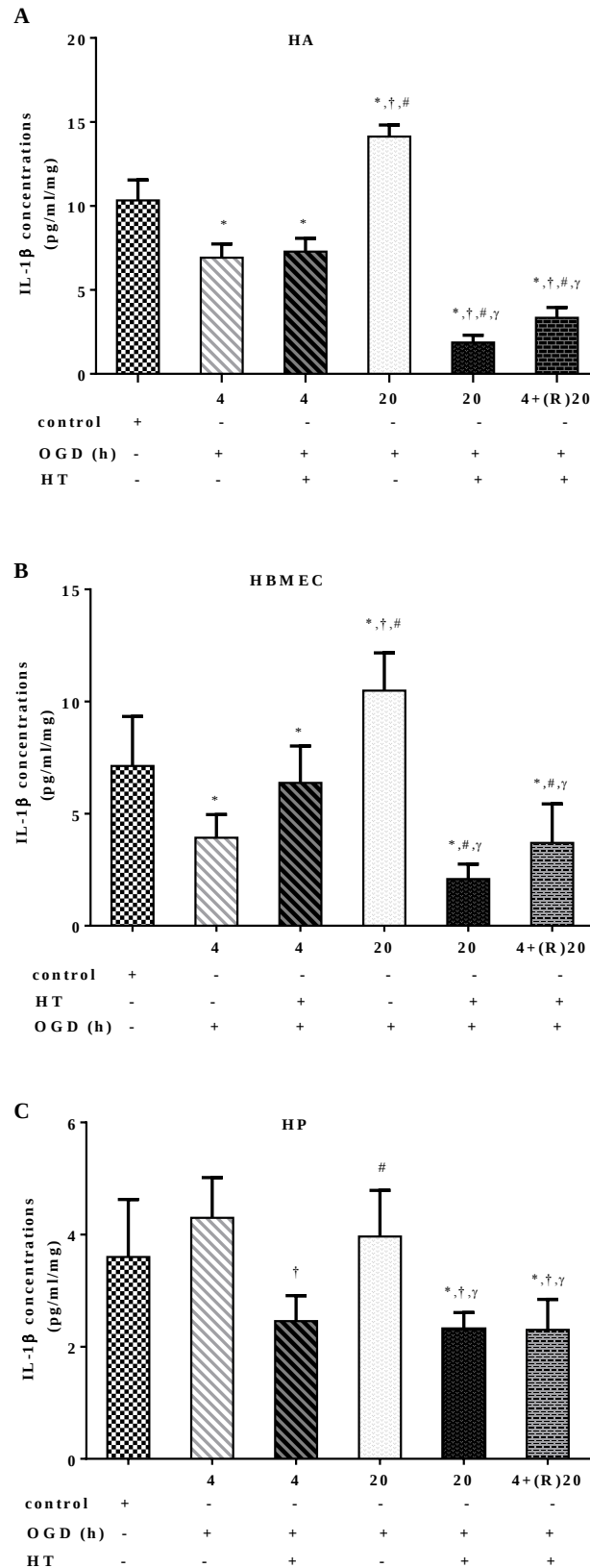


Figure 3-Effects of oxygen-glucose deprivation on IL-1 β levels in human astrocytes (HA, A), human brain microvascular endothelial cells (HBMEC, B) and human pericytes (HP, C) in the absence or presence of moderate hypothermia

Data represented as concentrations of IL-1 β on y-axis and time-dependent exposure of cells to the OGD in the absence or presence of hypothermia on x-axis. Results demonstrated from 6 independent experiments. * $p < 0.05$ vs controls, $^{\dagger}p < 0.05$ vs 4 hours OGD, $^{\#}p < 0.05$ vs 4 hours OGD + HT, $^{\gamma}p < 0.05$ vs 20 hours OGD; OGD-oxygen-glucose deprivation; HT-hypothermia; IL-1 β -interleukin-1 β ; (h)-hours

Effects of oxygen-glucose deprivation on TNF- α secretions in human astrocytes, human brain microvascular endothelial cells and human pericytes in the absence or presence of moderate hypothermia (35°C)

Using specific ELISA-based kit, TNF- α levels were measured in HA, HBMEC and HP. To mimic ischaemic insult in *in vitro* settings, the cells were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (intra-ischaemic episodes) and to mimic post-ischaemic settings, cells were subjected to 4 hours of OGD prior to induction of hypothermia for 20 hours. HA, HBMEC and HP when subjected to 4 hours of OGD in the absence of hypothermia showed transient increase in TNF- α level in comparison to controls. Conversely, cells under the influence of 4 hours of OGD in the presence of hypothermia significantly decreased the TNF- α level when compared to OGD alone groups. Moreover, cells when subjected to 20 hours of OGD in the absence of hypothermia showed an increase in TNF- α level, which was significantly reduced following an addition of hypothermia. High TNF- α concentration at 4 and 20 hours of OGD in the absence of hypothermia signifies the bi-phasic nature of the particular cytokine. Furthermore, in post-ischaemic episodes, hypothermia caused a decline TNF- α level in all three cell line, when compared to intra-ischaemic scenarios ($n=6$, $p < 0.05$).

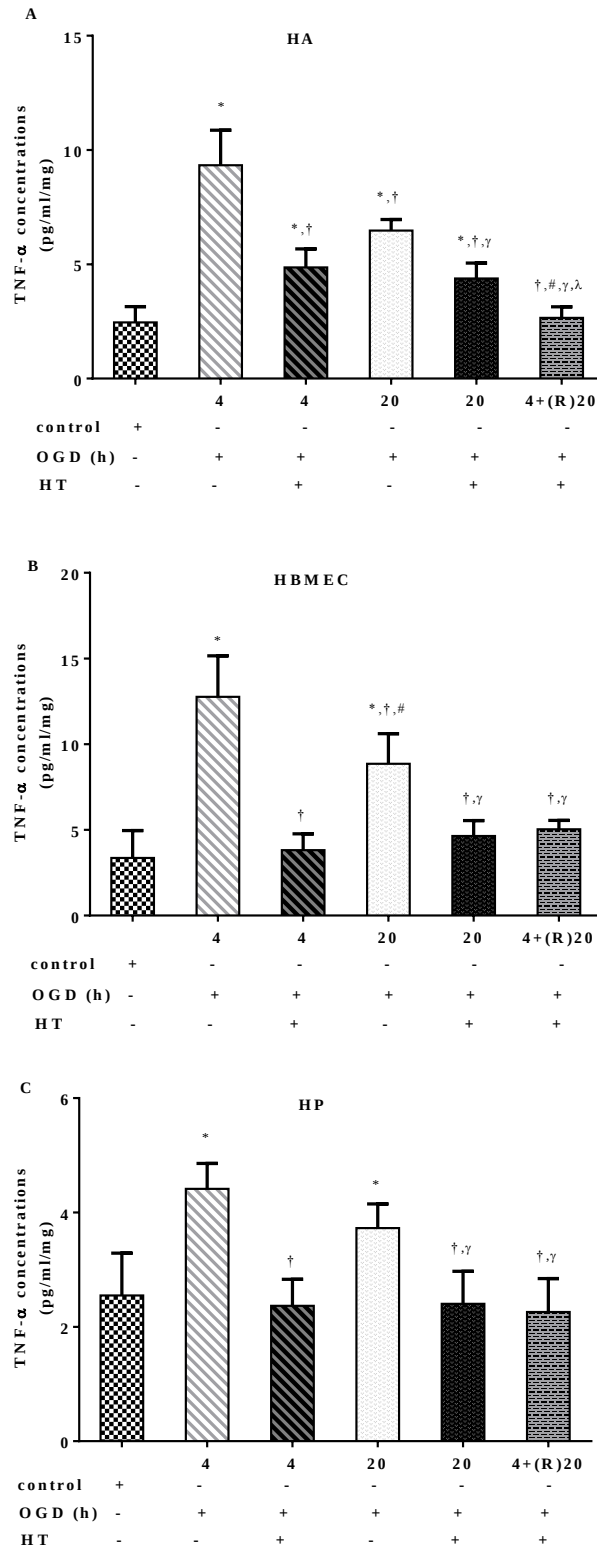


Figure 4-Effects of oxygen-glucose deprivation on TNF- α level in human astrocytes (HA, A), human brain microvascular endothelial cells (HBMEC, B) and human pericytes (HP, C) in the absence or presence of moderate hypothermia

Data represented as concentrations of IL-1 β on y-axis and time dependent exposure of cells to the OGD in the absence or presence of hypothermia on x-axis. Results demonstrated from 6 independent HBMEC, HA and HP experiments (n=6, p<0.05). *p<0.05 vs controls, †p<0.05 vs 4 hours OGD, #p<0.05 vs 4 hours OGD + HT, γ p<0.05 vs 20 hours OGD, λ p<0.05 vs 20 hours OGD + HT. TNF- α -tumor necrosis factor- α ; OGD-oxygen glucose deprivation; HT- hypothermia, (h)-hours.

Effects of oxygen-glucose deprivation on human *in vitro* triple culture blood-brain barrier models in the absence or presence of moderate hypothermia (35°C)

In order to assess the integrity and function of the BBB, *In vitro* human BBB models were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. BBB models were exposed to similar intra-ischaemic and post-ischaemic episodes in the absence or presence of hypothermia as mentioned earlier. Triple cultures when subjected to 4 hours of OGD in the absence of hypothermia demonstrated a decrease in TEER values when compared to controls. Results demonstrated loss of resistance across the barrier accompanied with 4 hours of OGD exposure. On the contrary, BBB models when subjected to 4 hours of OGD in the presence of hypothermia significantly showed an increase in TEER values compared to OGD alone groups. Furthermore, BBB models when exposed to 20 hours OGD in the absence of hypothermia further declined the TEER values as compared to 4 hours-OGD alone or control groups. Nevertheless the addition of hypothermia led to an increase in TEER values. Furthermore, post-ischaemic episodes of hypothermic treatments showed slight increase in TEER values when compared to intra-ischaemic OGD alone groups (n=11, p<0.05). To assess the degree of BBB destruction in terms of leakage of the barrier during intra- and post-ischaemic injury, two paracellular flux markers were used. Triple cultures when subjected to 4 hours of OGD in the absence of hypothermia showed concurrent increase in paracellular flux across the barrier as

documented with increased EBA and Naf cleared volumes. Signifying the amount of leakage befalls across the barrier during 4 hours of ischaemic insult. In contrast, addition of hypothermia resulted in reduction of paracellular flux values. Likewise, triple cultures subjected to 20 hours of OGD in the absence of hypothermia revealed significantly high amount of paracellular flux and introduction of hypothermia showed a significant decrease in paracellular flux values. Post-ischaemic episodes of hypothermic treatments showed a slight decrease in paracellular flux values when compared to OGD alone groups. Above-mentioned results signify the role of hypothermia in preserving the integrity of the BBB and delaying the damage.

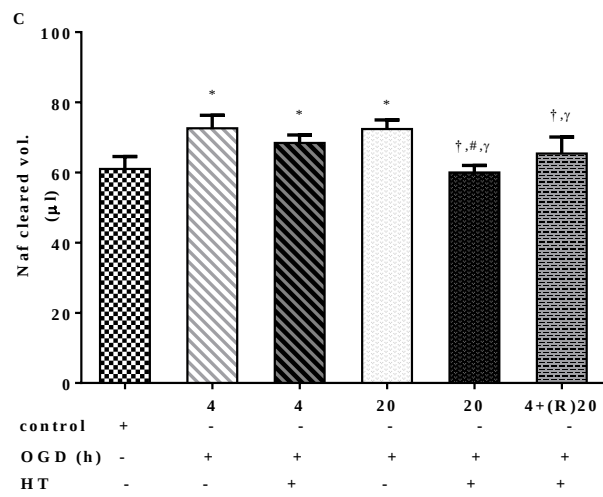
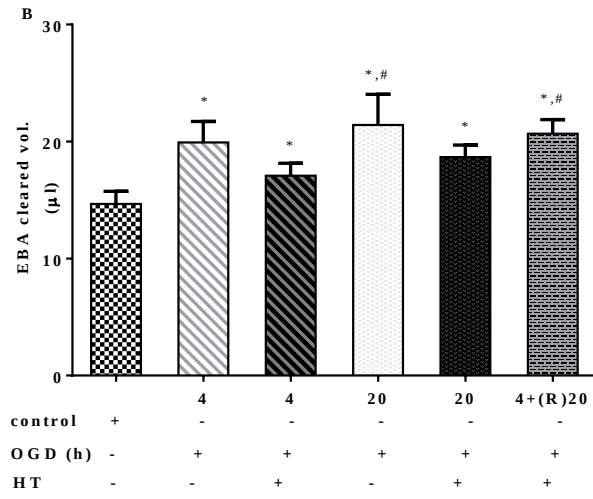
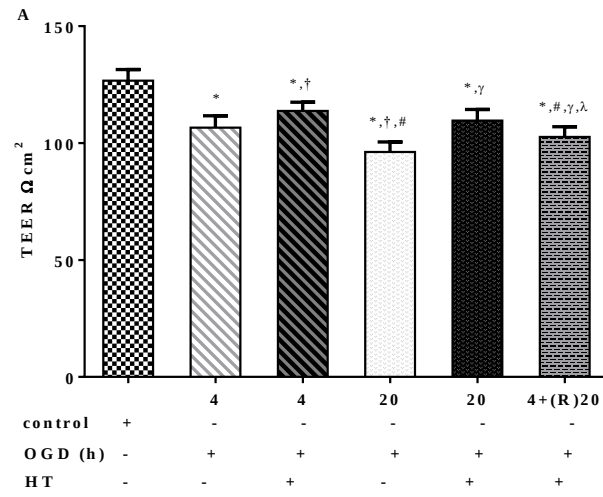


Figure 5-Effects of oxygen-glucose deprivation on the integrity and function of human *in vitro* triple culture blood-brain barrier models in the absence or presence of moderate hypothermia

Data on y-axis shows TEER (A) values and paracellular flux values EBA, (B) and Naf (C) and on x-axis data demonstrates time dependent exposure of triple culture *in vitro* BBB models to 4 and 20 hours of OGD in the absence or presence of hypothermia. Results for TEER demonstrated in 11 different experiments (n=11), EBA in 6 different experiments (n=6) and Naf in 5 different experiments (n=5). *p<0.05 vs controls, †p<0.05 vs 4 OGD, #p<0.05 vs 4 OGD + HT, †p<0.05 v/s 20 OGD, ‡p<0.05 v/s 20 OGD + HT. OGD-oxygen glucose deprivation, HT-hypothermia, TEER-transendothelial electrical resistance, EBA-Evan's blue paracellular flux marker, NaF- sodium Fluorescence paracellular flux marker.

Effects of oxygen-glucose deprivation on the tight junction protein expressions in the absence or presence of moderate hypothermia (35°C)

In order to assess claudin-5 tight junction protein expressions, HBMECs were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. OGD alone groups documented equal amount of protein expressions compared to the controls. Furthermore, cells subjected to OGD in the presence of hypothermia showed surge in protein expression at both 4 and 20 hours of exposure. Hence, suggesting the role of hypothermia in maintaining the tight junction anatomy and indirectly upholding the BBB architecture (n=3, p<0.05).

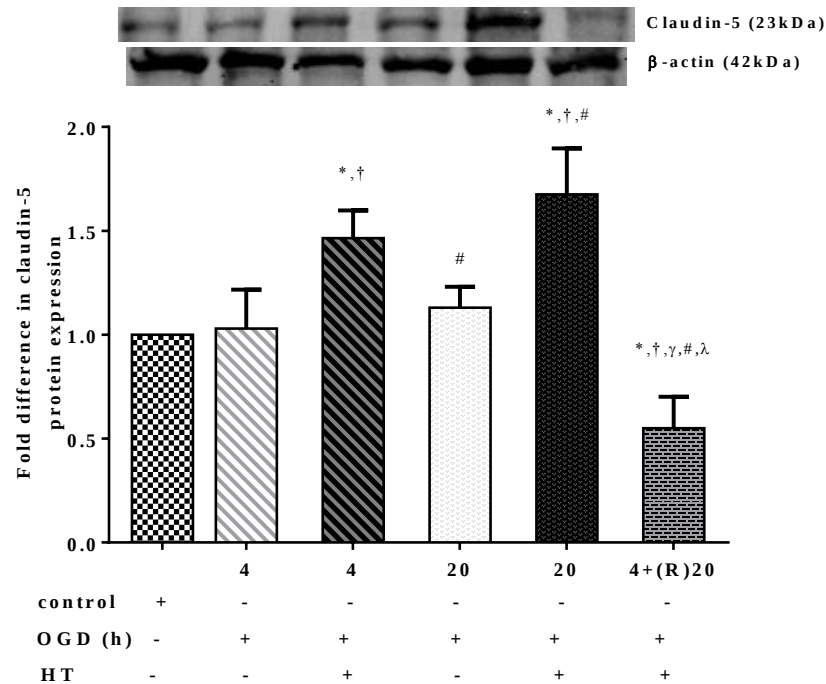


Figure 6-Effects of oxygen-glucose deprivation on the tight junction protein in the absence or presence of moderate hypothermia

Claudin-5 protein expression in HBMECs subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia. Data expressed in 3 different HBMEC experiments * $p < 0.05$ vs controls, † $p < 0.05$ vs 4 hours OGD, # $p < 0.05$ vs 4 OGD + HT, γ $p < 0.05$ vs 20 OGD, λ $p < 0.05$ vs 20 OGD + HT. OGD-oxygen glucose deprivation; HT- hypothermia; (h) - hours

Effects of oxygen-glucose deprivation on rate of apoptosis measured via caspase-3/7 enzyme activity in the absence or presence of moderate hypothermia (35°C)

In order to assess the rate of apoptosis, HA, HBMEC and HP were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (intra-ischaemic) and 4 hours of OGD prior to induction of moderate hypothermia for 20 hours (post-ischaemic). Cells when exposed to 4 hours of OGD in the absence of hypothermia documented transient upregulation in caspase-3/7 enzyme activities when compared to controls. On the contrary, cells subjected to 4 hours of OGD in the

presence of hypothermia documented a significant decrease in caspase-3/7 enzyme activities when compared to OGD alone groups. Furthermore, HAs exposed to 20 hours of OGD in the absence of hypothermia, results revealed increased amount of caspase-3/7 enzyme activity, which was significantly decreased by addition of hypothermia. Similarly, HBMECs subjected to 20 hours of OGD in the absence of hypothermia showed an insignificant increased in enzyme activity, while, subsequent use of moderate hypothermia led to decrease in caspase enzyme activity. Alternatively, HPs subjected to 20 hours of OGD showed less amount of enzyme activity in comparison to HPs exposed to 4 hours of OGD in either the absence or presence of hypothermia. Nevertheless, HP when subjected 20 hours of OGD in the presence of moderate hypothermia further declined the enzyme activity. Data suggests the role of moderate hypothermic treatments in declining the rate of apoptosis in HA, HBMEC and HP in intra-ischaemic episodes. Similarly, in post-ischaemic episodes hypothermia led to a decrease in caspase activities in all three cell lines.

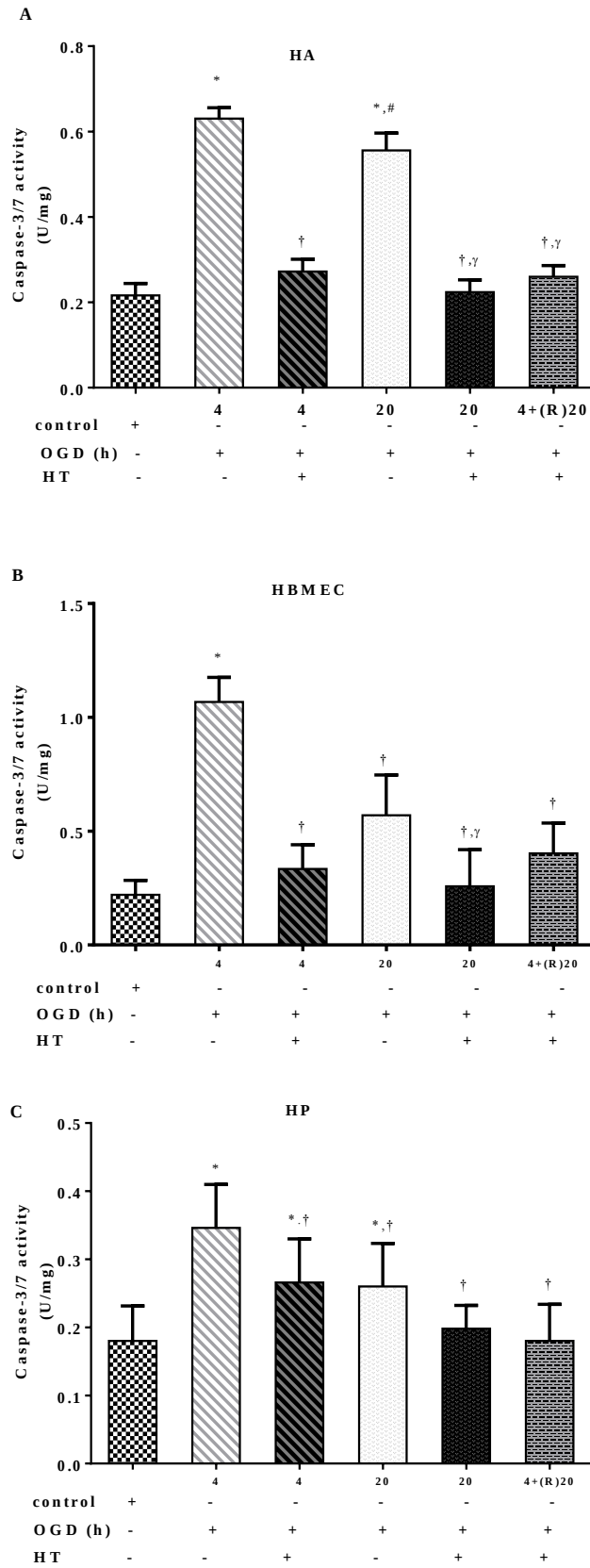


Figure 7 Effects of oxygen-glucose deprivation on rate of apoptosis via measuring caspase-3/7 activity in HA (A), HBMEC (B) and HP (C) the absence or presence of moderate hypothermia

Data on y-axis shows total caspase-3/7 activity and time dependent exposures of cells to OGD in the absence or presence of hypothermia on x-axis. Data represented from 5 different experiments for each cell line. * $p < 0.05$ vs controls, † $p < 0.05$ vs 4 hours OGD, # $p < 0.05$ vs 4 hours OGD + HT and $\gamma p < 0.05$ vs 20 hours OGD, OGD-oxygen glucose deprivation; HT-hypothermia; (h)- hours

Effects of oxygen-glucose deprivation on human astrocytes in the absence or presence of moderate hypothermia and assessment of rate of apoptosis via terminal de-oxynucleotidyl transferase biotin-dUPT nick-end labeling (TUNEL) assay

In order to assess rate of apoptosis via TUNEL-labelling assay, HAs were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. Cells subjected to 4 hours of OGD in the absence of hypothermia showed increase in number of TUNEL-positive cells when compared to controls and subsequent use of hypothermia manage to decrease the number of TUNEL-positive cells. Likewise, 20 hours of OGD exposure in the absence of hypothermia further increased TUNEL-positive cells and in addition of hypothermia for same duration insignificantly decreased such number of cells. In post-ischaemic settings, number of TUNEL-positive cells was increased when compared to intra-ischemic episodes

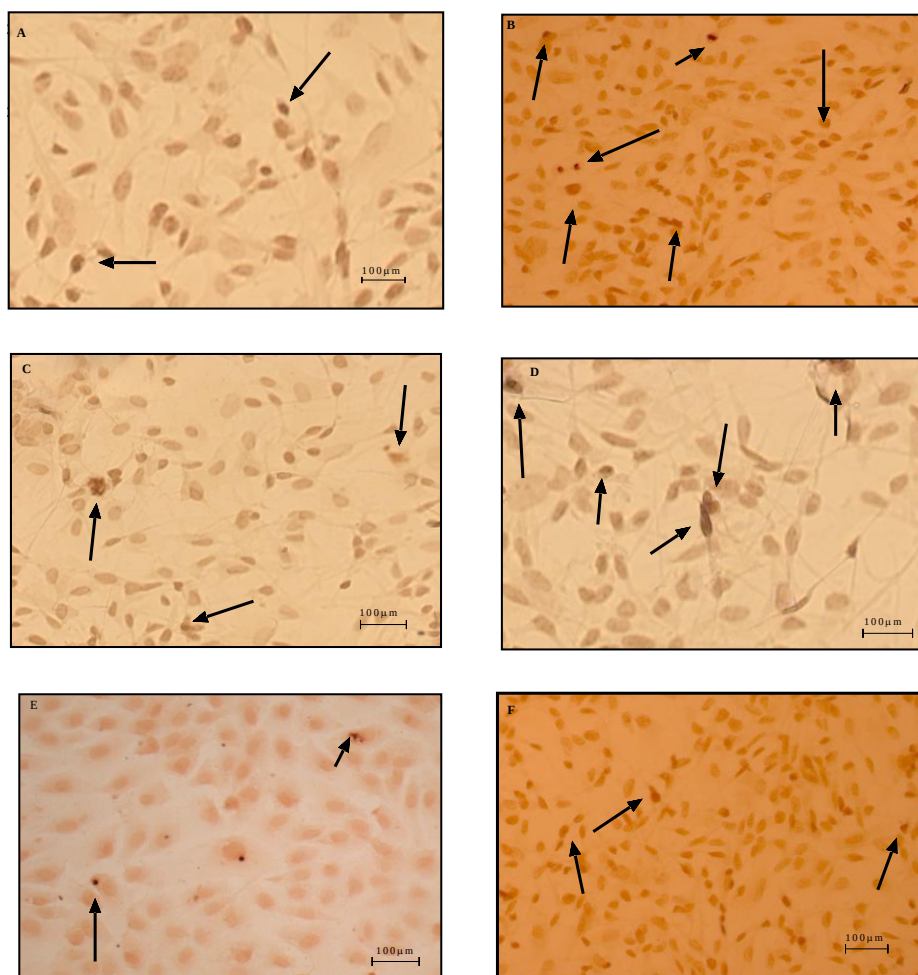
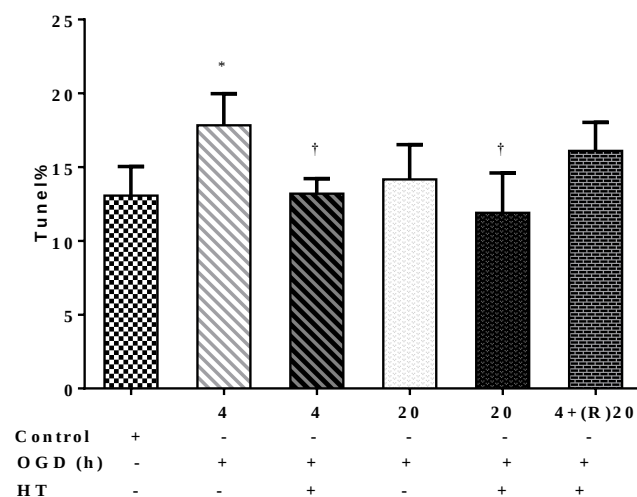


Figure 8-Human astrocytes subjected to 4 and 20 hours of oxygen-glucose deprivation in the absence or presence of hypothermia

Data represented in 3 different experiments. Graph presents percentage of TUNEL positive cells in HA when exposed to OGD in the absence or presence of hypothermia, y-axis showing percentage of cells and time dependent exposures of HA to OGD in the absence or presence of hypothermia on x-axis (n=3, p<0.05, magnification 20x). †p<0.05 v/s 4 hours of OGD, OGD-oxygen glucose deprivation; HT-hypothermia; (h)- hours

The effects of oxygen-glucose deprivation on human brain microvascular endothelial cells in the absence or presence of moderate hypothermia and assessment of rate of apoptosis via terminal deoxynucleotidyl transferase biotin-dUPT nick-end labeling (TUNEL) assay

In order to assess rate of apoptosis via TUNEL-labelling assay, HBMECs were subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. Cells subjected to 4 hours of OGD in the absence of hypothermia showed significant increase in number of TUNEL-positive cells when compared to controls and subsequent use of hypothermia manage to decrease the number of TUNEL-positive cells. Likewise, 20 hours of OGD exposure in the absence of hypothermia further increased TUNEL-positive cells and in addition of hypothermia for same duration insignificantly decreased such number of cells. In post-ischaemic settings, numbers of TUNEL-positive cells were decreased as evidenced by fall in TUNEL percentage when compared to intra-ischemic episodes. Results suggest the role of hypothermia in decreasing the rate of apoptosis during both intra- and post-ischaemic scenarios.

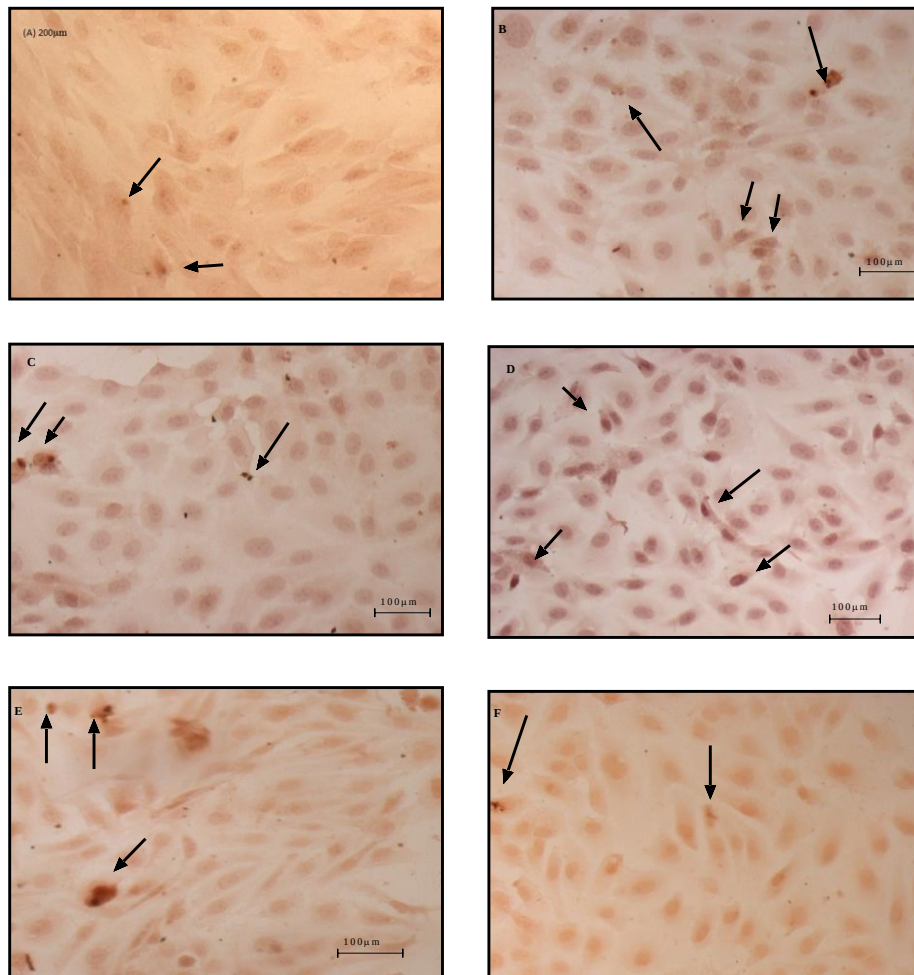
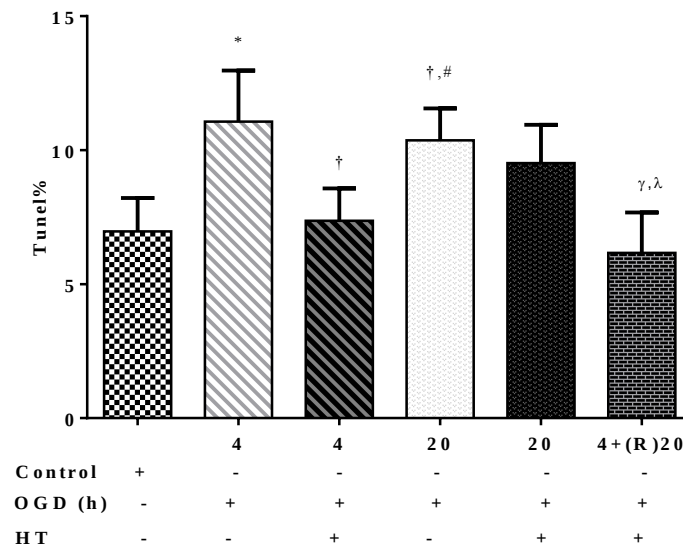


Figure 9-Human brain microvascular endothelial cells subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia

Data represented in 3 different experiments. Graph presents percentage of TUNEL positive cells in HBMEC when exposed to OGD in the absence or presence of hypothermia, y-axis showing percentage of cells and time dependent exposures of HA to OGD in the absence or presence of hypothermia on x-axis (n=3, p<0.05, magnification 20x). *p<0.05 vs controls †p<0.05 v/s 4 hours of OGD, #p<0.05 vs 4 OGD + HT, γp<0.05 vs 20 hours OGD, OGD-oxygen glucose deprivation; HT-hypothermia; (h)- hours

Effects of oxygen-glucose deprivation on cytoskeletal anatomy of human astrocytes when exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

In order to assess the effects of hypothermia on OGD led cytoskeletal changes, HAs were exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. Cells exposed to 4 hours of OGD in the absence of hypothermia experienced increased stress fibers as compared to controls. Layout documents human astrocytes showing actin filaments when subjected to OGD for 4 and 20 hours in absence or presence of hypothermia. HAs when subjected to 20 hours of OGD in absence of hypothermia showed much-increased thickening compared controls or 4 hours OGD groups, addition of hypothermia caused a decrease in stress fibers. In post-ischaemic settings, hypothermia manages to reduce the stress fibers when compared to intra-ischaemic OGD alone groups. These above results suggest beneficial role of hypothermia in decreasing fibers and indirectly maintain the BBB architecture.

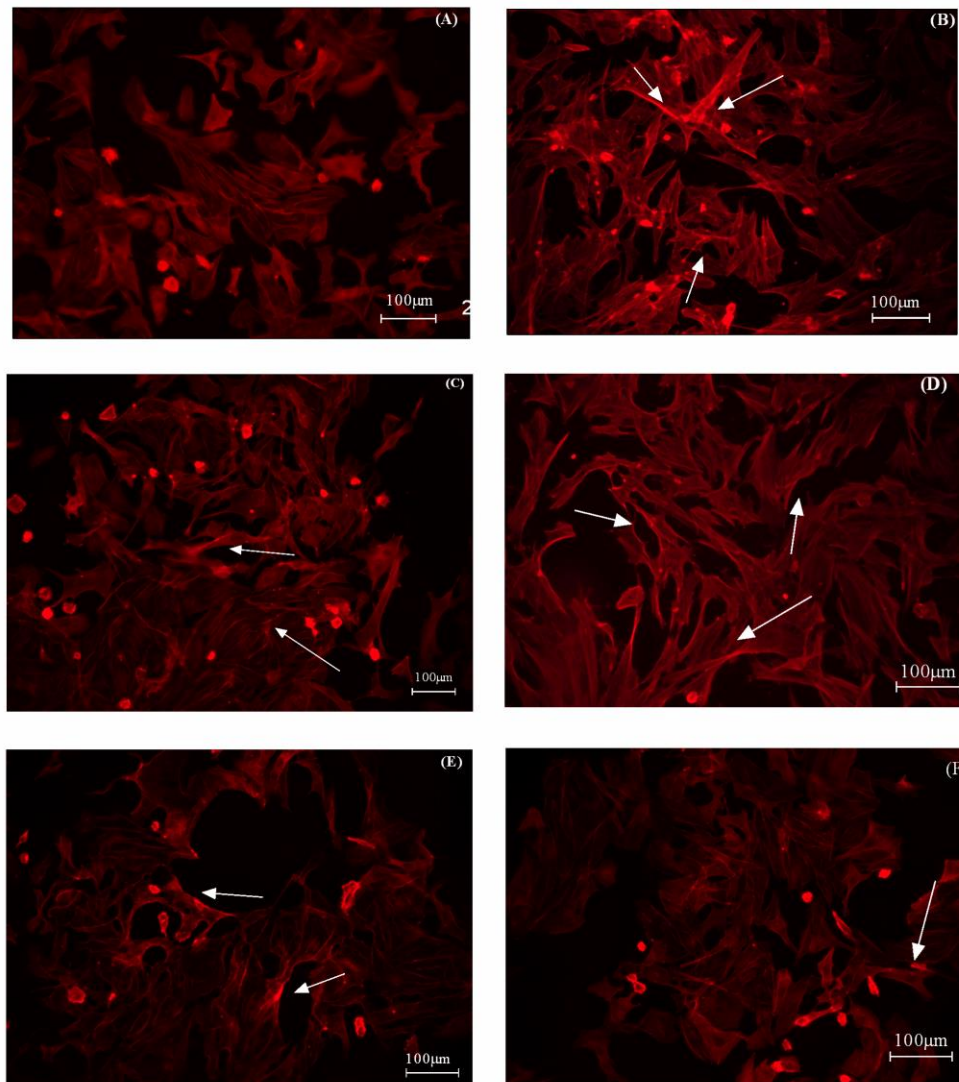


Figure 10-Demonstrates effects of moderate hypothermia on oxygen-glucose deprivation exposed human astrocytes showing stress induced cortical remodeling via F-Actin Scans.

Data stated by 3 different experiments of HA, (n=3). controls (A), 4 hours of OGD (B), 4 hours OGD + HT (C), 20 hours OGD (D), 20 hours OGD + HT (E) and 4 hours OGD + 20 hours HT (F), OGD-oxygen glucose deprivation, HT-hypothermia, pictures taken in 20x-magnification.

Effects of oxygen-glucose deprivation on cytoskeletal anatomy human brain microvascular endothelial cells when exposed to 4 and 20 hours of oxygen-glucose deprivation in the absence or presence of moderate hypothermia (35°C)

In order to assess the effects of hypothermia on OGD led cytoskeletal changes, HBMECs were exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. Cells exposed to 4 hours of OGD in the absence of hypothermia experienced increased stress fibers as compared to controls. Layout documents human astrocytes showing actin filaments when subjected to OGD for 4 and 20 hours in absence or presence of hypothermia. HBMECs when subjected to 20 hours of OGD in absence of hypothermia showed much increased thickening compared controls or 4 hours OGD groups, addition of hypothermia caused a decrease in stress fibers. In post-ischaemic settings hypothermia manages to reduce these fibers when compared to intra-ischaemic OGD alone groups. These above results suggest beneficial role of hypothermia in decreasing stress fibers and indirectly maintain the BBB architecture.

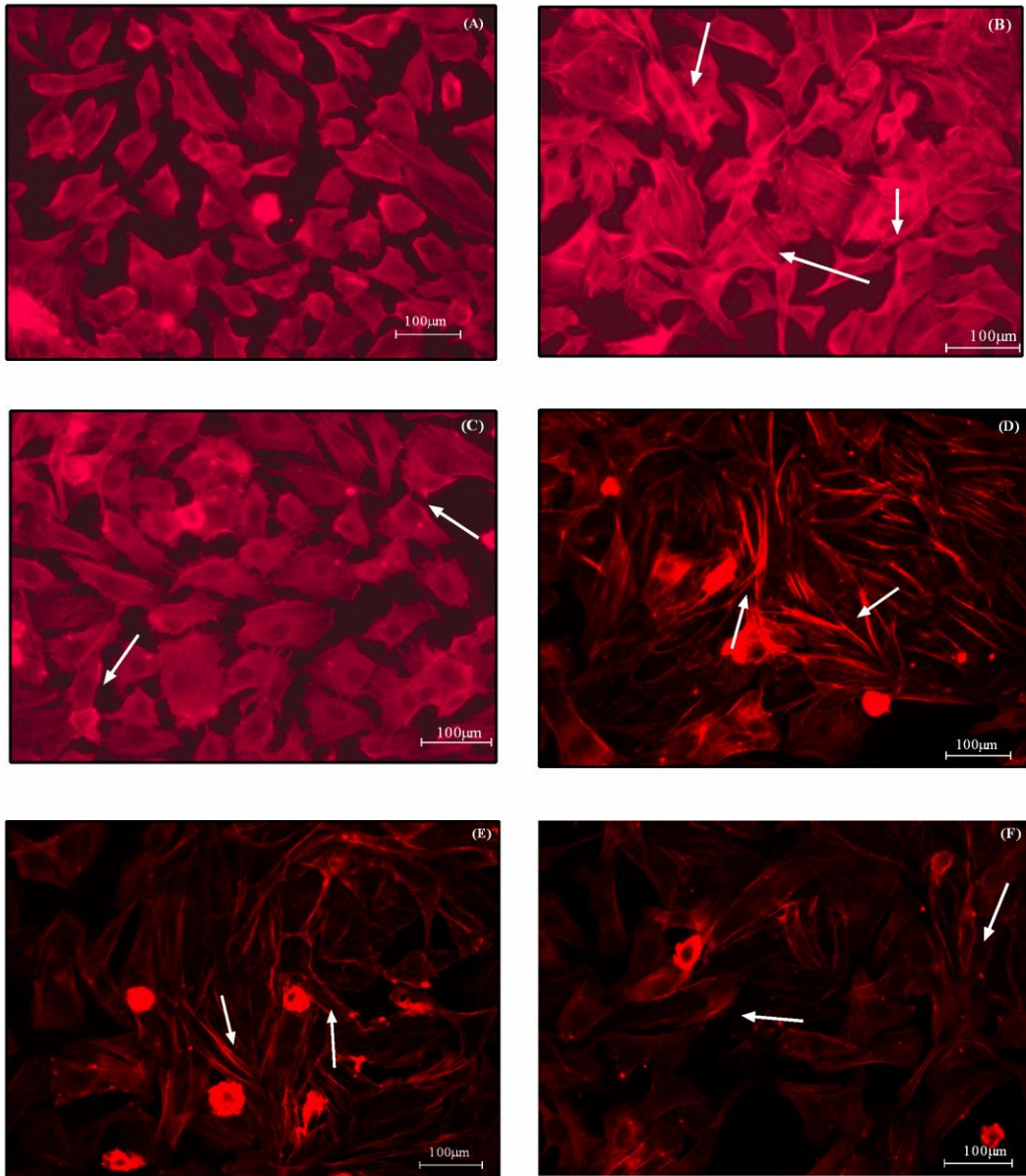


Figure 11-Demonstrates effects of moderate hypothermia on oxygen-glucose deprivation exposed human brain microvascular endothelial cells showing stress induced cortical remodeling via F-Actin Scans.

Data stated by 3 different experiments of HBMEC, (n=3). controls (A), 4 hours of OGD (B), 4 hours OGD + HT (C), 20 hours OGD (D), 20 hours OGD + HT (E) and 4 hours OGD + 20 hours HT (F), OGD-oxygen glucose deprivation, HT-hypothermia, pictures taken in 20x-magnification.

Effects of oxygen-glucose deprivation on cytoskeletal anatomy human pericytes when exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

In order to assess the effects of hypothermia on OGD led cytoskeletal changes, HPs were exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia. Cells exposed to 4 hours of OGD in the absence of hypothermia experienced increased stress fibers as compared to controls. Layout documents human astrocytes showing actin filaments when subjected to OGD for 4 and 20 hours in absence or presence of hypothermia. HPs when subjected to 20 hours of OGD in absence of hypothermia showed excess thickening compared controls or 4 hours OGD groups, addition of hypothermia caused a decrease in stress fibers. In post-ischaemic settings hypothermia manages to reduce the cortical thickening when compared to intra-ischaemic OGD alone groups. These above results suggest beneficial role of hypothermia in decreasing stress fibers and indirectly maintain the BBB architecture.

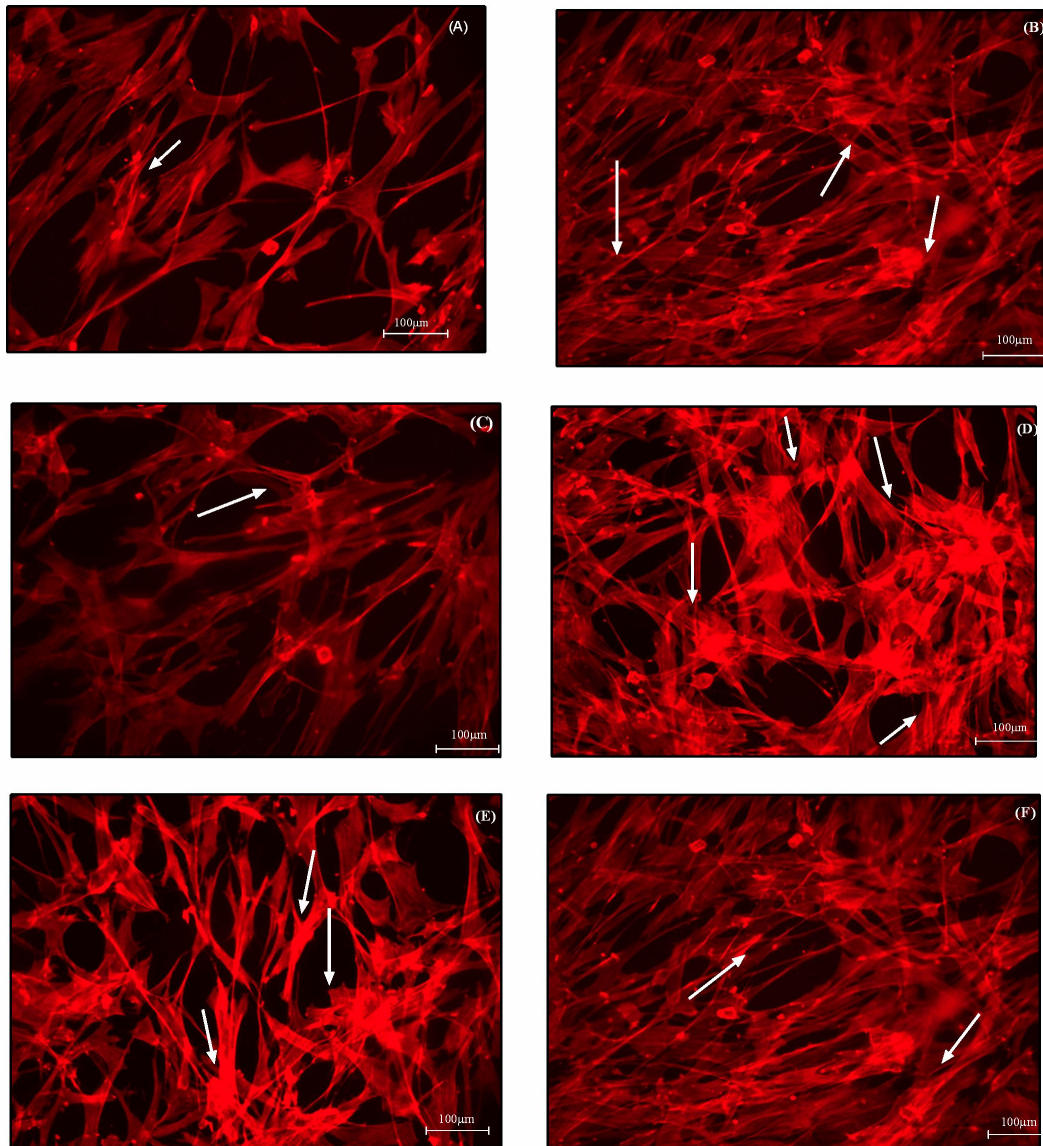


Figure 12-Demonstrates effects of moderate hypothermia on oxygen-glucose deprivation exposed human pericytes showing stress induced cortical remodeling via F-Actin Scans.

Data stated by 3 different experiments of HP, (n=3). controls (A), 4 hours of OGD (B), 4 hours OGD + HT (C), 20 hours OGD (D), 20 hours OGD + HT (E) and 4 hours OGD + 20 hours HT (F), OGD-oxygen glucose deprivation, HT-hypothermia, pictures taken in 20x-magnification.

Discussion

The BBB plays a significant role in maintaining nervous system homeostatic balance. Astrocytes, pericytes and the microglia surround the endothelial cells contributing to the efficacy of this barrier [54]. Specialised extracellular matrix is merely accountable for the connection between astrocytes, pericytes and endothelial cells. In this chapter, time dependent exposure of cells to the OGD in the absence or presence of moderate hypothermia manages to decrease the pro-inflammatory cytokine levels. For IL-1 β concentrations, cell subjected to 20 hours of OGD in the presence of moderate hypothermia were more promising and useful as compared to 4 hours of treatments. Also clarifies the late release property of IL-1 β . While, TNF- α showed high concentration at 4 and 20 hours of OGD exposure release, this was successfully reduced under the presence of moderate hypothermia. This does reflect the biphasic pattern associated with the release of TNF- α .

Presence of moderate hypothermia further delayed the activity of so called “executioner proteins” the caspase-3/7, likewise, effect of moderate hypothermia manages to show less TUNEL-positive cells signifying decreased rate of apoptosis during an acute ischaemic injury. Moreover, hypothermic treatments enhance tight junction protein expressions and all these above mentioned results jointly help in upholding the integrity and function of the BBB as evidenced by increase in TEER across the barrier and by decreasing the paracellular flux values in the presence of moderate hypothermia.

Multiple components of neurovascular unit respond rapidly and simultaneously to any kind of ischaemic injury [276]. Research have specified the defensive property of hypothermia in clinical settings for various pathologies such as traumatic brain injury,

ischaemic heart disease, cardiac arrest, intracerebral haemorrhage and ischaemic stroke [277-279]. Furthermore, inflammation is a key constituent in the development of ischaemic stroke. Within inflammation the main triggering components are the pro-inflammatory cytokines, rate of apoptosis and oxidative stress. Previous studies have also shown that abnormal expression of both TNF- α and IL-1 β is associated with increase in BBB permeability [261]. Administering IL-1 β to rats exposed to focal cerebral ischaemia led to deteriorated outcome, specifying a damaging role for this protein [280]. Similarly, mice deficient in IL-1 receptor had small size infarcts compared to wild type [112]. Our group has successfully demonstrated that TNF- α severely perturbs the integrity of the BBB [281]. Where, the results documented that exposure to TNF- α for 6 hours impaired BBB integrity as evidenced by marked decreases in TEER and concurrent increases in paracellular flux. Also, these cytokines are known to directly attack tight junctions by weakening the hydrophobic and disulfide bonds and inducing BBB leakage [282-284]. Kammersgaard et al., (2000), Schwab et al. (2001) and Georgiadis et al., showed on patients with moderate hypothermia have better outcome with fewer side effects such as thrombocytopenia [186, 285, 286]. The influence of hypothermia on infarct growth has been widely studied and analysed in rat and mouse models of middle cerebral artery occlusion [153, 287]. These studies showed that infarct size reduces under mild to moderate hypothermia (33 to 35°C) treatments in rat models of focal cerebral ischaemia [288, 289]. Mild hypothermia has been documented feasible in rats after intracerebral haemorrhage, by halting progression of oedema and BBB disruption, most likely by maintaining the tight junction and inhibiting cytokines [279]. It is generally acknowledged that hypothermia improves neurological outcome and reduces inflammation in animal models of focal cerebral ischaemia [191, 290].

Therefore, in light of above we assessed two primary pro-inflammatory cytokines responsible for the early and late stage damage following an ischaemic insult and promptly affecting the BBB. Therefore, to mimic ischaemic scenario, HA, HBMEC and HP were subjected to OGD for 4 and 20 hours in the absence or presence of moderate hypothermia. Deprivation worked as perfect example to put cells under hypoxic stress with 95% N₂, 5% CO₂ and 1% O₂. Results state that cells treated under presence of hypothermia versus non hypothermic groups suggests beneficial aspect of hypothermia by decrease in inflammatory cytokine concentrations and activated cells are the major source of cytokines as described in various studies [291, 292]. Precisely, concurrent influence of moderate hypothermia with 20 hours of OGD lowers IL-1 β concentrations (Figure 3) in all three cells and this late release pattern further explains time-dependent property of IL-1 β [293-296]. IL-1 β has been shown to increase following ischaemic injury [110], and also found to have a late release expression pattern with peaking release at a later stage of an attack (6 to 24 hours) [297]. This signifies our findings to be similar to the pattern found in the past research studies. Whereas, TNF- α is seen to have biphasic pattern, as documented by our results (Figure 4) and simultaneous induction of hypothermia results in decreases in the concentration in both 4 and 20 hours of OGD exposure. Furthermore, post-ischaemic hypothermic treatments further decrease the concentrations of both the cytokines when compared to OGD in the absence of hypothermia groups. Chang et al., and Offner et al., showed an increase of TNF- α level in serum at 6 and 24 hours after middle cerebral artery occlusion these findings signify our findings [298, 299]. Also, TNF- α is known to promote the release of other cytokines and chemokine at the site of ischaemic injury. Hence, hypothermia represented as an effective neuroprotectant in delaying the release of pro-inflammatory cytokines during intra-

and post-ischaemic episodes. Moreover, this decrease in concentrations of pro-inflammatory cytokines may further explain the role of hypothermia in maintaining the BBB.

Further, to study the effects of hypothermia on programmed cell death mediated by inflammation, caspase-3/7 activities were assessed in HA, HBMEC and HP. Cells subjected to 4 and 20 hours of deprivation resulted in increased rate of apoptosis as documented by surge in caspase-3/7 activity when compared to control group. Furthermore, parallel involvement of moderate hypothermia treatments to 4 and 20 hours of OGD decreased the enzyme activity when compared to OGD alone group in both intra- and post-ischaemic settings (Figure 7). These results are consonant with a study featuring inhibition of caspase-3/7 led to better outcome via decreased cell death and even in some cases of post-ischaemic treatments [300, 301]. Also, the later study has been able to provide a longer therapeutic window by inhibition of caspase. Furthermore, Maier et al., managed to rescue neurons from ischaemic-induced cell death by using caspase inhibitors [302]. In conclusion, hypothermia manages to reduce the rate of apoptosis mediated by inflammation when cells exposed to 4 and 20 hours of OGD via reducing the caspase-3/7 activities. Post-ischaemic treatments with hypothermia did manage to reduce Caspase-3/7 activities when compared to intra-ischaemic OGD in the absence of hypothermia groups. To further study apoptosis induced by acute ischaemic injury, DNA fragmentation was assessed by TUNEL-assay. Results documented that cells when exposed to OGD for 4 and 20 hours represented increase number of TUNEL-labeled cells when compared to control group. Cells when subjected to 4 and 20 hours OGD in the presence of moderate hypothermia showed lesser number of DNA fragmented TUNEL-positive cells compared to OGD alone group (Figure 8 & 9). These findings are consistent with

previous evidences as studied by Matsukawa et al., where they [303] demonstrated that 1 or 2 hours of post-ischaemic treatments with hypothermia revealed less amount of TUNEL-stained cell. Furthermore, splenic atrophy is usually attributed due to an acute ischaemic stroke, high numbers of TUNEL-positive cells are observed under splenic biopsy, which signifies and opens area of further research in presence of hypothermia over these cells [304]. Likewise, in a rat model study, where 2 to 4 hours of hypothermic exposure significantly decreases TUNEL-positive cells and this does connotes our findings [305]. Furthermore, there were more TUNEL-positive cells in 20 hours of exposure than compared to 4 hours irrespective of hypothermia. This may be due to longer exposure of OGD plays a dominant role in cell death. Also, it may highlight the beneficial effects of hypothermic neuroprotection at short intervals of time.

Tight junction proteins play a vital role in maintaining the anatomical structure of the BBB, therefore, in order to analyse the effects of moderate hypothermia, HBMECs were exposed to 4 and 20 hours of OGD in the absence or presence of hypothermia. Likewise, Sun et al., suggested that early hypothermia does helps in upregulation of tight junction such as Claudin-5 [279]. In relation to the findings as suggested earlier by Sun et al., our Claudin-5 expressions were upregulated in the presence of hypothermia compared to OGD alone groups (Figure 6). As mentioned by Knowland et al., [306] that major tight junction structural abnormalities occur at later phase of stroke progression, therefore, in this study post ischaemic exposure led to decrease in tight junction protein expression. Above listed authors also showed how the BBB disruption is carried in a manner of sequence. Where one phase involves upregulation of endothelial transcytosis and the later phase followed by major remodeling of the tight junction. Likewise, disruption of paracellular barrier in gut epithelial cells in

response to inflammation is also achieved by sequential manner [307]. These cellular events may therefore represent a general principle how they react to an inflammatory attack. Cerebral ischaemia has been shown to activate an extensive neurovascular remodeling which embraces the disruption of inter-endothelial tight junction, breakdown of the vascular basal lamina, endothelial proliferation, migration, and reorganisation to form new capillaries and microvessels [308]. Since changes in cytoskeleton may contribute to the ischaemic injury-induced barrier failure, the status of actin filament formation and distribution was comparatively studied in HBMEC, HA and HP (Figures 10-12). Cells cultured under normothermic conditions displayed a cortical actin staining while those subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia, possessed actin stress fibers traversing the cells and appeared predominantly elongated and somewhat cuboidal. However, these stress fibers were decreased when cells were exposed to OGD in the presence of hypothermia.

In order to assess the effects of hypothermia on the BBB, triple cultures were exposed to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (Figure 5). BBB models subjected to 4 and 20 hours of OGD in the absence of hypothermia documented low TEER values across the barrier, signifying the loss of resistance during an ischaemic insult. Furthermore, higher paracellular flux values were obtained in the OGD led exposure of the BBB models in the absence of hypothermia, signifying the loss of integrity. Furthermore, similar BBB models when exposed to OGD in the presence of hypothermia showed better TEER values across the barrier when compared to OGD alone groups and documented a decrease paracellular flux values, signifying the beneficial role of hypothermia in maintain the BBB architecture during intra- and post-ischaemic injury.

Chapter 2

Impact of oxidative stress on the blood-brain barrier with role of hypothermia

Introduction

It is known that cells respond to numerous stresses mainly through a number of precise and well preserved adaptive intercellular signaling pathways that contribute to ease the damage and maintain or re-establish the homeostasis, known as cellular stress response. This response necessitates a series of events leading to the accumulation of protective proteins that may counteract the stress-induced damage otherwise, cellular stress response may trigger programmed cell death to get rid of terminally damage cells [309]. As it is clear that during or after ischaemic insult, a reduced BBB and loss of tight junction integrity results in increased paracellular permeability, directly contributing to cerebral vasogenic oedema, haemorrhagic transformation and increased mortality. This loss of tight junction integrity happens in a phasic manner, depending on several codependent mechanisms like inflammation, oxidative and nitrosative stress, enzymatic activity, and angiogenesis. The close relationship between cerebral ischaemia and oxidative stress has generated considerable interest to develop antioxidant agents to combat the deleterious consequences of oxidative stress associated cerebral insults. Oxidative stress and mitochondrial damage are considered to be the key determinants with respect to the extent of injury during cerebral ischaemia. It is classified as, disturbance in the oxidant-antioxidant balance leading to the cellular damage, where it is subjected to excessive levels of molecular O₂ or its chemical derivatives, namely reactive oxygen species[310]. As the brain consumes about 20% of the body's total O₂, neuronal cells are more susceptible, than others, to oxidative injury [126]. Oxidative stress has been implicated in the ageing process and pathogenesis of numerous diseases such as, atherosclerosis, malignant tumours, neurodegenerative diseases and stroke [311]. Additionally, due to high O₂

consumption by the brain itself, extraordinary levels of peroxidisable lipids, low levels of protective anti-oxidants and high levels of iron that in turn act as pro-oxidants during ischaemic injury it turns out to be the target organ. One of the main contributors of oxidative stress are free radicals such as superoxide anion, hydroxyl radical, lipid radicals and reactive nitrogen species such as NO [127]. These free radicals are found to react with every type of molecule found in a living cell [312]. Basically, during and after ischaemic attack, lactic acid accumulates within neurons due to energy depletion, leading to acidosis. This acidic situation in turn endorses pro-oxidant effect by increasing H^+ ions. This further enhances rate of conversion of superoxide anion to hydrogen peroxide (H_2O_2) [310]. Not only in the central nervous system, there is evidence-supporting role for oxidative stress due to excessive production of reactive oxygen species in the pathophysiology of congestive heart failure. As stated, increased oxidative stress may contribute to contractile dysfunction, endothelial dysfunction, myocyte apoptosis and necrosis, resulting in remodeling of the extracellular matrix, and the progressive downward spiral of heart failure [313, 314]. Experimental studies also support a role for augmented oxidative stress in failing myocardium and in remote cardiac myocytes, which is due to a reasonable surge in reactive oxygen species, persuade a phenotype characterised by hypertrophy and apoptosis [313, 315]. Focusing back on the central nervous system, it has been shown that oxidative stress induces lipid peroxidation and brain damage. Animal experiments have supported the idea that free radical scavengers and antioxidants intensely decrease cerebral damage [316, 317].

Major enzymatic source of reactive oxygen species production in pathologically injured tissue is NADPH oxidase. *In vitro* studies have shown that brain macrophages are able to release glutamate and other various neurotoxins, including reactive oxygen

species [318, 319]. Therefore, such neuronal damage may represent a source of neurotoxic substances such as superoxide anion. It is well established that there are numerous conditions, including those mediated by nitric oxide, that have the potential in opening of the BBB, eventually leading to vasogenic oedema and secondary brain damage. Nitric oxide has been implicated in increasing permeability of the BBB in response to hypoxia [320]. Superoxide anion can be produced in phagosomes, which contain consumed bacteria and fungi, or it can be formed outside of the cell. It is a “foundation” radical that may lead to the formation of the reactive oxygen species [315]. Antioxidant enzymes, including superoxide dismutase, catalase, and peroxidases protect cells by maintaining superoxide anion and H_2O_2 at low levels [321]. Studies in animal models suggest that a long-lasting rise in oxidative stress in the myocardium, possibly due to deficiency of superoxide dismutase and other antioxidant pathways, could contribute to myocardial remodeling and failure [322]. Furthermore, Cheng et al., showed that mechanical stretch of papillary muscle increased superoxide anion and caused apoptosis and that together effects were reserved by NO, suggesting that superoxide anion responsible for stretch-induced apoptosis in cardiac myocytes [323]. An immunohistochemical study in mice using polyclonal antibodies discovered all the key components of NADPH oxidase (p22phox, Nox2, p47phox, p67phox and p40phox) mostly in the neurons of hippocampus, cortex, amygdala, striatum, and thalamus but not in the cerebellum [324]. This is vital because reactive oxygen species are reliably involved with certain cerebral vascular diseases such as hypertension and stroke.

Therefore, when NOX isoforms have been designated in the cerebral vasculature, cerebrovascular disease may acquire benefit from NOX as objective in the brain. NOX may also be a vital target in the prevention of reperfusion injury, a progressively

detected complication of re-canalising strategies used to treat post-ischaemic stroke. Upon reperfusion, the brain is swiftly exposed to oxygenated blood, and injured mitochondria due to the ischaemic insult are reduced and are unable to detoxify reactive species [325]. Moreover, immune cells, which penetrate ischaemic tissue are also capable of generating reactive species through numerous enzyme systems [268]. NOX is a membrane-bound enzyme complex with components found in the plasma membrane as well as in the cytoplasm. In the past, studies have shown up-regulation of NOX2 in microglia in cases of ischaemia [326], multiple sclerosis [327], and traumatic brain injury [328]. Catalysis of NOX occurs in the p91phox subunit and is commenced by relocating of electrons from molecular oxygen through redox coupling with NADPH, FAD and heme to produce superoxide anion [329]. The backbone of NADPH oxidase enzyme is the cell membrane bound cytochrome b558.

Similarly, small GTP-binding proteins belong to the Rho family standardises various phases of cellular life such as shape, motility, proliferation and apoptosis [330]. Rho kinase (ROCK) were the first downstream effectors of Rho discovered and was found to reunite actin cytoskeletal variations through effects on myosin light chain reaction (MLC) phosphorylation [331-333]. These are basically protein serine/threonine kinases that share 45% to 50% of homology to other actin cytoskeletal kinases such as myotonic dystrophy kinase and citron kinase [330]. Two ROCK isoforms have been recognised in mammalian system. ROCK1, which is also known as ROK β and p160ROCK, is located on chromosome 18 and encodes a 1354-amino acid protein. ROCK2, which is also known as ROK α or Rho-kinase is located on chromosome 12 [331, 332]. Despite of having similar domain both serve different functions and have varied targets. Furthermore, ROCK1 expression tends to be more universal, while ROCK2 is mostly expressed in cardiac and brain tissue. Inhibitors of ROCKs, such as

Y27632 or overexpression of dominant-negative mutants of ROCKs lead to the loss of stress fibers and focal adhesion complexes [334]. Other common pathology, which is associated with ischaemic stroke in 70 % cases, is diabetes. In 2010, after adjusting for population age differences, hospitalisation rates for stroke were 1.5 times higher among adults with diagnosed diabetes aged 20 years or older compared to those without diagnosed diabetes (Data from the National Diabetes Statistics Report, USA 2014 (released June 10, 2014). It is widely accepted that high levels of glucose in stroke carries high levels of PKC. Srivastva et al., have demonstrated in the past that *in vitro* ischaemic models in the presence of hyperglycemia accounted increased in the BBB damage [8].

Oxidative stress is an important underlying factor in delayed neuronal death induced by cerebral ischaemia reperfusion. Apocynin is a naturally occurring methoxy-substituted catechol that effectively inhibits NADPH oxidase through preventing the assembly of its multi-subunits [335]. This compound is derived from the rhizome of the medicinal herb *Picrorhiza kurroa*. *P. kurroa* has been used as a herbal medicine for centuries to treat inflammation and certain infectious diseases [336]. The foremost mechanism of action of apocynin is to prevent the translocation of the cytosolic subunit, p47phox, to the NOX catalytic membrane domain [337]. In some different models of middle cerebral artery occlusion, administration of apocynin has been shown to reduce infarct size in rats [338-341].

AIMS

1. To investigate the effects of OGD associated oxidative stress on HBMEC, HA and HP during intra- and post-ischaemic settings in the absence or presence of moderate hypothermia (35°C).
2. Further to examine impact of OGD induced oxidative stress on the integrity

and function of the blood-brain barrier.

Results

Measuring NADPH oxidase activity in human astrocytes, human brain microvascular endothelial cells and human pericytes subjected to 4 and 20 hours of oxygen-glucose deprivation in the absence or presence of moderate hypothermia (35°C)

Cells were subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia to measure the extent of oxidative stress via NADPH-oxidase activity in intra-and post ischaemic settings as mentioned earlier. Cells when exposed to OGD in the absence of hypothermia documented increased NADPH oxidase enzyme activity as compared to controls. However, this augmented increase was significantly decreased cells subjected to 4 hours of OGD in the presence of hypothermia. Likewise, HA, HBMEC and HP when subjected to 20 hours of OGD showed high amount of enzyme activity. High amount of enzyme activities documented in cells subjected to 4 and 20 hours of OGD in the absence of hypothermia signifies the excess amount of oxidative stress befalls an ischaemic injury. In contrast, hypothermic treatments for 20 hours managed to deliver a slight decrease in NADPH oxidase activity in all three cells. These results signify potent role of hypothermia in down regulating components of oxidative stress such as NADPH-oxidase enzyme during intra-ischaemic scenarios. Furthermore, HA acknowledged a slight decrease in enzyme activity during post-ischaemic treatments whereas, HBMEC and HP experienced an increase.

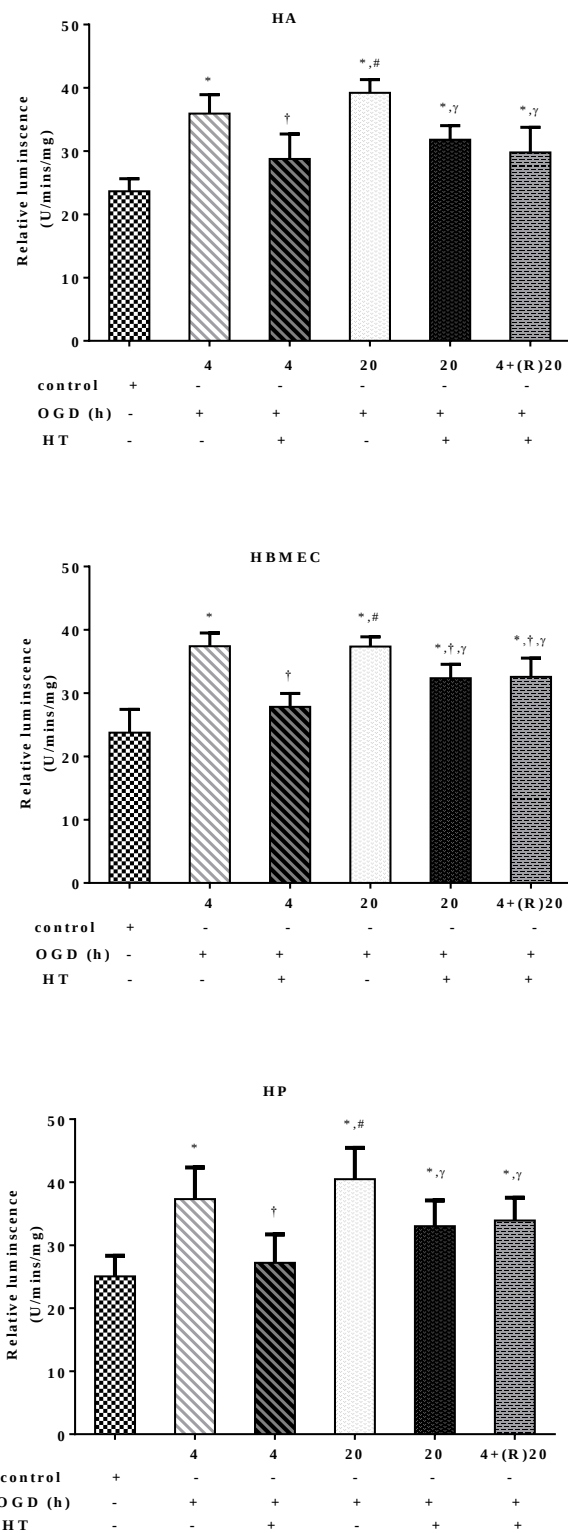


Figure 13-NADPH oxidase activity following oxygen glucose deprivation in HA (A), HBMEC (B) and HP (C) in the absence or presence of hypothermia

Data on y-axis shows units of NADPH oxidase activity and time dependent exposure of cells to OGD in the absence or presence of moderate hypothermia on x-axis. *p<0.05 vs controls, †p<0.05 vs 4 OGD, #p<0.05 vs 4 OGD + HT and ‡p<0.05 vs 20 OGD, OGD-Oxygen glucose deprivation; HT-Hypothermia; U-units; mg-microgram

Measuring superoxide anion generation in human astrocytes, human brain microvascular endothelial cells and human pericytes when subjected to oxygen-glucose deprivation in the absence or presence of moderate hypothermia (35°C)

In order to further evaluate the role of moderate hypothermia in OGD induced oxidative stress, cells were subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia to measure superoxide anion generation. HA, HBMEC and HP when exposed to 4 hours of OGD in the absence of hypothermia showed substantial increase in the rate of superoxide anion generation. Concurrent use of hypothermia for 4 hours resulted in a decrease in superoxide anion production in all three cells when compared to OGD alone groups. Furthermore, cells when exposed to 20 hours of OGD in the absence of hypothermia resulted in transient upregulation in superoxide anion generation when compared controls. This high amount of enzyme activity was significantly reduced in cells subjected to 20 hours of OGD in the presence of hypothermia. These results suggest the role of intra-ischaemic hypothermic treatments in lowering rate of free radical generation. Lastly, in post-ischaemic experimental settings HA experienced a fall in enzyme generation when compared to intra-ischaemic OGD groups and controls. Whereas, HP and HBMEC did experience a slight fall when compared to intra-ischaemic OGD groups.

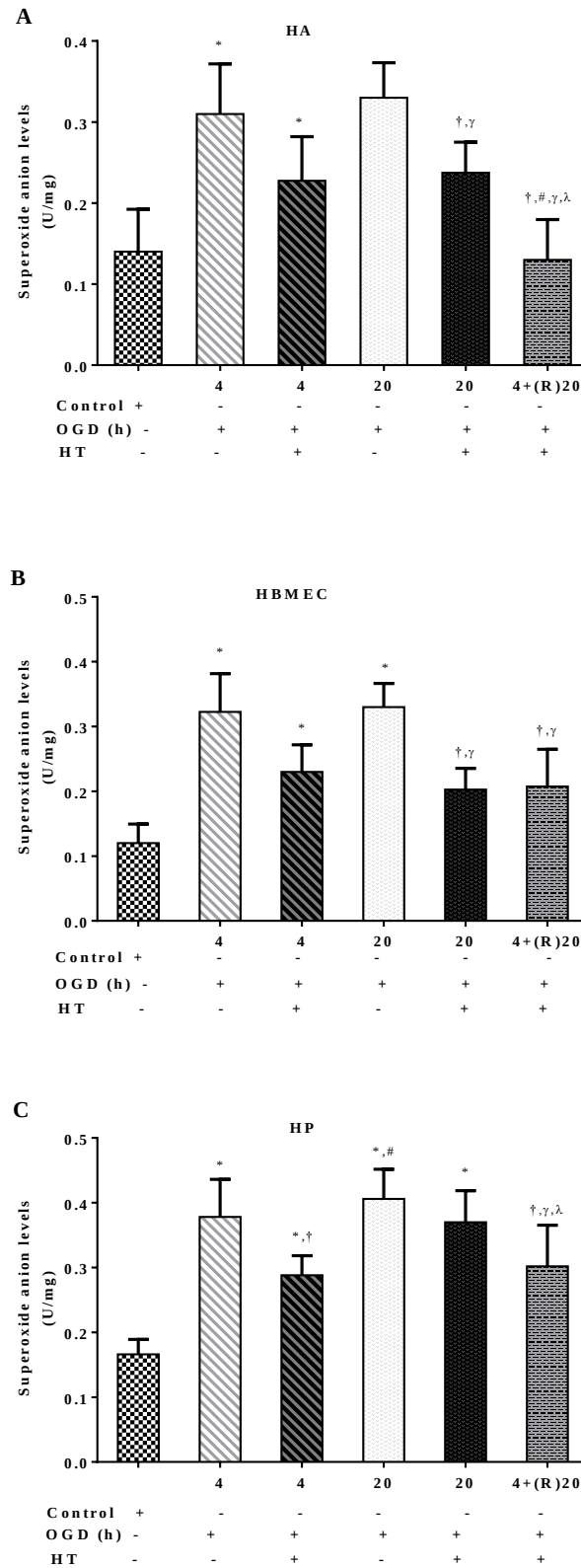


Figure 14-Measuring superoxide anion generation following oxygen glucose deprivation in HA (A), HBMEC (B) and HP (C) in the absence or presence of hypothermia

Graphs show superoxide anion generation in cells when subjected to OGD for 4 and 20 hours in the absence or presence of moderate hypothermia. Data on y-axis shows levels of radical generation and time dependent exposure of cells to OGD in the absence or presence of hypothermia on x-axis. * $p < 0.05$ vs controls, † $p < 0.05$ vs 4 hours OGD, # $p < 0.05$ vs 4 hours OGD + HT, † $p < 0.05$ vs 20 hours OGD, OGD-oxygen glucose deprivation; HT-Hypothermia; (h)-hours

Assessment of antioxidant mechanism via superoxide dismutase enzymes levels in human astrocytes subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

HA when exposed to 4 hours of OGD in the absence of hypothermia resulted in a decrease in total SOD levels as compared to controls. Alternatively, implication of moderate hypothermia for 4 hours in the presence of OGD reflected an increase in total SOD level. Furthermore, HA exposed to 20 hours of OGD in the absence of hypothermia documented a fall in total SOD levels, which was significantly increased under the influence of moderate hypothermia. Post-ischaemic hypothermia manages to increase total SOD expression as compared to intra-ischaemic. Similar results were documented in measuring Cu/Zn-SOD and Mn-SOD. Data suggests beneficial role of hypothermia in maintaining defense mechanism during an ischaemic insult.

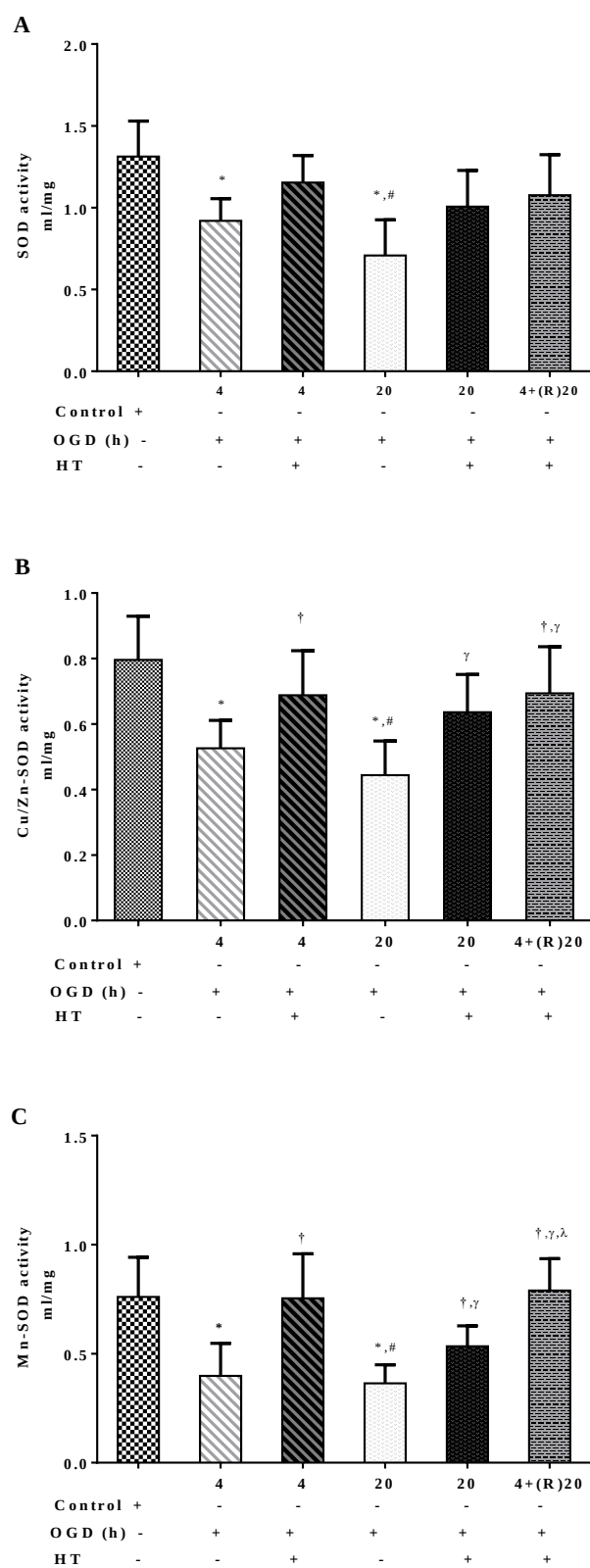


Figure 15-Superoxide dismutase levels in HA subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia

Graphs represented demonstrate total SOD levels **(A)**, Cu/Zn-SOD **(B)** and Mn-SOD **(C)** in HA subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia. Data presented on y axis demonstrate SOD levels in HA subjected to OGD and time dependent exposure of cells to OGD in the absence or presence of hypothermia on x axis (n=5, p<0.05). *p<0.05 vs controls, †p<0.05 vs 4 hours OGD, #p<0.05 vs 4 hours OGD + HT, γp<0.05 vs 20 hours OGD, OGD-Oxygen glucose deprivation; HT-Hypothermia; (h)-hours; Cu-copper; Zn-zinc; Mn- manganese; SOD-superoxide dismutase

Assessment of antioxidant mechanism via superoxide dismutase enzymes level in human brain microvascular endothelial cells subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

HBMECs when exposed to 4 hours of OGD in the absence of hypothermia resulted in a significant decrease in total SOD levels as compared to controls. Alternatively, implication of moderate hypothermia for 4 hours in the presence of OGD reflected an increase in total SOD level compared to OGD alone groups. Furthermore, HBMEC exposed to 20 hours of OGD in the absence of hypothermia documented a fall in total SOD levels, which was significantly increased under the influence of moderate hypothermia. Post-ischaemic hypothermia further manages to increase total SOD expression. Similar results were documented in measuring Cu/Zn-SOD and Mn-SOD levels. Data suggests beneficial role of hypothermia in maintaining defense mechanism during an ischaemic insult.

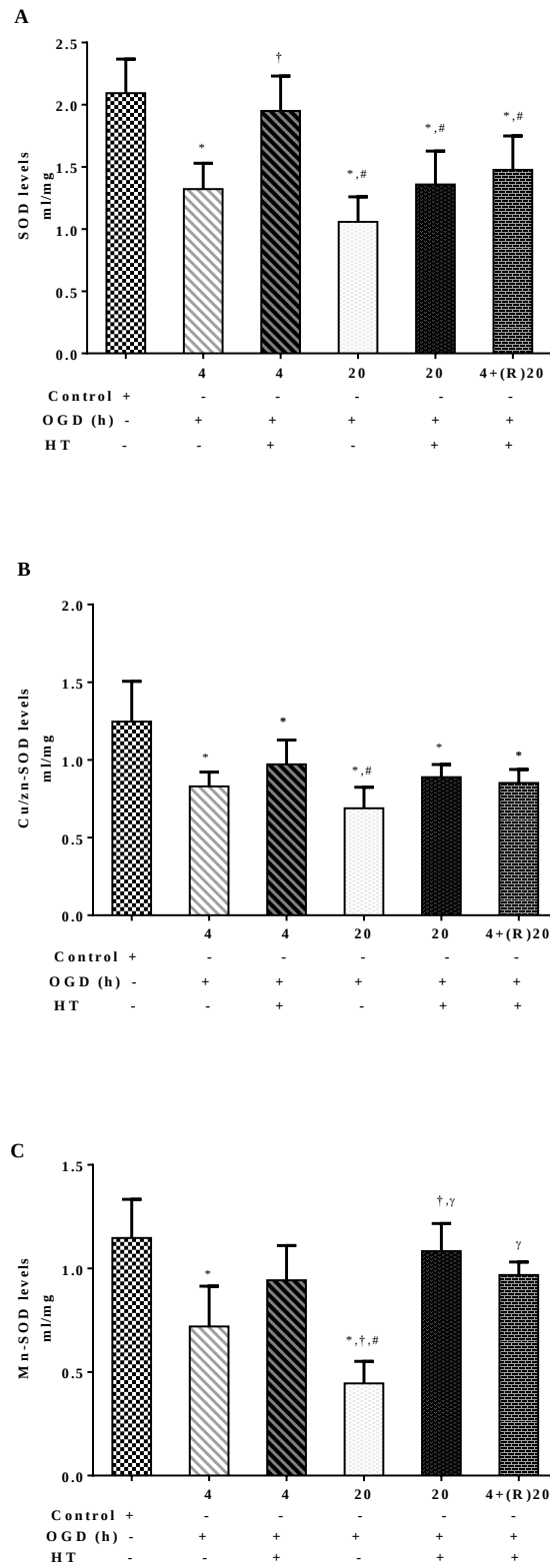


Figure 16-Superoxide dismutase levels in HBMEC subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia

Graphs represented demonstrate total SOD levels **(A)**, Cu/Zn-SOD **(B)** and Mn-SOD **(C)** in HBMEC subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia. Data presented on y-axis demonstrate SOD levels in HBMEC subjected to OGD and time dependent exposure of cells to OGD in the absence or presence of hypothermia on x-axis (n=5, p<0.05). *p<0.05 vs controls, †p<0.05 vs 4 hours OGD #p<0.05 vs 4 hours OGD + HT, #p<0.05 vs 20 hours OGD, OGD-Oxygen glucose deprivation; HT-Hypothermia; (h)-hours; Cu-copper; Zn-zinc; Mn- manganese; SOD-superoxide dismutase

Assessment of antioxidant mechanism via superoxide dismutase enzyme level in human pericytes subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

HP when exposed to 4 hours of OGD in the absence of hypothermia resulted in a significant decrease in total SOD levels as compared to controls. Alternatively, implication of moderate hypothermia for 4 hours in the presence of OGD reflected an increase in total SOD level compared to OGD alone groups. Furthermore, HP exposed to 20 hours of OGD in the absence of hypothermia documented a fall in total SOD levels, which was significantly increased under the influence of moderate hypothermia. Post-ischaemic hypothermia further manages to increase total SOD expression. Similar results were documented in measuring Cu/Zn-SOD and Mn-SOD levels. Data suggests beneficial role of hypothermia in maintaining defense mechanism during an ischaemic insult.

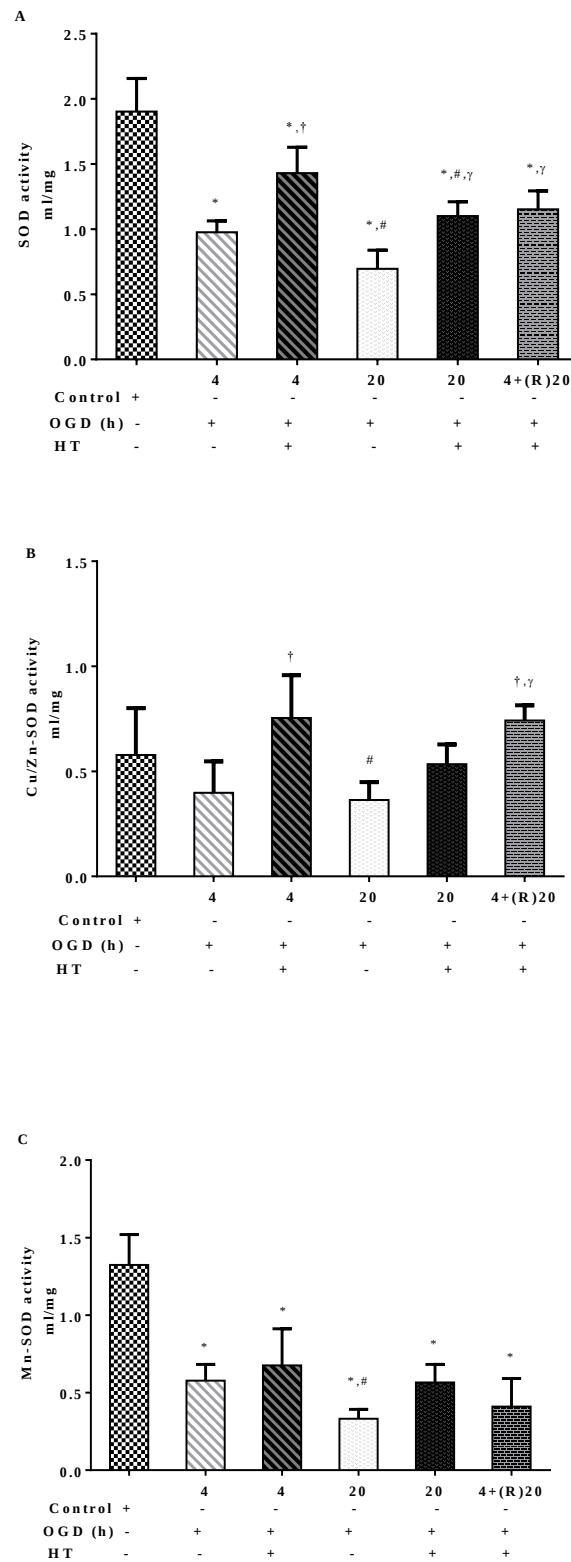


Figure 17-Superoxide dismutase levels in human pericytes subjected to 4 and 20 hours of OGD in the absence or presence of moderate hypothermia (35°C)

Graphs represented demonstrate total SOD levels **(A)**, Cu/Zn-SOD **(B)** and Mn-SOD **(C)** in HP subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia. Data presented on y-axis demonstrate SOD levels in HP subjected to OGD and time dependent exposure of cells to OGD in the absence or presence of hypothermia on x-axis (n=5, p<0.05). *p<0.05 vs controls, †p<0.05 vs 4 hours OGD #p<0.05 vs 4 hours OGD + HT, ‡p<0.05 vs 20 hours OGD, OGD-oxygen-glucose deprivation; HT-Hypothermia; (h)-hours; Cu-copper; Zn-zinc; Mn- manganese; SOD-superoxide dismutase

Effects of oxygen-glucose deprivation on human *in vitro* triple culture blood-brain barrier models in the absence or presence of moderate hypothermia (35°C) and apocynin

In order, to assess the damage attributed from oxidative stress on the BBB during intra- and post-ischaemic injury, *in vitro* BBB models were subjected to 4 hours of OGD in the absence or presence of hypothermia, with addition of apocynin as a treatment option. Moreover, this experimental setting also highlights the synergistic property of hypothermia when used in combination with an inhibitor. BBB models subjected to OGD alone showed a decrease TEER values as compared to controls. With addition of apocynin to the experimental conditions a rise in TEER values was documented as compared to OGD alone groups. Furthermore, use of apocynin on BBB models subjected to 4 hours OGD in the presence of hypothermia further increased TEER values. Similarly, post-ischaemic exposure of hypothermia manages to increase TEER values as compared to OGD alone in the presence of apocynin. To evaluate the degree of destruction attributed during an ischaemic insult, paracellular flux was measured. Triple cultures subjected to OGD in the absence of hypothermia documented a rise in paracellular flux values when compared to controls. Further addition of apocynin to OGD exposed BBB models documented a significant

decrease in paracellular flux values. BBB models subjected to 4 hours of OGD in the presence of hypothermia with apocynin showed slight decrease in paracellular flux values. In post-ischaemic treatments, hypothermia manages to limit leakage across the barrier in the presence of apocynin as compared to OGD alone groups. These results suggest synergistic property of hypothermia and beneficial effects in maintaining the integrity and function of the BBB during intra- and post-ischaemic insult.

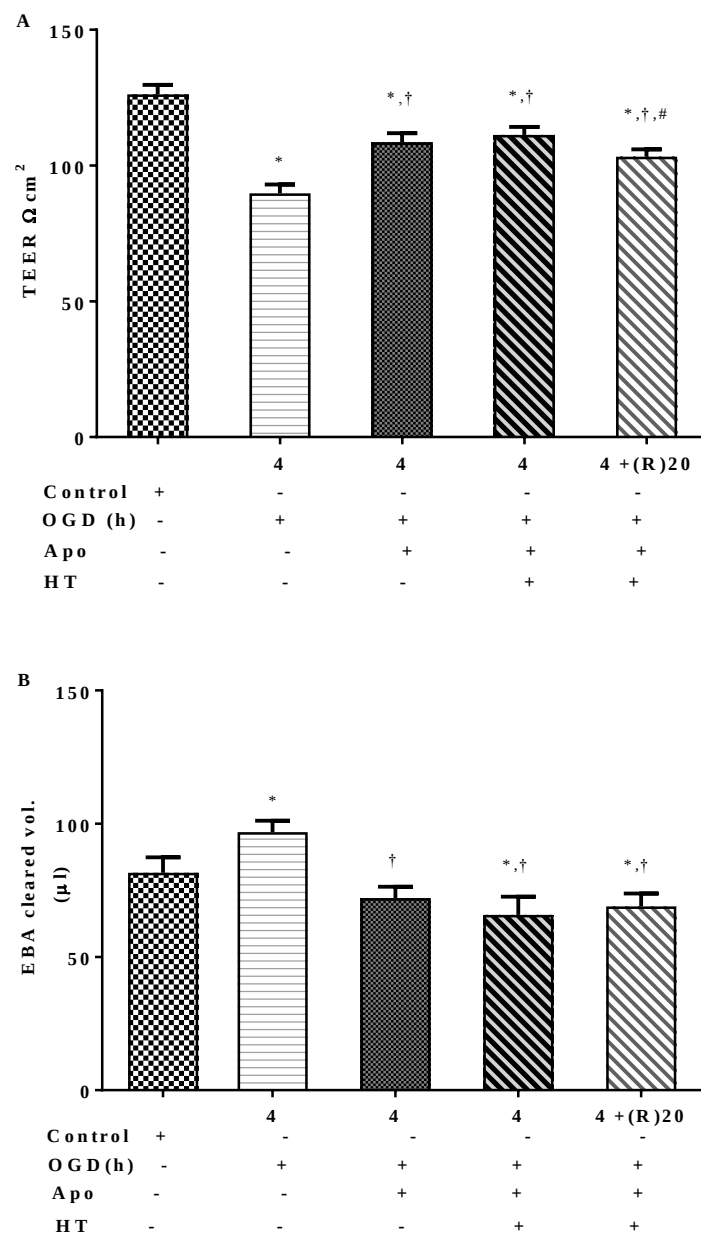


Figure 18-Effects of oxygen-glucose deprivation on triple culture *in vitro* BBB models subjected to 4 hours of OGD in the absence or presence of hypothermia and apocynin

Graphs demonstrate the integrity and function of the BBB during an intra- and post-ischaemic insult in the absence or presence of hypothermia with apocynin, as documented by TEER (**A**) and paracellular flux values (**B**, $n=3$, $p<0.05$). * $p<0.05$ vs controls, † $p<0.05$ vs 4 OGD, and # $p<0.05$ vs 4 OGD + apo + HT. OGD-oxygen-glucose deprivation; HT-Hypothermia; (h)-hours; BBB-blood-brain barrier; apo-apocynin

Effects of oxygen-glucose deprivation on human *in vitro* triple culture blood-brain barrier models in the absence or presence of moderate hypothermia (35°C) and Y27632

In order, to assess the damage attributed from neuroinflammatory processes, *in vitro* BBB models were subjected to 4 hours of OGD in the absence or presence of hypothermia, with addition of Y27632 a rho kinase inhibitor as a treatment option. Moreover, this experimental setting also highlights the synergistic property of hypothermia when used in combination with an inhibitor. BBB models subjected to 4 hours of OGD in the absence of hypothermia and Y27632 showed decreased TEER values as compared to controls. With addition of Y27632 to the experimental conditions a rise in TEER values was documented as compared to OGD alone groups. Furthermore, use of Y27632 on BBB models subjected to 4 hours OGD in the presence of hypothermia further significantly increased TEER values when compared to OGD alone groups. Similarly, post-ischaemic exposure of hypothermia manages to increase TEER values as compared to OGD alone intra-ischaemic groups. To evaluate the degree of destruction attributed during an ischaemic insult, paracellular flux was measured. Triple cultures subjected to OGD in the absence of hypothermia

documented a significant rise in paracellular flux values when compared to OGD alone groups. Further addition of Y27632 to BBB models exposed to 4 hours of OGD documented a significant decrease in paracellular flux values compared to OGD alone groups. Additionally, BBB models subjected to 4 hours of OGD with Y27632 in the presence of hypothermia showed further decrease in paracellular flux values. Lastly, post-ischaemic exposure of hypothermia manages to limit leakage across the barrier as compared to OGD alone groups but shows increased amount of leakage when compared to other groups. These results suggest synergistic property of hypothermia and beneficial effects in maintaining the integrity and function of the BBB during intra- and post-ischaemic insult.

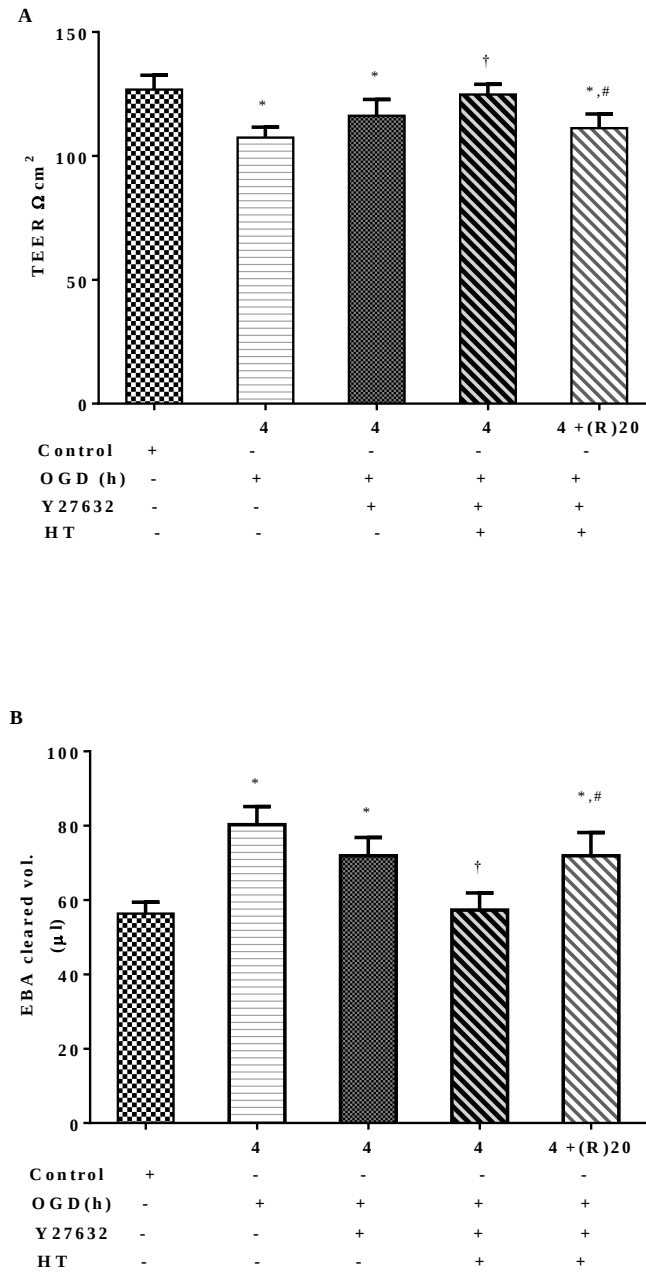


Figure 19-Effects of Y27632 on OGD associated BBB ischaemic insult in the absence or presence of hypothermia

Graphs demonstrate the integrity and function of the BBB during an intra- and post-ischaemic insult in the absence or presence of hypothermia and Y27632, as documented by TEER (**A**) and paracellular flux values (**B**, n=3, p<0.05). *p<0.05 vs controls, †p<0.05 vs 4 OGD, and #p<0.05 vs 4 OGD + Y27632 + HT. OGD-oxygen-glucose deprivation; HT-Hypothermia; (h)-hours; BBB-blood-brain barrier; Y27632-Rho kinase inhibitor

Effects of oxygen-glucose deprivation on human *in vitro* triple culture blood-brain barrier models in the absence or presence of moderate hypothermia (35°C) and LY333531

In order, to assess the PKC- β associated damage, *in vitro* BBB models were subjected to 4 hours of OGD in the absence or presence of hypothermia and LY333531 a PKC- β inhibitor as a treatment option. Moreover, this experimental setting also highlights the synergistic property of hypothermia when used in combination with LY333531. BBB models subjected to 4 hours of OGD in the absence or presence of hypothermia and LY333531 showed decreased in TEER values as compared to controls. Exposing triple cultures to 4 hours of OGD in the presence of LY333531 documented a significant rise in TEER values when compared to OGD alone groups. Furthermore, BBB models subjected to 4 hours OGD in the presence of hypothermia with LY333531 showed slight increase in TEER values when compared to OGD alone groups and groups treated with LY333531 in the absence of hypothermia. Alternatively, post-ischaemic exposure of hypothermia in the presence of LY333531 reduces TEER values as compared to intra-ischaemic hypothermia and LY333531 with OGD groups. To evaluate the degree of destruction attributed during an ischaemic insult, paracellular flux was measured across the barrier. Triple cultures subjected to OGD in the absence of hypothermia documented a significant rise in

paracellular flux values when compared to OGD alone groups. Further addition of LY333531 to OGD exposed BBB models documented a slight decrease in paracellular flux values compared to OGD alone groups. Furthermore, BBB models subjected to 4 hours of OGD with LY333531 in the presence of hypothermia showed further significant decrease in paracellular flux values when compared to OGD alone groups. Last of all, post-ischaemic hypothermic treatments shows an increase in paracellular flux values in the presence of LY333531 across the barrier compared to intra-ischaemic hypothermia and LY333531 with OGD groups. These results suggest synergistic property of hypothermia and beneficial effects in maintaining the integrity and function of the BBB during intra-ischaemic scenarios but not during post-ischaemic episodes.

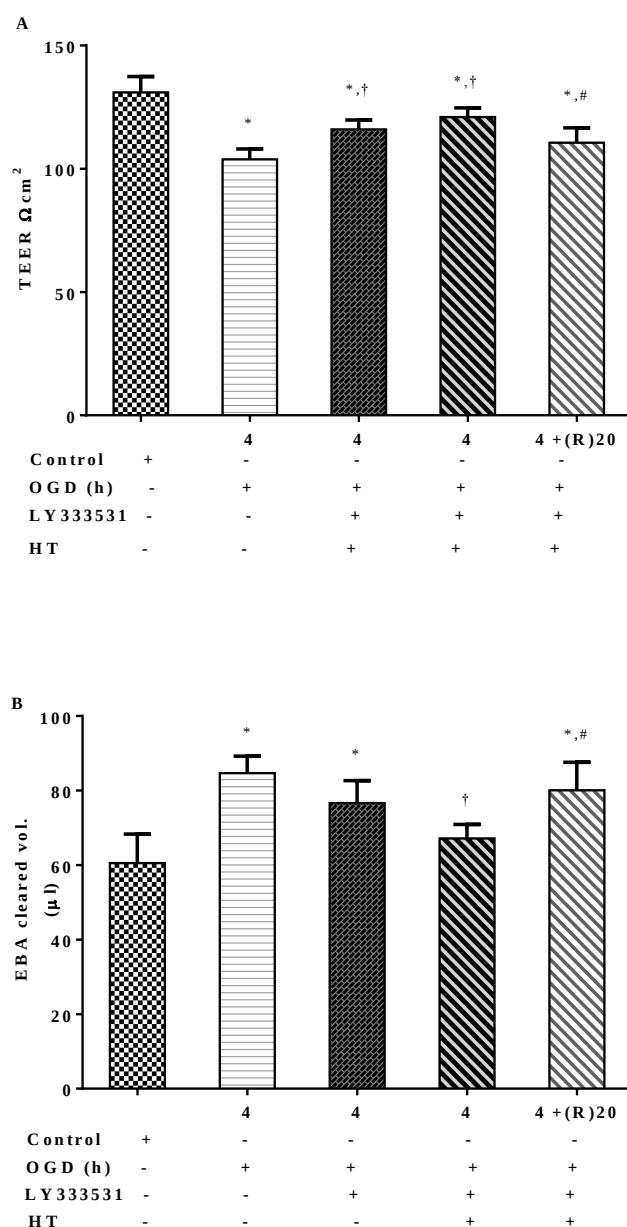


Figure 20-Effects of LY333531 on OGD associated BBB ischaemic insult in the SOD-absence or presence of hypothermia.

Graphs demonstrate the integrity and function of the BBB during an intra- and post-ischaemic insult in the absence or presence of hypothermia and LY333531 as documented by TEER (**A**) and paracellular flux values (**B**, $n=3$, $p<0.05$). * $p<0.05$ vs controls, † $p<0.05$ vs 4 OGD, and # $p<0.05$ vs 4 OGD + LY333531 + HT. OGD-oxygen-glucose deprivation; HT-Hypothermia; (h)-hours; BBB-blood-brain barrier; LY333531-PKC- β inhibitor

Discussion

Inflammation is a vital tool to protect oneself against infectious organisms. Yet, it becomes damaging when it is prolonged [272]. Over the past two decades, research has heavily stressed on an elementary mechanisms that irreversibly damage brain cells during intra- and post-ischaemic injury. Much attention has been focused on what makes these cells die easily and what strategies reduce their resistance to ischaemic injury. In the past few years, clinical experience with clot-lysing drugs has confirmed prospects that early reperfusion improves clinical outcome. With research highlighting ways to diminish tissue loss by both vascular and cell-based mechanisms, the attention is now shifting towards the study of how decreasing the brain temperature can preserve these cells. To answer this question, we assessed effects of moderate hypothermia on oxidative stress produced during intra- and post-ischaemic injury. Beneficial effects of hypothermia have already been documented in the past for the clinical setting for cerebrovascular disease and currently reported to be beneficial for various paediatric cardiac diseases [178, 342]. Stroke has a multifaceted aetiology, therefore, neuroprotectant should be able to demonstrate its putative effects at multi levels of ischaemic cascade. Hypothermia is one such candidate that fulfils the above-mentioned criteria [343]. To evaluate the effects of hypothermia on OGD associated oxidative stress levels, HBMEC, HA and HP were subjected to 4 and 20 hours of OGD in the absence or presence of hypothermia. In order to evaluate free radical generation, superoxide anion levels were measured in HA, HBMEC and HP subjected to OGD in the absence or presence of hypothermia (Figure 14). Results documented high levels of superoxide anion in cells subjected to 4 and 20 hours of OGD in the absence of hypothermia. Signifying the amount of

oxidative stress befalls during an ischaemic insult. Concurrent use of moderate hypothermia as a treatment option during intra-ischaemic scenarios significantly decreases the superoxide anion generation rate when compared to OGD alone groups. Hence, results do suggest the beneficial effects of intra-ischaemic hypothermia treatments in decreasing the oxidative stress in HA, HBMEC and HP. Post-ischaemic treatments with moderate hypothermia also manages to decrease the superoxide anion generation as compared to OGD alone groups. The production of superoxide anion and its dismutation to H_2O_2 provides the substrates for hydroxyl radical formation. Similar to our findings, Kataoka et al., found 30% reduction in superoxide anion production under hypothermia on microglial activation that is thought to cause the development of ischaemia-induced neuronal damage [344]. Also, Maier et al., showed that hypothermia manages to reduce generation of superoxide anion in peri-infarct areas [345]. Additionally, Riess et al., showed that hypothermia treatment resulted in moderate and reversible changes in mitochondrial function. However, hypothermia protects during ischaemia, as shown by preservation of mitochondrial NADPH energy balance and prevention of deleterious increases in calcium and ROS formation [346]. Furthermore, NADPH oxidases are a class of multicomponent enzymes whose exceptional function is the generation of reactive oxygen species in the vascular cells interacting with blood vessels [347]. The NADPH oxidases are a group of plasma membrane-associated enzymes found in primarily in neutrophils and microglia [348]. Therefore, to further assess oxidative stress components associated ischaemic injury, it is crucial to assess NADPH oxidase levels during intra- and post-ischaemic injury phase in the absence or presence of hypothermia (Figure 13). Our results documented an increase in NADPH oxidase enzyme activity in cells subjected to 4 and 20 hours of OGD in the absence of hypothermia presence of hypothermia. Simultaneous use of

hypothermia in experimental conditions resulted in a significant decrease in NADPH levels as compared to OGD alone groups. While, post-ischaemic hypothermia did manage to reduce the enzyme activity when compared to OGD alone groups but showed similar results to samples exposed to OGD in the presence of hypothermia. These findings stand with studies from the past where, hypothermia manages to decrease the oxidative stress levels during inflammation, further resulting in decreased free radical generation [349]. Tang et al., in their study found that pharmacologically inhibiting NADPH oxidase enzyme was protective against experimental stroke [350]. Consistent with this, Xian et al. also showed that the addition of microglia to BBB constituents potentiated injury due to OGD, and could be reversed by inhibiting NADPH oxidase [351].

In order to assess the effects of hypothermia on antioxidant mechanisms involved during an ischaemic injury, total SOD and its counterparts were studied in HA, HBMEC and HP exposed to 4 and 20 hours of OGD in the absence or presence of hypothermia. Results highlighted protective nature of moderate hypothermia, via documenting a decrease in SOD levels in cells when exposed to OGD in the absence of hypothermia. While an addition of hypothermia to the OGD exposed cells led to an increase in total SOD levels in both intra- and post-ischaemic episodes (Figure 15-17). These results stand along with findings of Liu et al., and Fukuhara et al., where they found SOD expressions increases after 6 hours of hypothermic exposure [352, 353].

To study various OGD induced inflammatory effects on the BBB in the absence or presence of hypothermia and specific inhibitors such as apocynin, LY333531 and Y27632 were added as a treatment options. *In vitro* human BBB models were subjected to 4 hours of OGD in the absence or presence hypothermia and inhibitors.

Furthermore, this experimental structure also helps to evaluate the synergistic property of hypothermia. Firstly, triple cultures exposed to OGD in the presence of apocynin (Figure 18) documented better values of TEER values and less leakage across the barrier as accounted by decreased paracellular flux values when compared to OGD alone groups. Additionally, following an introduction of hypothermia in the above mentioned setting results documented further upsurge in TEER values and significantly decreased paracellular flux across the barrier. Results suggest the beneficial effects of Apocynin as a treatment option and role of hypothermia in maintaining better outcome following an ischaemic injury.

Similarly, the use of Y27632 on new sets of triple culture alike to above-mentioned experimental settings, results showed better neurological outcome when compared to OGD alone groups. (Figure 19), TEER values were significantly increased when compared to OGD alone groups and the use of Y27632 was accompanied by a decrease in paracellular flux values. Further, hypothermia enhanced the outcome during an ischaemic injury episode, with further increasing TEER values and decreasing paracellular flux across the barrier. Lastly, to overcome PKC- β attributed damage on the BBB during an ischaemic insult, LY333531 was used as a treatment option. Results documented better outcome in maintaining the integrity and function of the BBB. Here, simultaneous use hypothermia with LY333531 further restored advantageous outcome (Figure 20).

Study conducted by our group found that inductions of PKC- β and Rho-kinase pathway are accountable for the hyperglycemia-induced barrier damage [8]. Therefore, our results also documented healthier outcome with use of LY333531 in the presence of hypothermia. Furthermore, increased rho-kinase activity is associated high Ca^{2+} levels during neuroinflammation specifically in conditions leading to

subarachnoid hemorrhage [354]. Additionally, rho and ROCK inhibitors have found to be effective in various trials focusing over stroke patients [355]. Our findings signify with Gibson et al., where they found that ischaemia led-BBB disruption was decrease with usage of Y27632 [356]. In some different models of middle cerebral artery occlusion, administration of apocynin has been shown to reduce infarct size in rats and mice [338-341]. Our results stand with the findings of Kahles et al., where they found inhibition of NADPH-oxidase with apocynin was very effective in maintaining the BBB [357]. In summary, moderate hypothermia manages to decrease the ischaemic induced oxidative stress and also works as a perfect synergistic candidate for future treatment options.

Chapter 3

Multiple Sclerosis and its effects on the integrity and function of the blood-brain barrier

Introduction

MS is characterised as an inflammatory demyelinating disease of the central nervous system that progresses in genetically susceptible individuals after exposure to unknown environmental trigger [250]. The order of proceedings in the development of the acute lesion in MS remains unresolved. Data from the past have been insufficient in explaining the concept behind the MS associated inflammatory attack. Although, postmortems or biopsies have been performed in the context of an acute relapse [247]. Inflammatory component has been recognised for many years but its precise role remains unclear. Though some authors suggest [358] it as a secondary response to myelin breakdown. Over the last 15 years evidence have been accrued showing perivascular inflammatory infiltrate being a characteristic component of the acute lesion in MS [359]. Nevertheless, such kind of perivascular inflammation has also been documented in normal-appearing white matter of the brain and in the absence of subjacent inflammation [360]. Thus leading to the assumption that inflammation in MS can occur in the absence of myelin breakdown, and the suggestion that a vascular event is a necessary preliminary to the development of structural damage. Basically, the most widely accepted hypothesis advises that attack mediated by T cell receptors on CD4-T lymphocytes, along with myelin antigens and presented by class II major histocompatibility complex (MHC) is expressed on macrophages/microglia, HA, and endothelial cells which further leads to an immune attack on the myelin-oligodendrocyte complex [247]. Further, these cross reactions between active CD4-T cells and myelin antigens, are apparently responsible in accumulating an inflammatory response and promotes further proliferation of T-and B-cells and macrophages. This infiltration further leads to penetration of the BBB by

activated leukocytes, which in turn initiates the production of cytokines and chemokines within the cerebral interstitium [240].

This chapter focuses on the effects of neuroinflammation during MS disease progression on the integrity and function of the BBB. Initial physiological studies in animals displayed the high electrical resistance of the cerebral capillary tight junction and discovered the complex transport mechanisms involved in the movement of glucose and essential amino acids into the brain. However, the recent stress in BBB studies is on the molecular biology of the blood vessels and surrounding cells and the applications to human disease [227]. MS attack destroys the axon in variable degrees and producing significant physical disability within 20 to 25 years in more than 30% cases. The classic clinical picture in MS consists of the symptoms such as sensory loss, spinal cord symptoms (motor and autonomic), cerebellar symptoms (dysarthria, ataxia and tremor), optic neuritis, facial myokymia, heat intolerance, and depression. Current concepts of pathophysiology of MS consider it a heterogeneous disease [237]. Recent data propose that in a subset of MS patients, the brain lesions show deep resemblances to tissue alterations found in vascular diseases, suggesting that a hypoxia-like metabolic injury is occurring in the inflammatory brain lesions. Both, vascular pathology as well as metabolic disturbances may be responsible for apoptosis of oligodendrocytes [238]. Early pathologists defended the concept of vascular lesions being a central module of the MS attack. Furthermore, Putnam and Adler in 1937 reconstructed serial brain sections from MS patients, and exhibited that fibrin deposits fenced the inflamed vessels [239]. Hence, the immune dysregulation in MS eventually represents a failure of the immune system to energise T-cells to myelin sheath components and a disparity among pro-and anti-inflammatory cytokines. TNF- α is elevated in peripheral blood mononuclear cells as well as in MS patients' serum and

its level associates with disease activity and secondary progression [241-243]. BBB associated endothelial cells express TNF receptor-1 (TNFR1), and TNF- α is also found to elevate other cytokine levels [244, 245]. In the periphery, TNF- α and other cytokine receptors affect the cellular distribution of tight junction proteins [99]. It has been well documented that IV administration TNF- α to mice resulted in an increased BBB permeability and high levels of cytokine in serum downregulate occludin expression [246]. These chemokines promote both adhesion of leukocytes to endothelial cells and migration of leukocytes across BBB. Clinically, the relapsing-remitting course of MS is considered to be a disturbance in cognitive, cerebellar, motor, sensory, bladder and bowel, brainstem, optic nerve, and cognitive functions. Besides demyelination, active MS lesions may display substantial axonal damage and loss [361]. Additionally, besides loss of the BBB solute barrier, enhanced trans-endothelial leukocyte migration into the central nervous system is detected early in the course of MS, and likely contributes to disturbances in neural integrity [247]. MRI-neuropathological studies of acute MS lesions have exposed that these focally enhanced areas entail active lesions, characterised by severe inflammation, loss of barrier, perivascular cuffing, and often extensive infiltration of tissue by monocytes [248, 249]. Disruption of the BBB is demonstrated *in vivo* in acute and chronic active MS lesions using contrast enhanced MRI.

Therefore, in light of above we assessed integrity and function of *in vitro* human BBB models when exposed to MS patients' serum. Further, to assess various inflammatory reactions occurring during MS disease progression, BBB models subjected to MS patients' serum were introduced to various inflammatory inhibitors such as rotenone, Y27632, LY333531, apocynin and MMP-2 inhibitor as a treatment options. Furthermore, MS patients' serums were used to measure the cytokine concentrations

namely TNF- α and IL-1 β and evaluated the caspase-3/7 activity to measure rate of apoptosis in MS.

AIMS

1. To investigate pro-inflammatory cytokine levels in multiple sclerosis patients' serum.
2. To explore integrity and function of blood-brain barrier when exposed to MS patients' serum.

Results

Measuring the degree of inflammation during multiple sclerosis disease progression via assessing IL-1 β concentrations in multiple sclerosis patients' serum

In order to assess the degree of inflammation befalling during MS disease progression, specific ELISA-based kit was used to assess the concentrations of IL-1 β in both remitting and relapse forms of MS patients' serum. Relapse MS documented high concentrations of IL-1 β in patients' serum as compared to controls. Whereas, remitting MS patients' serum documented slight less IL-1 β concentrations as compared to relapse-MS.

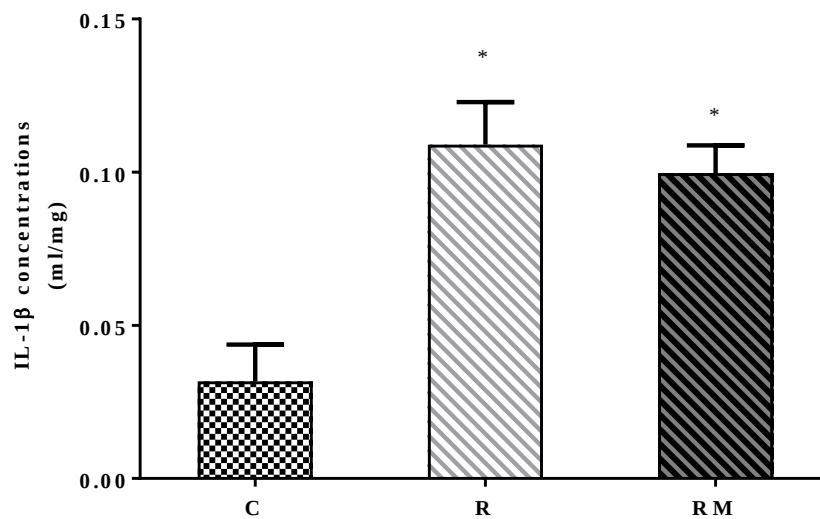


Figure 21-Degree of inflammation during multiple sclerosis disease progression via assessing IL-1 β concentrations in multiple sclerosis patients' serum

Graph represents IL-1 β concentrations in MS patients' blood serum. Data on y-axis shows IL-1 β concentration and x-axis demonstrates various forms of MS. (n=6, p<0.05). *p<0.05 vs controls, R-Relapse-MS; RM-remitting-MS; IL-1 β -interleukin-1beta

Measuring the degree of inflammation during multiple sclerosis disease progression via assessing TNF- α concentrations in multiple sclerosis patients' serum

In order to assess the degree of inflammation befalling during MS disease progression, specific ELISA-based kit was used to assess the concentrations of TNF- α in both remitting and relapse-MS patients' serum. Relapse MS documented significantly high amount of TNF- α level as compared to controls. While, TNF- α level in remitting MS patients' serum documented decreased concentrations as compared to relapse-MS.

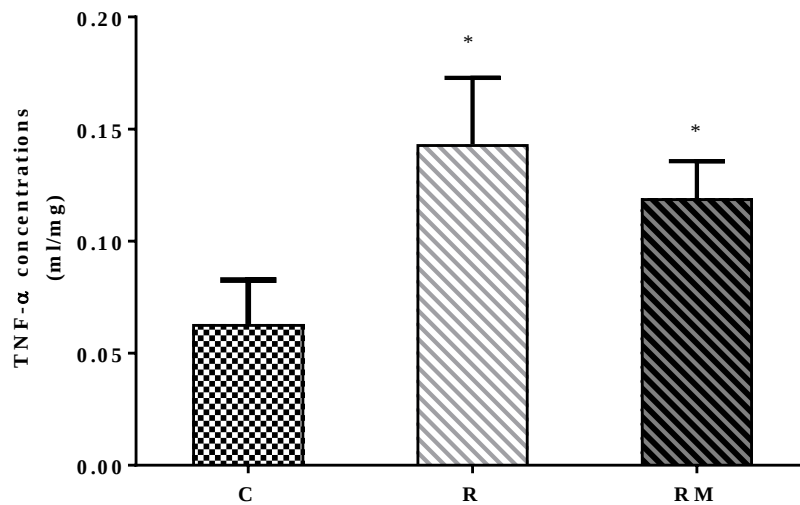


Figure 22-Degree of inflammation during multiple sclerosis disease progression via assessing TNF- α concentrations in multiple sclerosis patients' serum

Graph represents TNF- α concentrations in MS patients' blood serum. Data on y-axis shows TNF- α concentration and x-axis demonstrates various forms of MS. (n=6, p<0.05). *p<0.05 vs controls, C-Controls; R-Relapse-MS; RM-remitting-MS; TNF- α -tumor necrosis factor-alpha

Assessing the rate of apoptosis arising during multiple sclerosis disease progression via measuring caspase-3/7 enzyme activity in multiple sclerosis patients' serum

Both forms of MS revealed high amount of Caspase-3/7 activity when compared to controls, signifying high amount of rate of apoptosis throughout MS disease progression. However, relapse MS patients' serum accounted for highest level of caspase activity in comparison to remitting MS patient's serum. Suggesting increased neuroinflammatory devastation associated with relapse MS.

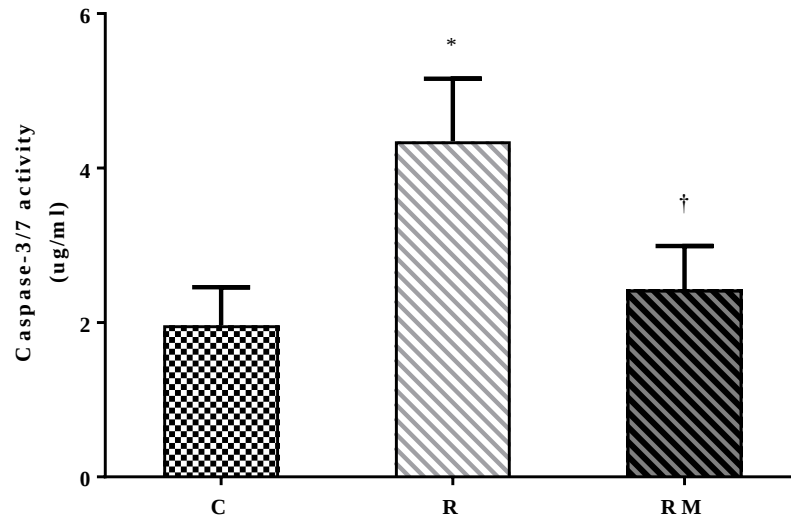


Figure 23-Rate of apoptosis via total caspase-3/7 enzyme activity in multiple sclerosis patients' serum

Graph represents total caspase-3/7 enzyme activities in multiple sclerosis disease progression. Data on y-axis shows total caspase activity in MS patients' serum and x-axis demonstrates various forms of MS and controls (n=6, p<0.05). *p<0.05 vs controls, †p<0.05 vs relapse-MS; C-Controls; R-Relapse-MS; RM-remitting-MS

Assessment of degree of neuroinflammation accounted during multiple sclerosis disease progression and its effects on integrity and function of blood-brain barrier in the absence or presence of various inflammatory inhibitors

In order, to assess the MS-associated effects on the integrity and function of the BBB, MS patients' serums were introduced on triple cultures for 6 hours. Following exposure, TEER and paracellular flux were measured across the barrier. Results documented a decrease in levels of TEER in both forms of MS as compared to controls. Results with relapse-MS showed further decline in TEER values when

compared to remitting MS group. Furthermore, following addition of apocynin as a treatment option to the experimental conditions results documented a significant increase in the TEER values in both relapse and remitting-MS as compared non-inhibitor groups. Similarly, addition MnTBAP to the experimental conditions, results documented upsurge in TEER values as compared to non-inhibitor groups and equivalent to groups under apocynin treatments. Lastly, when rotenone was introduced to the triple cultures exposed to MS patients' serums, data showed a slight increase in TEER values but less effective than previously used inhibitors. To further evaluate the degree of MS associated BBB destruction, paracellular flux across the barrier were measured in the absence or presence of inhibitors. EBA cleared volume was calculated across the barrier following the exposure to MS patients' serum for 6 hours. Results in the absence of inhibitors documented significant increase in paracellular flux values when compared to controls in relapse and remitting MS. Specifically, relapse-MS showed highest amount of leakage across the barrier compared to remitting MS. Following addition of inhibitors such as apocynin, both treatment groups showed a significant decrease in paracellular flux values when compared to non-inhibitor groups. Similarly, MnTBAP, also reduced paracellular flux across the barrier in both forms of MS. Lastly, results with rotenone treatments unfortunately documented high amount of paracellular flux across the barrier. Results suggest high amount of BBB destruction associated during MS disease progression, specifically in relapse-MS. Apocynin and MnTBAP did manage to reduce the destruction and maintain the integrity of the BBB, while rotenone failed to show protective effects towards the BBB.

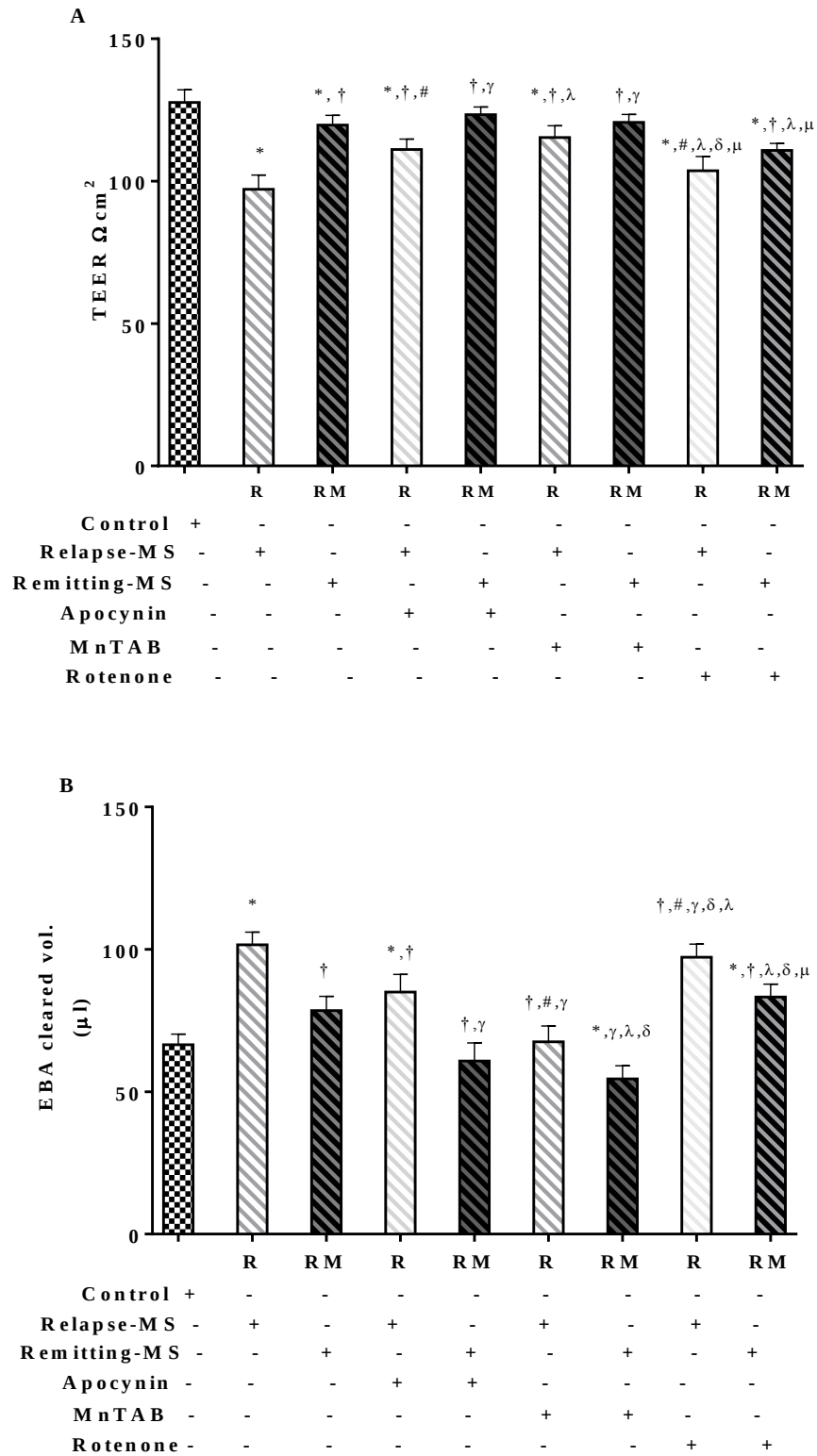


Figure 24-Inflammatory effects of multiple sclerosis disease progression on the integrity and function of blood-brain barrier in the absence or presence of inhibitors of the oxidative stress

Graphs represent data reflecting integrity and function of human BBB in MS disease progression and in the absence or presence of specific inflammatory cascade inhibitors of oxidative stress. Data on y-axis shows values of transendothelial electrical resistance TEER **(A)** across the BBB following exposure of MS disease progression and x-axis demonstrates various forms of MS with controls in absence or presence of inhibitors. (n=6, EBA **(B)** n=3, p<0.05). *p<0.05 vs controls; †p<0.05 vs relapse-MS; #p<0.05 vs remitting-MS; ‡p<0.05 vs relapse-MS + apocynin; §p<0.05 vs remitting-MS + apocynin; ¶p<0.05 vs relapse-MS + MnTBAP; ¶p<0.05 vs remitting-MS + MnTBAP. R-Relapse-MS; RM-remitting-MS; BBB-blood-brain-barrier; MnTBAP-Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride

Inflammatory effects of multiple sclerosis disease progression on the integrity and function of blood-brain barrier in absence or presence of specific inflammatory inhibitors

In order, to assess the MS-associated effects on the integrity and function of the BBB, MS patients' serums were introduced on triple cultures for 6 hours. Following exposure, TEER and paracellular flux were measured across the barrier. Results documented a decrease in levels of TEER in both forms of MS as compared to controls. While relapse-MS showed further decline in TEER values when compared to remitting MS group as discussed in the last study. Furthermore, following addition of Y27632 as a treatment option to the experimental conditions results documented a significantly increase in the TEER values in both relapse and remitting-MS as compared non-inhibitor groups. Similarly, addition of LY333531 to the experimental conditions, results documented no increase in both remitting and relapse MS in terms of TEER when compared to controls or non-inhibitor groups and to groups under Y27632 treatments. Lastly, when MMP-2 inhibitor was introduced to experimental conditions, results documented a slight rise in TEER values when compared to MS

groups in the absence of inhibitors. When compared to other inhibitor treatment groups their TEER values remain the same. To measure the degree of destruction, paracellular flux across the barrier were measured in the absence or presence of inhibitors. EBA cleared volume was calculated across the barrier following the exposure to MS patients' serum for 6 hours. Results in the absence of inhibitors documented significant increase in paracellular flux values when compared to controls. Specifically, relapse form of MS showed highest amount of leakage across the barrier compared to remitting MS. Following addition of inhibitors such as Y27632, both treatment groups showed a significant decrease in paracellular flux values when compared to non-inhibitor groups. While, addition of LY333531 showed an increase in paracellular flux value when compared to controls and groups under Y27632 treatments. Lastly, with MMP-2 inhibitor as a treatment option in MS-associated BBB damage, results documented a decrease in paracellular flux values when compared to other treatment groups. These above mentioned results suggest the role of inhibitors in suppressing the inflammatory effects on the BBB damage when exposed to MS inflammatory insult, however LY333531 did not manage to show beneficial aspects towards the BBB architecture.

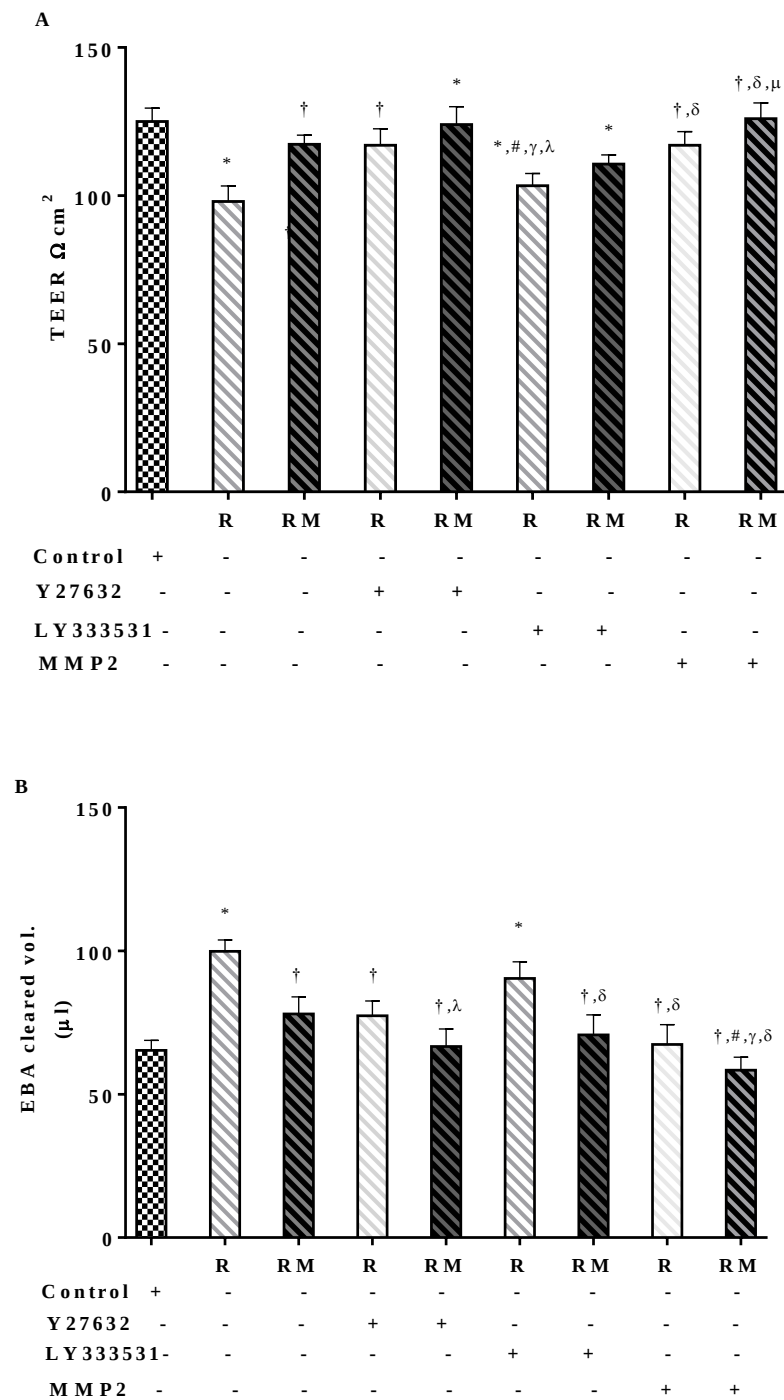


Figure 25-Inflammatory effects of multiple sclerosis on integrity and function of blood-brain barrier in absence or presence of inhibitors

Graphs represent data reflecting integrity and function of human BBB in MS disease progression and in absence or presence of specific inflammatory cascade inhibitors

such as Y27632, LY333531 and MMP-2. Data on y-axis shows values of TEER values across the barrier when exposed to MS patients' serum for 6 hours **(A)** and x-axis demonstrates various forms of MS with controls in the absence or presence of inhibitors. **(B)** EBA cleared volume across the barrier in relation to MS disease progression *p<0.05 vs controls, †p<0.05 vs relapse-MS, #p<0.05 vs remitting-MS, ‡p<0.05 vs relapse-MS+Y27632, §p<0.05 vs remitting-MS+Y27632, ¶p<0.05 vs relapse-MS+LY333531, ††p<0.05 vs remitting-MS+LY333531. R-Relapse-MS, RM-remitting-MS, BBB-blood-brain-barrier, Y27632- Rho kinase inhibitor, MMP-matrix metalloproteinases, PKC-protein kinase-C, LY333531-PKC inhibitor

Discussion

MS is considered as an inflammatory demyelinating disease, exclusively containing focal demyelinated plaques in the white matter of the central nervous system. This also includes demyelination in the cortex and also embraces the normal-appearing white matter [362]. The mechanism involved for the formation of these lesions is highly complex and heterogeneous. Moreover, this heterogeneity is reflected by different clinical presentations of the disease, such as relapsing or progressive MS. In this study, we have evaluated the effects of MS associated inflammation on the integrity and function of BBB by exposing triple cultures to MS patients' serum and further investigated other inflammatory factors resulting in the BBB destruction such as pro-inflammatory cytokines and rate of apoptosis. Furthermore, integrity of the BBB was assessed via suppressing various inflammatory stress pathways using precise inhibitors. There are varied treatment options for MS but there are two major modalities available such as immunomodulatory therapy and therapies to relieve or modify symptoms such as interferon- β 1a or 1b etc.

The occurrence of inflammatory episodes in the early stages of MS has been linked with late neurodegeneration [363]. Augmented glutamate is thought to play a role in the inflammation associated neurodegenerative process of MS and in turn, enhanced

glutamate release is associated with high pro-inflammatory cytokine levels [364], hence this study investigates concentrations of inflammatory cytokines such as TNF- α and IL-1 β released during acute MS attacks. Our results suggest high concentrations of the cytokine IL-1 β in relapse form of MS as compared to remittent MS (Figure 21). Results are significant with study conducted by Cacabelos et al., where they found high amount of IL-1 β in cerebrospinal fluid of MS patients [365]. This is significant and also elaborates deleterious effects of such high concentrations over the BBB in MS [240]. Furthermore, Rossi et al., documented high levels of IL-1 β in cerebrospinal fluid of MS patients and related oedema present which signifies BBB destruction associated with high levels of cytokine in MS [363]. Furthermore, TNF- α levels were assessed using specific ELISA-based kit (Figure 22) and results suggested high concentrations of cytokine in relapse form of MS when compared to remittent and controls. Moreover, above demonstrated results were significant with a study Sharief et al., where they found similar high levels of TNF- α in active-MS associated with disruption of the BBB [366]. Also, immune cells of MS subjected to high levels of inflammatory cytokines and enzymes that can facilitate their migration to the central nervous system by influencing BBB function [367]. Minager et al., also described that various cells in active lesions release Th1 cytokines, T lymphocytes expressing CD4 are also recognised as helper T cells, and these are regarded as being the most productive cytokine producers. This subset can be further subdivided into Th1 and Th2, macrophages/microglia, and astrocytes [368]. The major Th1 cytokines involved in pathogenesis of MS are interferon-gamma, TNF- α and IL-1 β .

Additionally, apoptosis plays a significant role in the development of MS, presumably via the immune-regulation activated-induced T-cell death and in local processes of tissue damage [369]. To analyse rate of apoptosis accounted to during the MS disease

progression, we measured caspase-3/7 enzyme activity in MS patients' blood serum (Figure 23). Results suggested high levels of caspase-3/7 activity accounted in relapse form of MS compared to remittent MS. This is significant and verifies robust role of apoptosis in MS, similarly shown by Zipp et al., and Cudrici et al., [369, 370]. Moreover, Barbara et al., postulated that early stage of MS disease progression is associated with oligodendrocytes apoptosis resulting in loss of cells in plaques [371]. Lastly, to study the effect of MS on the BBB integrity and function, *in vitro* human BBB models were subjected to MS patients' blood serum for the duration of 6 hours. Following the exposure, results documented decreased TEER values and increased paracellular flux in both remittent and relapse-MS when compared to controls signifying the barrier destructive effects associated with MS. Furthermore, remittent MS showed better TEER values and decreased paracellular flux when compared to relapse-MS suggesting decreased proportion of damage accounted in remitting MS. This damage may be accompanied by an increase in rate of apoptosis and upsurge in pro-inflammatory cytokine levels as discussed before. Also, results documented may signify findings of Catherine et al., where they found migration of activated leukocytes due to inflammation during the MS results in destruction of the BBB via enhancing the permeability and accompanied by increase cytokine levels [367]. Also, Suidan et al., showed increase in vascular endothelial growth factor is associated with an increase in the BBB leakage [372]. Some studies have also documented the role of oxidative stresses in damaging the BBB during MS disease progression [373, 374]. Therefore, in light of above, *in vitro* human BBB models were subjected to MS patients' serum in the presence of various inhibitors such as LY333531, Y27632, MnTBAP, apocynin, rotenone, MMP 2 (Figure 24). Recently, our group evaluated that specific NADPH-oxidase suppression leads to better BBB outcome following

hyperglycaemia aggravated BBB disruption [375]. Likewise, to inhibit NOX, via NADPH-oxidase specific inhibitor, apocynin was introduced to the experimental models. MS associated BBB groups in the presence of apocynin documented better resistance values and significantly decreased paracellular flux values across the barrier when compared to groups in absence of apocynin. Thus, signifying oxidative stress related BBB damage during a MS inflammatory insult. To further expand the study in relation to oxidative stress over the MS associated BBB damage, a broad spectrum super oxide dismutase mimicking inhibitor, MnTBAP was introduced. This inhibitor is found to inhibit neuronal apoptosis and to prevent generation of reactive oxygen species [376]. In light of this MS associated BBB group in presence of MnTBAP revealed better resistance values and lesser paracellular flux when compared to the group without an inhibitor.

Furthermore, to study mitochondrial chain reactions during MS associated BBB insult, rotenone, which specialises in inhibiting mitochondrial electron at NADH-ubiquinone oxidoreductase, was introduced to similar experimental conditions. Additionally, rotenone has been helpful in decreasing inflammation through subsiding the activity mitochondrial complex 1 and further found helpful in diseases such as Parkinson's by declining inflammation [377]. Inhibitors used have already been explained before and the results we found were encouraging in terms of increase in resistance values signifying better integrity when oxidative stress mechanism is suppressed. Likewise, Qing et al., in their study suggested treatments with mitochondrial chain complex 1 inhibition may be the new prospects in controlling neuro-inflammation and also, not only here rotenone also managed to decrease pro-inflammatory cytokine levels in hepatocytes [378]. Therefore, when we exposed MS-

BBB to rotenone, results suggested better TEER values and lesser paracellular flux signifying the protective role of rotenone towards MS-BBB destruction.

From the past it is known that high levels of MMP-2 are documented in MS pathology [379] and their association with disruption of the BBB. Therefore it becomes essential to analyse MS associated BBB damage via suppression of MMPs activities through introduction of MMP specific inhibitor. Our results have shown significant increase in TEER values and decrease in paracellular flux across the barrier (Figure 25). Therefore, it signifies with our prior group study, which shows that MMPs are responsible for digestion of extracellular matrix, and also contributes to enhance TNF- α level evoked cerebral barrier dysfunction [281]. One of the major factor associated in aggravation of MS associated BBB disruption and pathology is antibody-induced demyelination cross linkage of oligodendrocytes. This effect was decreased by Marta et al., by help of a potent Y27632 which further led to better outcome in MS [380]. Similarly, Steven et.al., in their study also documented that by use of this potent inhibitor axonal degeneration is delayed and may characterised as a novel therapeutic target [381]. Although these were not in human clinical trials, therefore in light of above *in vitro* human BBB were subjected to MS patients' serum in presence of Y27632 and the result documented better TEER values when compared to MS associated BBB in absence of Y27632 group. Thus, signifying better outcome via blocking rho-kinase activities during a MS initiated insult.

Furthermore, PKC isoforms has been recognised as a key factor in development of neuroinflammation and affecting the barrier properties not just in MS but also in diseases like Alzheimer's and ischaemic stroke [375, 382]. Therefore it becomes crucial to evaluate MS-barrier properties by inhibiting PKC- β via LY333531, the

results documented higher TEER values as compared to groups in the absence of inhibitors and reduced paracellular flux across the barrier.

General Discussion

Cerebral ischaemic and MS both are characterised as an acute neurodegenerative disorders and the leading cause of death. There is a prompting awareness that inflammatory processes are vital in the brain, precisely in pathophysiological conditions. Further, comprising of cytokines, which are of diverse group of polypeptides comprising of multiple properties on many different brain cells. To our understanding, the directive of cytokines bioactivity is enormously multifaceted with control applied at the levels of transcription, translation and cellular release with additional inflection via soluble receptors. On the whole there is an extensive countenance of many cytokines in response to an ischaemic insult one of which is destruction of the BBB during and after an ischaemic insult. Overall, the effects of pro-inflammatory cytokines in recovery appear to be as vague as their roles as mediators of acute damage. MMPs over-expression is majorly involved in neuroinflammation that result in opening of the BBB and increased risk of haemorrhage. Studies with increased expression of MMPs have been documented with higher degree of BBB destruction. Synthetic inhibitors of MMP help in attaining the integrity and function of the BBB, suggesting that they may be used therapeutically to reduce neuroinflammatory response in both MS and ischaemic stroke models.

The endothelial cells of the BBB are involved in pathogenesis of inflammatory demyelinating diseases such as MS. The HBMECs form the inmost coating of all the blood vessels and therefore are majorly responsible for synthesis of a number of pathophysiological events such as apoptosis, oxidative stress, nitric oxide during and after an ischaemic attack. Nevertheless, HA are also equally important in triggering inflammation and found to release inflammatory cytokines at various stages of destruction [383]. In CNS, these cells including HP form tightly sealed barrier termed

as the BBB, which limit the paracellular permeability to a number of selective blood-driven solutes. However, the pathological conditions like ischaemic stroke and MS are found to alter the integrity and function of the BBB by activating many intercellular stress-complex and inflammation-sensitive signalling pathways. The outlined studies in the thesis set out to highlight neuroprotectant “hypothermia” and its effects on intra-and-post-ischaemia-mediated BBB disruption, by investigating the OGD± hypothermia. Vasogenic oedema has been stated as a result of hypoxia-induced cerebral injury linked with BBB disruption and is also a leading cause of death during or after ischaemic stroke [384]. Hence, the current OGD studies focused on some of the key molecular mechanisms responsible for the hypoxia related brain injury which include pro-inflammatory cytokine release, cytoskeletal remodelling, activation of pro-oxidant NADPH oxidase enzyme complex, superoxide anion, anti-oxidant mechanisms with SODs and modulation of the tight junction proteins. Moreover, the implication of these molecular mechanisms was also studied by evaluating their effects on the BBB integrity and the functionality. The overall results generated throughout these studies confirm these hypotheses and have shown that OGD compromises the BBB integrity by activation of various inflammatory cascades and hypothermia delivers neuroprotection against OGD-induced injury. Also, hypothermia can be implicated with other treatment measures for ischaemic stroke. This thesis has demonstrated that specific inhibition of various stages and counterparts of ischaemic cascade under the presence of hypothermia. Also, we evaluated various effects of underwritten inflammatory process and by inhibiting different pathways our result documented better outcome in MS.

Lastly, these findings may help in; (a) improve post-stroke survival rate, (b) reduce the occurrence of the secondary strokes, and (c) Help in discovering other new modalities for acute neurodegenerative disorders such as MS and ischaemic stroke.

Future Prospective

Hypothermia is a notable neuroprotection associated treatment option that preserves tissues and limits inflammatory injury. It is possibly the most robust neuroprotectant studied in the laboratory and has also been shown to have efficacy in humans. It affects nearly every metabolic, molecular and cellular event in cell death to endorse tissue preservation. This multi-faceted feature of therapeutic hypothermia may explain the need multi-stage approaches. As hypothermia manages to work effectively when added with other treatment options does open new conduits to extend the therapeutic window for the management strategies. In spite of the healthy protective effects of therapeutic hypothermia validated in the laboratory, there are still several obstacles, including how to achieve effective cooling in humans, how to prevent untoward effects and how to identify patient populations that are most likely to benefit from therapeutic hypothermia. Animal models of ischaemic injury and the methods of cooling used in the laboratory are quite different from those used in the clinic. In addition, investigations to overcome complications of systemic hypothermia, such as shivering, infection and coagulopathies, are lacking. These complications are serious at the clinical level, but are largely ignored in the laboratory; therefore this requires further studies on how hypothermia influences signalling pathways. Additionally, MS study highlighted BBB destruction associated and thus becomes the main target for future therapy and the reason behind inflammatory symptoms. In summary, therapeutic hypothermia has its place in the laboratory, especially when it is managing to preserve BBB integrity and function for longer durations. However, it is clear that more research is still needed to understand

its biological significance as well as how it can be effectively applied in additional clinical conditions.

Conclusion

Hypoxia is the end point in many disorders such as stroke, cardiac arrest, carbon monoxide poisoning, respiratory diseases as well as at high altitude associated sickness. It leads to the BBB disruption resulting in increased permeability, vasogenic oedema and tissue damage. It being the main counterpart of ischaemic neuronal injury during ischaemic stroke episodes and evokes a pressing need for multi-staged neuroprotectant as treatment option. Factors such as high amount of pro-inflammatory cytokine release, increased apoptosis rate and oxidative stress all contribute to ischaemia associated BBB damage. Furthermore, only small percentage of stroke attack survivors can be treated with thrombolytic agents, there is an urgent need to develop new alternative treatments such as neuroprotection. Hypothermia has successfully managed to suppress inflammatory factors at various stages of inflammation, thus contributing in maintaining the BBB and delaying after effects. The current knowledge from both experimental and clinical research suggests that hypothermia should be initiated as soon as possible after stroke onset in order to achieve the best outcome. Prolonged cooling should be considered if the initiation of the hypothermia treatment is delayed. As the rewarming phase strongly influences the outcome after stroke, it is crucial that all studies use slow rewarming phases. Currently, it can be considered that mild to moderate hypothermia is found to be neuroprotective and produces fewer complications when compared to deep or profound cooling. At the same time, controlling physiological parameters and the temperature during the treatment are essential for successful clinical or experimental trials. Therefore in light of this study it is very much crucial to further research the effects of hypothermia both in molecular and clinical settings. Similarly, immune-mediated myelin inflammation in MS does affect the BBB. As documented in this

study, relapse form of MS stands alone equipped with factors upsetting the MS associated integrity and function of the BBB.

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Appendix

Equipment

Materials	Company	Origin
6-well tissue culture plates	Corning Life Sciences	UK
12-well tissue culture plates	Corning Life Sciences	UK
96- well tissue culture plates	Corning Life Sciences	UK
Tissue culture Flasks	Corning Life Sciences	UK
12mm diameter, 0.4µm pore size, polyester membrane transwell inserts	Corning Life Sciences	UK
Cell Scrappers	TPP	Switzerland
Fluorescence plate reader	TECAN GENios	USA
Absorbance/luminescence plate reader	BMG-LABTECH Omega	USA
Zeiss fluorescence microscope	Carl Zeiss GmbH	Germany
Odyssey IR imaging system	Li-Cor Biosciences	Germany
PAGE apparatus	Bio-Rad	UK
PVDF membrane	GE Healthcare	UK
N ₂ cylinders	Airproducts	UK

Buffers

The following buffers were used detected the protein bands by Western blotting technique in cell lysates.

✓ **10X Running buffer (1 Litre)**

24.2 g Tris base

144.0 g Glycine

10.0 g SDS

1000 mL Distilled water

✓ **1X Running buffer (1 Litre)**

100 mL 10X Running buffer

900 mL distilled water

✓ **10X Transfer buffer (1 Litre)**

24.2 g Tris base

144.0 g Glycine

1000 mL Distilled water

✓ **1X Transfer buffer (2 Litres)**

200 mL 10X Transfer buffer

400 mL methanol (100%)

1400 mL Distilled water

✓ **10X TBST (1 Litre)**

24.2 g Tris base
87.6 g Sodium chloride
1000 mL Distilled water, pH 7.4-7.6
10 mL Tween-20

✓ **1X TBST (1 Litre)**

100 mL 10X TBST
900 mL distilled water

✓ **Blotto blocking buffer (5%)**

2.5 g Blotto milk
50 mL 1X TBST

✓ **Ammonium persulphate (APS; 10%)**

0.5 g Ammonium persulphate
5 mL distilled water

✓ **L-Tris buffer (1.87 M, 0.5 Litres)**

113.6 g Tris-base
500 mL distilled water
pH 8.8 adjusted with Conc. Hydrochloric acid

✓ **U-Tris buffer (1.25 M, 0.5 Litres)**

75.7 g Tris-base
500 mL distilled water
pH 6.8 adjusted with Conc. Hydrochloric acid

✓ **2x Laemmli Sample Buffer**

65.8 mM Tris-HCl
26.3% (w/v) glycerol
2.1% SDS

0.01% bromophenol blue

Polyacrylamide gel electrophoresis (PAGE) recipe

Gel components	Resolving (7.5%) mL	(10%) mL	(15%) mL	Stacking (4%) mL
Ultra-pure water	7.600	6.201	3.405	3.680
40%w/v Acrylamide	2.740	3.653	5.475	.490
2% Bis-acrylamide	1.460	1.946	2.920	.260
L-Tris (1.87 M)	3000	3000	3000	-
U-Tris (1.25 M)	-	-	-	.500
10% SDS	.150	.150	.150	.050
10% APS	.100	.100	.100	.040
TEMED	.015	.015	.015	.010

“Jai Shri Ram”