Elucidating the role of hyperglycaemia in endothelial cell and pericyte morphology, function and crosstalk, and blood-brain barrier integrity

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Declaration

I confirm that this is my own work and the use of all material from other sources have been fully acknowledged.

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Abstract:

Diabetes is a complex metabolic disorder associated with a myriad of neurovascular complications, including disruption of the blood-brain barrier (BBB) and endothelial dysfunction. The intricate relationship between hyperglycaemia and cells of the neurovascular unit is of significant importance and yet remains inadequately understood. This study aims to delve into the impact of elevated glucose levels on three key areas: endothelial cell function, pericyte-endothelial interaction, and the integrity of the BBB in vitro.

Our study employed human brain microvascular endothelial cells and pericytes, cultured under normal glucose conditions (5mM) and high glucose conditions (25mM). A comprehensive evaluation of endothelial cell morphology, stress fibre formation, DNA damage responses, tube formation, and the secretome profile was performed. Furthermore, pericytes were exposed to endothelial cell-conditioned media, and the consequent cytokine production was measured. An in vitro BBB model was harnessed to assess the localization of tight junction proteins and paracellular/transcellular permeability.

Exposure to high glucose levels led to cytoskeletal remodelling in endothelial cells, although it did not noticeably modify cell morphology or directly cause DNA damage. The endothelial secretome exhibited an increase in angiogenic factors under high glucose conditions. Interestingly, pericytes exposed to high glucose-conditioned endothelial secretome displayed reduced expression of inflammatory cytokines. High glucose conditions also resulted in increased localization of tight junction proteins and augmented transcellular permeability across the BBB model.

Our findings stress that acute hyperglycaemia, while capable of initiating internal cellular changes, does not drastically alter the apparent phenotype of endothelial cells. Nevertheless, it significantly influences their secretome profiles, leading to the disruption of pericyte-endothelial crosstalk and compromising the integrity of the BBB. This research sheds light on the neurovascular dysfunction associated with diabetes and proposes multiple potential targets for the development of therapeutic interventions aimed at mitigating these complications.

Abbreviations:

ACs:	Astrocytes
AGEs:	Advanced glycation end products
AJs	Adherens junctions
AMT	Adsorptive-mediated transcytosis
AMT	Adsorptive mediated transcytosis
Ang1	Angiopoietin
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BRB	Blood retinal barrier
cAMP	Cyclic adenosine mono phosphate
CBF	Cerebral blood flow
CCL2/MCP-1	Monocyte chemoattractant protein-1
CMT	Carrier-mediated transcytosis
CNS	Central nervous system
CVD	Cardiovascular disease
CXCL2/GRO	Chemokine (CXC motif) ligand 2
DAMPs	Damage-associated molecular patterns
DM	Diabetes Mellitus
EBA	Evans blue albumin
ECs	Endothelial cells
EPC	Endothelial progenitor cells

ERK	Extracellular signal regulated kinase
ET-1	Endothelian-1
FGF-basic	Fibroblast growth factor basic
GLUT-1	Glucose transporter 1
HBMEC	Human brain membrane endothelial cells
HBSS	Hanks balance salt solution
HRMECs	Human retinal membrane endothelial cells
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule 1
IL-1	Interleukin-1
IL-6	Interleikin-6
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MIF	Migratory inhibitory factor
MMP9	Inflammatory matrix metalloprotease 9
NaF	Sodium fluorescence
NO	Nitric oxide
NVU	Neurovascular unit
OEC	Outgrowth endothelial cells
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PCs	Pericytes
PDGF	Platelet-derived growth factor

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PDGF-BB	Platelet derived growth factor beta
PDGFR-β	Platelet derived growth factor receptor beta
PIGF	Placental growth factor
PPP	Pentose phosphate pathway
RAC1/PHOA	Guanine nucleotide exchange factor- ?
RAGE	Receptor for advanced glycation end products
RMT	Receptor-mediated transcytosis
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
rtPA	Recombinant tissue plasminogen activator
sPLA2-IIA	Phospholipase A2-IIA
T2DM	Type 2 Diabetes Mellitus
TBI	Traumatic brain injury
TCA	Tricarboxylic acid
TEER	Trans-endothelial electrical resistance
TGF- α	Transforming growth factor alpha
TJs	Tight junctions
TNF-α	Tumour necrosis factor-alpha
uPA	Urokinase plasminogen activator
VE-caherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens
α-SMA	Alpha-smooth muscle actin

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γH2AX	Gamma H2A histone family X

1 Introduction

Stroke is the second leading cause of death and significant disability in the world (Huang, Wang et al. 2023). Reportedly, every year, more than 15 million people suffer a first stroke; of these, two-thirds die or are left with a perpetual disability (Katan, 2018). Stroke has impacted many families and their livelihood, with over 113,000 in UK people experiencing strokes each year. There are more than 1.3 million stroke survivors in the UK alone and with projected increase in the rate of first-time strokes among people aged 45 and over in the UK, and this will rise from 117,600 in 2015 to 148,700 in 2025 and 187,000 in 2035- an increase of 59% in two decades (Patel, n.d.) . Additionally, one in four stroke survivors experience recurrent stroke, which contributes to a significantly higher mortality rate and inferior functional recovery when compared to patients with a first stroke (Jerrgensen, Nakayama et al. 1997). There are many risk factors that contribute to stroke occurrence, including age, atrial fibrillation, high blood pressure, smoking, obesity, and diabetes. Ageing is considered to be one of the most significant risk factors for stroke. As with age the risk of developing the above-mentioned risks increases, as evident by the fact that incidence rate of stroke is doubled every 10 years after 45, and more than two-thirds of strokes are in late adulthood above the age 65. (Kelly-Hayes 2010)This can be attributed to blood vessels and the heart becoming more stiff, and hence not being able to cope with the fluctuating blood flow demands.

There are two main types of strokes: (i) Ischemic stroke, which is the most prominent type of stroke that occurs when a blood clot forms in the artery and blocks blood flow to the brain. This type of stroke can be further divided into two subtypes: thrombotic and embolic stroke. Thrombotic stroke occurs when a clot is formed in the artery suppling to the brain. Embolic stroke occurs when a clot forms in another region, for example the heart, and travels to the brain. (ii) Haemorrhagic stroke occurs due to rupture of weakened blood vessel in the brain, causing bleeding in the brain. This can be further divided into two subtypes: intracerebral and subarachnoid haemorrhage. Intracerebral haemorrhage is when a blood vessel is ruptured and bleeds into the brain tissue. Subarachnoid haemorrhage occurs when a blood vessel is ruptured on the surface around the brain, known as the subarachnoid space. Ischemic stroke and haemorrhagic accounts to 85% and 15% of all reported stroke cases reported, respectively (Lau, Lew et al. 2019).

To this day, the treatment for ischemic stroke is limited to thrombolysis and thrombectomy. Thrombolysis is the recanalization of the blood vessels by administrating recombinant tissue plasminogen activator (rtPA) and thrombectomy is the mechanical removal of the clot using a catheter, and these are the only clinically approved treatments to reduce the impact of

ischemic stroke (Roaldsen MB, 2021) However, less than 1% of patients benefit from thrombolysis due to strict inclusion criteria and the short therapeutic window (within 4.5 hours of stroke onset), and only a small proportion of patients with blood clots in large arteries can benefit from thrombectomy (Wardlaw JM, 2014). While thrombectomy is a highly effective treatment for ischaemic stroke, there are risks associated with the procedure including: exacerbation of the internal bleeding, damage to blood vessels and risks of further cases of recurrent strokes. This is evident in the figures published by Stroke (Kleindorfer DO, et al (2021)), in which the incidence of internal bleeding among patients one week post thrombectomy is reported to be one in 25 patients. In addition to this, the mortality rate associated with this procedure is one in 40. However, it is important to note that the benefits of thrombectomy far outweigh the risks and have greatly impacted and improved the chances of a positive outcome for patients with ischemic stroke. Albeit the risks combined with only a small fraction of vulnerable patients eligible for these treatments due to strict inclusion criteria, highlights the need for safer and more efficacious novel therapeutic strategies to be developed by optimising modern day technological advancements and ever prominent artificial intelligence tools.

Pre-clinical studies have been conducted to identify novel therapeutic approaches for the treatment of stroke. These studies have led to the development of new therapeutic strategies, such as the infusion of tissue plasminogen activator analogues- like alteplase, streptokinase, (Nikitin D, et al (2021)) fibrinolytic-bearing nanoparticles (Lippi, Mattiuzzi et al. 2013), and platelet glycoprotein IIb/IIIa antagonists, such as tirofiban and abciximab (Lippi, Mattiuzzi et al. 2013). These emerging therapeutic strategies in thrombolysis have the potential to enhance treatment efficacy and reduce the risk of systemic bleeding, expanding the number of eligible patients. However, it is essential to acknowledge that only a few of these novel techniques have been approved for clinical use, underscoring the importance of continued research to create therapeutic options that address multiple pathophysiology's in the post-acute phase of stroke, including inflammation, ecotoxicity, oxidative damage, and apoptosis (Topol, Moliterno et al. 2001, Hisham and Bayraktutan 2013, Shao and Bayraktutan 2014, M. Mathur 2017).

In this study, we aim to investigate the role of pericyte and endothelial crosstalk in hyperglycaemic conditions to better understand the mechanism underlying stroke recovery in diabetic patients. Previous studies have demonstrated the significant role of pericytes in promoting functional outcome following stroke induced damage by contributing to angiogenic and vasculogenic capabilities, as well as immune support (Liao, Luo et al. 2017) though cytokine secretion such as VEGS and TNF-α (Abdullah and Bayraktutan 2014). These findings

highlight the potential of pericytes as a primary candidate for cell-based therapy. By investigating the role of pericyte-endothelial cell crosstalk in hyperglycaemic conditions, our findings may contribute to the development of therapeutic strategies aimed at improving stroke outcomes in patients with diabetes.

1.1 The Neurovascular Unit

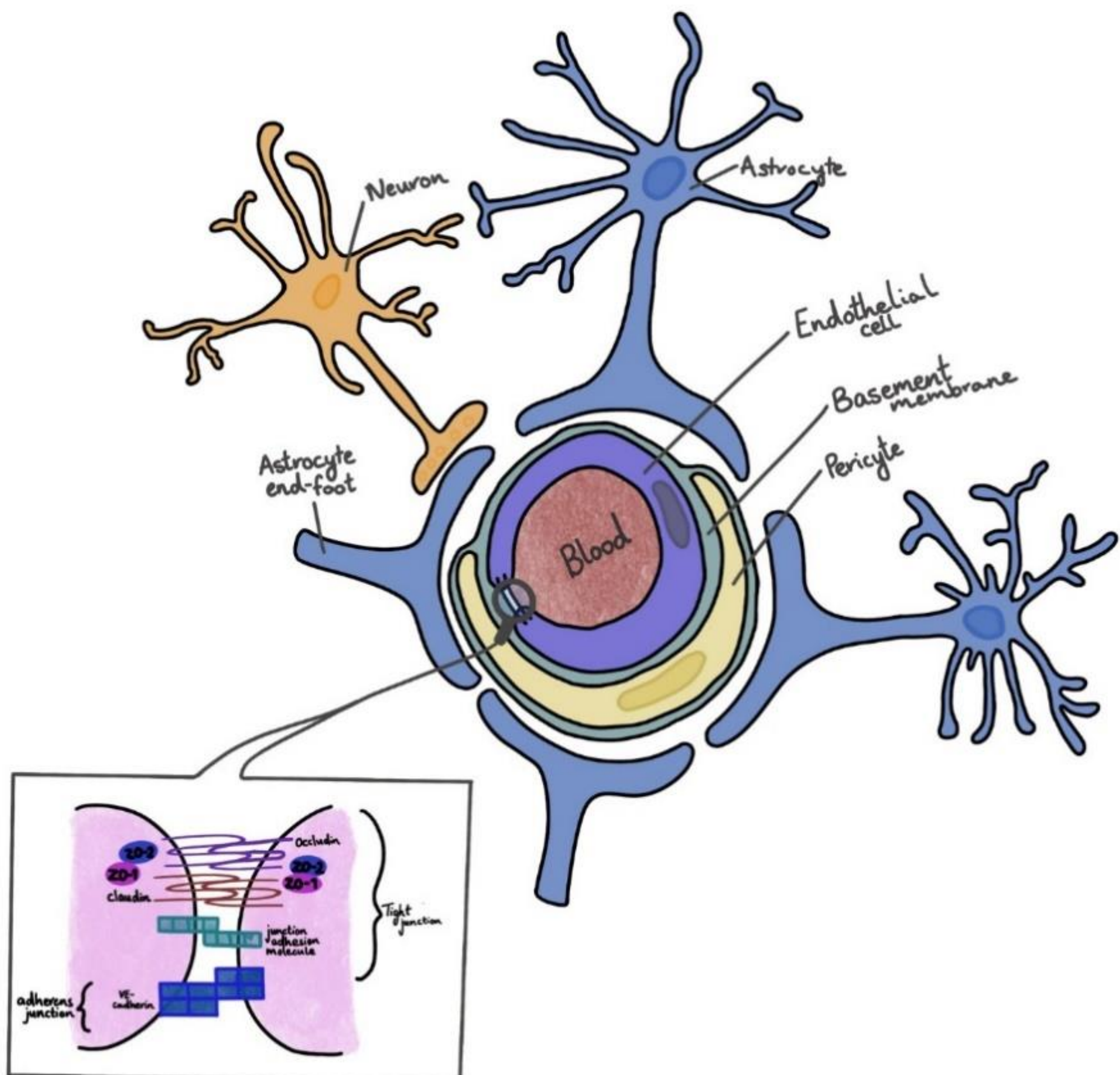


Figure 1 Schematic representation of the Neurovascular Unit (NVU) showing the complex interactions between neurons, astrocytes, pericytes, and endothelial cells*. Adopted from (Iadecola 2017).*

The concept of the neurovascular unit (NVU) has gained significant recognition in the fields of neuroscience and cardiovascular medicine due to its potential to offer a more comprehensive understanding of brain physiology and pathology. The NVU was formalized by the National Institute of Neurological Disorders and Stroke during the 2001 stroke progress review group meeting (Iadecola 2017). Since then, an increasingly considerable number of studies have been developing across a relatively short period of time, describing the crucial functional and structural role of the brain and the blood vessels. The NVU plays important role in maintaining brain function, particularly in the regulation of cerebral blood flow (CBF) and the formation of the blood-brain barrier (BBB)(Brown, Foster et al. 2019).

The neurovascular unit is a complex and interconnected system comprising endothelial cells (ECs), pericytes, astrocytes, glial cells and neurons that work together to maintain the structural integrity of blood vessels, regulate cerebral blood flow, and provide metabolic support to the nervous system. As described in a review article by Ladecola and Nedergaard (2007), any disruption in the function of ECs in the NVU can lead to pathological conditions such as stroke, neurodegeneration, and cerebral oedema (Iadecola 2017). Therefore, gaining a better understanding of the complex interplay between the various components of the NVU, including the endothelium, is crucial for developing effective treatment and prevention strategies for cerebrovascular diseases (Roh and Sohn 2018).

Its recognition as a relatively new but essential concept is attributed to its ability to provide a better understanding of the complex interactions between neurons, glia, and vascular cells in the brain (Daneman and Prat 2015). This understanding has opened up new avenues for the development of therapeutic approaches that target the NVU to treat stroke and other neurological disorders. Therefore, the concept of the NVU has provided a more comprehensive understanding of the complex interactions between different cell types in the brain, which has opened up new avenues for the development of therapeutic approaches for cerebrovascular diseases and other neurological disorders. As research in this area continues to progress, it is expected that the importance of the NVU in neuroscience and cardiovascular medicine will only continue to increase.

1.1.1 Endothelial cells

Endothelial cells (ECs) are a crucial component of the NVU as they serve various functions, including the regulation of cerebral blood flow in response to the changing demands of the brain. In addition, ECs play a significant role in the formation and maintenance of the blood-brain barrier (BBB), a highly selective membrane that separates circulating blood from the brain's extracellular matrix. (Daneman and Prat 2015). ECs are involved in the regulation of

immune responses in the brain through the expression of adhesion molecules and the secretion of cytokines. They also play a crucial role in maintaining the integrity of the blood-brain barrier by regulating the transport of nutrients, ions, and other molecules across the BBB (Daneman and Prat 2015). Dysfunction of the endothelium, which lines the blood vessels in the brain, can lead to increased permeability of the blood-brain barrier. This can result in the accumulation of toxic substances and immune cells in the brain, leading to neuroinflammation and neuronal damage. Endothelial dysfunction has been linked to a range of other pathological conditions, including hypertension, atherosclerosis, and diabetes (Hadi, Carr et al. 2005). It can also contribute to the development of ischemic stroke and haemorrhagic stroke. In addition, the endothelium plays a crucial role in regulating blood flow and maintaining vascular homeostasis in the brain. Therefore, understanding the underlying mechanisms of endothelial dysfunction is essential for developing effective treatments and prevention strategies for these conditions.

Previous experiments have demonstrated that EPCs in culture form two cell types: early EPCs (eEPCs) and late EPC (or outgrowth endothelial cells, OECs) (Kadir, Alwjwaj et al. 2022). They can be identified and differentiated using their specific cellular morphology, including cell surface proteins and possess distinct genomic profiles. Spindle-shaped eEPCs appear early in the culture (4-7days) and survive up to four weeks in culture. They show limited proliferative potential as they do not form a colony or vessels in vitro in Matrigel assay, yet they do have the tubulogenic capabilities, furthermore, they express hematopoietic markers and inflammatory markers (Rehman, Li et al. 2003). In contrast, cobblestone shaped OECs appear later in culture (2 to 3 weeks) and survive up to 12 weeks in culture, exhibiting strong proliferative, angiogenic and tubulogenic capacity (Abdulkadir, Alwjwaj et al. 2020) and thus are the focus of many clinical trials and show promising therapeutic potential in the post-ischemic restoration of neurovascular integrity.

The close communication between pericytes and endothelial cells is facilitated by the intercellular gap, (Hisham and Bayraktutan 2013) which is often smaller than 20 nm (Rouhl, van Oostenbrugge et al. 2008). This reciprocal communication is crucial for maintaining vascular homeostasis and regulating blood flow in the brain. Studies have shown that transforming growth factor-α (TFG-α) is one such cytokine that is secreted by both pericytes and endothelial cells and has a significant impact on cellular processes such as proliferation, migration, and differentiation (Abdullah and Bayraktutan 2014).

TGF-α is known to inhibit endothelial cell development by pericytes, which plays a crucial role in the regulation of vascular morphogenesis and stabilization (Hisham and Bayraktutan 2013, Abdullah and Bayraktutan 2014). This cross-regulatory mechanism between pericytes and

endothelial cells is essential for the proper formation and maintenance of the BBB and is critical for neurological functions.

Endothelial dysfunction is a critical indicator of BBB dysfunction, which is characterized by the leakage of blood and its constituents into the brain parenchyma (Bayraktutan 2019). This process can lead to vasogenic oedema and haemorrhagic transformation, which are the main causes of stroke-induced death within the first week of an ischemic stroke (Kadir, Alwjwaj et al. 2022). Even when endothelial dysfunction is not the primary insult, many of the secondary events associated with it impact the overall severity and outcome of a stroke (Kadir, Alwjwaj et al. 2022). Therefore, understanding the principal mediators of BBB damage and the molecular mechanisms involved in BBB disruption is of paramount importance for developing novel therapeutic approaches to treat ischemic stroke.

While EPCs have shown promise in clinical applications and the treatment of acute conditions such as ischemic stroke, their limited availability in bone marrow or systemic circulation poses a challenge. In vitro cell culture systems can address this limitation by allowing the controlled growth and expansion of isolated mononuclear cells to produce large quantities of EPCs for therapeutic purposes (Bayraktutan 2019). The importance of these in vitro systems is further highlighted by the critical role of the intricate crosstalk between pericyte and endothelial cells in regulating blood flow, maintaining BBB integrity, and supporting neurological functions. Co-culture studies in a physiologically relevant environment can help elucidate these complex interactions and identify new therapeutic interventions, ultimately leading to a better understanding of the underlying mechanisms involved.

1.1.2 Pericyte

Pericytes were first described by Eberth and Rouget in the late 19th century (Attwell, Mishra et al. 2016) as a distinct cell type associated with the capillary wall, with soma projections that wrapped around the underlying channel and were implanted in the basement membrane at both straight portions and branch sites of capillaries, and the term "pericyte" was coined by Zimmerman in the 1920s (Haddad-Tóvolli, Dragano et al. 2017); however, there is still disagreement on what exactly defines a pericyte due to the widespread usage of the aforementioned terminology in studies examining pericyte function, encompassing a wide range of cell morphologies, including those that were in transition from endothelial to vascular smooth muscle cells (Hill, Tong et al. 2015).

Pericytes are a unique type of cell found in the walls of small blood vessels. They possess various capabilities, including angiogenic and vasculogenic capabilities. In addition to these

capabilities, pericytes are also known to secrete a variety of cytokines that could impact their proliferative, angiogenic, and vasculogenic properties (Bergers and Song 2005). Following ischemic insult, pericytes are known to induce apoptotic and immune support responses, secreting growth factors such as VEGF and TNF-α (Abdullah and Bayraktutan 2014). These factors significantly promote functional outcome and regeneration after ischemic injury. Overall, pericytes play an essential role in maintaining the integrity and function of small blood vessels, making them a promising target for the treatment of ischemic diseases.

The significant role of pericytes in the recovery process after a stroke has been well-documented, underscoring their potential as a vital primary candidate for cell-based therapy. The extensive research on pericytes and their involvement in stroke recovery has shown promising results, making them an attractive option for developing novel treatments (Hisham and Bayraktutan 2013). However, while there is a growing body of evidence supporting the use of pericytes in cell-based therapy, further studies and clinical trials are still needed to establish their safety and efficacy as a therapeutic approach for stroke patients. It is therefore essential to continue conducting research in this area to fully understand the potential of pericytes in stroke recovery and to develop safe and effective therapies that can improve outcomes for stroke patients.

In the past 15 years, significant advances in technology and more sophisticated approaches for identifying and studying pericytes have accelerated research into pericyte function. This has led to a better understanding of the critical role that pericytes play in maintaining the health and function of the central nervous system, including their interactions with endothelial cells, regulation of blood flow, and support of neuronal functions (Cheng, Korte et al. 2018). These advancements have opened up new avenues for the development of therapeutic approaches targeting pericytes to treat various neurological disorders, making the study of pericytes a crucial area of research in the field of neuroscience and cardiovascular medicine.

1.1.2.1 Pericyte subtypes

There are different classifications of pericytes (PCs), however no single classification is universally accepted, and different studies have proposed different pericyte subtypes and functions based on different characteristics. The most prominent and widely accepted categorisation is the classification of pericytes into three subtypes based on their location along the capillary bed and the levels of (αSMA) expression (Hartmann, Underly et al. 2015, Martinez-Quinones, McCarthy et al. 2018), these subtypes are:

(i) PCs closer to the arteriole end that express higher levels of α-smooth muscle actin and are involved in regulating cerebral blood flow.

(ii) (ii) PCs in the middle of the capillary bed that express lower levels of αSMA and are critical for maintenance of BBB integrity and function (Nirwane and Yao 2022).

(iii) (iii) PCs at the venule end, expressing low levels of αSMA, with a critical role in regulating immune cell entry into the brain parenchyma (Jeske, Albo et al. 2020).

Other studies have used the presence or absence of Nestin to classify pericyte subtypes and their ability to differentiate into different cell types (Birbrair, Zhang et al. 2013). Finally, another study suggested the use of desmin (Geranmayeh, Rahbarghazi et al. 2019) as a marker for different pericyte subtypes into desmin positive and desmin negative pericytes, which are thought to be involved in regulation of blood flow and regulation of angiogenesis, respectively (Geranmayeh, Rahbarghazi et al. 2019). Due to the complexity of these issues and the limited resources available in this study, the investigation of hyperglycaemia's role in pericyte classification or the classification of pericytes based on desmin expression cannot be fully addressed (Geranmayeh, Rahbarghazi et al. 2019). However, it remains an important area of research to better understand the role of hyperglycaemia in the development of diabetes-related complications and to improve the classification of pericyte subtypes.

1.1.2.2 Function of pericytes

Pericytes perform a vital role in the formation, stabilisation, and maintenance of the blood brain barrier, regulate blood flow, regulate vessel integrity (Gerhardt and Semb 2008, Cooke, LeBleu et al. 2012) and aid the recruitment, differentiation and maintenance of endothelial cells. They also have an impact on wound healing and immune response (Bodnar, Satish et al. 2016).

Pericytes have a vital role in formation of new vessels known as angiogenesis, which is essential for tissue growth and repair. Studies by Peter C et al (Stapor, Sweat et al. 2014) have concluded that during angiogenesis, pericyte and endothelial cell crosstalk is essential for both stabilisation and promotion of capillary sprouting. This was attributed to combination of several mechanisms that worked simultaneously to regulate pericyte recruitment, improve endothelial cell viability and pericyte differentiation by platelet-derived growth factor (PDGF-BB/PDGF receptor-β), Angiopoietin 1/tyrosine kinase (Ang1)/Tie2 paracrine communication pathway (Thalgott, Dos-Santos-Luis et al. 2015). Furthermore, in vitro studies have illustrated that loss of pericytes exacerbated vascular dysfunction by reducing brain microcirculation, loss of blood flow in response to brain activation and accumulation of neurotoxic molecules, and therefore BBB breakdown have concluded that during angiogenesis, pericyte and endothelial

cell crosstalk is essential for both stabilisation and promotion of capillary sprouting (Nakisli, Lagares et al. 2023).

Pericytes have shown to be crucial for the neuronal function and essential for healthy development and maintenance of the cerebral micro-vasculature (Bennett and Kim 2021). Due to their proximity to endothelial cells and their ability to possess contractile properties (Thalgott, Dos-Santos-Luis et al. 2015), they can regulate the cerebral blood flow by modulating the blood vessels diameter and communicate with endothelial cells, meaning they are able to act as chemical sensors which enable communication between the two groups. Studies have illustrated that only a minor change in the diameter of the blood vessel can result in a significant change in blood flow rate. For example, a small increase of capillary size by 4-μm led to doubling of red blood cell velocity (Dessalles, Babataheri et al. 2021). The specific mechanism for pericyte constriction and how it regulates blood flow remains a highly debated subject.

Recent studies have shown that pericytes are able to actively relax or contract in response to changes in neuronal activity, which can alter cerebral blood flow (CBF) (Brown, Foster et al. 2019). Studies have demonstrated that only a subset of pericytes, known as ensheathing pericytes located at the arteriole-capillary transition, express higher concentrations of α-SMA compared to pericytes located in the middle of the capillary bed or at the end of the capillary by the venule transition (Brown, Foster et al. 2019). This contractile property has been attributed to the presence of contractile proteins α-SMA and myosin in pericytes (Alarcon-Martinez, Yilmaz-Ozcan et al. 2018). Although only a subset of pericytes, the unsheathing pericytes, present at the arteriole capillary transition, express higher concentration of α-SMA than the pericytes at the middle of the capillary bed or the end of capillary by the venule transition.

While this evidence supports the role of pericytes in regulating CBF, there is ongoing controversy surrounding the exact mechanism of pericyte contraction and its contribution to blood flow regulation. There are many experiments that have investigated whether pericytes have an innate ability to influence blood vessel constriction and dilation (Bergers and Song 2005). A hypothesis is that pericyte contraction increases vessel wall stiffness and hence modulate blood vessel diameter to adapt to increased blood pressure and alteration in the environmental conditions (Dessalles, Babataheri et al. 2021). Another hypothesis is that pericytes do not control contractility themselves, but that it is controlled by arteriolar vascular smooth muscle cells. This can be attributed for insufficient understanding of different pericyte subtypes' function, and inadequate nomenclature for pericytes. For instance, Attwell and colleagues (Attwell, Mishra et al. 2016) stated that various cell types have similar morphology

and secretory molecules in comparison to pericytes, and the incorrect usage of nomenclature between the studies have given rise to this debate. It is important to address the insufficient understanding of different pericyte subtypes' functions, and inadequate nomenclature for pericytes, which can lead to the misinterpretation of results and inconsistent findings across studies. Therefore, the development of a universal pericyte classification system could facilitate the standardization of research and facilitate comparisons between studies.

Further research into pericyte contraction and their contribution to blood flow regulation is necessary to develop new therapeutic targets and improve outcomes for patients with neurological conditions.

1.2 Hyperglycaemia

Epidemiological studies have provided compelling evidence that diabetes is a significant modifiable risk factor for both ischemic and haemorrhagic stroke. This chronic metabolic disorder is characterized by abnormally high levels of glucose in the blood, which can result from a deficiency in insulin secretion or resistance to insulin action (Yan, Zhou et al. 2020). The number of people living with diabetes has increased significantly over the past decade, with an estimated 463 million people living with diabetes worldwide in 2019; Unfortunately, this number is projected to rise to 700 million by 2045 (Saeedi, Petersohn et al. 2019), the projected increase in the number of people with diabetes is a significant challenge for both public health and the economy, highlighting the urgent need for effective prevention and management strategies.

This dramatic increase has primarily been attributed to an increase in the rate of obesity levels both in developing and developed countries (Chen, Ovbiagele et al. 2016). It is imperative to note that even after numerous studies and clinical trials that research in this field is still ongoing to better understand the relationship between these conditions and to develop effective novel treatment and prevention strategies. Type 2 diabetes mellitus (T2DM) is the most common subtype of diabetes, accounting for about 95% of all cases (Association 2011). It is a progressive disease that may not present with any symptoms in the early stages, which makes it difficult to diagnose. However, if left uncontrolled, the long-term high blood glucose levels in T2DM patients can lead to various vascular and neural complications, including diabetic retinopathy, nephropathy, peripheral neuropathy, and encephalopathy (Huang, Shi et al. 2020).

It is important to note that diabetes is not just a disease in itself, but it is also a risk factor for other chronic diseases, including cardiovascular diseases. People with diabetes have a higher

risk of developing stroke, which is a leading cause of death and disability worldwide (Allen and Bayraktutan 2009). Moreover, diabetes also poses a significant economic burden on individuals and healthcare systems. The cost of healthcare for individuals with diabetes is estimated to be 3.6 times that of the rest of the population, which can lead to financial strain on individuals and families (Saeedi, Petersohn et al. 2019).Recent research has highlighted the significance of pericytes in the progression of microvascular complications associated with diabetes, including conditions like diabetic retinopathy, nephropathy, and peripheral artery disease (Saini, Kochar et al. 2021) . However, the scientific community remains divided on the exact role that pericytes play in the development of these diseases, and further investigation is needed to fully understand their contribution.

The occurrence of hyperglycaemia has the potential to induce pericyte loss because of the downregulation of platelet-derived growth factor receptor-β (PDGFR-β) signal transduction both in vivo and in vitro (Giri, Dey et al. 2018). Additionally, studies have found that in diabetic models induced by a high-fat diet, an increase in thrombin due to diabetic conditions can cause dysfunction in the blood-brain barrier, leading to complications in the central nervous system (CNS) related to diabetes. Furthermore, in STZ-induced diabetic mice, pericytes have been observed to retract from brain endothelial cells, resulting in remodelling and retraction of both pericytes and astrocytes (Yan, Zhou et al. 2020).

Understanding the underlying mechanisms of hyperglycaemia and its effects on the body can lead to the development of better treatments and preventative measures for these diseases. It can also aid in identifying individuals who are at risk for developing hyperglycaemia-related complications, allowing for early intervention and management.

Glucose is a vital energy source for cells, which is transported into the cytoplasm through various glucose transporters. Once inside the cell, glucose is broken down into lactate through a series of enzymatic reactions, which produces ATP, water, and carbon dioxide (Wu and Bai 2023). The generation of ATP from glucose metabolism is crucial for cellular viability, as numerous proteins, ion channels, receptors, and kinases require it to function appropriately in cells source (Lin and Hardie 2018). Moreover, protein changes such as acetyl-CoA-induced acetylation and lactate-mediated lactation are likewise driven by the by-products generated by glucose catabolism, as shown by multiple sources (Wu and Bai 2023). Therefore, the efficient metabolism of glucose is essential for cell survival and function.

The transport of glucose, other nutrients, and oxygen to tissues is largely dependent on the availability and functionality of the vasculature, which allows for their efficient delivery to cells through blood vessels (Lin and Hardie 2018). Oxygen can diffuse freely through vascular walls

down a concentration gradient, while glucose and other nutrients are transported across the vascular endothelium by specific transporters (Daneman and Prat 2015). And as such, a denser vascular network is a hallmark of tissues with greater metabolic demand.

The three main pathways for glucose catabolism include glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle, which have been studied for over a century (Hui, Ghergurovich et al. 2017). Glycolysis and the TCA cycle produce the majority of ATP, providing the necessary energy for cellular function and protein activity. These pathways also produce NADH, which plays a critical role in regulating cellular redox processes (Wu and Bai 2023). Thus, understanding endothelial glucose metabolism is crucial for maintaining cellular homeostasis and proper function.

1.3 Communication Mechanism between PC and ECs

Pericytes play a critical role in vascular remodelling by surrounding the abluminal surface of endothelial cells with their cytoplasmic processes. This is achieved through a variety of mechanisms including direct cell-to-cell contact via peg socket, tight, and adherence junctions, as well as paracrine signalling mediated by key modulatory pathways such as PDGF- PDGFR-β, VEGF, TNF-α , and Ang1/tie2 (Sweeney, Ayyadurai et al. 2016).

The abundance of pericytes relative to endothelial cells varies depending on the size and location of the vessel. Generally, there is a higher proportion of pericytes at the start of the venous capillary transition and post-capillary venules (Armulik, Genové et al. 2011). In the CNS, particularly the retina, the pericyte to endothelial cell ratio is 1:1, which underscores the importance of pericytes in neuronal tissue where there is a greater need for strict regulation of blood flow due to high metabolic activity (Sims 1986). In normal tissue, the ratio of pericytes to endothelial cells can vary from 1:1 to 1:10, whereas the skeletal musculature has the lowest ratio of pericytes: endothelial cells to a ratio of 1:10 (Geevarghese and Herman 2014). Collectively, these findings highlight the critical role of pericytes in various tissues, particularly in neuronal tissue where strict regulation of blood flow is essential due to its high metabolic activity.

Pericytes play a vital role in the maintenance of the function and integrity of blood vessels by regulating the microenvironment of the vasculature (Payne, Zhao et al. 2019). Pericyte ratio varies across different regions of the vasculature and hence the degree that pericytes can alter the microenvironment can differ from region to region. The density of pericytes has been found to directly influence the stability of blood vessels. An increase in pericyte density leads to increased stability of blood vessels, thereby promoting the proper functioning of the vascular

system. This can aid in maintaining the robustness of the BBB and prevent unwanted permeability. Conversely, a decrease in pericyte density can result in increased instability of blood vessels. This instability can lead to serious complications, including an increased risk of vessel rupture and BBB disruption (Wu and Bai 2023).

Pericytes and ECs communicate through various mechanisms, including paracrine signalling involving growth factors such as angiopoietin-1 and PDGF and their receptors, such as Tie-2 and PDGFR-β, as well as juxtacrine signalling mediated by molecules such as Jagged1-Notch3 (Scheppke, Murphy et al. 2012, Lippi, Mattiuzzi et al. 2013, Perrot, Herrera et al. 2020). These signalling pathways play crucial roles in regulating the development, stability, and angiogenic properties of pericytes (Gaengel, Genové et al. 2009), ultimately influencing their interactions with ECs and their impact on the microvascular environment. Understanding these signalling mechanisms is important in furthering our understanding of the role of pericytes in regulating blood flow and maintaining the blood-brain barrier, as well as in developing targeted therapies for diseases involving pericyte dysfunction.

Paracrine signalling is an important mechanism for maintaining blood vessel development and function. Growth factors such as angiopoietin-1 and Platelet-Derived Growth Factor (PDGF) are released by both pericytes and endothelial cells and bind to their respective receptors, including Tie-2 and PDGFR-β, on the surface of the other cell type. This process stimulates the growth, migration, and differentiation of pericytes and endothelial cells, contributing to the development and maintenance of blood vessels (Raica and Cimpean 2010).

Juxtacrine signalling, on the other hand, involves direct cell-to-cell interaction between pericytes and endothelial cells. This interaction occurs when specific ligands, such as Jagged1, are present on the surface of one cell type and bind to their corresponding receptors, such as Notch3, on the surface of the other cell type (Siebel and Lendahl 2017). This mechanism regulates the differentiation of pericytes and endothelial cells and plays a critical role in the formation of new blood vessels. These signalling mechanisms are essential for maintaining the proper function and development of blood vessels in the body (Raica and Cimpean 2010).

Pericytes and endothelial cells work together through direct cell-to-cell contact and a range of paracrine signalling pathways to regulate vascular functions such as angiogenesis, vascular remodelling, and blood flow. These functions are vital for maintaining the integrity of blood vessels and ensuring proper tissue perfusion. Any imbalance in these signalling pathways can

lead to pathological conditions such as tumour angiogenesis, diabetic retinopathy, and atherosclerosis (Brown, Foster et al. 2019), which can ultimately result in tissue damage and organ dysfunction. Therefore, understanding the interactions between pericytes and endothelial cells and their associated signalling pathways is crucial for developing therapies to treat vascular-related diseases.

1.3.1 Basal lamina

The vascular basement membrane, also known as the basal lamina, is a thin layer composed of glycoproteins including laminin, collagen type IV, nidogens, and heparan sulphate proteoglycans. Located on the abluminal surface of the endothelium, it surrounds the endothelial cells and pericytes, facilitating their adhesion to the basement membrane through specialized receptors such as integrins (Hermann and ElAli 2012). The basal lamina is crucial for providing mechanical support, facilitating cell migration and attachment, and separating neighbouring cells. Its absence can hinder the ability of pericytes and endothelial cells to maintain adequate cell-cell contact, alter signalling between them, and disrupt blood flow. One notable example of the importance of the extracellular matrix in vascular function is the role of heparan sulphate proteoglycan agrin in glioblastoma vessels. Studies have shown that the absence of agrin leads to decreased levels of occludin-5, a tight junction protein critical for maintaining the integrity of the blood-brain barrier in brain endothelial cells (Barber and Lieth 1997). This disruption in tight junction formation can have significant consequences, including increased permeability of the blood-brain barrier and the potential for pathogenic cells to invade the brain tissue. Once in the brain, these cells can proliferate and form secondary brain tumours, known as brain metastases, which are often more difficult to treat than primary brain tumours (Singh, Saxena et al. 2022). Furthermore, the increased permeability of the BBB can also exacerbate neuroinflammatory conditions, as it allows pro-inflammatory substances from the blood to enter the brain.

In addition to its role as a mechanical anchor for cell binding, the basal lamina functions as a migratory pathway and an interface among neighbouring tissues. It can also potentially obstruct the passage of macromolecules. Integrins, which are a class of transmembrane receptors, including heterodimers comprising a and b subunits, play a role in cell attachment to the basal lamina (Barber and Lieth 1997). These findings highlight the crucial importance of the basal lamina in providing structural support to cells and tissues and its role in the interactions between pericytes and endothelial cells, which play critical roles in maintaining the integrity of blood vessels and regulating blood flow.

Advances in technology and research methods, such as high-resolution imaging techniques and genetic engineering, provide new opportunities to study the basal lamina's functions and mechanisms at the molecular and cellular levels (Xu, Nirwane et al. 2019). Furthermore, research on basal lamina may also contribute to the development of novel therapies and interventions for diseases related to its dysfunction.

1.3.2 Peg and socket

Peg and socket are specialised structures formed between pericyte and endothelial cells, characterised by protrusion or “pegs” extending from pericytes to invagination or “sockets” in the endothelial cells (Caruso, Fedele et al. 2009). They are also characterised by the presence of N-cadherin-dependent adherence junctions and connexin-43 (Cx43)-dependent gap junctions (Winkler, Bell et al. 2011), which are critical for adequate mechanical support, proper cell-to-cell communication, and signalling.

N-cadherin-dependent adherence junctions are protein complexes that mediate cell-cell adhesion between adjacent cells that possess critical roles in maintaining the structural integrity of blood vessels and regulating cell behaviour by mediating cell-cell adhesion. This is evident in studies by (Perrot, Herrera et al. 2020) Perrot and colleagues that clarify the role of Rac1/RhoA guanine nucleotide exchange factor that helped to facilitate the recruitment of Vascular endothelial (VE)-cadherin to EC junction and hence stabilise the endothelial barrier.

Conversely, Cx43-mediated gap junctions enable the exchange of various molecules include ions, secondary messengers like cyclic AMP (cAMP), metabolic products, and signalling molecules (Perrot, Herrera et al. 2020). Ions, such as sodium, potassium, and calcium, are essential for maintaining cellular homeostasis and driving the bioelectric phenomena that underlie muscle contraction and neuronal signalling. (Armulik, Abramsson et al. 2005). These gap junctions play a critical role in numerous biological processes such as EC-induced differentiation of mural cell progenitors. This differentiation is only possible with the help of Cx43-mediated gap junctions, ensuring metabolic cooperation between cells and allows for the synchronization of cellular responses to various stimuli. This process is essential for maintaining the integrity of blood vessels and regulating blood flow.

The importance of gap junctions in maintaining vascular tone is highlighted by their absence in certain pathologies. For example, connexin43-deficient mice have impaired vascular tone regulation and are more susceptible to hypertension (Armulik, Abramsson et al. 2005). Additionally, disruption of gap junctions between pericytes and endothelial cells has been linked to impaired contractility and increased permeability of the blood-brain barrier (Haefliger,

Nicod et al. 2004). In contrast, mechanical signals, such as contractile forces from pericytes to endothelial cells, are transmitted through adhesion plaques (Kruse, Lee et al. 2018). These plaques serve as a physical link between cells and allow for the transmission of forces that contribute to the overall tone of the vessel. While adhesion plaques have been shown to transmit mechanical signals between pericytes and endothelial cells, the precise mechanisms of this communication and its role in vascular tone regulation are still not well understood. Further research could help shed light on the signalling pathways involved and identify potential therapeutic targets for conditions such as hypertension and stroke.

Despite the known significance of these adhesion plaques in cell-cell communication and vascular regulation, the precise mechanisms underlying these processes remain elusive. How exactly do mechanical signals transmitted through adhesion plaques influence pericyte and endothelial cell behaviour? What specific signalling pathways are involved? How does this interplay contribute to the regulation of vascular tone?

Answering these questions could have significant implications for understanding and treating vascular diseases. Conditions like hypertension and stroke, which involve abnormalities in vascular tone and blood flow, could potentially be targeted through therapies that modulate the signalling pathways associated with adhesion plaques. As such, future research in this area is not only interesting from a basic science perspective, but also holds promise for clinical applications.

1.3.3 Tight junction, Adherence junction

Tight junctions (TJs) and adherens junctions (AJs) are integral components of the cellular structures that constitute the BBB and other endothelial barriers throughout the body. These junctions are responsible for maintaining the selective permeability of these barriers, hence playing a crucial role in bodily homeostasis and protection (2002, Wu, Chen et al. 2023). TJs are specialized structures that form between adjacent endothelial cells and contribute to the formation of a barrier that seals inter-endothelial cell gaps, preventing the passage of ions and molecules between cells, thus lowering paracellular permeability and creating a high endothelial electrical resistance (Chen, Luo et al. 2018). Similarly, AJs are primarily involved in mediating cell-cell adhesion. They are crucial in maintaining the structural integrity of tissues. AJs are formed by the calcium-dependent homophilic interaction of cadherins (like VE-cadherin in endothelial cells) from adjacent cells, which are connected to the actin cytoskeleton via catenins (Chen, Luo et al. 2018). The proper function of these structures is

essential for the maintenance and stabilization of the cells, preventing the passage of harmful substances from the blood and into the surrounding tissue, and allowing for the diffusion of ions and small water-soluble solutes through the paracellular pathway.

The stability of TJs and AJs is ensured through the anchoring of junction proteins to the actin cytoskeleton, a key structural component within cells such as zonula occludens (ZO), alpha- and beta-catenins (Dejana and Giampietro 2012). This anchoring is facilitated by various accessory proteins, creating a complex network of interactions that maintain the integrity and functionality of these junctions, particularly the ZO family of proteins (ZO-1, ZO-2, and ZO-3). The ZO proteins serve as scaffolding molecules that link the transmembrane proteins of the TJs to the actin cytoskeleton (Venkat, Chopp et al.); This arrangement allows the actin-myosin cytoskeleton to be distributed as short filaments and diffuse monomers between the ECs, providing structural support and playing roles in signal transduction pathways.

During hypoxic stress, the actin filaments within endothelial cells polymerize into linear stress fibres, a process that leads to the contraction of the actin-myosin cytoskeleton through phosphorylation of the myosin light chain. This results in increased cytoskeletal tension, which weakens the seals formed by TJs, thereby increasing the permeability of the BBB (Harris and Nelson 2010). This process was observed in a study where, after a reperfusion event, the TJ proteins were not evenly distributed in vascular endothelial cells and their staining intensity had reduced, indicating a disruption in TJ structure and function. This disruption and subsequent increase in BBB permeability were more pronounced in the HG group than the NG group, suggesting that conditions like hyperglycaemia could exacerbate the effects of hypoxic stress on the BBB. (Komarova, Kruse et al. 2017). However, TJ proteins re-established uniformity across the blood vessels and elevated staining intensity at 3- and 7-days following reperfusion, with the recovery process being significantly less rapid in HG than in NG groups (Komarova, Kruse et al. 2017). Therefore, it is important to maintain the proper function of TJs and AJs to ensure the integrity and stability of the blood-tissue barrier, particularly during hypoxic stress.

Studies have shown that the BBB permeability can be altered by various stimuli, including oxidative stress, inflammation, and hypoxia Therefore, understanding the regulation of TJs and AJs and their role in maintaining endothelial barrier integrity is crucial for developing.

By comprehending the molecular processes that control the regulation of TJ and AJ, and how these mechanisms are affected by external factors like as oxidative stress, inflammation, and hypoxia, researchers could identify novel targets for intervention (Małkiewicz, Szarmach et al. 2019). This may result in the emergence of medications or alternative treatment methods

aimed at maintaining or repairing the integrity of the BBB, thereby protecting the brain from harmful substances in the bloodstream and promoting neurological health.

1.3.4 Paracrine communications:

1.3.4.1 Platelet-derived growth factor (PDGF)

Pericytes are highly sensitive to the PDGF-BB:PDGFRβ signalling pathway, as even a partial deficiency of this pathway has been shown to result in overwhelming BBB leakage and cognitive impairment (Bell, Winkler et al. 2010).Studies have shown that PDGF-BB is necessary for proper BBB development, as mice deficient in PDGF-BB have shown a decrease in pericyte coverage and subsequently increased microaneurysms development (Sagare, Bell et al. 2013). In addition, studies on PDGFR-β deficient mice have demonstrated that pericytes are required for the maintenance of BBB integrity, as the loss of pericytes results in BBB disruption and the formation of microhaemorrhages (Ramsauer 2006).

The negatively charged heparin sulphate proteoglycans secreted by endothelial cells bond electrostatically with the positively charged C-terminal of PDGF-BB, causing the latter to be held in place (Winkler, Bell et al. 2011). For pericyte recruitment, attachment, and migration to occur, there must be a sufficient concentration gradient of PDGF-BB (Hosaka, Yang et al. 2013). The binding of PDGF-BB to PDGFR-β triggers many signalling pathways, including extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-Akt. The ERK pathway has been shown to be involved in pericyte proliferation and differentiation, while the PI3K-Akt pathway is involved in regulating pericyte survival and metabolism (Hosaka, Yang et al. 2013, Johnson, Stanfield et al. 2019).

The level of PDGF-BB- PDGFR-β signalling, which has been proven to improve pericyte survival, is inversely proportional to the total number of pericytes in the neural tube (Winkler, Bell et al. 2011, Jansson, Scotter et al. 2016). These pathways involved in pericyte recruitment, proliferation, differentiation, metabolism, and survival ultimately lead to changes in gene expression and cellular responses that are vital for regulating microvascular function (Jansson, Scotter et al. 2016). Notably, PDGF-BB: PDGFR-β signalling plays a crucial role in the formation and maintenance of the blood-brain barrier, and targeting this pathway may have therapeutic potential in diabetic retinopathy and other microvascular disorders.

The development of the vascular system and the BBB in cerebral vessels relies on PDGFs signalling, with PDGF-BB secreted by endothelial cells and PDGFR abundantly expressed in pericytes, vSMCs, and vascular-associated fibroblast-like cells in the vasculature (Nakisli, Lagares et al. 2023). Sequestered in the basement membrane from endothelial cells, PDGF-

BB forms a concentration gradient that promotes mural cells to blood vessels (Ido, McHowat et al. 1994), with pericytes surrounding capillaries subsequently stabilizing them and resulting in the development of the BBB. PDGF-BB and PDGFR-β deficient mice exhibit various vascular abnormalities, including impaired BBB development, microaneurysm development, haemorrhages, and a lack of mural cell saturation, with animals having minor defects in PDGF-BB:PDGFR signalling demonstrating BBB leakage, pericyte insufficiency, and cognitive impairment (Xia, Inoguchi et al. 1994). Pericytes rely heavily on PDGFR-β signalling for their survival, and they are substantially more vulnerable to PDGFR-β depletion compared to SMCs (Xia, Inoguchi et al. 1994).

In PDGF-BB or PDGFR-β knockout embryos, where PDGF-BB signalling is disrupted, the expression of vascular endothelial growth factor A (VEGF-A) is upregulated, leading to the dilation of micro-vessels and subsequent oedema development characterized by the accumulation of excess fluid in the tissues due to increased vascular permeability (Hellström, Gerhardt et al. 2001). VEGF-A binds to and activates the receptor tyrosine kinase VEGFR2 on endothelial cells, triggering a cascade of signalling events that promote vascular expansion and endothelial cell proliferation, resulting in the formation of new blood vessels and an increase in vascular permeability, allowing for the leakage of fluid and proteins into the surrounding tissue. The disrupted PDGF-BB signalling in PDGFR-β knockout embryos exacerbates this process, leading to the formation of oedema and other vascular abnormalities (Hirschi, Rohovsky et al. 1998).

Overall, the PDGF-BB: PDGFR-β signalling pathway is essential for proper BBB development and maintenance, as well as pericyte function. Disruptions in this pathway can lead to BBB leakage, cognitive impairment, and other neurological disorders (Armulik, Genové et al. 2010). Thus, further understanding of this pathway and its role in pericyte-endothelial cell interactions may provide valuable insights for the development of novel therapies for BBB-related diseases.

1.3.4.2 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF), as the name suggests, is a prominent angiogenic growth factor, crucial in the formation and maintenance of blood vessels (Shibuya 2011). They are also important for the survival and function of both pericytes and endothelial cells, dysregulation of which did impact their blood brain barrier formation capabilities and has led to BBB disruption after stroke in mice (Bai, Zhu et al. 2015). Following an ischemic insult, VEGF levels increase, which subsequently leads to the formation of oedema (Silvia, Peck-Lin et al. 2011). Furthermore, VEGF upregulation in astrocytes increased endothelial permeability,

an effect that was significantly nullified by treating the cells with VEGF inhibitors(Deng, Zhou et al. 2020).

Finally, loss of PDGF- PDGFR-β in embryos lead to various changes in the vascular system, including an increase in the number of blood vessels (vascular hyperplasia), widening of small blood vessels (micro-vessel dilation), and an increase in the expression of VEGF-A (Eilken, Diéguez-Hurtado et al. 2017).

After stroke, pericytes can contribute to BBB breakdown in mice by VEGF which directly stimulates endothelial cell proliferation and migration, while also playing a crucial role in angiogenesis processes (Hu, Zheng et al. 2022). However, in the acute phase of stroke, the increased VEGF levels can lead to BBB leakage, cerebral haemorrhage, and infarction volume (Hu, Zheng et al. 2022), despite its positive effects when delivered before or at a later time point following stroke. Hypoxia, a condition that occurs after stroke, is one of the main factors that upregulate VEGF expression and leads to BBB breakdown and brain oedema Upregulation of VEGF expression in astrocytes, as noted in a previous study, can result in increased endothelial permeability. This is because VEGF can modify the properties of endothelial cells, including those that make up the BBB, leading to changes in the tightness of the junctions between these cells. (Deng, Zhou et al. 2020). VEGF has been shown to have beneficial effects on post-ischemic BBB integrity and angiogenesis. In another study, it was shown that delivering VEGF over a span of 21 days following middle cerebral artery obstruction (a model for ischemic stroke) led to improvements in BBB integrity and an increase in the density of capillaries in the brain at 10 days post-injury (Zechariah, ElAli et al. 2013). This suggests that VEGF may have a protective effect on the BBB and promote vascular regeneration in the context of cerebral ischemia. Additionally, the metabolic penumbra, a region of tissue in the brain where protein production has been diminished but ATP synthesis is maintained, was preserved in ischemic regions that demonstrated improved angiogenesis as evident by increased cerebral blood flow (Zechariah, ElAli et al. 2013).

Moreover, VEGF was found to boost N-cadherin expression in cerebral micro-vessels. This increase in N-cadherin expression, in turn, increased pericyte surrounding of brain endothelial cells (Zechariah, ElAli et al. 2013). This increase in pericyte coverage is crucial for maintaining the BBB, as pericytes play a crucial role in regulating BBB permeability. These findings suggest that VEGF may be a promising therapeutic target for enhancing angiogenesis and BBB integrity following ischemic stroke.

1.3.4.3 Glucose transporter-s (GLUTs)

Polysaccharides such as starch, maltose, and cellulose are an important source of energy for animals, including humans (Chen, Cheung et al. 2015). These complex carbohydrates contain sugar, which is essential for various metabolic processes in the body. When consumed, digestive enzymes break down the polysaccharides into simpler sugars, such as monosaccharides, which can then be absorbed by cells and tissues (Chen, Cheung et al. 2015).

Of all the monosaccharides, glucose is one of the most crucial since it is used as the primary energy source by nearly all cells and tissues. However, glucose cannot cross the hydrophobic cell membrane without the assistance of specialised glucose transporters (Custódio, Paulsen et al. 2021).

GLUT1 and GLUT3 are two isoforms of glucose transporters expressed in the brain. The blood-brain barrier expresses high levels of GLUT1 while GLUT3 is the predominant isoform in neuronal cells (Custódio, Paulsen et al. 2021). Overall, the differential expression of GLUT1 and GLUT3 in the blood-brain barrier and neuronal cells plays a crucial role in maintaining the brain's energy balance and function.

Knockdown experiments have demonstrated that GLUT1 and GLUT3 are crucial for endothelial transmembrane glucose transport, with a reduction of 35% and 28%, respectively, in glucose uptake when either transporter is knocked out (Tumova, Kerimi et al. 2016). Furthermore, when both transporters are knocked down, glucose uptake is decreased by 60%. Surprisingly, GLUT6 knockdown enhances endothelial glucose uptake by 120%. While previous research has mainly focused on GLUT1, recent findings reveal that GLUT3 is also widely expressed in human endothelial cells and plays a significant role in glucose uptake, similar to GLUT1 (Tumova, Kerimi et al. 2016). These results suggest that targeting multiple GLUTs may be a promising approach for modulating endothelial glucose uptake in various pathologies.

The study conducted by Tumova et al. (Tumova, Kerimi et al. 2016), highlighted the importance of pericytes in maintaining the blood-brain barrier. They found that pericytes utilize lactate obtained from the endothelium as a source of energy, and depletion of endothelial GLUT1 in the brain and retina blood vessels could lead to pericyte damage, thereby compromising the integrity of the blood-brain barrier and causing abnormal permeability. Moreover, the study showed that administering oral lactate could reverse the adverse effects

(Lee, Xu et al. 2022), underscoring the crucial role of lactate metabolism in sustaining the blood-brain barrier.

1.3.4.4 Angiopoietin/ Tyrosine Kinase

Two key molecules in controlling blood vessel development and maintenance are tyrosine kinase (TK) and angiopoietin (Shibuya 2011).The angiopoietins are a group of growth factors that play a critical role in the development and function of blood vessels (Thurston 2002). These factors bind to the Tie2 receptor, which is found on the surface of both endothelial cells and pericytes. Angiopoietins are produced by a variety of cell types, including endothelial cells, pericytes, and smooth muscle cells (Barton, Dalton et al. 2014). While Angiopoietin-2 promotes vascular destabilization and inhibits endothelial cell survival, Angiopoietin-1 encourages endothelial cell proliferation and survival, as well as the development of new blood vessels (Yu and Ye 2020).

The family of enzymes known as TKs is also crucial in controlling cell proliferation and differentiation. These enzymes are involved in transmitting signals from growth factor receptors such as the VEGF receptor (Barton, Dalton et al. 2014). TKs mediate intracellular protein tyrosine phosphorylation, which initiates signalling pathways that regulate cell proliferation and differentiation. In the development of new blood vessels (Lemmon and Schlessinger 2010), TKs play a critical role in transmitting signals from growth factor receptors on the surface of endothelial cells to promote their survival and proliferation.

Pericytes express functional Tie2 receptors in both mice and humans (Cai, Kehoe et al. 2008). Knockdown of Tie2 in pericytes promotes migration. Studies on pericyte spheroids (Chang, Andrejecsk et al. 2013), both in vitro and in vivo, demonstrate that Tie2 regulates sprouting angiogenesis. Downstream signalling of Tie2 in pericytes involves calpain, Akt, and FOXO3A. In mice, deletion of Tie2 driven by the Ng2-Cre leads to transient suppression of postnatal retinal angiogenesis. However, Tie2 deletion in pericytes has a pro-angiogenic effect that promotes cancer development (Teichert, Milde et al. 2017). These findings contribute to our understanding of the Angiopoietin/Tie signalling pathway in the vascular system and suggest a reciprocal EC-pericyte model of Tie2 signalling.

The interaction between endothelial cells (EC) and pericytes is regulated by Ang/Tie signalling, which plays a crucial role in vascular development and function (Daly, Wong et al. 2004). However, our understanding of the underlying chemical mechanisms is limited. Pericyte-expressed Ang1 activates EC-expressed Tie2 to help maintain the EC quiescent phenotype (Teichert, Milde et al. 2017). The Tie2 receptor, upon phosphorylation by Ang1, initiates

downstream signalling pathways that play crucial roles in regulating cellular survival, proliferation, migration, and anti-inflammatory responses. In addition, AKT-dependent FOXO1 regulation contributes to vessel stabilization and survival (Zhou, Guo et al. 2022). The second angiopoietin ligand, Ang2, is a partial receptor agonist of Tie2 that suppresses Tie2 signalling in the presence of Ang1 but slightly stimulates Tie2 in its absence (Teichert, Milde et al. 2017). As an autocrine regulator of Ang/Tie2 signalling, Ang2 is generated, stored, and released by EC in response to stimulation. The loss of pericytes and subsequent vascular instability in the mouse retina after treatment with recombinant or transgenic Ang2 has been observed (Yuan, Khankin et al. 2009). Overall, the Ang/Tie2 signalling pathway plays a crucial role in the EC-pericyte interaction and contributes to vascular development and maintenance.

1.4 Neuroinflammation and pericytes:

Neuroinflammation, also known as central nervous system inflammation, refers to the local and systemic immune response, and is the central common pathology of a variety of acute brain diseases, including stroke, traumatic brain injury, infection, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Amor, Puentes et al. 2010).

The inflammatory response is coordinated by interaction and activation of several types of immune cells, including astrocytes, and microglia, signalling molecules including nitric oxide, lipid compounds for instance prostaglandins, and a plethora of cytokines, including TNF-α, interleukin (IL)-1β, IL-8, LPS, and α-synuclein (Takata, Nakagawa et al. 2021). Recently, pericytes have been shown to be able to perform the immune cell’s function. Activation of pericytes can lead to phagocytosis and, can exacerbate the inflammatory response by expressing pro-inflammatory molecules and cytokines, and act as antigen presenting cells to facilitate the function of immune response. For instance, when exposed to cytokines such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), pericytes have been found to amplify the inflammatory response. This is primarily achieved through the production and release of matrix metalloprotease 9 (MMP9) (Yang, Hawkins et al. 2019), an enzyme known for its role in inflammation and tissue remodelling. Leading to degradation to TJ- associated proteins and degradation of these TJ-associated proteins by MMP9 results in increased permeability of the BBB, leading to a breakdown of this critical neuroprotective barrier (Hawkins, Lundeen et al. 2007).

1.4.1 Neuroinflammation

Neuroinflammation has a complex role in the brain, and its impact can be either positive or negative, depending on the extent and duration of the inflammatory response (Yang, Hawkins

et al. 2019). Activation of the neuroinflammatory cascade can induce a neuroprotective effect by reducing the extent of injury, clearing damaged cells, and promoting neural and tissue proliferation. For instance, low level of transient inflammation, that is common during infection, propagates the release of interluckin-1 (IL-1), which increases the "surveillance" by microglia and hence propagate the clearance of the infected area (Liu, Pan et al. 2017). Additionally, following a CNS traumatic injury, increase in expression of interluckin-4 (IL-4) has expressed beneficiary effects on rate of recovery by promoting axonal growth.

On the other hand, excessive and prolonged neuroinflammation has led to the maladaptive inflammatory response by amplification of the inflammatory cascades, leading to additional damage and/or death of cells (Matsumoto, Takata et al. 2014). These factors create an inhospitable environment for neural repair and also contribute to the development of secondary mechanisms of injury, such as oxidative stress and excitotoxicity that can extenuate the damage to the brain. For instance, prolonged exposure to low levels IL-1 and IL-4 after a traumatic injury, has reduced neuronal plasticity and exacerbated cognitive impairment (Liu, Pan et al. 2017). This reduction in neuronal plasticity can exacerbate cognitive impairment and hinder neural repair. Therefore, it is important to closely monitor and manage neuroinflammation after a brain injury to promote optimal neural repair and recovery.

Neuroinflammation is primarily initiated by microglia, astrocytes, and infiltrating leukocytes, although pericytes may also function as immune cells (Jansson, Rustenhoven et al. 2014). When exposed to cytokines such as interleukin-1 beta (IL-1b) and tumour necrosis factor alpha (TNF-α) (Herland, van der Meer et al. 2016). TNF-α and IL-1b also increase albumin clearance in MVEC/PC co-cultures, and cause dose-dependent relaxation of PCs (Kerkar, Williams et al. 2006). The relaxation of PCs is significantly inhibited by sPLA2-IIA and pLA2 inhibitors, but only partially blocked by COX-II inhibitors (Herland, van der Meer et al. 2016). Hence, targeting pericytes and their responses to inflammatory mediators and cytokines may offer a promising approach to prevent or reduce BBB disruption and subsequent neuroinflammatory response. More research is needed to understand the role of pericytes in neuroinflammation and BBB dysfunction and identify potential new treatments for better patient outcomes.

1.4.2 Tumor necrosis factor- alpha (TNF-α)

TNF-α is a proinflammatory cytokine that plays a crucial role for regulating various biological processes including immune response by activating and recruitment of other inflammatory mediators, inducing apoptosis by activating the intrinsic apoptotic pathway, regulating metabolic function implicated in obesity and other metabolic disorders, maintaining tissue

homeostasis and reducing tissue damage (Dohgu, Takata et al. 2019), compromising BBB integrity which can further exacerbate cell death.

Brain pericytes are involved in the generation of inflammation-related compounds and express a number of receptors for endogenous cytokines, pathogens, and pathogenic substances such TNF-, interleukin (IL)-1, IL-8, LPS, and –synuclein (Matsumoto, Takata et al. 2014).

TNF-α also been shown to play a critical role on the regulation and function of pericytes and endothelial cells and can induce the migration and proliferation of pericytes through the activation of TNF-α receptors (TNFRs) on the pericyte membrane. Moreover, they have been shown to be able to induce pericyte relaxation mediated by plasmin, effects of which was completely nullified by the inhibitory secretory phospholipase A2-IIA (sPLA2-IIA) (Matsumoto, Takata et al. 2014).

1.5 Blood-Brain Barrier

The blood-brain barrier is a complex structure composed of tightly linked endothelial cells of capillaries, enveloped by astrocyte end-feet and supported by pericytes within the basal lamina. It acts as a barrier between the circulating blood and the extracellular fluid of the nervous system, safeguarding the brain from harmful substances and maintaining a stable environment for proper neuronal function (Jia, Yang et al. 2023).

The BBB allows for the passage of small molecules through passive diffusion, which is a process where molecules naturally move from an area of high concentration to an area of low concentration without the aid of any external energy (Alahmari 2021). This means that small molecules can cross the BBB without the need of any specific transporters or channels. Additionally, the BBB plays an indispensable role in maintaining brain health by selectively controlling the transport of crucial elements from the bloodstream into the brain's neural tissue. These include essential nutrients, ions, organic anions, and vital macromolecules. (Devraj, Klinger et al. 2011). This selective transport is mediated by specific transporters and channels that are present on the endothelial cells of the BBB. For example, glucose is transported across the BBB through a specific glucose transporter, GLUT-1 (Wu and Bai 2023). Similarly, amino acids are transported across the BBB through specific amino acid transporters. These transporters and channels are selective in nature, meaning that they only allow the passage of specific molecules, thus ensuring the maintenance of a stable environment for the neural tissue. The BBB also plays an important role in maintaining the homeostasis of the brain, by regulating the influx and efflux of ions and molecules (Alahmari 2021), thus allowing for proper neural function.

Transcytosis refers to the process by which molecules and other substances are transported across a cellular barrier, such as the BBB and the blood-retinal barrier (BRB). This process is essential for the maintenance of balance of singular molecules, hormones and nutrients between different compartments within the body. There are three main mechanisms by which transcytosis occurs (Au - Bora, Au - Wang et al. 2022): (I) receptor-mediated transcytosis (RMT), which occurs when a receptor on the surface of the cell recognises a complimentary ligand and facilitates their transport across the cell barrier, (Au - Bora, Au - Wang et al. 2022) carrier-mediated transcytosis (CMT), which occurs when substances are transported across the cell barrier by diffusion through transmembrane transporters, and (iii) adsorptive-mediated transcytosis (AMT), which occurs when substances are transported across by binding the specific receptors on the surface of the barrier cells which are then transported across the cell membrane using vesicles (Pulgar 2019, Pawar, Vasdev et al. 2022). Of these, RMT is the most prominent type.

Clathrin mediated transcytosis, caveolae mediated transcytosis, and macropinocytotic transcytosis are types of mechanisms that mediate the internalisation of extracellular substances into the cell. Clathrin mediated transcytosis occurs when substances are internalised into the cell through clathrin-coated pits, which are formed at the plasma membrane (Pulgar 2019). Clathrin-coated pits then pinch off to form intracellular vesicles containing internalised substances. (Au - Bora, Au - Wang et al. 2022), Caveolae-mediated transcytosis occurs when substances are transported across the cell barrier by passing through small invaginations in the plasma membrane known as caveolae. Macropinocytotic mediated transcytosis occurs when the cell engulfs the extracellular matrix by forming an invagination in the plasma membrane, forming an intracellular vesicle (Pulgar 2019).

Despite significant progress, there are still gaps in our understanding of the multifaceted nature of diabetes and its associated diseases, necessitating ongoing research and innovative approaches. More work is needed to identify the interactions between insulin resistance, hyperglycaemia, oxidative stress, inflammation, and other factors in diabetic complications. Additionally, the emergence of new subtypes, such as type 3c diabetes (Ewald and Hardt 2013), presents challenges for diagnosis and management. Continued research efforts are essential for the development of targeted therapies and preventive strategies. Furthermore, as the prevalence of diabetes and its related complications continues to rise globally, the need for novel and effective treatments becomes more urgent. With new advancements in technology and medicine, it is important to stay up to date on the latest research to continue improving patient outcomes and reducing the burden of these conditions on both individuals and society as a whole.

1.5.1 Secretome and its significance in stroke therapies

The secretome, which includes all the proteins, growth factors, cytokines, and enzymes that cells actively produce (Gallina, Turinetto et al. 2015), is becoming an increasingly important notion in our understanding of BBB function. HBMECs take part in this secretory activity within the BBB, releasing an extensive array of signalling chemicals into the surrounding neurovascular milieu. Intricate communication between HBMECs and their surrounding cellular neighbours such as astrocytes, neurons, and pericytes is facilitated by this secretome. Not only do these secreted substances control BBB function, but they also affect neuronal function, inflammation, and repair.

The therapeutic potential of unravelling the BBB secretome's dynamics is enormous. By decoding the unique proteins and signalling molecules generated by HBMECs, researchers expect to find new targets for therapeutic intervention in ischemic stroke and other neurological diseases. These circulated chemical substances contribute in maintaining BBB integrity and, conversely, may increase barrier breakdown and neuroinflammation if they are not well controlled in pathological conditions like stroke.

Proteomic and genomic developments in recent years have given researchers unprecedented access to the BBB secretome and its complexities (Cattaneo, Banfi et al. 2021). Researchers are not only uncovering the underlying principles driving BBB function by characterising the intricate interaction between HBMECs and their released proteins but are also proposing possible treatment routes. The idea of developing precision medicines that can modulate BBB permeability and mitigate neuroinflammation, both of which are critical in the setting of ischemic stroke therapy, is tantalising.

Scientists are getting closer to realising the full therapeutic potential contained in the cellular signalling language as research at the interface of the BBB and secretome progresses. The continued exploration of the EC secretome not only deepens our understanding of neurovascular interactions but also holds the key to innovative, targeted therapies that could revolutionise the landscape of ischemic stroke treatment and pave the way for novel interventions in various neurological disorders.

Disruptions to the BBB, particularly in the acute phase following stroke, are known to increase the permeability of the barrier, allowing for the infiltration of harmful molecules and immune cells into the brain. This BBB breakdown is among the early stages that precede numerous neurological disorders such as inflammation, oedema, and neuronal damage. Furthermore, an enhancement of BBB permeability, or "opening" of the barrier (Daneman and Prat 2015)

has been linked to an increase in endothelial transcytosis, the mechanism by which molecules are transported across the barrier. This increase in transcytosis may be involved in the brain's response to injury and disease, as well as the progression of neurological disorders. Therefore, it is essential to comprehend the mechanisms underlying BBB disruption and permeability in order to devise new treatments for stroke and other neurological disorders.

1.6 Aims

The main aims are:

-To examine pericyte and endothelial cells function and phenotype in normal and hyperglycaemic conditions.

-To elucidate the role of hyperglycaemia in the pericyte and endothelial cell crosstalk mechanisms.

-To assess the impact of hyperglycaemia on the integrity and function of BBB.

The following steps are taken to achieve the aims:

- To attain and analyse pericyte proteome/secretome for the three groups after 3 days: normal glucose (NG; 5.5mM D-glucose), high glucose (HG; 25mM D-glucose) and control (D-mannitol; 5.5mM glucose + 19.5mM mannitol).
- To assess the proteome profile expression of ECs subjected to normal and hyperglycaemic conditions using a human angiogenesis array kit and a cytokine array kit.
- To evaluate the impact of hyperglycaemia on barrier formation, and integrity of the blood-brain barrier.
- To integrate the results obtained from our experiments to draw conclusions about the impact of hyperglycaemia on pericytes and endothelial cell structure and function at the blood-brain barrier.

2 Methodology

2.1 Cell culture:

Human brain microvascular endothelial cells (HBMEC, Neuromics, USA),human pericytes (HP, Neuromics, USA), and human astrocytes (HBMEC, Neuromics, USA),from passages 4-8 were cultured in their respective complete media, supplemented with 100 units/mL of penicillin and 100 µg/mL of streptomycin, their respected growth factors and FBS, under optimal conditions (37°C, 5% CO2, 70% N2 and 25% O2) until they reached ~90% confluency. The cells were maintained at optimal culture conditions to ensure that they maintained their characteristic phenotype and function.

The procedure for passaging, splitting, and seeding cells was conducted as follows:

Cells were initially thawed and seeded in a T-75 flask. The following day, the media was changed to remove the Dimethyl sulfoxide (DMSO), and the cells were allowed to grow until they reached almost full confluence. The cells were then washed with phosphate buffered saline (PBS) to remove any dead cells and incubated with 3.5mL of 0.25% trypsin-EDTA solution for 5 minutes. After confirming that the cells had detached using a light microscope (Leica DFC3000 G, Wetzlar, Germany), the trypsin was neutralized with x1.5 of the respective media. The cell suspension was collected in a universal tube and centrifuged at 250 *g for 5 minutes. The supernatant was carefully aspirated, taking care not to disturb the cell pellet. The pellet was then utilized for seeding or freezing. To freeze the cells, the pellet was thoroughly mixed in 1mL of the respective freezing medium and aliquoted into a 1mL cryogenic vial. The vial was placed in Mr. Frosty and then into the -80°C freezer. The following day, the vial was transferred into liquid nitrogen for safe long-term storage.

2.2 Treatment:

HBMEC were grown in three T-75 flasks until fully confluent. Cells were washed with PBS twice and incubated for 72 hours under normoxic conditions and were exposed to normal glucose (NG; 5.5 mM D-glucose), hyperglycaemic (HG, 25 mM D-glucose) or D-mannitol control (5.5 mM D-glucose + 19.5 mM D-mannitol).In order to confirm that the observed changes in response to HG were not simply a result of an increase in osmolality, additional experiments were performed using D-mannitol as an osmotic control. D-mannitol, a sugar alcohol with the same molecular weight as glucose, does not induce insulin secretion or have any metabolic effects, but can be used to increase the osmolality of the medium without affecting the glucose concentration.

2.3 Tubulogenic Analysis

The study employed a tubulogenesis assay to examine the effects of experimental conditions on the cells' ability to form tubule-like structures, indicative of angiogenesis.

Cell Culture and Treatment Conditions

Human brain microvascular endothelial cells were cultured in T-25 flasks with endothelial cell medium (ECM) supplemented with growth factors at 37°C in a humidified 5% CO2 incubator. At 80-90% confluence, cells were treated with either normal glucose (NG, 5mM D-glucose), high glucose (HG, 25mM D-glucose), or an osmotic control of mannitol (5mM D-glucose + 20mM D-mannitol) for 3 days to mimic hyperglycaemic conditions.

Matrigel Preparation

To evaluate endothelial tubule formation capacity, Matrigel matrix (Thermo Fisher Scientific, Massachusetts, USA) was used. Matrigel mimics the extracellular matrix environment to promote three-dimensional angiogenesis. The day before the experiment, 50µl pipette tips and a 96-well plate cooling unit were placed at -20°C to solidify but not freeze the Matrigel. On the day of the experiment, 50µl of Matrigel was slowly pipetted into wells of a pre-chilled 96-well plate (Sigma, Missouri, United States) housed on the cooling block in the tissue culture hood to prevent premature gelling. The plate was then transferred to a 37°C incubator for 1 hour to allow the Matrigel to solidify prior to cell seeding.

Cell Seeding

Thirty minutes prior to seeding, endothelial cells were harvested from flasks using 0.25% trypsin-EDTA (Thermo Fisher Scientific, Massachusetts, USA) and cell counts with trypan blue exclusion were performed using a haemocytometer Cells were re-suspended in medium at densities of 10,000 and 20,000 cells/100µl. 100µl suspensions were seeded directly onto the surface of polymerized Matrigel and incubated at 37°C for 4 hours.

Following the seeding process, the cells were incubated for a period of 12 hours to facilitate tubule formation. Upon completion of the incubation period, images of the cells were captured with an inverted microscope fitted with a camera (Leica DFC3000 G, Wetzlar, Germany). Uniformity in the microscope settings was maintained across all samples to ensure the accuracy of comparisons.

The captured images were subsequently analysed using the Angiogenesis Analyzer plugin for ImageJ, a freely available image processing program. This software tool enabled the objective

quantification of various parameters indicative of angiogenesis, including the total tube length, the number of branches, and the number of junction nodes. The total tube length provided an indication of the extent of angiogenesis, while the number of branches and junction nodes represented the complexity of the angiogenic response.

2.4 Wound scratch assay

The wound scratch assay, also known as the in vitro scratch assay or migration assay, is a commonly used experimental technique to assess cell migration and wound healing capabilities. It is primarily used to study the processes involved in angiogenesis, tissue repair, and cell migration.

The cells were cultured in a 6-well plate (Sigma, Missouri, United States) until they reached full confluence. Standard cell culture protocols were followed, including regular media changes and monitoring for confluency. Once the cells were fully confluent, the wounds were created using a 1000 µl pipette tip. Care was taken to ensure consistent scratch size and depth across all wells to maintain reproducibility.

Lines were added at the bottom of the well plate, perpendicular to the desired direction of wound scratching. These lines served as reference markers to picture the same areas during each imaging time point. By including these reference lines, any potential variations in the positioning or orientation of the well plate during imaging were minimized. This allowed for accurate and consistent comparisons of the same areas across different time points.

To remove any detached cells or debris, the scratched cell monolayers were gently washed with PBS. The PBS was carefully aspirated, taking care not to disturb the remaining cells or the created scratch. This step helped to clean the scratch area and provide a clear field of view for subsequent observations.

For the experimental conditions, a specific medium was prepared according to the requirements of the study. The medium composition was determined based on established protocols or formulations. The cells in each well of the 6-well plate were subjected to their respective experimental medium, applied at a 1:1 ratio to ensure complete coverage of the scratch area. Concurrently, the respective secretory medium was added to its designated wells in a 1:1 ratio, maintaining the appropriate concentrations.

To document the progress of cell migration and wound closure, images were captured at different time points using a microscope equipped with a built-in camera (Leica DFC3000 G, Wetzlar, Germany). The time points selected for image acquisition were immediately after creating the scratch 0 hours and 12 hours post-scratch creation. Care was taken to use consistent imaging settings, including magnification, exposure, and focus, to ensure comparability between the images captured at different time points. Multiple images were taken per well to cover the entire scratch area and ensure representative data.

Following image acquisition, the acquired images were processed and analysed using appropriate image analysis software. The extent of wound closure or cell migration was quantified by measuring the scratch area or calculating the percentage of wound closure.

2.5 Secretome

The secretome, the collection of molecules secreted by cells into the extracellular space, plays a pivotal role in cell-to-cell communication and significantly influences the cellular microenvironment. Changes in the composition of the secretome can provide insights into cellular responses to various stimuli or conditions.

Secretome collection:

To obtain the secretome, the supernatants of the treated cells was condensed by centrifugation (Amicon Ultra-15 Centrifugal Filter Units, Merck Group, Darmstadt, Germany) at 4000g for 15 minutes to condense the filter centrifuge filter column with the pore size of 10kDa. We then exposed healthy pericytes to the secretome and evaluated pericyte cytokine expression using specific proteome profiler kit.

2.6 Protein profiler:

The Proteome Profiler Human Angiogenesis Array Kit (Amicon Ultra-15 Centrifugal Filter Units, Merck Group, Darmstadt, Germany) was selected for the proteomic profiling experiments because it provided researchers with a targeted, sensitive, user-friendly, and validated membrane-based platform for high-throughput analysis of 55 key angiogenesis-related proteins in the endothelial cell secretome in response to hyperglycaemia using an established antibody-based sandwich immunoassay format.

For analysis of cytokine expression changes induced by hyperglycaemia, Proteome Profiler Human Cytokine Array Kit (R&D systems, Minnesota, United States) was chosen .as it allows

for simultaneous quantitative measurement of the levels of 103 different human cytokines and chemokines in cell culture supernatants using an antibody-based detection method. The kit provides a high-throughput, validated approach to broadly profile how hyperglycaemia altered endothelial secretome impact inflammatory microenvironment of pericytes, complementing the angiogenic protein data obtained from the Angiogenesis Array Kit, helping elucidate the underlying mechanisms driving glucose-induced vascular dysfunction and inflammation from a multi-analyte screening perspective.

For accurate results, the supplier's instructions were strictly followed when preparing reagents and samples. Precise measuring and mixing of buffers and detection antibodies is crucial.

To initiate the blocking experiment, 2.0 mL of Buffer 7 was dispensed into each well of a 4-well multi-dish (Sigma, Missouri, United States) . Careful consideration was taken to ensure that the buffer was evenly distributed across the well to create an optimal environment for the subsequent stages of the experiment. Then, using a flat tweezer, each membrane was carefully removed and placed in a well of the 4-well multi-dish with the number facing upwards. To ensure uniform mixing, it is crucial to position the multi-dish on a rocking platform shaker that whirls the chemicals from end to end in the well. Under these circumstances, the membrane was incubated for an hour.

Next, 1 mL of each sample was mixed with 0.5 mL of buffer 4 in a separate tube. After aspirating buffer 7, 15 µL of the reconstituted detection antibody cocktail was added to the sample. This mixture was then transferred to a 4-well plate and required thorough mixing. It was incubated for an hour before being applied to the membrane. The plate was incubated overnight at 2-8 degrees C on a rocking platform shaker. This allowed the detection antibody cocktail to bind to any protein of interest.

The next day, the membranes were carefully removed from the multi-dish and placed in a plastic container with 20 mL of wash buffer for 10 minutes. This step was repeated two more times. The used 4-well multi-dish was then rinsed and dried with distilled water.

After the final wash, the membranes were removed from the wash buffer and returned to the 4-well multi-dish containing 2 mL of diluted Streptavidin-HRP. This was incubated on the rocking platform shaker for 30 minutes at room temperature. The membranes were washed three times with wash buffer and then incubated at room temperature for 1 minute with 1 mL of prepared Chemi Reagent Mix on each membrane.

The membrane was thoroughly dried to get rid of any extra chemi reagent mix and a chemiluminescent scanner and imaging analysis software was used to quantify the proteins expressed in the sample.

2.7 Formation of the Blood-Brain Barrier:

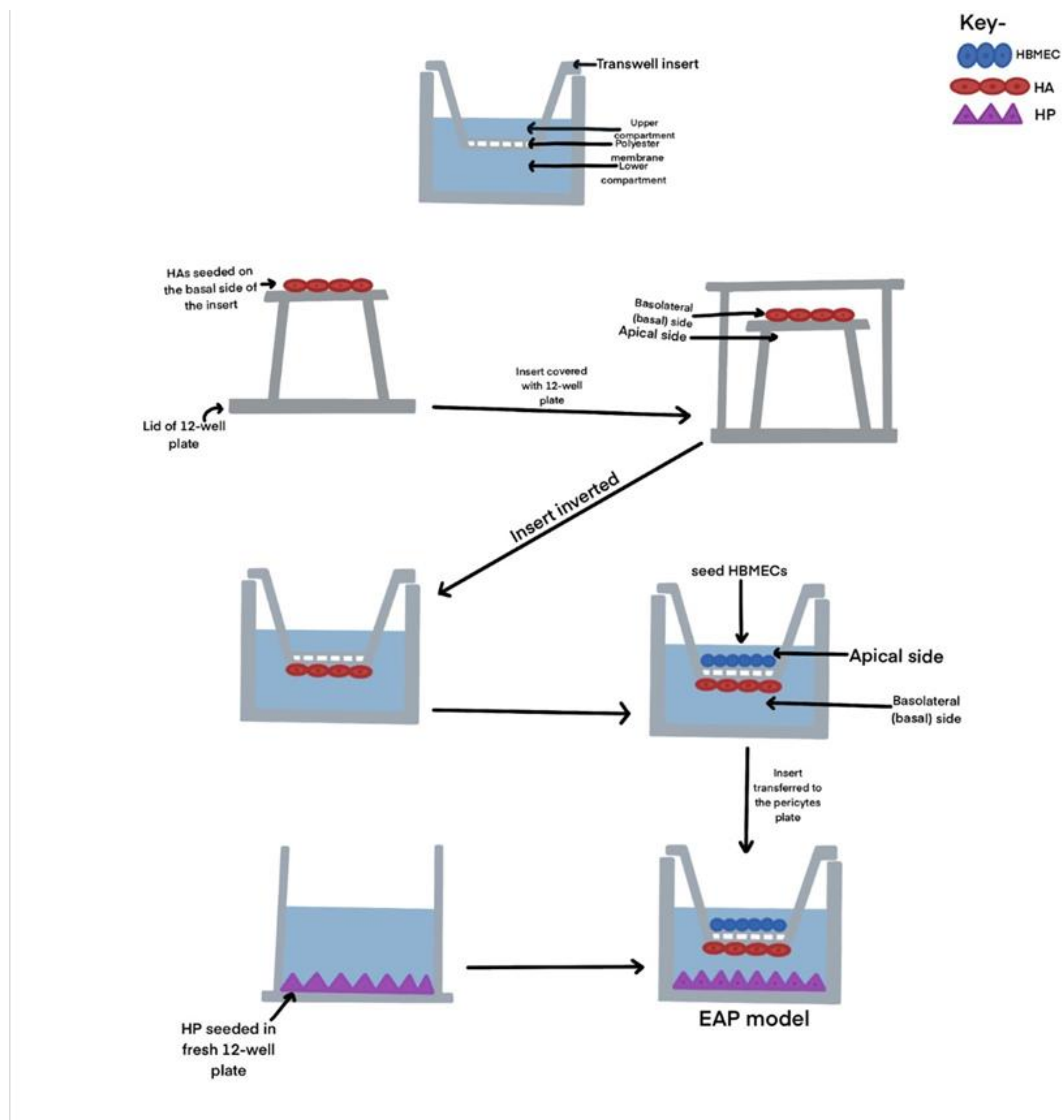


Figure 2 The illustration shows the steps for seeding HAs and HBMECs.

To seed HAs, the transwell insert was placed in the well plate, and HAs with growth media were added. They were incubated for a few hours and then transferred to a new plate until they became fully confluent. To seed HBMECs, the cells were washed and detached, then seeded in specialized media.

The HBMECs were then added to the prepared well plate and incubated with growth media. Finally, HPs were seeded in a new well plate with specialized media and incubated until they became confluent. Adopted from (Au - Bora, Au - Wang et al. 2022)

Pericytes are another essential component for the formation of the BBB. Several studies have demonstrated that pericytes or their extracellular matrix can enhance the integrity of the BBB, tightening the barrier and producing a higher TEER value. Therefore, we adopted the HMBEC, HA, and HP contact model to closely replicate and monitor the cells as closely as possible to their in vivo counterparts.

A frozen vial containing astrocytes was thawed and seeded into a T-75 flask in a humidified atmosphere at 37°C. The media was changed the following day to remove the DMSO, and the media was changed every other day following this. The same procedure was utilised for HBMEC and HPs. When the cells reached approximately 90% confluency, they were washed with PBS and detached using trypsin. The number of cells was counted, and the percentage of viable cells was calculated.

To seed HAs on the basolateral side of the insert

First the lid of a 12-well plate was opened and positioned upside down. Using sterilized tweezers, the sterilized transwell insert was carefully placed onto the bottom of the well plate, as per the instructions provided in the diagram. Next cells were seeded at a density of 7.5×10^4 on the basolateral side of the insert, using 25 µL of astrocyte growth media. For consistency and reliability, it was ensured each group was replicated three times concurrently The cells were incubated on the transwell membrane for 3-4 hours. Once the cells fully attached to the membrane, any remaining media was aspirated. The insert was then flipped and placed into a fresh 12-well plate and incubated until fully confluent.

To seed the HBMECs

The same procedure was followed for thawing and seeding for both pericytes and endothelial cells. Once fully confluent in the T-75 flask, the cells were washed with PBS and detached using trypsin. Their respective numbers were determined, and they were seeded accordingly in their specialized media.

The 12-well plate already prepared with astrocytes was taken out and placed in the hood. The astrocyte media was then removed from both chambers. 4×10^4 HBMECs were seeded on the apical side of the chamber and 750 µL of ECM growth media was added to the luminal chamber. Next, 1 mL of astrocyte growth media was added to the abluminal chamber and incubated accordingly.

Then, 1.5 × 10^5 HPs were seeded in a new 12-well plate with pre-warmed pericyte media. This was incubated until approximately 90% confluent.

Once the desired confluency was reached, the 12-well plate containing HBMEC and HA, and the pericyte-containing well plate were taken out and placed inside the hood.

All three-cell media were then removed from the abluminal and luminal chambers, washed with PBS, and the 12-well plate containing the HBMEC and HA was very gently transferred to the 12-well plate containing HPs. After this, 750 µL of both pericyte and astrocyte media was added in the abluminal chamber, and 750 µL of endothelial cell media was added into the luminal chamber. The media was changed every day while measuring the TEER.

2.7.1 To assess BBB integrity, TEER measurement

The transepithelial/trans endothelial electrical resistance (TEER) technique is a highly sensitive and reliable method for measuring the electrical resistance across a cellular monolayer. It provides an indication of the integrity and permeability of the BBB. The formation of robust tight junctions and signalling between neighbouring cells are critical for tight junction formation and lower permeability. A higher TEER value indicates better BBB integrity and function.

TEER was calculated using the following formula:

TEER = Resistance x Effective Membrane Area (cm2)

The resistance was the reading from the EVOMX meter (World Precision Instruments, Hertfordshire, UK). The effective membrane area represented the area available for the cells to grow, which was 1.12 cm^2.

While TEER measurements are good indicators of monolayer conductance, it is important to note that these results cannot be extrapolated to reflect the permeability of in vitro BBB models alone. Therefore, functional analysis is required to validate and extrapolate the data gained.

2.7.2 BBB function, molecular markers (EBA/NaF)

To evaluate the function of the BBB, the transmembrane flux of high and low molecular weight markers is commonly used. In this experiment, the markers used were Evans blue labelled with 0.1% albumin (EBA, 67 kDa) and low molecular weight sodium fluorescein (NaF, 375 Da).

The first step in the procedure was to subject the cells to the experimental conditions, which could include exposure to drugs or other compounds that might affect the BBB. After this, the cells were washed twice with Hank's Balanced Salt Solution (HBSS) and placed in a new 12-well plate containing 2 mL of HBSS.

Next, a solution of either EBA or NaF was prepared in HBSS. For EBA, 500 µL of a 165 µg/mL solution was added to the luminal chamber. For NaF, 500 µL of a 10 µg/mL solution was added to the luminal chamber.

The plate was then incubated for one hour After this time, samples were taken from both the abluminal (basolateral) and luminal (apical) chambers for analysis.

The concentration of EBA in the abluminal chamber was measured by absorbance at 610 nm using a plate reader (BMG Labtech Ltd, UK). For NaF, fluorescence was measured with excitation at 485 nm and emission at 520 nm using a plate reader.

Finally, the flux of EBA and NaF across the BBB was calculated using the following formula:

Cleared volume = (Concentration abluminal reading x Volume abluminal) / Concentration luminal reading

This formula considers the volume of the abluminal chamber, the concentration of the marker in the abluminal and luminal chambers, and the amount of time the experiment was conducted. The cleared volume represented the amount of fluid cleared of the marker per unit time and was utilised as an indicator of the permeability of the BBB to that particular marker.

2.8 Immunohistochemistry and actin visualisation

The main purpose of performing immunohistochemistry/immunostaining in this study was to visualize specific protein markers and structures in endothelial and pericyte cells under different treatment conditions (normal glucose, high glucose, mannitol control).

Immunostaining enabled analysis of molecular and structural changes linked to BBB dysfunction under hyperglycaemic conditions, complementing functional permeability assays. This helped elucidate specific pathogenic mechanisms of diabetes-induced vascular impairment.

Qualitative and quantitative comparisons between treatment groups was allowed by visualising protein targets within individual cells using fluorescence microscopy. The cells were cultured in a 24-well plate and stained for PDGFR-β, γH2AX, ZO-1, and actin, each marker offering unique insights. PDGFR-β indicated proliferative activity, γH2AX revealed DNA damage, ZO-1 assessed the health of tight junctions, and actin, visualized with phalloidin, reflected the structural integrity of the cells.

Procedure

First, it had to be ensured that the cells on coverslips in the 24-well plate were fully confluent and free of contaminants. The cells' morphology was examined using an inverted microscope to confirm their health.

Next, the cells were moved from tissue culture to the molecular lab, the media was removed, and they were washed with 1 mL of warm PBS.

The cells were fixed in 500 µl of 4% formaldehyde and incubated for 15 minutes at room temperature. After fixing, the cells were washed twice with PBS and permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Then, the cells were washed twice with 1 mL of PBS.

The cells were blocked with 1% BSA in PBST (0.1% Tween 20 and PBS) for 30 minutes at room temperature and washed with PBS once after blocking. The cells were incubated with primary antibody (ThermoFisher, UK) diluted with 1% BSA overnight at 4°C.

The cells were washed three times with 1mL of PBS for 5 minutes each, incubated with secondary antibody (ThermoFisher, UK) diluted in 1% BSA at room temperature for 1 hour, and washed again three times with 1mL of PBS for 5 minutes each.

Following the incubation with secondary antibody, the cells were washed three times for five minutes each with 1 mL of PBS, then the nuclei was stained with DAPI diluted in PBS for three minutes at room temperature and washed once with 1 mL of PBS.

Finally, a drop of mounting media (Vector Laboratories, Peterborough, UK) was put on a slide. The coverslip was removed from the 12-well plate and the slide cell was placed side down on top of the mounting media. This was covered with aluminium foil and incubated at room temperature for 15-30 minutes for the mounting medium to dry before storing or carrying out imaging under a fluorescence microscope (pE-300white, Andover, Hampshire). By following these steps, the actin cytoskeleton, and the location of specific proteins of interest was able to be visualised.

2.9 Statistical analysis

All quantitative results were expressed as ± standard mean error of three independent experiments (n=3). Comparison between two experimental conditions or time points was analysed using the unpaired Student's t-test for parameters following a normal distribution. For multiple comparisons among treatment groups, one-way analysis of variance (one-way ANOVA) was performed to determine statistically significant differences. For assays involving two variables such as treatment and cell density, two-way ANOVA was used to assess any interaction effects. When ANOVA indicated significant differences, Tukey's multiple comparisons test was applied as a post-hoc analysis to discern precisely which group means were significantly different from others. This allowed further investigation into the exact effects of glucose exposure versus controls at each seeding density or time interval analysed. The α level denoting significance was defined as $p < 0.05$ for all tests. All statistical analyses were conducted using GraphPad Prism 9 software.

3 Results:

This chapter is a detailed presentation of our experimental findings, pivotal to our objective of understanding the effects of hyperglycaemia on endothelial cells, pericytes, and the BBB. The insights we gather hold potential to shed light on the neurovascular complications associated with diabetes.

The results are systematically segmented into distinct sections, each corresponding to the various assays undertaken. The first part of this chapter explores the morphological changes in endothelial cells and pericytes under normal glucose, high glucose, and mannitol control conditions, as assessed through light microscopy. We also examine alterations in key proteins such as PDGFR-β and markers of DNA damage.

Subsequently, we delve into the impact of these conditions on tubulogenesis, evaluated via in vitro tube formation assays. This is followed by an analysis of wound healing capabilities using the scratch wound assay model.

Further, we present the findings from the proteome profiling of angiogenic factors and cytokines. Here, a key aspect is the analysis of the pericyte secretome, offering insights into the range of proteins secreted by pericytes under different conditions. Additionally, we explore how pericytes respond to endothelial-derived secretome under varying conditions, focusing on the modulation of cytokine expression. This intersection of pericyte and endothelial cell interactions at the secretome level provides a novel perspective on the cellular response to hyperglycaemic conditions.

The chapter concludes with findings relating to changes in barrier integrity over time, assessed through trans-endothelial electrical resistance measurements and permeability assays. We also characterize the localization patterns of tight junction proteins, offering insights into the structural alterations in the BBB under hyperglycaemic conditions.

3.1.1 Cellular Morphological Alterations in Endothelial Cells and Pericytes Under Hyperglycaemic Conditions

Cellular morphology was assessed using an integrated light microscope camera following experimental treatments. Both pericytes at passage 7 and endothelial cells at passage 9 were grown to confluence in T-75 flasks. Prior to imaging, cells were subjected to either normal glucose (NG, 5mM), high glucose (HG, 25mM), or mannitol control conditions for three days, as outlined in the Methods section.

Figure 3 presents images of endothelial cell morphology under each treatment condition. Regardless of the condition, endothelial cells maintained their typical cobblestone-like appearance, with clear cell borders and a polygonal shape.

Figure 4 provides representative images of pericyte morphology under each of the treatment conditions. Across all conditions, pericytes preserved their characteristic elongated, stellate appearance.

Morphological assessments conducted via light microscopy demonstrated that both pericytes and endothelial cells maintained their characteristic cellular features under HG, NG, or mannitol conditions over a three-day period.

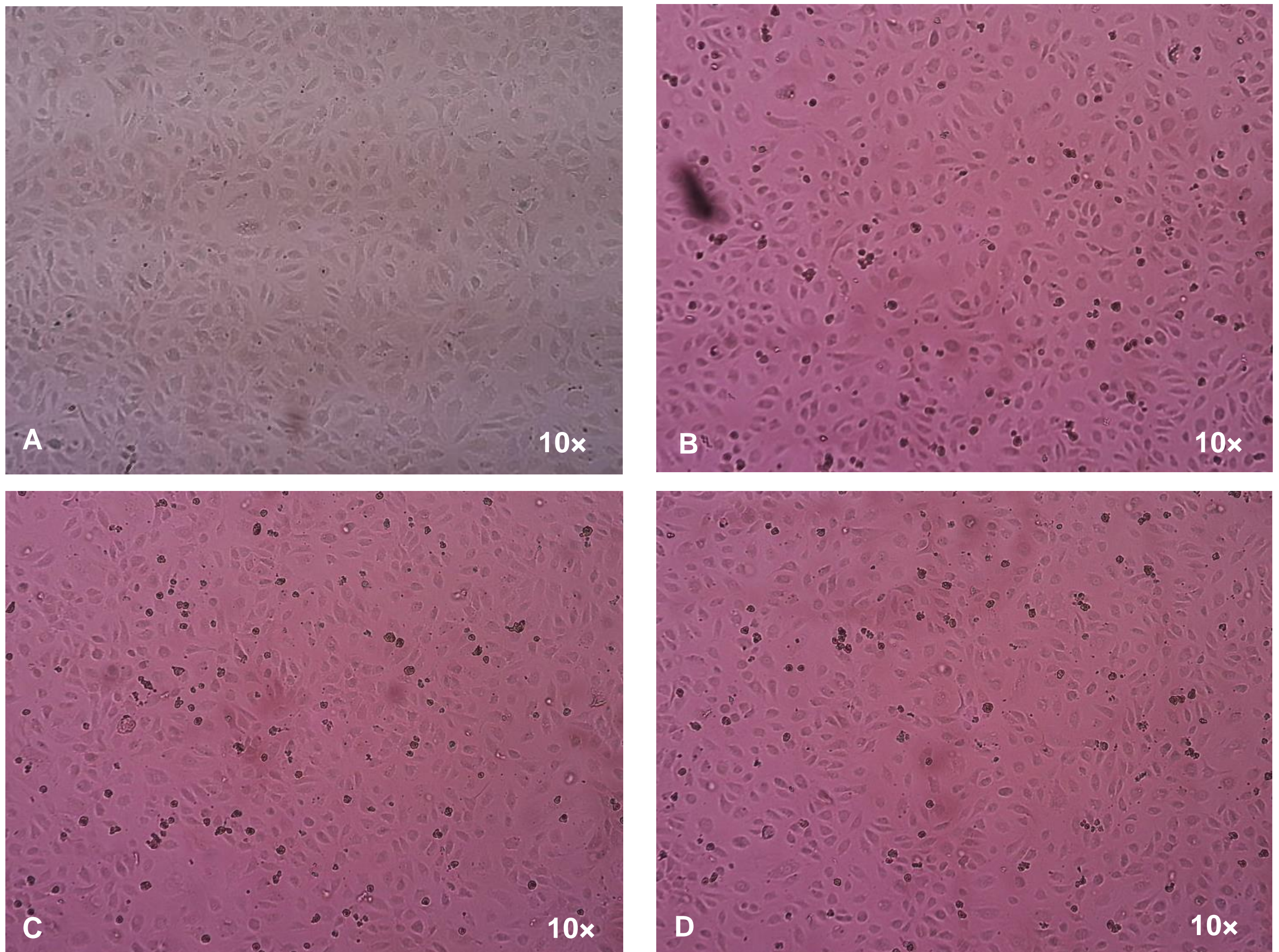


Figure 3 Morphological characteristics of passage 9 endothelial cells under various conditions.

(A) Endothelial cells at full confluency, displaying typical morphological characteristics. B) Endothelial cells following exposure to high glucose conditions for a duration of three days. (C) Endothelial cells treated with normal glucose levels for three days. (D) Endothelial cells subjected to D-mannitol treatment for osmotic control. Each image was captured using light microscopy, offering a visual comparison of endothelial cell morphology under different experimental conditions.

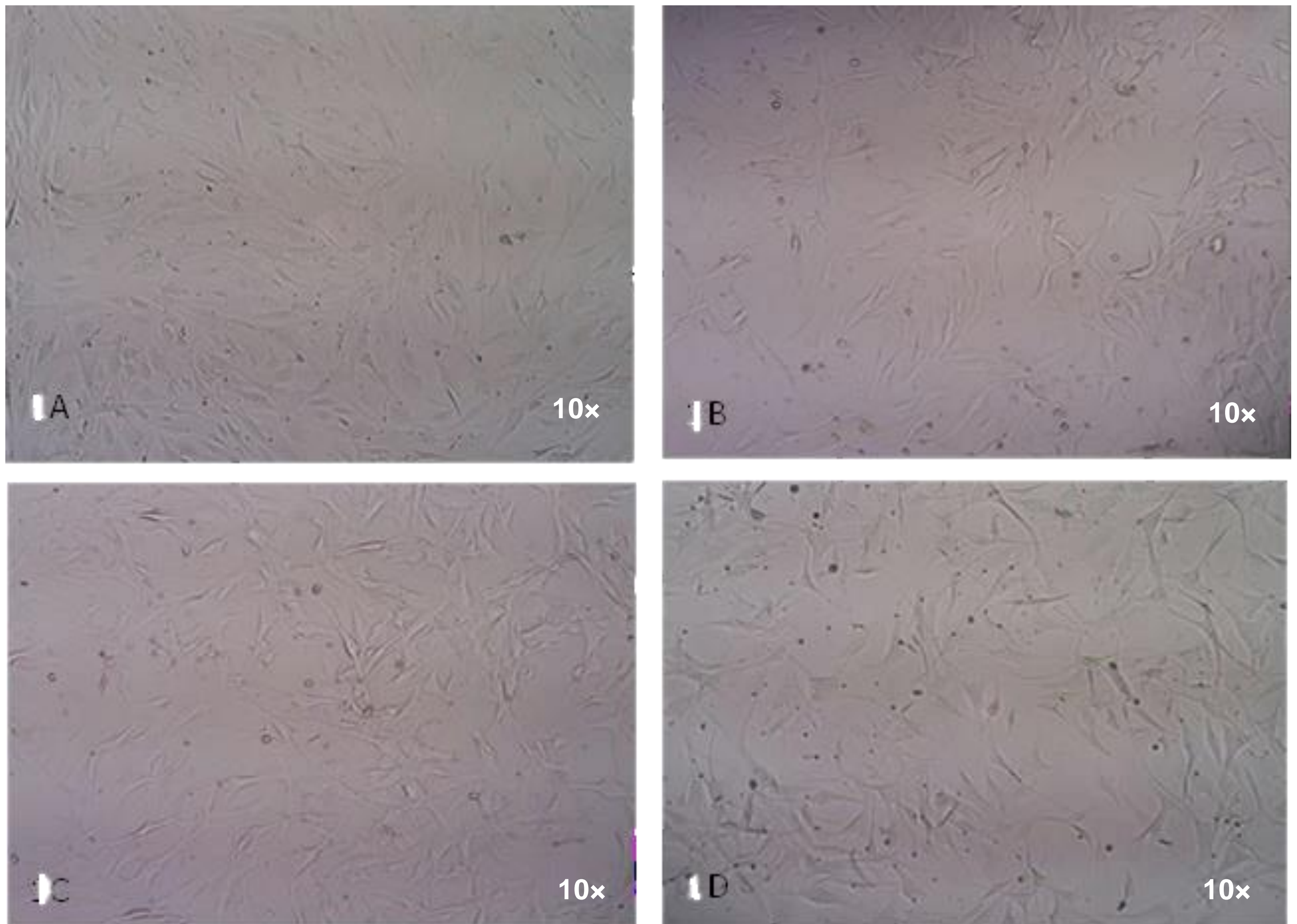


Figure 4 PC Morphological characteristics of passage 7 pericytes under various conditions.

(A) Fully confluent pericytes in control conditions. (B) Pericytes subjected to high glucose treatment for three days. (C) Pericytes treated with normal glucose for three days. (D) Pericytes treated with D-mannitol as an osmotic control. Each image was captured using light microscopy, offering a visual comparison of endothelial cell morphology under different experimental conditions.

3.1.2 Differential Expression of Platelet-Derived Growth Factor (PDGF) in Response to Hyperglycaemic Conditions

This chapter presents the results from the investigation into the effects of glucose treatment on PDGFR-β fluorescence intensity in primary human pericytes. The cells were treated with normal glucose (5mM), high glucose (25mM), or mannitol (5.5mM NG+19.5mM D.mannitol) control for 72 hours before being stained for PDGFR-β and fluorescence intensity quantified by confocal microscopy.

An unpaired t-test revealed a significant difference in PDGFR-β intensity between the normal and high glucose treatments (p=0.0025, Figure 5). High glucose-exposed pericytes exhibited markedly higher PDGFR-β expression (mean 26.81) compared to those under normal glucose control (mean 16.68). The mean difference of 10.13 (95% CI -16 to -4.26) indicates a substantial increase in PDGFR-β expression under high glucose conditions.

A one-way ANOVA demonstrated a significant effect of treatment condition on PDGFR-β levels (F=19.95, p<0.0001), with the model explaining 4.46% of the total variation. This indicates that the treatment condition significantly influences PDGFR-β expression levels.

Post-hoc Tukey's comparisons revealed that high glucose treatment significantly increased PDGFR-β expression compared to both the normal glucose (p=0.0008) and mannitol control (p<0.0001). Furthermore, there was no significant difference observed between the normal glucose and mannitol (p=0.1141).

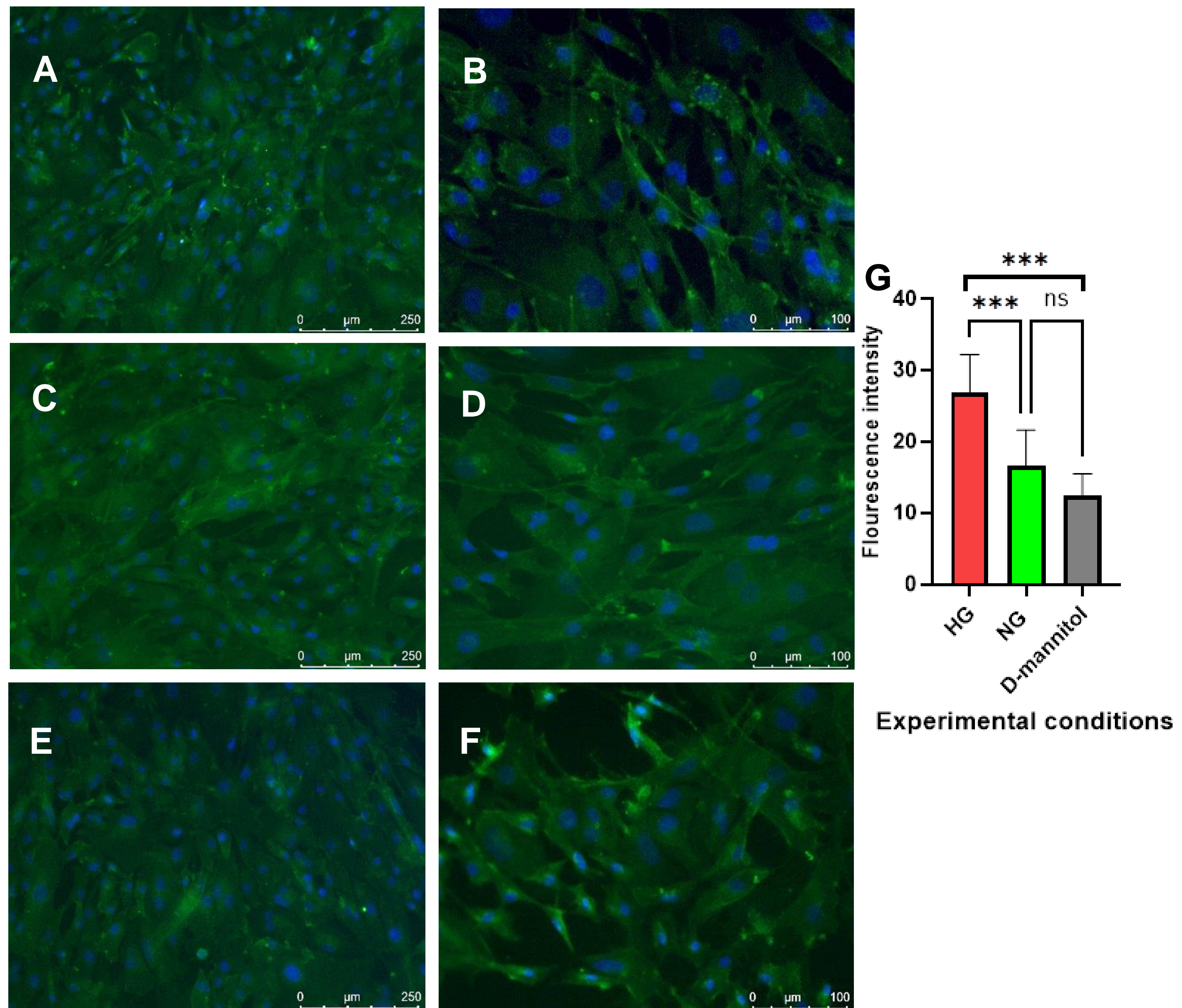


Figure 5 Assessment of PDGFRβ Expression in Pericytes Under Different Conditions.

This figure includes immunofluorescence images and a bar chart. The images (A-B), (C-D),(E-F) depict PDGFRβ expression in pericytes treated with High Glucose (HG), Normal Glucose (NG), and D-mannitol, respectively. The bar chart (G) quantifies the fluorescence intensity of PDGFRβ in each treatment condition, providing a comparative measure of PDGFRβ expression levels. Each data point represents the mean of three independent replicates (n=3), and the error bars represent standard mean error.

3.1.3 Differential expression of actin filaments in response to hyperglycaemic conditions

Stress fibres are actin filament bundles that contribute to endothelial cell cytoskeletal architecture and regulated vascular permeability.

This chapter presents the results obtained from the visualization and quantification of stress fibres in endothelial cells under hyperglycaemic, normoglycaemia, and D-mannitol osmolar control conditions. Stress fibres were stained with phalloidin and quantified using fluorescent microscopy.

A one-way ANOVA revealed a significant effect of treatment condition on stress fibre formation ($F(2,6)=5.345$, $p=0.0465$). This indicates the presence of substantial differences in stress fibre counts across treatment conditions, suggesting that the treatment conditions have a significant impact on stress fibre formation.

Post-hoc analyses using Tukey's multiple comparisons test showed that high glucose stimulation resulted in significantly more stress fibres compared to the normal glucose controls. The mean difference was 34 fibres (95% CI 18-50, $p=0.004$), indicating that high glucose conditions can stimulate the formation of a significant number of additional stress fibres.

High glucose treatment also resulted in significantly more stress fibres compared to D-mannitol treatment, with a mean difference of 28 fibres (95% CI 13-43, $p=0.007$). This suggests that high glucose conditions can stimulate stress fibre formation even more than D-mannitol treatment.

Specifically, stress fibre quantification showed that high glucose treatments prompted a mean count of 76 fibres per cell, compared to 42 fibres under normal glucose conditions and 32 fibres under D-mannitol conditions.

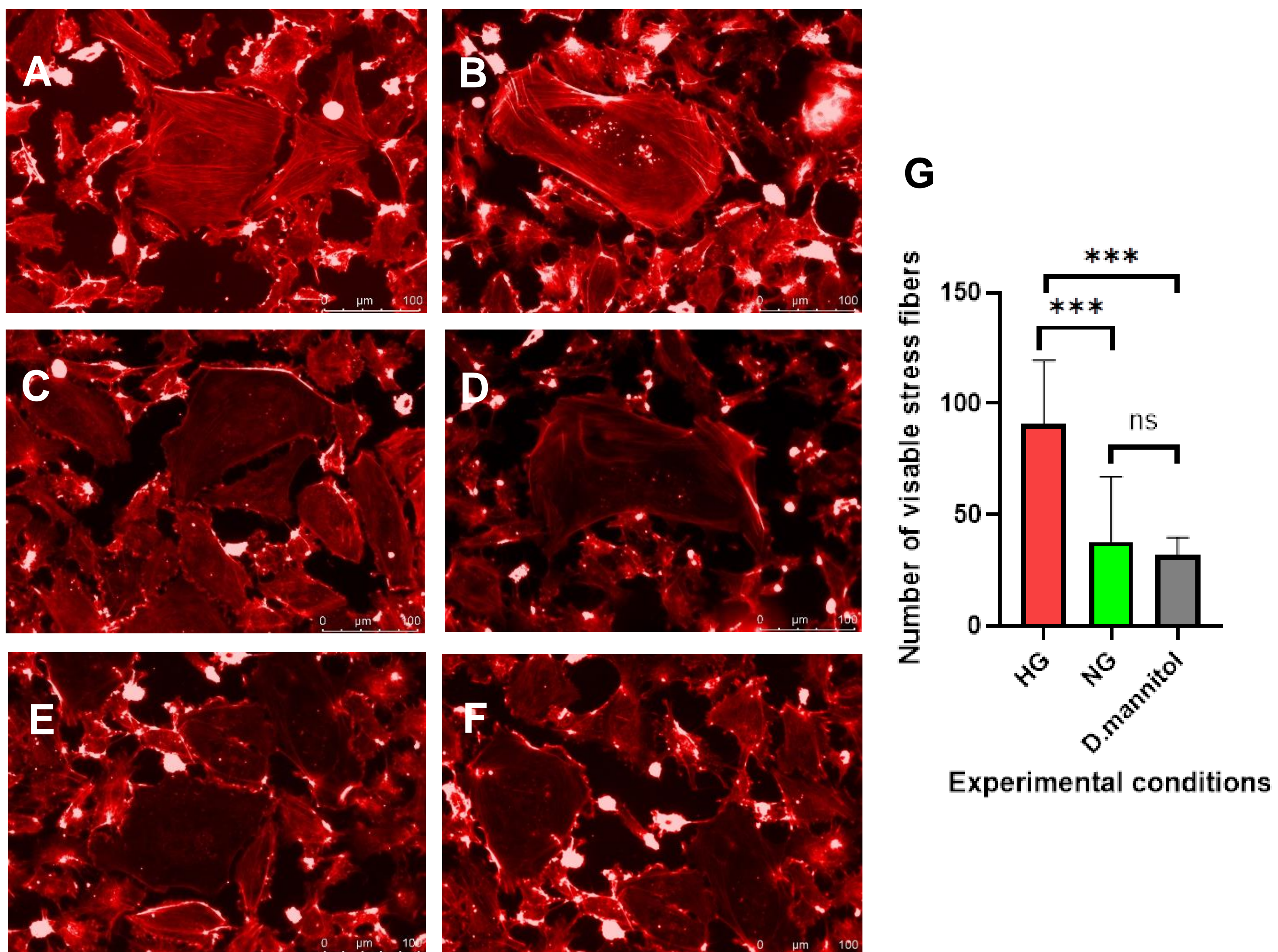


Figure 6 Immunocytochemistry depictions of F-actin stress fibre distribution under different treatment condition*.*

Endothelial cells were subjected to treatments with high glucose (A-B), normal glucose (C-D) and D-mannitol (E-F) over a span of 3 days. The bar char (G) offers a quantitative representation of the numbers of visible stress fibre distribution under each condition. Fluorescent microscopy was employed to visualize the influence of diverse glucose conditions and osmotic stress on the cytoskeletal organization within the endothelial cells. These illustrative depictions contribute significantly to our understanding of cellular adaptation mechanisms in response to varying glucose environments. Each data point represents the mean of three independent replicates (n=3).

3.1.4 Hyperglycaemia induced γ-H2AX expression: A marker of DNA damage in Endothelial Cells

Gamma H2AX (γH2AX) is known for its rapid phosphorylation at the sites of DNA breaks, which makes it a reliable marker for detecting both recent and ongoing DNA damage. The levels of γH2AX can be objectively quantified utilizing microscopy or immunoblotting, facilitating a comparative analysis between different treatment groups.

Results gathered from the measurement of γH2AX, a DNA damage marker, in endothelial cells under HG, NG, and d-mannitol osmolar control conditions are Mean γH2AX expression were compared among the three treatment groups.

The data show that γH2AX levels are similar among the hyperglycaemic, normoglycemic, and d-mannitol osmolar control groups. Gamma H2AX under the defined experimental settings, the treatment conditions do not significantly affect the DNA damage marker in endothelial cells. That is to say that any changes in γH2AX levels across the groups are more likely to be the result of chance than of any actual therapeutic efficacy.

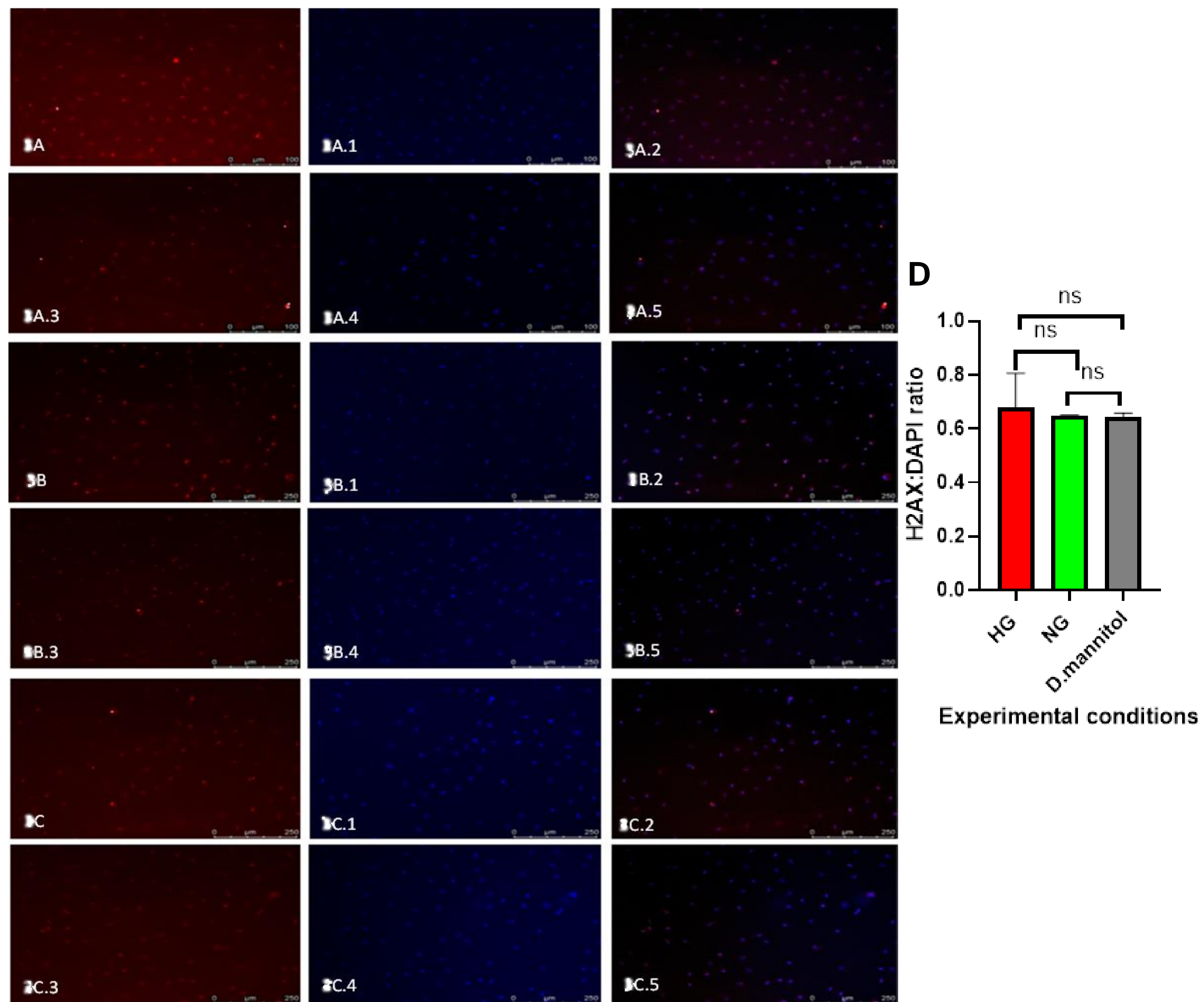


Figure 7 Assessment of DNA damage markers via γ-H2AX staining under different conditions.

This figure includes immunocytochemistry images and a quantification graph. The endothelial cells were treated with High Glucose (HG), Normal Glucose (NG), and D-mannitol for three days and processed for γ-H2AX staining. The images show γ-H2AX staining for HG (3A,3A.3), NG (3B, 3B.3), and D-mannitol (3C, 3C.3). DAPI-γ-H2AX staining for HG (3A.1, 3A.4), NG (3B.1, 3B.4), and D-mannitol (3C.1, 3C.4) is depicted, marking the nuclei in blue. Additionally, merged images of γ-H2AX and DAPI staining are shown for HG (3A.2, 3A.5), NG (3B.2, 3B.5), and D-mannitol (3C.2, 3C.5). The bar chart (D) quantifies the DNA damage markers under each condition, represented by red dots. The blue dots in the graph correspond to DAPI staining, indicating the presence of nuclei. Each data point represents the mean of three independent replicates (n=3), and the error bars represent standard mean error.

3.1.5 Impact of hyperglycaemia on Tubulogenesis: In vitro tube formation assay findings

This chapter presents and analyses the results of a tubulogenesis study aimed at investigating the effects of high glucose, normal glucose, and D-mannitol treatments on key parameters related to tubular network formation. The parameters analysed include the number of nodes and junctions, both integral to the complexity and connectivity of the tubular structure. Additionally, the mesh area was evaluated to assess changes in the overall structure and density of the tubular network. To further understand the scale and extent of tubular network formation, the total length of all tubules was measured. Lastly, the total segment length, indicating the length of individual segments between nodes, was assessed to uncover any modifications in the architecture of the tubular network.

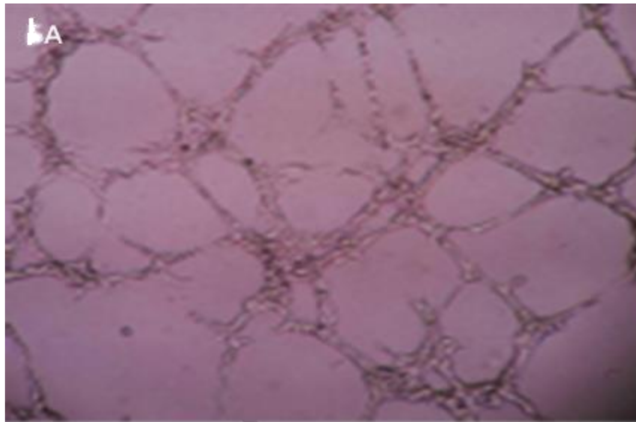
The analysis of the number of nodes revealed no statistically significant differences among the treatment groups. The mean difference between HG and NG was -The adjusted p-values for these comparisons were 0.8207, 0.8518, and 0.5003, respectively.

The analysis of the number of junctions also indicated no statistically significant differences among the treatment groups. The adjusted p-values for these comparisons were >0.9999, 0.9996, and 0.9998, respectively.

Similar to the previous parameters, the analysis of the mesh area did not reveal any statistically significant differences among the treatment groups. The adjusted p-values for these comparisons were 0.9707, 0.9540, and 0.9980, respectively.

The analysis of the total length revealed no statistically significant differences among the treatment groups. The adjusted p-values for these comparisons were 0.9888, >0.9999, and 0.9871, respectively.

The analysis of the total segment length also indicated no statistically significant differences among the treatment groups. The adjusted p-values for these comparisons were 0.8418, 0.2994, and 0.5924, respectively.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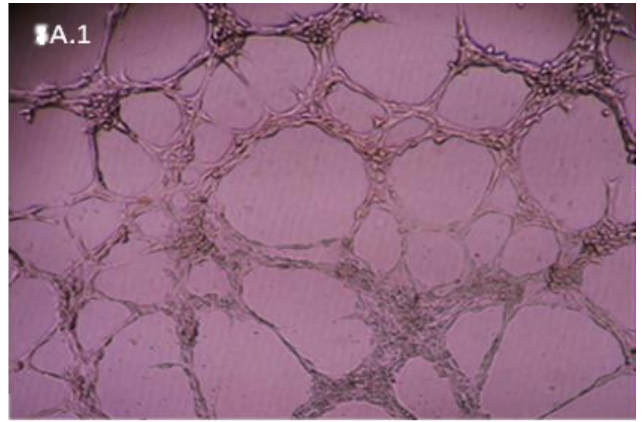
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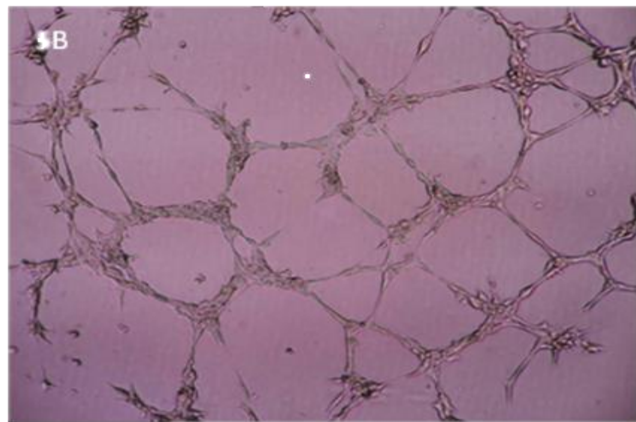
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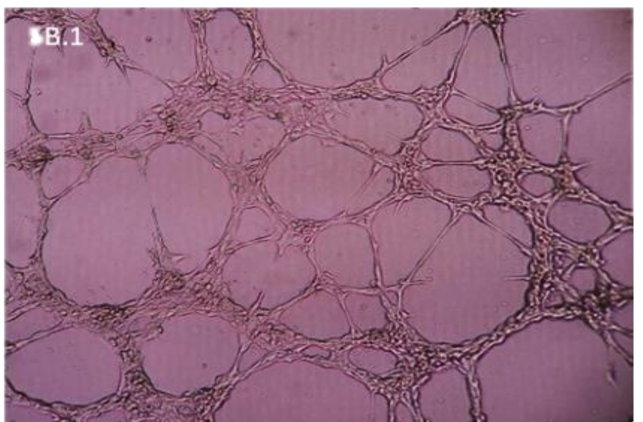
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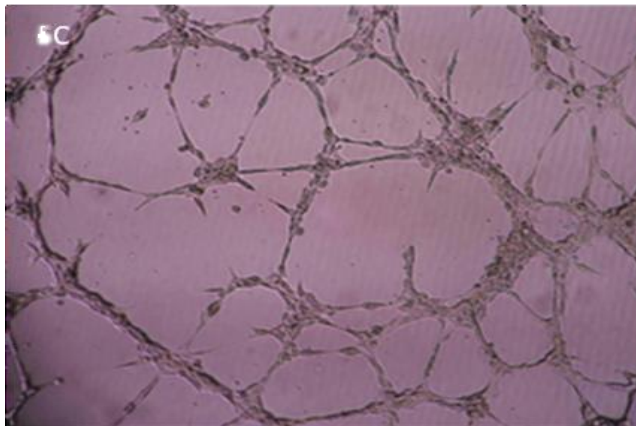
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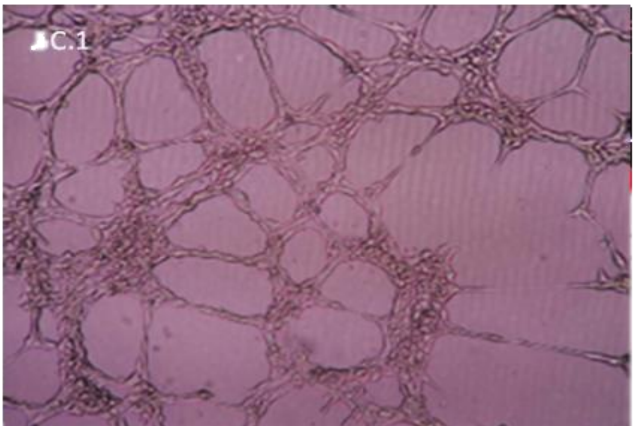
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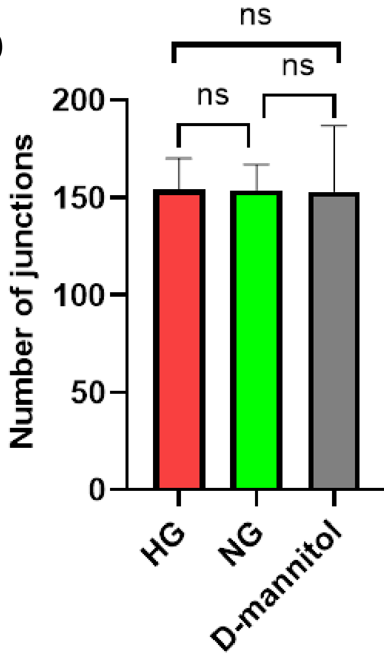

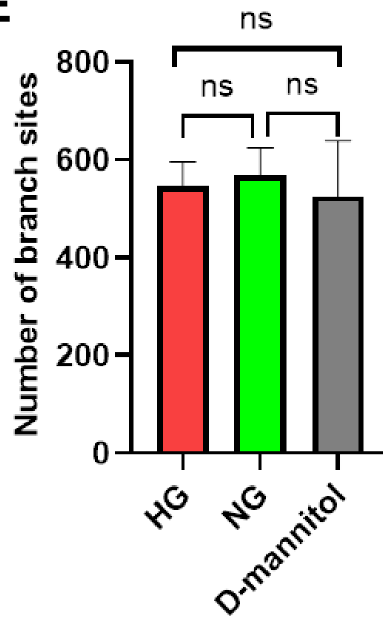

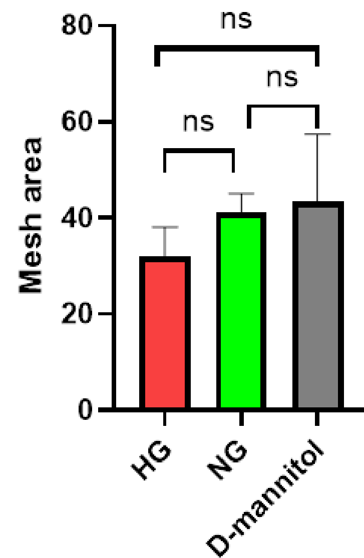

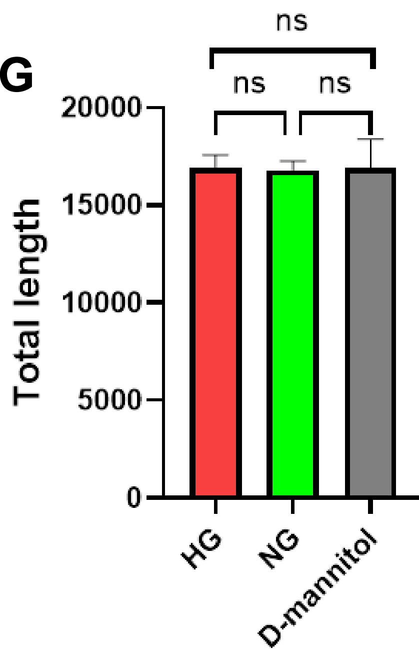

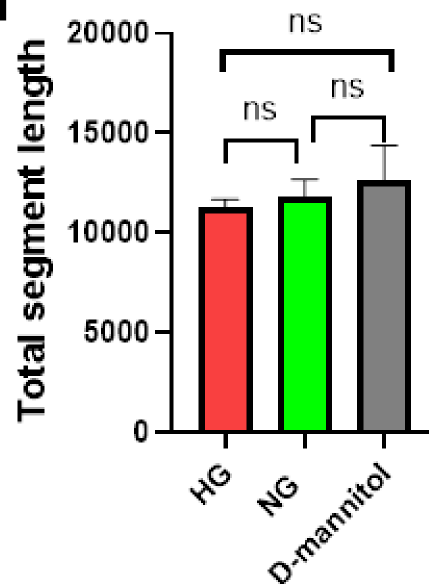

Figure 8 Tubulogenesis Analysis Under Different Conditions.

The images depict tubulogenesis in cells under the following conditions: High Glucose (HG) with 10,000 cells (A), HG with 20,000 cells (A.1), Normal Glucose (NG) with 10,000 cells (B), NG with 20,000 cells (B.1), D-mannitol with 10,000 cells (C), and D-mannitol with 20,000 cells (C.1). The bar charts quantifies key parameters of tubulogenesis, including the number of junctions(D), number of branching points (E), mesh area (F), total length (G), and total segment length (H) for each condition, providing a comparative measure of angiogenic capacity under varying conditions. Each data point represents the mean of three independent replicates (n=3)., and the error bars represent standard mean error.

3.1.6 Insights from wound scratch assay in Endothelial Cells:

The assay involves creating a scratch or gap in a confluent monolayer of cells, typically using a sterile pipette tip or a specialized tool. The scratch creates an artificial wound, mimicking a tissue injury. The cells are then allowed to migrate into the gap and close the wound over time. By monitoring the closure of the gap, researchers can evaluate the migratory and wound healing abilities of the cells under different experimental conditions.

The wound scratch assay provides several advantages. It is relatively simple, cost-effective, and can be performed with a wide range of cell types. It allows for the assessment of collective cell migration, where cells move as a group to close the gap. Moreover, it enables the investigation of the effects of various factors, such as growth factors, drugs, or different culture conditions, on cell migration and wound healing.

The wound healing process over time was evaluated by performing a wound scratch assay. The percentage of wound closure was measured at two time points: 0 hours (immediately after wounding) and 12 hours post-wounding.

The mean percentage of wound closure at 0 hours was 51.63%, and at 12 hours, it was 40.17%. The difference between these means was -11.47%, with a standard error of the mean (SEM) of ± 5.246%.

The results from the unpaired t-test demonstrated no significant difference in the percentage of wound closure between 0 hours and 12 hours ($t=2.186$, $df=4$, $P=0.0941$). The two-tailed P-value was above the threshold of significance ($P > 0.05$), suggesting that the differences observed could be due to chance.

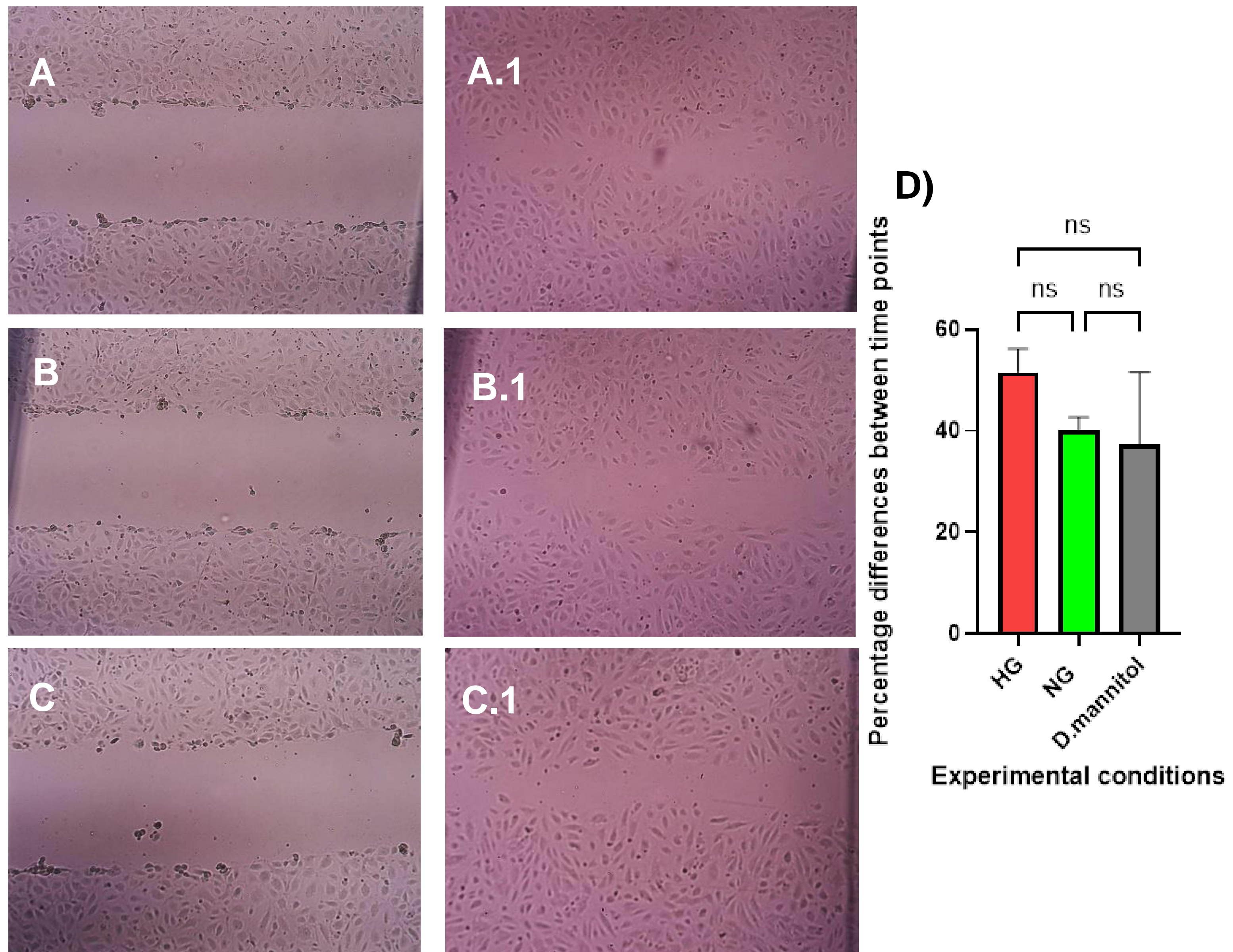

***Figure 9 Wound Scratch Assay Under Different Conditions and Time Points**.*

This figure includes bright-field images and a bar chart. The images show the wound area in cells under High Glucose (HG) conditions at 0 hours (A) and 12 hours (A.1), Normal Glucose (NG) conditions at 0 hours (B) and 12 hours (B.1), and D-mannitol conditions at 0 hours (C) and 12 hours (C.1). Wounds were created using a 1ml pipette tip, with careful control to ensure similar wound sizes across all experimental conditions. The bar chart (D) represents the percentage of wound healing between the two time points under each condition. This provides a comparative measure of cellular migration and proliferation, key aspects of wound healing, under varying conditions. Each data point represents the mean of three independent replicates (n=3), and the error bars represent standard mean error.

3.1.7 Deciphering the impact of hyperglycaemia on EC Angiogenesis: Results from Proteome Profiling

The study sought to understand the impact of hyperglycaemia on the secretory profile of brain endothelial cells. Conditioned media from human brain microvascular endothelial cells (HBMECs) that were treated with NG or HG for 72 hours were examined using the Proteome Profiler Human Angiogenesis Array Kit.

As presented in Figure 10, HG treatment significantly increased the endothelial secretion of angiogenin compared to NG treatment p-value ≤ 0.01. Similar significant enhancements were observed in the levels of basic Fibroblast Growth Factor (bFGF), with a p-value of ≤ 0.01.

Endothelin-1 and, exhibited a very significant increase with HG treatment, p-value of ≤ 0.001. On the other hand, Placental Growth Factor (PIGF) exhibited a very significant decrease expression with p-values of ≤ 0.001

In contrast, Vascular Endothelial Growth Factor (VEGF) and Angiopoitin-2 expression remained unaffected by glucose variations.

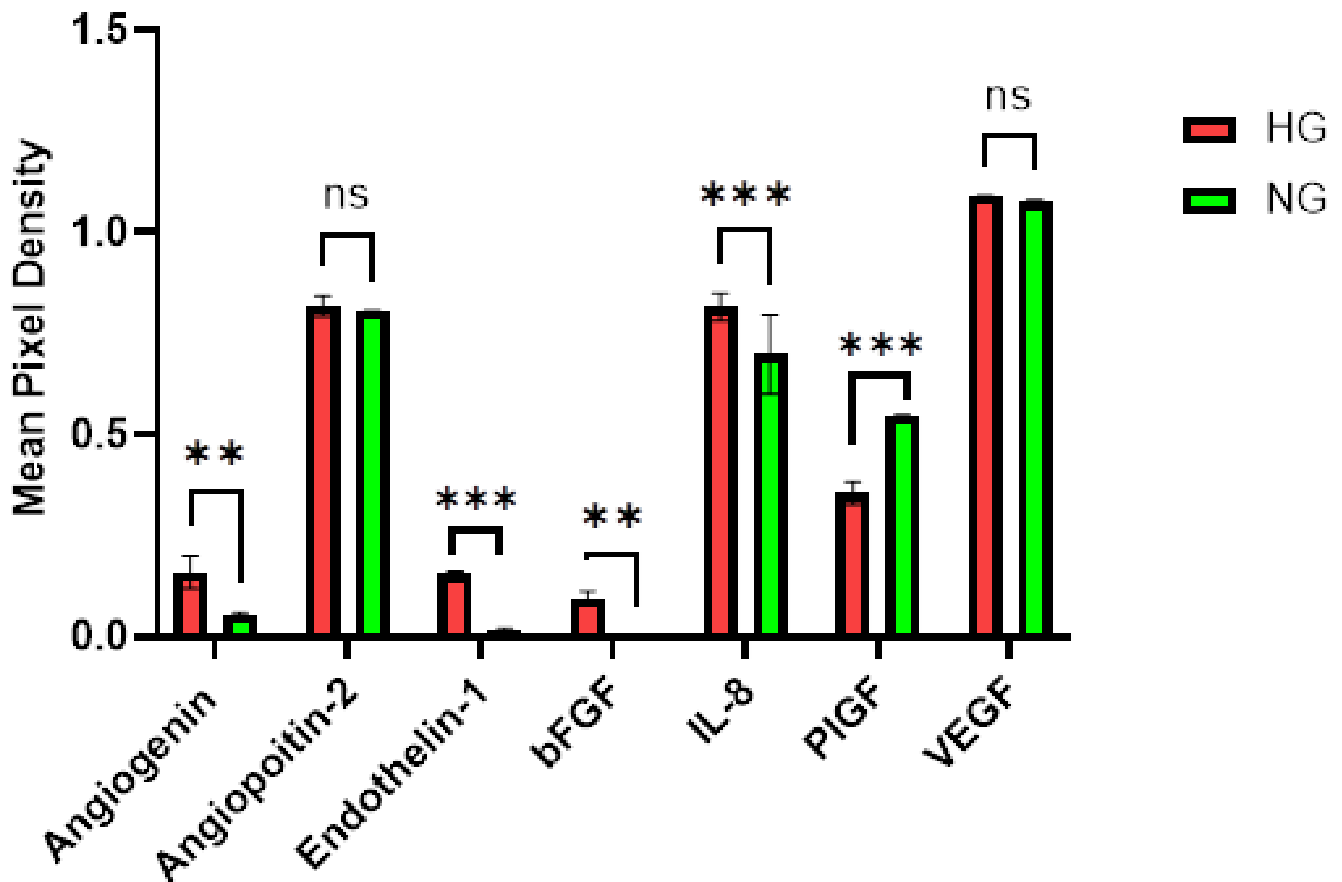

***Figure 10 Comparison of angiogenic factors expression under different conditions**.*

This bar chart illustrates the expression levels of different angiogenic factors, which are represented on the x-axis. The y-axis shows the normalized pixel density or fold difference, serving as a measure of the expression level of each angiogenic factor. Two bars are shown for each factor, representing the High Glucose (HG) and Normal Glucose (NG) conditions, respectively. This comparison offers insights into how glucose levels may influence the expression of various angiogenic factors. Each data point represents the mean of three independent replicates (n=3), with error bars indicating the standard error mean.

3.1.8 Responses of Pericytes to Endothelial Cell-derived secretome under hyperglycaemic conditions: Analysis of cytokine expression

The study aimed to evaluate the influence of the secretome (secreted molecules) from endothelial cells exposed to hyperglycaemic conditions on the release of cytokines by pericytes. The secretome was obtained by subjecting endothelial cells to experimental conditions for three days, condensing the secretome, and then diluting it in bulk. The diluted secretome was subsequently exposed to fully confluent pericytes.

As demonstrated in Figure 11, exposure to the HG condition significantly decreased the secretion of CCL2/MCP-1, ICAM-1, and IL-8 by pericytes in response to the HG-conditioned secretome, with p-values of ≤ 0.001. Conversely, Macrophage Migration Inhibitory Factor (MIF) levels were significantly elevated under HG conditions, also with a p-value of ≤ 0.001.

No significant changes were noted in the secretion of CXCL2/GRO, IL-6, and Serpin levels.

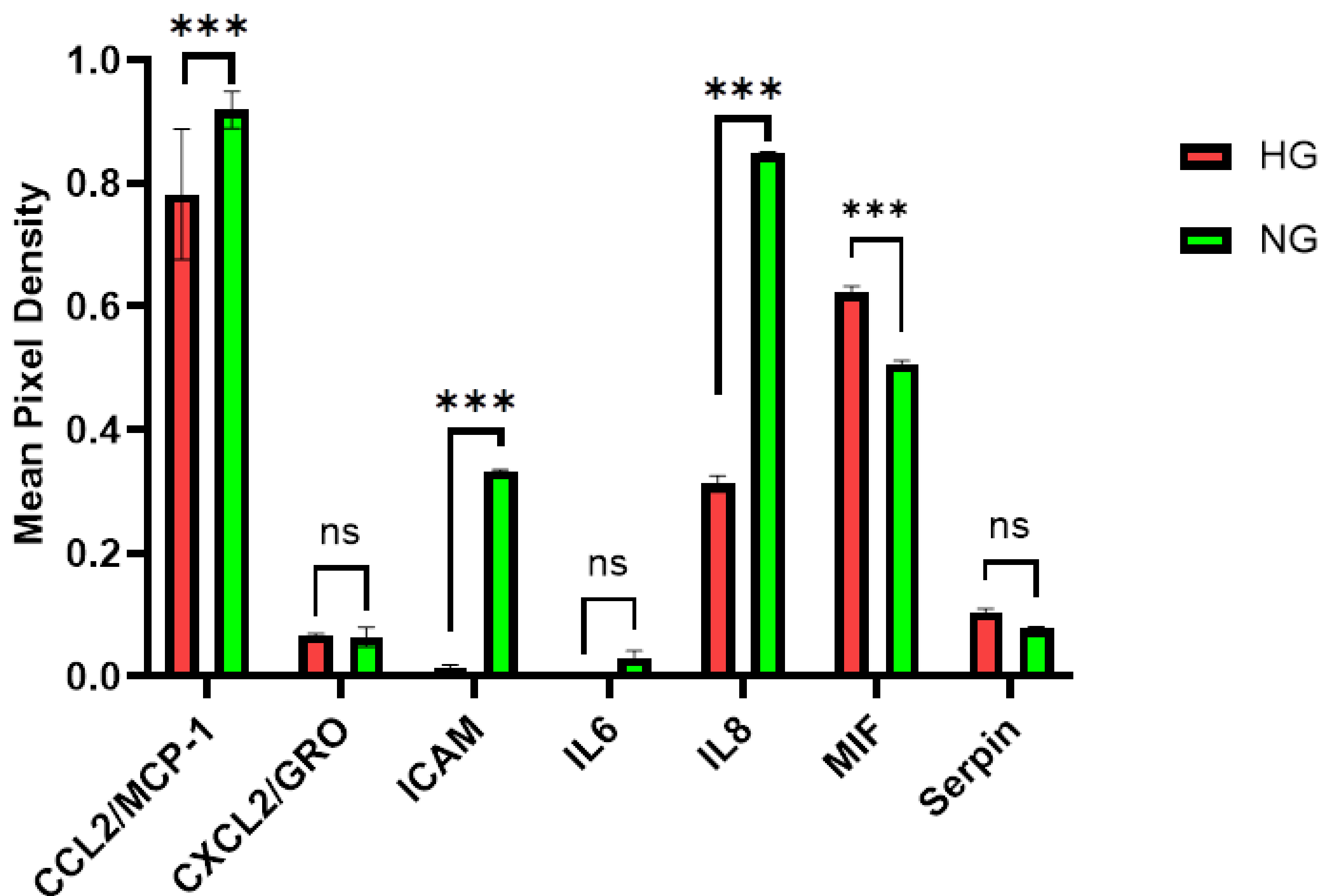

Figure 11 Cytokine Profiling of Pericyte secretory molecules when exposed to treated Endothelial Cell Secretome.

This bar chart displays the expression levels of various cytokines, represented on the x-axis, in the secretome of endothelial cells (EC) acting on pericytes. The y-axis shows the normalized pixel density or fold difference, serving as a measure of the expression level of each cytokine. Two bars are shown for each cytokine, representing the High Glucose (HG) and Normal Glucose (NG) conditions, respectively. Data have been normalized to the NG condition to facilitate direct comparison. Each data point represents the mean of three independent replicates (n=3), with error bars indicating the standard error mean. This assay was conducted using a protein profiler kit, providing insights into the influence of glucose conditions on the cytokine profile of the EC secretome on pericytes.

3.1.9 Evaluating the Impact of hyperglycaemia on blood-brain barrier structure and function: Findings from TEER measurements and permeability to Evans Blue Albumin and Sodium Fluorescence

The study aimed to investigate the effects of HG, NG, and D-mannitol on the permeability of BBB over three days. The mean differences between the groups were compared using Tukey's multiple comparisons test.

On Day 2, the study drew several comparisons between the HG, NG, and D.mannitol groups.

Firstly, between the HG and NG groups, the mean difference in blood-brain barrier (BBB) permeability was -3.778, with a 95% Confidence Interval (CI) of -6.136 to -1.419. The adjusted p-value was 0.0012, indicating a statistically significant difference. Interestingly, the BBB permeability was found to be lower in the HG group compared to the NG group.

Secondly, comparing the HG and D.mannitol groups, the mean difference in BBB permeability was -7.833, with a 95% CI of -10.19 to -5.475. The adjusted p-value was less than 0.0001, signifying a highly significant difference. Again, the BBB permeability was lower in the HG group compared to the D.mannitol group.

Lastly, between the NG and D.mannitol groups, the mean difference in BBB permeability was -4.056, with a 95% CI of -6.414 to -1.697. The adjusted p-value was 0.0006, suggesting a highly significant difference (***). The BBB permeability was lower in the NG group compared to the D.mannitol group.

On Day 3, similar comparisons were conducted.

In the HG vs. NG comparison, the mean difference in BBB permeability was -11.11, with a 95% CI of -13.47 to -8.752. The adjusted p-value was less than 0.0001, indicating a highly significant difference (***). The BBB permeability was lower in the HG group compared to the NG group.

In the HG vs. D.mannitol comparison, the mean difference in BBB permeability was -13.89, with a 95% CI of -16.25 to -11.53. The adjusted p-value was less than 0.0001, indicating a highly significant difference (***). The BBB permeability was lower in the HG group compared to the D.mannitol group.

Finally, in the NG vs. D.mannitol comparison, the mean difference in BBB permeability was -2.778, with a 95% CI of -5.137 to -0.4191. The adjusted p-value was 0.0183, indicating a significant difference (*). The BBB permeability was lower in the NG group compared to the D.mannitol group.

EBA

The results from the analysis conducted on the Evans Blue Albumin (EBA) measurements. EBA is a marker for macromolecular passage across cellular barriers, and understanding its behaviour under different treatment conditions is imperative.

The F-value is 0.09280. Since the p-value associated with this F-value is 0.9127, which is higher than the typical significance level of 0.05, we can conclude that there is no statistically significant difference among the means of the analysed data sets.

Since the p-value is not less than 0.05, we can conclude that there is no significant difference among the means. Therefore, the treatments or experimental conditions being compared do not appear to have a significant effect on the functional assessment of macromolecular passage by Evans blue albumin.

NAF

The sodium fluorescence (NaF) analysis was conducted to evaluate the micromolecular passage of molecules.

An analysis of variance (ANOVA) was performed to determine the significance of the differences among the means of the analysed data sets. The ANOVA summary revealed a highly significant result, with an F-value of 477.8 and a p-value of <0.0001. This indicates a statistically significant difference among the means of the treatments.

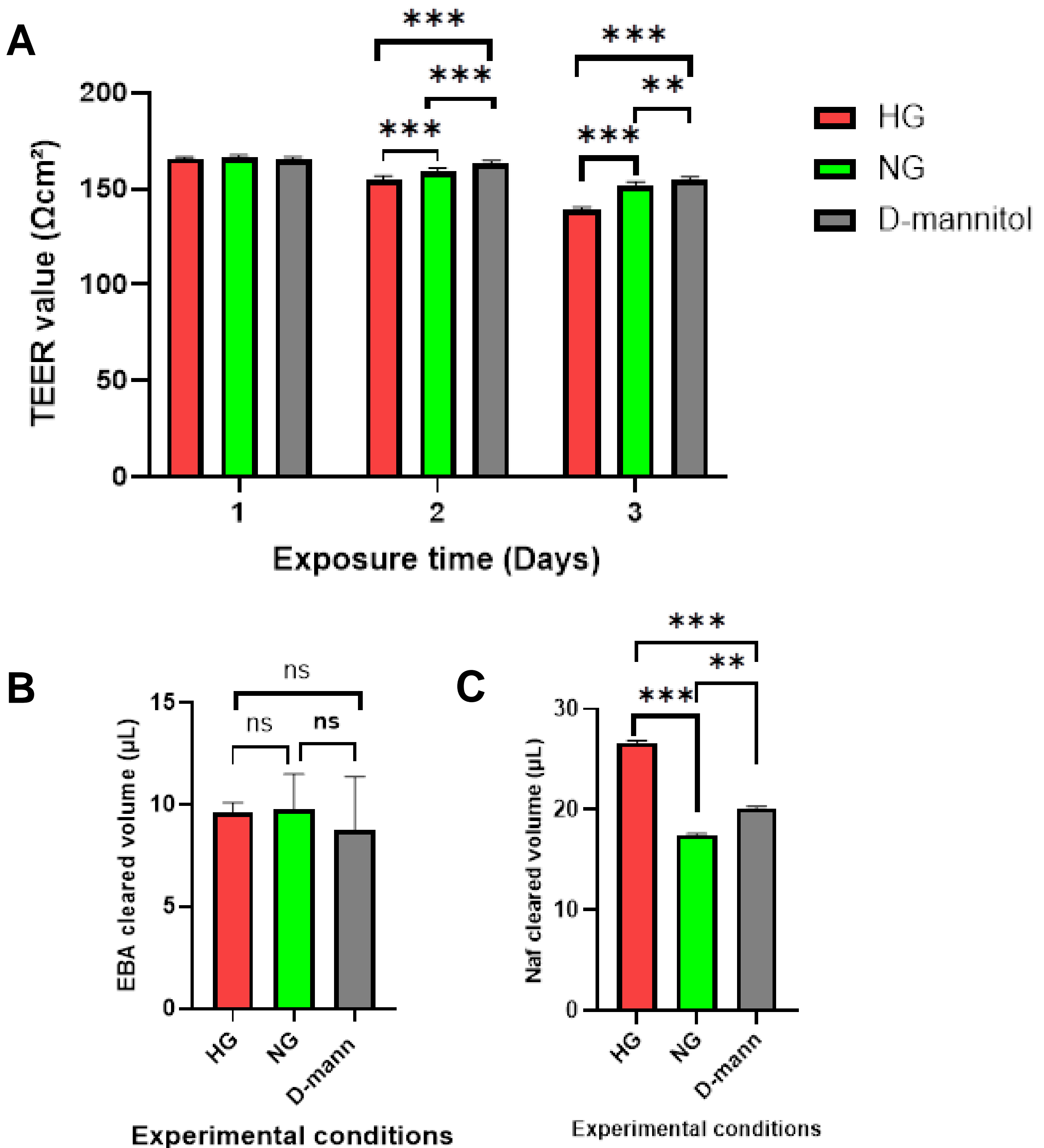

Figure 12 Impact of different conditions on BBB structure and permeability.

The first chart (A) shows the impact of High Glucose (HG), Normal Glucose (NG), and D-mannitol conditions on the structure of the blood-brain barrier (BBB), as measured by trans-endothelial Electrical Resistance (TEER, in Ohm cm^2). The y-axis represents TEER values, and the x-axis shows the exposure time in days. The second chart (B) presents Evans Blue Albumin (EBA) clearance (in microliters) under HG, NG, and D-mannitol conditions. The y-axis represents EBA clearance values, and the x-axis shows the experimental conditions. The third chart (C) displays Sodium Fluoride (NaF) clearance (in microliters) under HG, NG, D-mannitol. The y-axis represents NaF clearance values, and the x-axis shows the experimental conditions. Each data point represents the mean of three independent replicates (n=3), and the error bars indicate mean error. Asterisks (*) denote statistically significant differences between conditions. These charts provide insights into the effects of glucose levels and D-mannitol on BBB integrity and permeability.

3.1.10 Deciphering the Influence of Treated Endothelial Cell-Derived Secretome on Blood-Brain Barrier Integrity and Function: Findings from TEER measurements and permeability to Evans Blue Albumin and Sodium Fluorescence

The results of Tukey's multiple comparisons test provide additional insights into the specific pairwise differences between the experimental conditions (HG, NG, and d-mannitol) at different time points (2 hours, 6 hours, 10 hours, and 21 hours).

These findings provide insights into the specific time-dependent effects of glucose and d-mannitol on the in vitro BBB model under investigation.

The results of these comparisons are:

At the 2-hour mark, no significant differences were observed in any of the pairwise comparisons, namely HG vs. NG (mean difference = -1.833, 95.00% CI = -7.554 to 3.887, ns, adjusted p = 0.6444), HG vs. D.mannitol (mean difference = -5.222, 95.00% CI = -11.08 to 0.6371, ns, adjusted p = 0.0798), and NG vs. D.mannitol (mean difference = -3.389, 95.00% CI = -7.529 to 0.7515, ns, adjusted p = 0.1110).

However, at 6 hours, significant differences emerged between HG and NG (mean difference = -9.833, 95.00% CI = -12.83 to -6.838, ***, adjusted p < 0.0001), and HG and D.mannitol (mean difference = -8.167, 95.00% CI = -10.42 to -5.915, ***, adjusted p < 0.0001). Notably, NG and D.mannitol did not exhibit any significant differences (mean difference = 1.667, 95.00% CI = -1.425 to 4.758, ns, adjusted p = 0.3327).

At the 10-hour point, significant differences persisted between HG and NG (mean difference = -4.889, 95.00% CI = -9.506 to -0.2719, *, adjusted p = 0.0389), and HG and D.mannitol (mean difference = -5.278, 95.00% CI = -10.55 to -0.004676, *, adjusted p = 0.0498). As before, no significant difference was observed between NG and D.mannitol (mean difference = -0.3889, 95.00% CI = -4.912 to 4.134, ns, adjusted p = 0.9684).

Finally, at 21 hours, significant differences were observed between HG and NG (mean difference = -8.111, 95.00% CI = -14.28 to -1.942, *, adjusted p = 0.0123), and HG and D.mannitol (mean difference = -9.833, 95.00% CI = -15.25 to -4.418, **, adjusted p = 0.0028). Yet again, no significant difference was observed between NG and D.mannitol (mean difference = -1.722, 95.00% CI = -6.567 to 3.123, ns, adjusted p = 0.5761).

EBA

An Analysis of Variance was performed to assess the significance of differences among the data sets. The ANOVA summary revealed an F-value of 0.08814, with a corresponding p-value of 0.9168 The non-significant p-value suggests that there is no statistically significant difference among the means of the EBA measurements across the various treatment conditions. In other words, the differences observed in EBA measurements are likely due to random chance rather than the effect of the treatment conditions.

NaF

The findings of the sodium fluorescence analysis were conducted to assess the micromolecular passage of molecules. This analysis was crucial to understand the impact of various treatment conditions on the micromolecular transport across the cellular barriers.

To determine the statistical significance of the differences among the means of the analysed data sets, an analysis of variance (ANOVA) was performed. The ANOVA summary revealed an F-value of 2.026 with a corresponding p-value of 0.2267. The p-value was above the commonly accepted threshold for statistical significance ($p > 0.05$). This non-significant p-value indicates that there is no statistically significant difference among the means of the treatment groups.

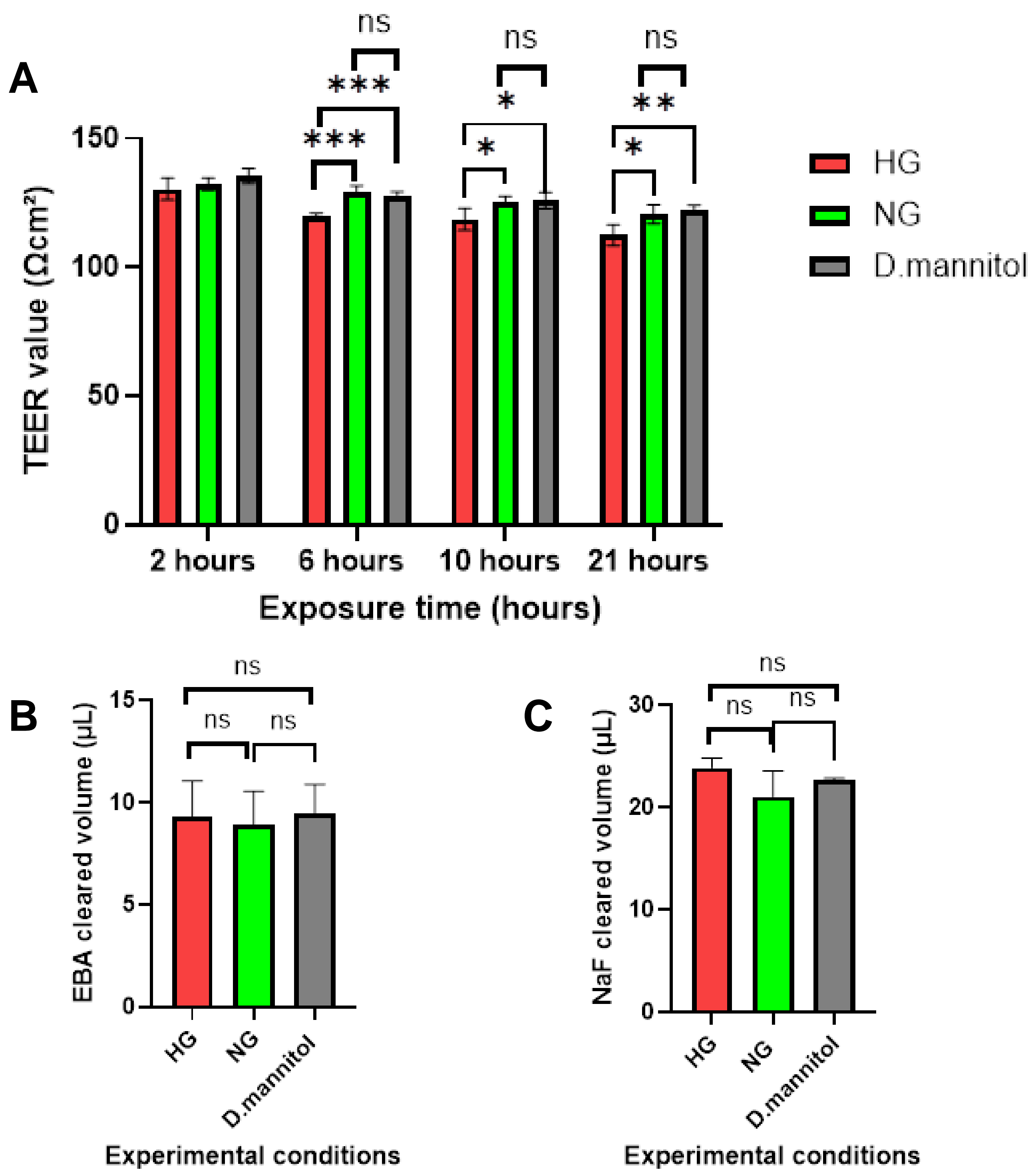

Figure 13 Impact of HMEC Secretome on BBB Structure and Permeability.

The first chart (A) illustrates the effect of the Human Microvascular Endothelial Cell (HMEC) secretome on the structure of the blood-brain barrier (BBB), as measured by trans-endothelial Electrical Resistance (TEER, in Ohm cm^2). The y-axis represents TEER values, while the x-axis denotes the exposure time in hours. The second chart (B) demonstrates Evans Blue Albumin (EBA) clearance (in microliters) following exposure to the HMEC secretome. The y-axis displays EBA clearance values, and the x-axis represents the experimental conditions. The third chart (C) depicts Sodium Fluoride (NaF) clearance under the same conditions. The y-axis shows NaF clearance values, and the x-axis outlines the experimental conditions. Each data point represents the mean of three independent replicates (n=3), and the error bars indicate mean error. Asterisks (*) denote statistically significant differences between conditions. These charts provide insights into the effects of the HMEC secretome on BBB integrity and permeability.

3.1.11 Evaluating the Localization of Zonula Occludens-1 under Normal and hyperglycaemic Conditions

The localization of zonula occludens-1 (ZO-1), a key tight junction protein, was qualitatively assessed in endothelial cells. These cells were cultured under four different conditions: HG, NG, D.mannitol control, and endothelial cell medium (ECM) conditions. The assessment was performed via immunofluorescence staining and imaging to understand the impact of these different conditions on ZO-1 localization.

In the endothelial cells cultured under ECM conditions, ZO-1 displayed a prominent linear localization at the cell borders, outlining intact cell-cell contacts. This pattern indicates well-organized tight junctions and suggests that the ECM conditions support normal tight junction structure.

The endothelial cells cultured under NG and mannitol conditions exhibited similar ZO-1 junctional patterns to those seen under ECM conditions. The uniform ZO-1 localization further suggests that these conditions do not disrupt the normal organization of tight junctions.

However, the endothelial cells cultured under HG conditions revealed a different pattern. The ZO-1 staining was abnormal, possessing discontinuous patterns, with visible gaps between cells. This disrupted pattern suggests that high glucose conditions might lead to disruption of tight junctions, affecting their normal functioning.

The quantitative analysis of ZO-1 fluorescence intensity in different experimental settings showed a marked reduction in the quantities of this particular protein in cells subjected to HG (Figure 14 I). In contrast, no statistically significant changes were observed between HG and the D-mannitol groups.

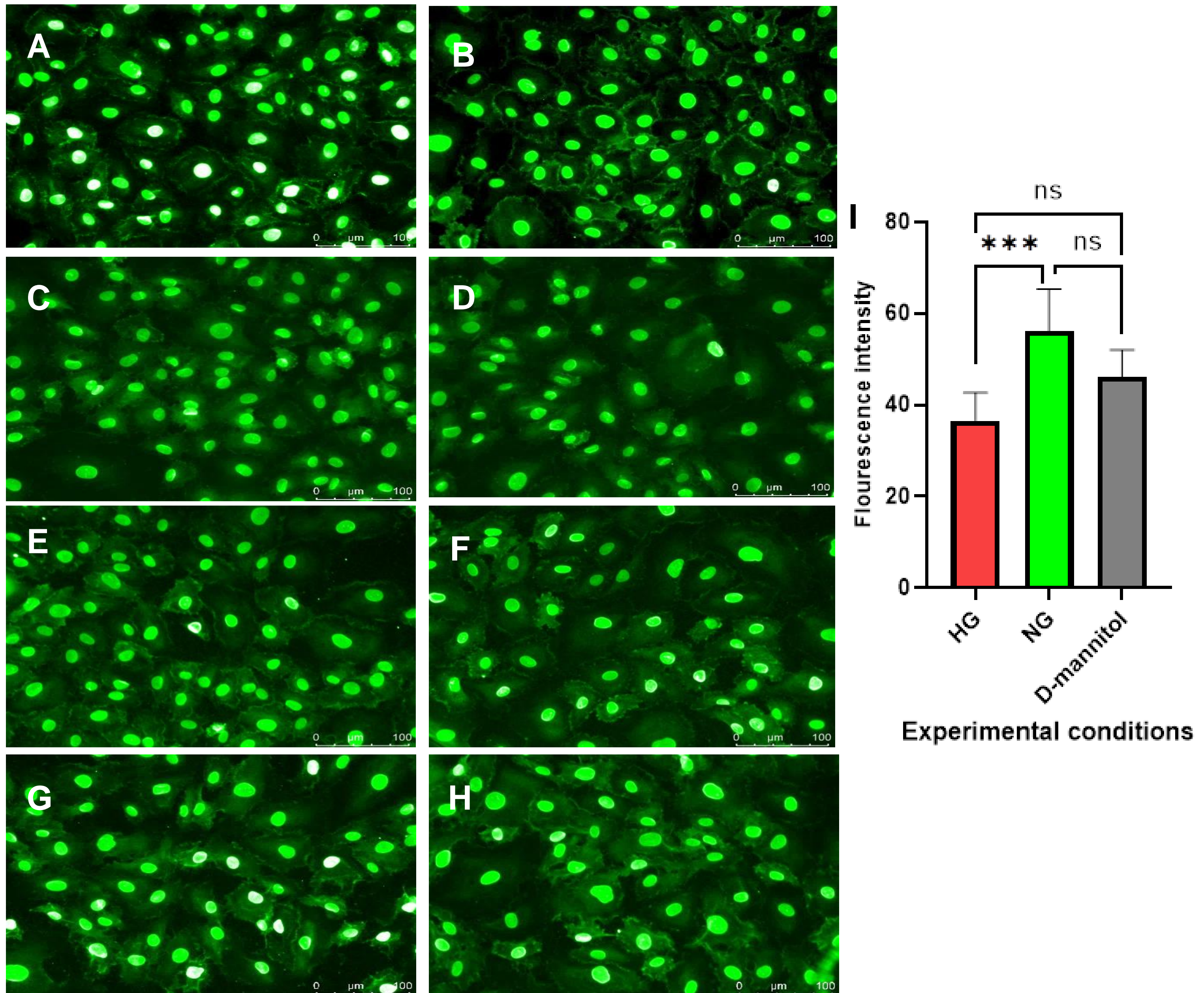

Figure 14 Analysis of ZO-1 Localization Under Various Conditions.

This figure includes immunofluorescence images of ZO-1 localization in cells under Endothelial Cell Medium (A,B), High Glucose (C,D), Normal Glucose (E,F), and D-mannitol (G,H) conditions. The graph (I) displays the comparative ZO-1 fluorescence intensity in cells treated with High Glucose, Normal Glucose, and D-mannitol. The images aim to provide a visual comparison of ZO-1 distribution and localization under these different conditions. In a typical healthy situation, ZO-1, a tight junction protein, should exhibit a continuous and linear distribution along the cell borders, indicating well-structured and intact tight junctions. Fluorescence intensity, reflecting ZO-1 expression levels, is plotted against the treatment groups. Each data point represents the mean of three independent replicates (n=3), and the error bars indicate mean error

4 Discussion

The escalating prevalence of hyperglycaemia, particularly in the context of diabetes, presents a critical concern for public health globally. Forecasts suggest that the diabetic population could exceed 600 million individuals by 2040 (Ogurtsova, da Rocha Fernandes et al. 2017), with a notably high prevalence among individuals suffering from cardiovascular diseases. This trend is concerning, especially considering the adverse outcomes associated with hyperglycaemia in patients with cardiovascular complications (Elgebaly, Ogbi et al. 2011). Through comprehensive analysis, the research aims to uncover mechanisms underlying vascular health compromise due to hyperglycaemia, potentially guiding targeted therapeutic strategies. our observations indicate no discernible morphological differences, a reduction in PDGF in pericytes, an increase in number of stress fibers, and no changes in DNA damage markers, tubulogenic parameters, or wound healing. However, alterations in endothelial cell angiogenic markers and subsequent changes in pericyte cytokine marker expression were noted, along with modifications in the structural and functional capabilities of the blood-brain barrier and ZO-1 localization and distribution.

4.1.1 Hyperglycaemia and cellular morphology: Absence of observable changes in Endothelial Cells and Pericytes under light microscopy:

After conducting a thorough study of Human Brain Microvascular Endothelial Cells and pericytes in different experimental conditions, we observed no discernible differences in morphology between the HG, NG, and D-Mannitol groups for both cell types. To be more specific, HBMEC maintained their typical endothelial cell cobblestone shape in all experimental conditions. Normal endothelial cells, which have a cobblestone shape, were stable despite changes in glucose levels and the presence of D-Mannitol. This surprising consistency in HBMEC shape highlights their powerful resistance to morphological modifications even under diverse glucose circumstances and contradicts the traditional notion of how glucose fluctuations could affect the structural integrity of endothelial cells. Our findings are in line with those of an external research group, who also investigated the effects of high glucose conditions on endothelial cell morphology. In their study, similar to ours, they found no substantial changes in the morphology of endothelial cells when exposed to high glucose conditions. (Turner, Ball et al. 2001).

We found a similar pattern when we looked at PC. Despite being exposed to different glucose concentrations, pericytes maintained a consistent and uniform morphology regardless of whether they were in the HG, NG, or D-Mannitol groups. PCs are a key part of the circulatory system, and the fact that their shape doesn't change much over time demonstrates their intrinsic flexibility and robustness.

However, we did notice a degree of cell loss in all treatment groups compared to initial seeding densities. This cell loss could potentially be attributed to using DMEM as the culture medium. DMEM is a less defined medium that may not fully support long-term cell health and viability compared to specialized endothelial or pericyte growth media.

Interestingly, pericytes appeared much more resilient to this cell loss induced by DMEM. PC cultures exhibited very minimal changes in cell numbers between treatment groups. This suggests pericytes have an enhanced ability to withstand suboptimal culture conditions relative to endothelial cells.

The consistent morphology but observed cell loss indicates that while HBMECs and PCs can maintain their characteristic cellular shapes under different glucose environments, external factors like medium composition can still negatively impact long-term cell survival. Further experiments optimizing culture conditions are needed to draw definitive conclusions about hyperglycaemia's direct effects on vascular cell integrity over longer time periods.

4.1.2 hyperglycaemia-Induced PDGFR-β Upregulation in Pericytes

To investigate the effect of high glucose treatment on the expression of platelet-derived growth factor receptor beta (PDGFR-β), particularly in the context of pericyte involvement in diabetes. The results have significantly expanded our understanding of the impact of hyperglycaemia on PDGFR-β expression and the resulting implications for capillary health and the development of diabetes and stroke.

The most significant finding from this study is the marked increase in PDGFR-β expression in high glucose conditions. This increase was statistically significant when compared with both the normal glucose ($p=0.0008$) and mannitol control groups ($p<0.0001$), which suggests that elevated glucose levels exert a profound effect on PDGFRβ expression. This observation supports the hypothesis that chronic hyperglycaemia, a defining characteristic of diabetes, can lead to enhanced expression of PDGFR-β, thereby influencing pericyte recruitment and function.

Our research aligns with previous studies that have demonstrated the crucial role of PDGF-BB, the ligand for PDGFR-β, in pericyte recruitment during vascular development (Winkler, Bell et al. 2010). Pericytes, specialized cells that encase blood vessels, are critical for maintaining the stability and function of the micro-vasculature. Their loss or dysfunction has

been implicated in the pathogenesis of various vascular diseases, including diabetic retinopathy (Wang and Lo 2018).

The increase in PDGFR-β expression in high glucose conditions could suggest an attempt by endothelial cells to recruit more pericytes in response to hyperglycaemic stress. This may be a compensatory mechanism aimed at preserving the structural integrity of blood vessels. However, further studies are required to elucidate the precise mechanisms behind the increased PDGFR-β expression in high glucose conditions and its impact on pericyte behaviour.

Pericytes are crucial in maintaining the stability and functionality of various vascular beds in the body, including the brain, kidney, heart, lung, and adipose tissue (Corliss, Ray et al. 2020). Studies using mice deficient in PDGF-BB or PDGF-receptor-β have shown a notable absence of microvascular pericytes in brain vessels, leading to the formation of capillary microaneurysms (Levéen, Pekny et al. 1994). These microaneurysms can cause cerebral haemorrhages during late gestation (Smyth, Highet et al. 2022).

When diabetes is induced in rodents, there is a decrease in the number of pericytes in retinal capillaries, followed by the formation of microaneurysms, and thickening of the vascular basement membrane (Levéen, Pekny et al. 1994). As the disease progresses in the human diabetic eye, further vascular occlusions can occur, leading to increased vascular permeability and the development of macular oedema. Additionally, new blood vessels can form and proliferate into the vitreous, changes which can ultimately result in vision loss.

The precise cause of pericyte loss in the early stages of diabetic retinopathy remains unclear. One theory is that it may be related to the accumulation of toxic byproducts, such as sorbitol or advanced glycation end products (AGEs), within the pericytes (Ahmed 2005). Pericyte loss is thought to be a prerequisite for the formation of microaneurysms, potentially due to the local weakening and bulging of the capillary wall (Hammes, Lin et al. 2002). Pericytes also regulate the proliferation of endothelial cells, the cells lining the inner surface of blood vessels. This suggests that loss of pericytes may contribute to the development of proliferative diabetic retinopathy, a more advanced stage of the disease characterized by the proliferation of new blood vessels.

4.1.3 Unaltered DNA damage response in hyperglycaemic conditions: A discussion on the non-significant differences in γ-H2AX levels in Endothelial Cells

The analysis of gamma H2AX data presented a nuanced perspective on the genomic stability of diabetic endothelial cells under HG, NG, and D-mannitol osmolar control conditions. The ANOVA summary indicated that there were no significant differences in γH2AX levels among the treatment groups, with an F-statistic of 0.2250 and a p-value of 0.8050.

The non-significant differences in γH2AX levels suggest that, under the experimental conditions tested, alterations in glucose concentrations did not induce significant DNA damage in ECs. This unexpected result prompts further exploration into the mechanisms underlying genomic stability in diabetic ECs. It is possible that ECs possess robust DNA repair mechanisms, allowing them to mitigate DNA damage effectively even under hyperglycaemic conditions.

These findings have significant implications for our understanding of diabetic vascular complications. While hyperglycaemia is widely recognized as a contributor to DNA damage and subsequent complications, our results suggest that the relationship between glucose variations and DNA damage in ECs might be more complex than previously thought. The absence of observable differences in γH2AX levels challenges the conventional understanding of hyperglycaemia-induced DNA damage in ECs, prompting a re-evaluation of the factors contributing to genomic stability in diabetic conditions.

One possible explanation for these results could be the efficient DNA repair mechanisms in ECs that counteract the potential damage induced by high glucose levels. It is essential to investigate the specific DNA repair pathways and repair proteins involved in maintaining genomic stability in diabetic ECs. Additionally, exploring the role of oxidative stress, glycolysis-dependent repair mechanisms, and DNA damage response pathways in this context could provide valuable insights into the cellular adaptations that prevent excessive DNA damage in diabetic conditions.

Furthermore, these findings underscore the need for a comprehensive understanding of the interplay between metabolic abnormalities, DNA repair processes, and genomic stability in diabetic ECs. The intricate balance between these factors likely determines the overall health of the vascular system in diabetes. Future studies should focus on elucidating the specific pathways involved in preserving genomic stability in ECs under hyperglycaemic conditions. Additionally, investigating the impact of long-term exposure to hyperglycaemia on DNA repair

and genomic stability could provide critical information for developing targeted therapeutic interventions aimed at preserving vascular health in diabetic individuals.

4.1.4 Hyperglycaemia induced actin cytoskeletal reorganization and its effects on endothelial cell barrier function

Stress fibres, composed of actin microfilaments, play a crucial role in the cell's structural framework. They interact intimately with microtubules and intermediate filaments to dictate cell shape (Prasain and Stevens 2009)). In quiescent endothelium, a state when the cells are at rest and not activated, actin forms a cortical rim (Lee and Gotlieb 2002).This rim interacts with both cell-cell and cell-matrix adhesion complexes, tethering these structures to intracellular organelles. This process is essential for maintaining a functional endothelial cell barrier (Phillips, Lum et al. 1989).

Our experimental investigations have shed light on the impact of different glucose conditions on stress fibre formation in endothelial cells. The evidence gathered suggests that high glucose conditions significantly enhance the formation of actin stress fibres. These actin stress fibres are known to reorganize from a cortical actin distribution into fibres that traverse the entire cell body in response to inflammatory agonists. This reorganization causes an increase in centripetal tension, which in turn instigates the retraction of cell-cell borders into discernible gaps, thereby compromising the integrity of the endothelial cell barrier (Prasain and Stevens 2009).

Stress fibres, by affecting the rate and size of inter-endothelial cell gaps, play a pivotal role in either maintaining or disrupting the barrier function. Our findings underscore that high glucose conditions can initiate this disruptive process, thus emphasizing the importance of glucose regulation in preserving vascular health. Further research in this area could steer the development of therapeutic strategies aimed at mitigating the vascular complications associated with hyperglycaemia.

4.1.5 The unexpected lack of significant differences in γH2AX expression between treatment groups

The goal of this study was to understand the impact of hyperglycaemia on the DNA damage response in brain endothelial cells by quantifying γH2AX levels under normal and high glucose conditions. Contrary to our expectation, the results did not indicate significant differences in γH2AX expression between the treatments.

Several limitations of our study may have contributed to these findings. Firstly, our in vitro model employed a static 2D system, which does not account for the flow shear stresses that affect endothelial physiology in vivo. Secondly, our analyses were restricted to a single timepoint, potentially missing dynamic changes over the progression of the disease.

Moreover, our monoculture model did not include interactions with supporting cells like pericytes and astrocytes, which contribute significantly to endothelial responses. A more advanced neurovascular co-culture platform incorporating flow might more accurately recapitulate the in vivo neurovascular niche.

The lack of a significant effect in our study does not definitively prove that hyperglycaemia does not cause DNA damage. Other mechanisms not involving γH2AX phosphorylation could be at play. Chronic hyperglycaemia experienced clinically could accumulate DNA lesions over time through various pathways, which were not captured in our acute exposure model. Additionally, DNA damage response may vary depending on the cell type, and testing other relevant cells such as pericytes or tubular epithelial cells could provide more comprehensive insights.

In conclusion, while our study did not find evidence of DNA damage based on γH2AX levels following acute high glucose exposure, more evidence is required before we can conclude that glucose is completely benign to the endothelial genome in the short term.

4.1.6 Observations from tube formation assay reflecting no significant impact on key parameters of tubulogenesis:

Angiogenesis, the formation of new blood vessels, is a finely orchestrated event in the vast terrain of vascular biology. It's regulated by a complex interplay of pro- and anti-angiogenic signals. Over the past two decades, significant progress has been made in understanding the underlying molecular mechanisms of angiogenesis. Integrins, chemokines, angiopoietins, oxygen-sensing agents, junctional molecules, and endogenous inhibitors all play crucial roles in regulating angiogenesis (Bouïs, Kusumanto et al. 2006). Proangiogenic signals including basic fibroblast growth factor, vascular endothelial growth factor, platelet-derived growth factor, and epidermal growth factor, activate receptors on endothelial cells, triggering a cascade of reactions (Ausprunk and Folkman 1977).Activated endothelial cells secrete proteases, enzymes that degrade the basement membrane, permitting cell migration. This removal of the barrier enables rapid proliferation and migration of endothelial cells, resulting in sprouts that can extend several millimetres within a day (Chung, Lee et al. 2010).

Angiogenesis is underpinned by the remarkable ability of endothelial cells to proliferate, migrate, and differentiate in response to angiogenic cues.

The tube formation assay is a cornerstone in angiogenesis research. Developed in 1988, it leverages the inherent abilities of endothelial cells to proliferate and migrate when exposed to angiogenic stimuli (Kubota, Kleinman et al. 1988, Arnaoutova and Kleinman 2010). Cells are placed in a Matrigel-coated well, where Matrigel, composed of natural extracellular matrix components, provides a nutrient-rich environment promoting cell proliferation. The tubes that form, characterized by a central lumen surrounded by interconnected endothelial cells linked by junctional complexes, are indicators of angiogenic activity.

The results from the tube formation assay revealed no significant differences among the HG, NG, and D-mannitol treatment groups across all examined parameters of tubulogenesis. Both the number of nodes/branching sites and junctions, representing key components of vascular branching, showed no significant variation attributable to the treatments. Additionally, the size of mesh areas, representing the enclosed spaces within the tubular network, and the total length and total segment length of the tubes, indicative of the overall extent and complexity of the tubular network, also did not significantly differ among the treatments. Adjusted p-values for all comparisons were notably high, further supporting the notion that the treatments did not significantly influence tubular network formation and organization under the experimental conditions. These findings suggest that HG, NG, and D-mannitol treatments may not substantially affect angiogenesis in the context of the parameters measured in this study.

Although the current study did not demonstrate significant effects of the treatments on tubulogenesis parameters they actually contribute valuable information to our understanding of angiogenesis. They suggest that the process is robust under a variety of conditions, and that changing glucose levels or using osmotic controls like D-mannitol may not be sufficient to disrupt it. This may inform future research directions, suggesting a need to explore other regulatory factors or conditions that can more significantly influence angiogenesis. It's also possible that these treatments might have more profound effects under different experimental conditions, or that they might affect other aspects of angiogenesis not captured by the parameters measured in this study.

4.1.7 Wound scratch assay reveals no significant progress in wound closure during the Initial 12 Hours

The wound scratch assay serves as a valuable tool for investigating the cellular and molecular mechanisms underlying cell migration and wound healing. Despite its simplicity and cost-effectiveness, the assay offers important insights into complex biological processes such as tissue repair and angiogenesis (Liang, Park et al. 2007). The assay involves creating a 'scratch' or 'wound' in a monolayer of cells, simulating a tissue injury, and then observing the cells' migration into the gap, thus evaluating their wound healing capabilities.

In the current study, our results showed no significant difference in the percentage of wound closure between 0 hours and 12 hours. This suggests that within the first 12 hours of the experiment, the wound healing process, as measured by the wound scratch assay, had not significantly progressed. However, it should be noted that the sample size was quite small (n=3), which might have limited the statistical power of our tests.

Furthermore, we observed the levels of pro-angiogenic factors including Angiogenin, Angiopoietin-2, FGF basic, and VEGF in our samples. suggesting an upregulation of these factors in our samples. Conversely, FGF basic showed a decrease in the HG group compared to the NG group

Interestingly, these results corroborate the findings from our wound scratch assay. The observed increase in pro-angiogenic factors, particularly Angiogenin and Angiopoietin-2, is consistent with the initiation of the wound healing process, which involves angiogenesis or the formation of new blood vessels. The upregulation of these factors is likely to promote cell migration and wound closure, which is captured in our wound scratch assay. Therefore, despite the lack of a significant difference in wound closure between 0 and 12 hours, the overall trend in our data suggests a positive correlation between the wound healing process and the increase in pro-angiogenic factors.

The wound scratch assay, also known as the in vitro scratch assay or migration assay, is a commonly used experimental technique to assess cell migration and wound healing capabilities. It is primarily used to study the processes involved in angiogenesis, tissue repair, and cell migration (Lamalice, Le Boeuf et al. 2007).

It is important to note that the wound scratch assay has certain limitations. The scratch itself can induce cell damage, which may trigger additional cellular responses unrelated to migration. Additionally, the assay primarily assesses two-dimensional cell migration, which may not fully represent the complex processes involved in in vivo tissue repair and

angiogenesis. Therefore, complementary assays, such as transwell migration assays or three-dimensional culture models, are often used to provide a more comprehensive understanding of cell migration and angiogenic processes.

4.1.8 Delving into the angiogenic landscape: A detailed discussion on the insights and implications from proteome profiler angiogenesis array results

In our endeavour to understand the intricate mechanisms of angiogenesis modulation, we adopted an experimental approach that involved exposing Human Brain Microvasculature Endothelial Cells to high glucose conditions for a period of three days. The subsequent analysis of the secretome, performed using a Human Angiogenesis Proteome Profiler Kit, provided intriguing insights into the cellular changes instigated by this modified environment. Chronic hyperglycaemia has been proven to initiate a series of events, including oxidative stress, inflammation, and endothelial dysfunction, which collectively create a microenvironment that supports aberrant angiogenesis, vascular leakage, and endothelial cell damage (Poznyak, Grechko et al. 2020).

Our study's key findings include the overexpression of angiogenin, a potent angiogenic factor, within the HBMEC secretome under HG conditions. Under HG stress, there was a noticeable change in angiogenin expression levels, suggesting a dynamic modulation of angiogenesis. This modulation could influence endothelial cell migration, proliferation, and the formation of capillary-like structures, indicating a cellular response aimed at stimulating angiogenesis (Tello-Montoliu, Patel et al. 2006). This response potentially serves as a compensatory mechanism to counteract the detrimental effects of HG, underscoring the secretome's role in enhancing angiogenic processes amidst metabolic challenges.

In a hyperglycaemic environment, the nuclear translocation of angiogenin orchestrates ribosomal RNA (rRNA) transcription, ribosome biogenesis, and cell growth, serving a crucial role in endothelial cell proliferation and neovascularization (Kishimoto, Liu et al. 2005). Downregulation of angiogenin hampers these processes, underscoring its essential role in angiogenesis. Moreover, the inhibition of angiogenin attenuates angiogenesis induced by other angiogenic factors, implying that angiogenin acts as a linchpin, coordinating the action of various angiogenic molecules in the diabetic microenvironment(Kishimoto, Liu et al. 2005).

Despite angiogenin's relatively weak activity compared to classical endothelial cell mitogens, it plays a significant role in stimulating endothelial cell migration and proliferation (Tello-Montoliu, Patel et al. 2006)). An interesting mechanism of action proposed for angiogenin is

that it exerts its proliferative effects by undergoing nuclear translocation in endothelial cells. This nuclear translocation is not a passive event but a critical process essential for angiogenin's biologic activity (Kishimoto, Liu et al. 2005). The inhibition of nuclear translocation abolishes angiogenin's angiogenic activities, highlighting the importance of this unique cellular event.

Angiopoietin-2 (Ang-2), recognized for its role in destabilizing blood vessels and promoting angiogenesis, hints at the intricate balance between vessel stabilization and formation in response to high-glucose stress. Elevated levels of Ang-2 in hyperglycaemic conditions could exacerbate these issues by promoting abnormal angiogenesis and vascular remodelling (Whipple and Korc 2010). The capacity of Ang-2 to disrupt the balance between vessel stabilization and destabilization could lead to leaky vessels, impaired blood flow regulation, and increased inflammation susceptibility. Over time, this vascular instability may contribute to the development and progression of diabetic complications, such as retinopathy, nephropathy, and cardiovascular diseases (Cade 2008).

Despite the high-glucose stress, Ang-2, a guardian of vascular integrity, displayed consistent expression (0.817 HG vs. 0.805 NG), emphasizing endothelial cells' dedication to maintaining vascular stability even under adverse conditions. This sustained expression of Ang-2 underscores its role in fine-tuning angiogenic responses, ensuring endothelial cell equilibrium and preventing excessive vessel growth or regression. When upregulated, Ang-2 destabilizes blood vessels, making them more susceptible to inflammatory cues and facilitating angiogenesis.

The angiopoietin-Tie system, consisting of angiopoietins (Ang-1 and Ang-2) and their corresponding receptors Tie-1 and Tie-2, is a pivotal regulator of endothelial cell survival, vascular maturation, and angiogenesis (Zhang, Kontos et al. 2019). This system plays a crucial role in the development of blood and lymphatic vasculature, making it a key player in various physiological and pathological processes, including placental development and angiogenesis in the context of diabetes.

In the broader context of vascular biology, the angiopoietin-Tie system is crucial for vessel maturation, maintaining blood vessel quiescence, and regulating vascular smooth muscle cell recruitment. Ang-2, in particular, plays a significant role in these processes. Angiopoietin-1 (Ang1) is widely recognized as a strict agonist of Tie2 receptor activation, promoting pro-survival signalling and the quiescence of the endothelium (Davis, Papadopoulos et al. 2003). When Ang1 binds to the Tie2 receptor, it triggers receptor dimerization and phosphorylation, thereby activating downstream signalling pathways that initiate angiogenesis. On the other

hand, Angiopoietin-2 (Ang2) has been shown to competitively inhibit Ang1-induced Tie2 activation, suggesting that it shares a single ligand-binding site on Tie2 with Ang1, and plays an antagonistic role. This means that when Ang2 binds to Tie-1/Tie-2 heterodimers, the receptor phosphorylation and subsequent Tie2 activation that typically follow Ang1 binding do not occur (Barton, Dalton et al. 2014). This contrast between the roles of Ang1 and Ang2 in Tie2 activation and endothelial cell signalling underscores the complexity of angiopoietin-Tie interactions.

Considering the critical roles of Ang-2 in angiogenesis and vascular stability, its dysregulation in hyperglycaemic conditions can have profound implications. Chronic hyperglycaemia in diabetes can disrupt the delicate balance of angiopoietin-Tie signalling, potentially contributing to vascular complications associated with the disease.

Our research has brought to light the dramatic increase in the levels of Endothelin-1 and FGF basic within the secretome of HBMEC when subjected to HG conditions. Renowned for its potent vasoconstrictive properties, Endothelin-1 also has multifaceted roles in angiogenesis, which include the regulation of endothelial cell proliferation and migration (Salani, Taraboletti et al. 2000). The marked increase in the presence of Endothelin-1 underscores the strong angiogenic signalling initiated by HBMEC in the face of high glucose levels. Conversely, another study reports that overproduction of this ET-1 contributes to the onset and progression of various cardiovascular disorders. These include tissue remodelling, inflammation, aberrant cell proliferation, and vasomotor dysfunction(Brunner, Brás-Silva et al. 2006). This dichotomy in ET-1's roles underscores the complexity of its function within the vascular system and highlights the importance of understanding its context-specific impacts.

Furthermore, our study revealed diverse expression patterns of Endothelin-1, under HG conditions (0.154 HG vs. 0.011 NG). This variation in expression patterns highlights the intricate role of the secretome in maintaining a balance between vasoconstriction and vasodilation, which could be of significant influence in blood flow regulation. The upregulated expression of Endothelin-1 in HG environments suggests a heightened vascular response, thereby indicating the role of ECs in mitigating the alterations in blood vessel dynamics caused by metabolic stress.

Endothelin-1 emerged as a significant player in microvascular function in our study. The elevated levels of Endothelin-1 in diabetes have been linked to increased vasomotor dysfunction, inflammation, and capillary fluid filtration. This is in line with another study that

showed hyperglycaemia enhances the constriction of retinal venules induced by ET-1, thus exacerbating retinal complications. Importantly, this effect was found to be mediated by the Endothelin Receptor A (ETAR) (Chen, Rosa et al. 2020), underscoring the therapeutic potential of targeting this receptor to alleviate diabetic microvascular dysfunction.

As diabetes progresses, there is a surge in ET-1, both in the vitreous fluid and plasma. These observations suggest that ET-1 could be a crucial molecular target for therapeutic interventions aiming to mitigate vascular complications in diabetes. Future research should continue to explore the complex mechanisms of ET-1 in diabetic pathology, paving the way for advancements in treatment strategies.

The Fibroblast Growth Factor Basic (bFGF), a growth factor crucial for the process of forming new blood vessels, or angiogenesis, has shown a significant upsurge under HG conditions. This increase is noteworthy as it implies that despite the challenging physiological environment, the cells are attempting to promote angiogenic activities. The altered expression of FGF basic (0.095 HG vs. 0 NG) emphasizes its role in orchestrating cellular proliferation, which is a cornerstone of angiogenesis.

Vascular Endothelial Growth Factor and basic Fibroblast Growth Factor are key players in the family of angiogenic growth factors. They are classified as direct angiogenic factors due to their capacity to stimulate the proliferation of endothelial cells, both in the laboratory and in living organisms. On the other hand, indirect angiogenic growth factors, such as PDGF and Transforming Growth Factor-β (TGF-β) (Stavri, Zachary et al. 1995), support angiogenesis in living organisms but do not directly stimulate endothelial cell proliferation in a lab setting. Instead, they modulate the expression and functionality of direct angiogenic factors, adding another dimension to the intricate regulatory network that governs angiogenesis.

Interestingly, bFGF, besides promoting endothelial cell proliferation and angiogenesis, has a wide range of activities. It can stimulate the proliferation of Vascular Smooth Muscle Cells (VSMCs) and fibroblasts (Burgess and Maciag 1989). Research has also revealed its capability to indirectly regulate VEGF expression.

The absence of a secretory signal peptide in bFGF adds complexity to our understanding of how it becomes biologically active. One proposed mechanism for its activation involves its release following cell death and degradation of the extracellular matrix. This highlights the crucial role of the tissue microenvironment in controlling the availability of angiogenic factors.

Our study has presented compelling insights into the role of the secretome, particularly in relation to Endothelial Cell migration, a critical function in tissue repair and regeneration. One of the key findings was the abundant presence of Interleukin-8 (IL-8), a powerful chemoattractant, within the Human Brain Microvascular Endothelial Cells secretome. Given the importance of IL-8 in creating a chemoattractant gradient, particularly under conditions of inflammation and oxidative stress, its abundant presence within the secretome is noteworthy.

High concentrations of growth factors within the secretome, including IL-8, create an environment conducive to the mobilization and migration of ECs. This observation underscores the intricate role of the secretome in promoting EC functionality, even in challenging microenvironments. The expression levels of IL-8, an inflammation mediator, showed significant changes under HG stress, indicating the secretome's role in controlling inflammatory processes (Bickel 1993) This could have implications for immune cell recruitment and endothelial cell activation.

Endothelial cells are key regulators of inflammation and injury, releasing a variety of cytokines, adhesion molecules, and chemo-attractants. Previous research has consistently shown a rapid increase in pro-inflammatory cytokines like TNF-α at Traumatic Brain Injury (TBI) sites, suggesting their role in initiating an inflammatory response (Kinoshita, Tanjoh et al. 2008). Under HG conditions, TNF-α-induced IL-8 synthesis in endothelial cells can intensify the inflammatory environment. This intensified inflammatory response, along with resulting endothelial cell damage, can exacerbate tissue injury and foster neutrophil accumulation at an injury site (Tsao, Hsu et al. 1999). As such, managing early glycaemic levels could be a strategy to reduce secondary tissue damage and inflammation following trauma.

In the context of diabetes, the increased secretion of IL-8 under HG conditions signals heightened inflammation in the microvasculature. In turn, this increase in IL-8 levels suggests a robust immune response triggered by hyperglycaemia, which could set off a cascade of inflammatory events in retinal endothelial cells. This heightened inflammatory state is often seen in diabetic complications, contributing to the progression of retinopathy.

Our findings also point to endothelial cell activation in response to HG conditions, a precursor to pathological changes observed in diabetic retinopathy, such as increased vascular permeability and leukocyte adhesion. Furthermore, IL-8's role as a potent chemoattractant suggests intensified recruitment of inflammatory cells to the retinal microvasculature, contributing to retinal inflammation and damage.

Our study suggests a positive feedback loop, where TNF-α activates endothelial cells to produce IL-8, which contributes to the recruitment and activation of neutrophils and other inflammatory cells. The implications of this study are significant, especially in the management of trauma patients. Early hyperglycaemia, characterized by glucose levels exceeding 200 mg/dL, correlates with higher infection and mortality rates in trauma patients, independent of injury characteristics (Eakins 2009).

Inflammatory cell recruitment into the damaged brain is a complex process involving leukocyte/endothelial adhesion molecules and specific chemotactic gradients. Our experiments revealed heightened IL-8 production by vascular endothelial cells under HG conditions, accentuated by TNF-α influence. This indicates the critical role of adhesion molecules, cytokines, and chemo-attractants in mobilizing peripheral inflammatory cells into damaged tissue. Understanding these mechanisms could pave the way for targeted therapeutic interventions aimed at modulating these inflammatory responses post-TBI.

Our study found a modest increase in VEGF, a renowned pro-angiogenic factor (Shibuya 2011) suggesting an effort by HBMEC to enhance angiogenesis under challenging conditions. This subtle elevation demonstrates the complex cellular mechanisms at work, where precise regulation of angiogenic responses becomes critical. Notably, VEGF, as a master regulator of vascular growth, showed minor changes in expression levels (1.090 HG vs. 1.074 NG) under HG stress. This nuanced modulation points to the secretome's role in precisely regulating vascular growth and permeability, both critical aspects of angiogenesis. The slight upregulation of VEGF suggests a subtle enhancement of angiogenic processes, indicating the secretome's adaptive mechanisms in response to HG-induced challenges.

VEGF plays a prominent role in the intricate landscape of diabetic vascular pathology. It has multiple functions, including angiogenesis, vascular permeability, and endothelial cell survival, making it a promising therapeutic target. The delicate balance between VEGF's physiological and pathological effects relies on complex signalling pathways and interactions with other molecules within the retinal microenvironment. Our study provides important evidence linking hyperglycaemia-induced VEGF upregulation to retinal complications, reinforcing VEGF's significance as a therapeutic target.

VEGF, a known stimulator of EPC proliferation and differentiation, emerged as a powerful element within the secretome. Its ability to activate Ras signalling and the MAPK/ERK pathway

emphasizes its role in promoting EPC proliferation, making it a crucial element in the intricate network of angiogenic signalling.

Research by Ghassemifar et al. (Ghassemifar, Lai et al. 2006) revealed that VEGF significantly upregulates ZO-1α+ and ZO-1α− transcripts and proteins, resulting in a considerable increase in their TEER. However, it's noteworthy that increased VEGF levels can lead to BBB leakage, cerebral haemorrhage, and increased infarction volume. This highlights the complexity of the pathways influenced by VEGF and the need for finely balanced mechanisms in their regulation.

Our study interestingly revealed a decrease in the expression of Placental Growth Factor (PIGF) within the HBMEC secretome under HG conditions. Traditionally, PIGF is recognized as a pro-angiogenic factor, contributing to the process of new blood vessel formation. However, under HG stress, PIGF displayed an unexpected downregulation, which points to a complex interplay of angiogenic factors at work.

This intriguing finding suggests that in certain circumstances, elements of angiogenesis could be suppressed. This suppression may represent a cellular attempt to fine-tune angiogenic responses or perhaps to mitigate excessive angiogenesis, which could lead to pathological conditions.

The expression levels of PIGF varied under HG stress (0.355 HG vs. 0.548 NG). This fluctuation indicates the secretome's role in modulating angiogenic signalling pathways, which could in turn impact endothelial cell behaviour and vessel formation. The differential expression of PIGF highlights its intricate involvement in the regulation of angiogenesis, suggesting its contribution to the adaptive responses orchestrated by the secretome under challenging metabolic conditions.

Contrary to what we might expect given its pro-angiogenic role, the downregulation of PIGF under HG conditions suggests that the cellular response to stress is multifaceted and complex. It's possible that this downregulation represents a compensatory response, attempting to balance out the effects of other factors within the secretome that promote angiogenesis, such as VEGF. This complex interplay of factors underscores the finely tuned nature of angiogenic regulation and the need to better understand the roles of individual factors within this process.

However, the counterintuitive decrease in PIGF, alongside the subtle adjustments in angiopoietin-2 and VEGF levels, underscores the complexity of angiogenic regulation under

high-glucose stress. These findings highlight the need for further investigation into the intricate signalling pathways and molecular interactions orchestrating angiogenesis in response to metabolic challenges.

Understanding the nuances of angiogenic modulation under pathological conditions, such as hyperglycaemia, holds immense therapeutic potential. By deciphering these intricate cellular responses, we pave the way for targeted interventions aimed at restoring angiogenic balance and mitigating the adverse consequences of conditions like diabetes. As we delve deeper into these mechanisms, the prospect of developing precise and effective therapies for angiogenesis-related disorders comes closer to fruition, ushering in a new era of therapeutic strategies in the field of vascular biology and regenerative medicine.

4.1.9 Unveiling the influence of Endothelial Cell-derived secretome on Pericyte Cytokine Expression: An In-Depth Analysis Derived from Proteome Profiler Outcomes

In this experiment, we aimed to delve deeper into the interactions within the neurovascular unit, specifically between endothelial cells and pericytes. We achieved this by exposing pericytes to the secretome derived from HBMEC cultured under HG and NG conditions. The integrity of the neurovascular unit is essential for brain function and is implicated in various neurological disorders. By investigating how the secretome of endothelial cells influences pericytes, which are vital regulators of blood-brain barrier stability, we hoped to gain insights into the complex cellular communication that governs brain health.

We then analysed cytokine expression in the pericyte secretome following exposure to the HBMEC-derived secretome. Cytokines are key mediators of cell signalling, with roles in inflammation, immune response, and tissue repair (Zhang and An 2007). By profiling cytokine expression patterns, we aimed to provide a comprehensive view of the paracrine signalling signatures under different glucose conditions. This approach could offer potential clues about the molecular language these cells use to communicate and how this communication is impacted during a hyperglycaemic state.

Dysregulated neuroinflammation is a hallmark of many neurological disorders. Therefore, understanding how endothelial cells, particularly under hyperglycaemic conditions, modulate pericyte responses through secreted factors can help unravel the mechanisms underlying neuroinflammation (Ramesh, MacLean et al. 2013).

Our findings revealed elevated levels of pro-inflammatory cytokines (CCL2, CXCL2, ICAM, IL6, IL8, MIF) and a thrombotic marker (PAI-1) in pericytes. This indicates an intensified inflammatory response and impaired fibrinolysis, potentially exacerbating tissue damage, neuroinflammation, and thrombus formation post-stroke. These findings underscore the significant role of pericytes in stroke-induced neuroinflammation and thrombotic events, further emphasizing the importance of understanding the complex interplay between endothelial cells and pericytes within the neurovascular unit.

Monocyte Chemoattractant Protein-1 (CCL2/MCP-1) plays a pivotal role in the recruitment of monocytes and macrophages, indicating ongoing inflammation. In stroke, elevated CCL2 levels contribute to immune cell infiltration, which exacerbates tissue damage. Furthermore, in diabetes, increased CCL2 levels have been linked to vascular complications and atherosclerosis (Girbl, Lenn et al. 2018), thereby escalating the risk of stroke.

Our recent experiments depicted an unexpected decrease in the expression of CCL2/MCP-1 under HG conditions (0.782), compared to NG conditions (0.919). This result suggests potential dysregulation in the chemotactic signalling pathway associated with monocyte recruitment and inflammation, which has several implications:

The decrease in CCL2/MCP-1 expression under high glucose conditions could disrupt chemotactic signalling pathways related to monocyte recruitment and inflammation, potentially impairing immune responses, exacerbating vascular complications in diabetes, and contributing. While excessive inflammation is detrimental, a balanced inflammatory response is necessary for proper tissue repair and immune defence (Amor, Puentes et al. 2010).

The study underscores the need for cell-specific generation of defined chemokines. Disrupted CCL2/MCP-1 signalling could upset the delicate balance in chemokine production, leading to irregular behaviour of neutrophils and other immune cells within tissues.

Therapeutic interventions targeting the CCL2/MCP-1 pathways could not only restore monocyte recruitment but also ensure proper guidance for other immune cells, thereby maintaining a balanced and effective immune response. The observed decrease in CCL2/MCP-1 levels under HG conditions is indicative of potential impairment in immune cell recruitment, particularly monocytes and macrophages.

In conditions where inflammation and tissue repair are necessary, reduced CCL2/MCP-1 expression could slow down the arrival of immune cells at the site of injury or infection. This

delay might compromise the body's ability to mount an effective defence against pathogens, leading to prolonged infections or inadequate tissue repair.

CCL2/MCP-1 plays a significant role in regulating chronic inflammation. Altered expression of this chemokine may disrupt the delicate balance between pro-inflammatory and anti-inflammatory signals, leading to sustained inflammation. Chronic inflammation is a characteristic of various diseases, including diabetes and its complications. Prolonged, unresolved inflammation can lead to tissue damage, exacerbating the progression of diseases such as diabetic nephropathy, retinopathy, or neuropathy. Hence, the diminished CCL2/MCP-1 levels observed in the HG environment could contribute to this sustained inflammatory state.

Our research findings highlight a notable difference in the levels of CXCL2/GRO under high glucose (HG) conditions (0.066730425) as compared to NG conditions (0.063953). The role of CXCL2, a Growth-Related Oncogene, is central to the recruitment and activation of neutrophils, key players in inflammation (Girbl, Lenn et al. 2018). The observed elevated CXCL2 levels in HG conditions may correspond to increased neutrophil activation, which augments the potential for tissue damage following stroke. In the context of diabetes, this could signify a state of intensified inflammation, potentially detrimental to vascular health and possibly raising the risk of stroke.

The effectiveness and resolution of inflammatory responses hinge on the precise coordination of neutrophil emigration from the bloodstream. Previous research has contributed significant mechanistic insight into the role of chemokine signals, specifically CXCL2, in guiding neutrophils through the venular wall, consisting of the endothelial monolayer and pericyte sheath. This body of work underscores the unique role CXCL2 plays in driving neutrophil migration through these tissue planes, facilitated by interactions with CXCR2 (Girbl, Lenn et al. 2018).

The formation of localized CXCL2 gradients within endothelial cell-cell junctions, mediated by ACKR1, creates directional cues that promote efficient neutrophil movement from the luminal to the abluminal side. Intriguingly, prior studies indicate that migrating neutrophils could be the main source of CXCL2, setting up an autocrine feedback loop that enhances transmigration (Shulman, Cohen et al. 2011). Without stringent control over CXCL2 signalling, neutrophil emigration could become disarrayed, potentially leading to sustained inflammation.

Our research results reveal a significant decrease in the expression of ICAM (Intercellular Adhesion Molecule) under HG conditions (0.014643901) compared to NG conditions (0.333554). This observation carries profound implications for our understanding of pathophysiological processes, especially in chronic diseases such as diabetes. ICAMs are integral to immune responses, inflammation, and vascular integrity. Therefore, any alterations in ICAM levels are highly relevant in the context of numerous diseases.

ICAMs play a critical role in the immune system, facilitating leukocyte adhesion and transmigration to sites of infection or injury. The reduced ICAM expression observed in the HG environment could potentially impair the immune response by hindering the effective recruitment of immune cells to sites of inflammation or infection (Ley, Laudanna et al. 2007). This impairment in immune cell adhesion and transmigration might compromise the body's ability to combat pathogens, possibly leading to prolonged infections or inadequate immune responses.

Moreover, ICAMs are essential for maintaining vascular integrity and endothelial function. They contribute to endothelial cell adhesion and communication, playing a significant role in vascular homeostasis (Stark, Eckart et al. 2013). The alterations we observed in ICAM expression—specifically, the decreased levels in the HG condition—may disrupt endothelial integrity. Endothelial dysfunction is a prominent feature of various vascular complications associated with chronic conditions like diabetes. Thus, impaired ICAM expression could contribute to increased vascular permeability, inflammation, and oxidative stress, which are factors known to exacerbate the progression of vascular complications such as atherosclerosis and diabetic vasculopathy (Ramesh, MacLean et al. 2013).

Our findings underscore the need for further research to explore the exact mechanisms by which hyperglycaemia affects ICAM expression and function. Understanding these pathways could provide new therapeutic targets for managing immune responses and vascular complications in diabetes and other chronic conditions.

Our research results indicate a significant decrease in the expression of IL-6 and IL-8 under HG conditions compared to NG conditions. The levels for IL-6 dropped from 0.030092 (NG) to 0.0000777927 (HG) and for IL-8, from 0.849658 (NG) to 0.312589388 (HG). These observations carry significant implications for our understanding of immune responses and angiogenesis, especially in the context of chronic diseases such as diabetes.

IL-6 is traditionally considered proangiogenic and exhibits a dual nature in angiogenesis. High concentrations of IL-6 can induce local VEGF expression, promoting angiogenesis. However, chronic exposure to excessive IL-6 levels can lead to defective angiogenesis, characterized by diminished pericyte coverage (Gopinathan, Milagre et al. 2015). The observed decrease in IL-6 levels in the HG condition highlights the context-dependent effects of IL-6 on angiogenesis, influenced by concentration and duration of exposure. In a diabetic state, such altered IL-6 levels may disrupt finely tuned angiogenic processes, potentially contributing to the impaired angiogenesis observed in diabetes-related complications.

IL-8, also known as CXCL8, primarily functions as a chemoattractant for neutrophils and other immune cells to sites of inflammation or injury. The significant decrease in IL-8 expression in the HG condition is noteworthy (Bickel 1993). Reduced IL-8 levels might impede the recruitment of immune cells to inflammatory sites, impairing the early stages of the immune response. This impaired chemotactic response could delay the clearance of pathogens or damaged tissues, prolonging the inflammatory process. In the context of diabetes, impaired IL-8-mediated immune cell recruitment might contribute to delayed wound healing, increased susceptibility to infections, and the development of chronic, non-resolving inflammation, all of which are common complications associated with diabetes.

These findings underscore the need for further research into how hyperglycaemia specifically influences IL-6 and IL-8 expression and their resultant effects on immune responses and angiogenesis. Unravelling these mechanisms could provide potential therapeutic targets for managing diabetes and associated complications.

The results from this study reveal a significant increase in the levels of Macrophage Migration Inhibitory Factor (MIF) under HG conditions (0.623354614) compared to NG conditions (0.506616). This is significant because MIF, a proinflammatory cytokine, is known to play a critical role in immune responses and inflammatory processes. The elevated MIF levels under hyperglycaemia suggest a heightened inflammatory response, a common feature of diabetes-related complications.

Increased MIF levels in high glucose conditions, indicative of an exaggerated immune response, can lead to chronic inflammation, a factor in various diabetes-related complications such as cardiovascular diseases, nephropathy, and retinopathy (Ramesh, MacLean et al. 2013). MIF's role in promoting immune cell recruitment and activation exacerbates tissue damage caused by the oxidative stress and inflammation associated with hyperglycaemia.

Furthermore, MIF is implicated in the development of atherosclerosis, a common diabetes condition. By enhancing monocyte adhesion to endothelial cells, higher MIF levels contribute to atherosclerotic plaque formation and the acceleration of cardiovascular complications in a diabetes setting (Poznyak, Grechko et al. 2020).

The role of MIF in neutrophil transmigration, a critical process in the inflammatory response, is multifaceted and complex. It involves the intricate interactions among endothelial cells, the extracellular matrix basement membrane (BM), and pericytes. Proinflammatory cytokines like TNF-α stimulate ECs to increase the expression of adhesion molecules, facilitating the transmigration process (Ley, Laudanna et al. 2007). MIF, expressed by various cell types, including ECs, is a key player in this scenario. Elevated MIF levels are associated with diseases characterized by acute inflammation and injury, including acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS) (Wu, Sun et al. 2016).

The involvement of MIF in mediating the crosstalk between ECs and PCs and its impact on neutrophil transmigration underscores its significance in the regulation of vascular barrier function during inflammation. Understanding the precise mechanisms through which MIF influences these processes will provide valuable insights into the development of targeted interventions for inflammatory disorders.

Our exploration of Plasminogen Activator Inhibitor-1 (PAI-1) has surfaced a critical biological narrative. Elevated levels of PAI-1 point towards a hindered fibrinolysis process and an augmented risk of thrombosis. This scenario, when placed in the context of stroke, could potentially amplify clot formation. In diabetes, high PAI-1 levels hint at a contribution to atherosclerosis, thereby raising the risk of stroke.

In our study, we observed a significant uptick in the expression of Serpin E1/PAI-1 under high glucose conditions (0.105035566) when compared to normal glucose conditions (0.077367). This observation underscores the substantial alterations that hyperglycaemia can induce in the regulation of the fibrinolytic system.

PAI-1, an important player in the fibrinolytic system, mediates the breakdown of blood clots. The role of PAI-1 becomes even more pivotal when we dive into the process of angiogenesis. This complex, multi-step process involves endothelial cell activation, migration, proliferation, and ultimately, the maturation of new capillary tubes (Binder, Mihaly et al. 2007).

Endothelial activation triggers proteinases like urokinase plasminogen activator (uPA), which converts plasminogen to plasmin upon binding to its receptor (uPAR) on the cell surface. This triggers a proteolytic cascade involving matrix metalloproteinases (MMPs), leading to extracellular matrix degradation, release of growth factors, and modification of cell surface proteins (Binder, Mihaly et al. 2007). The interaction of uPA and uPAR induces conformational changes in uPAR, influencing gene expression, signalling events, and cell-matrix adhesion.

PAI-1 tightly regulates the activity of uPA and the uPA/uPAR complex, playing a pivotal role in angiogenesis regulation. Elevated expression of PAI-1, particularly during active angiogenesis, can be found in newly forming vessels. PAI-1 interacts with vitronectin, an extracellular matrix protein, stabilizing its active form (Basu, Menicucci et al. 2009). A decrease in PAI-1 function modifies cell migration by retaining higher levels of αv integrin on the cell surface, leading to increased cell-substrate adhesion. This interplay between PAI-1, uPA, and their counterparts influences angiogenesis by modulating endothelial cell behaviour and the extracellular matrix environment.

In conclusion, our experiment holds substantial implications for understanding the complex communication networks within the neurovascular unit, deciphering neuroinflammatory processes, discovering potential biomarkers, and identifying therapeutic targets. By examining the cytokine expression profiles in pericytes exposed to HBMEC secretome under different glucose conditions, we have contributed valuable knowledge to the fields of stroke, diabetes pathology, and neurobiology. This could potentially guide the development of novel therapeutic strategies for neurological disorders associated with vascular dysfunction and neuroinflammation.

Our research findings have recently unveiled novel data that lends evidence to the critical role of pericytes in counteracting the detrimental effects of HG conditions on endothelial cells ECs). This investigation into the interplay between EC and pericytes under hyperglycaemic conditions and the subsequent alterations in the expression of key molecules, specifically interleukin-8 (IL-8), has provided intriguing insights.

Pericytes have long been recognised for their role in maintaining the integrity of the BBB. Our study, however, suggests that their role might be even more significant, particularly in the context of mitigating the deleterious effects of hyperglycaemia on endothelial cells.

When exposed to HG conditions, EC showed a notable increase in IL-8 expression. The levels rose from 0.6989 under NG conditions to 0.8155 under HG conditions. IL-8 is a pro-

inflammatory cytokine and has been linked to increased inflammation and vascular dysfunction, which are characteristic of hyperglycaemic states. Hence, this upregulation of IL-8 in EC under HG conditions is indicative of a heightened inflammatory response and vascular instability that could compromise the function of the BBB.

Interestingly, our observations revealed a contrasting response when pericytes were exposed to the secretome of endothelial cells treated under high glucose conditions. The pericytes demonstrated a decrease in IL-8 expression levels, dropping from 0.8497 under NG conditions to 0.3126 under HG conditions. This significant drop in IL-8 expression suggests that pericytes may have a unique capacity to dampen the heightened inflammatory response triggered by hyperglycaemia when exposed to signals from stressed endothelial cells.

These results provide a novel perspective on the cellular responses to hyperglycaemia, emphasizing the dynamic interplay between endothelial cells and pericytes. However, this is just the tip of the iceberg. The exact pathway through which pericytes downregulate IL-8 expression under HG conditions remains to be determined, indicating that further research is necessary for a more comprehensive understanding of the underlying molecular mechanisms. It would also be of interest to investigate whether pericytes can counteract other hyperglycaemia-induced changes in endothelial cells.

4.1.10 Decoding the Consequences of hyperglycaemia on Blood-Brain Barrier stability: A thorough discussion on findings from trans endothelial Electrical Resistance Measurements and Evans Blue Albumin/Sodium Fluorescein Permeability Assays

The purpose of this study was to investigate the impact of different experimental conditions and time points on the structure of the BBB. The results of the study demonstrated significant effects of both the experimental conditions and time points on BBB structure, as well as subject variability.

The research underscores the profound impact of glucose dysregulation on the integrity of the BBB. It has been demonstrated that hyperglycaemia can induce alterations in BBB structure, with sustained effects visible at up to 21 hours. This is of particular relevance for conditions like diabetes, where elevated glucose levels could potentially contribute to BBB disruption and subsequent neurovascular complications.

The alterations in BBB structure were mainly evidenced by the downregulation of TJ proteins, which are pivotal in maintaining the impermeability of the BBB. This downregulation led to an increased permeability of the BBB, thereby allowing substances to pass through the barrier more easily. These structural changes were found to be linked with cognitive impairment, as demonstrated in a mouse model of Type 2 diabetes mellitus.

Furthermore, the study revealed increased levels of inflammatory markers in the brain tissue of these diabetic mice, indicating the presence of inflammation and interactions between leukocytes and the brain endothelium. These inflammatory responses could be mitigated by either blocking the angiotensin II type 1 receptor or activating the peroxisome proliferator-activated receptor-γ (Zhong, Wang et al. 2012).

In patients with Type 1 diabetes experiencing diabetic ketoacidosis (DKA), brain oedema was a common occurrence, attributed to neuroinflammation and compromise of TJ proteins. Chronic Type 2 diabetes in rats led to increased BBB permeability and reduced expression of TJ proteins in the hippocampus. Recent studies have also linked BBB dysfunction and cognitive impairment with obesity, connecting these conditions to a high-fat/high-sugar diet (Bogush, Heldt et al. 2017).

Overall, these findings highlight the potential role of glucose dysregulation in BBB dysfunction and its possible implications for cognitive impairment and neurovascular complications in conditions such as diabetes. Further investigations into the specific mechanisms underlying these effects are warranted. This research may have significant implications for the treatment and management of diabetes and other conditions characterized by glucose dysregulation.

Other research teams have observed similar effects in their studies using in vitro models of the BBB exposed to HG conditions, which mirror the findings from human and animal studies. High glucose conditions have been demonstrated to undermine BBB integrity and precipitate the partial loss of TJ proteins (ZO-1, occludin, and claudin-5) in co-cultures of human brain microvascular endothelial cells (BMVECs) and astrocytes (Bogush, Heldt et al. 2017). This degradation of the BBB is accompanied by escalated activity of NADPH oxidase and the production of superoxide anions, both indicative of oxidative stress, which appear to rise in tandem with BBB permeability (Ahmed 2005). These alterations become evident after a five-day exposure to 25 mM D-glucose and are not seen with normoglycemic osmotic insult. The BBB permeability can be reversed by inhibiting p38 MAPK, a signalling pathway involved in inflammation, while the induction of superoxide anions and degradation of TJ proteins caused

by HG can be prevented by activating AMP-activated protein kinase (Bogush, Heldt et al. 2017).

Continuing from where we left off, the BBB is a highly specialized structure formed by endothelial cells lining the brain's blood vessels. It plays a vital role in maintaining the brain's microenvironment by stringently controlling the passage of substances between the bloodstream and the brain tissue. Tight junction proteins are integral components of the BBB, contributing to its integrity and function. These include occludin, claudins, and zonula occludens proteins.

In pathological conditions such as diabetic encephalopathy, a downregulation or loss of these TJ proteins has been observed, resulting in an increased permeability of the BBB (Huber et al., 2006). This increased permeability allows substances, including potentially harmful agents and inflammatory molecules, to cross the BBB more freely, leading to neuroinflammation and brain dysfunction.

Animal models of Type 2 diabetes have demonstrated an association between downregulation of TJ proteins in the BBB and cognitive impairment, as gauged by tests such as the Morris Water Maze. Moreover, markers of inflammation, including monocyte chemoattractant protein 1 (MCP-1)/CCL2, tumour necrosis factor α (TNFα), interleukin 6 (IL-6), and superoxide ion, are increased in the brain tissue of these animals (Serban, Stanca et al. 2016). These findings suggest that inflammation, leukocyte-brain endothelium interaction, and oxidative stress contribute significantly to the pathogenesis of diabetic encephalopathy.

In patients with Type 1 diabetes experiencing diabetic ketoacidosis (DKA), a severe complication characterized by high blood glucose and ketone levels, brain oedema is a common occurrence. The compromised TJ proteins and neuroinflammation in the BBB have been implicated in the development of this brain oedema in DKA (Zhong, Wang et al. 2012). Studies have shown elevated levels of inflammatory markers, such as IL-6, and the presence of molecules associated with inflammation, including CCL2, NF-κB, and nitro-tyrosine, in the brain tissue of DKA patients (Yorulmaz, Kaptan et al. 2015). Moreover, an absence of TJ proteins, like ZO-1, occludin, JAM-1, and claudin-5, in the brain tissue correlates with the development of oedema (Zhong et al., 2012).

In conclusion, understanding these mechanisms is crucial for developing therapeutic strategies to target and mitigate the BBB dysfunction, inflammation, and oxidative stress observed in diabetic encephalopathy. Approaches aimed at preserving or restoring the integrity of the BBB, reducing neuroinflammation, and controlling oxidative stress may hold

promise for preventing or treating the neurological complications associated with diabetes mellitus. However, research in this area is ongoing, and further studies are needed to fully understand the complex mechanisms involved in diabetic encephalopathy and develop effective therapeutic interventions.

In our experiment, we did not observe a statistically significant change in Evans Blue Albumin (EBA) clearance between the HG, NG, and D-mannitol groups. However, we did notice a significant increase in Sodium Fluoride (NaF) clearance volume in the HG group compared to both the NG and D-mannitol groups. These findings provide important insights into the effects of high glucose levels on the permeability of the BBB. The lack of significant change in EBA clearance suggests that the paracellular pathway of the BBB remained largely intact under high glucose conditions.

On the other hand, NaF, a small molecule that typically crosses the BBB via the transcellular pathway, showed a significantly increased clearance volume in the HG group. This suggests that high glucose conditions may heighten the permeability of the BBB for small molecules, possibly by affecting the function or expression of transporters or channels involved in the transcellular pathway.

Our results also highlight the importance of the osmotic control group (D-mannitol). The lack of significant change in EBA clearance volume in this group indicates that the changes observed in the NaF clearance volume in the HG group are likely attributable to the high glucose levels rather than a general osmotic effect. This underscores the specificity of the effects of high glucose on BBB permeability then secretome bb with EBA and NaF

4.1.11 Assessing the Blood-Brain Barrier under the Influence of Treated Endothelial Cell Secretome: A Comprehensive Discussion on Findings from Transendothelial Electrical Resistance Measurements and Evans Blue Albumin/Sodium Fluorescein Permeability Assays

The aim of our study was to examine the impact of EC treated secretome on the structure of the BBB, as assessed through trans-endothelial electrical resistance. TEER is a widely used quantitative technique to measure the integrity of tight junction dynamics of the BBB. We compared the effects under HG, NG, and D-mannitol conditions at different time points: 2 hours, 6 hours, 10 hours, and 21 hours.

At the 2-hour time point, there were no significant differences in TEER measurements between any of the experimental conditions. This suggests that the EC treated secretome does not elicit rapid changes in BBB structure, at least within the first 2 hours. This could be due to the time required for ECs to respond to the high glucose or osmotic conditions and subsequently secrete factors that might affect BBB integrity.

At the 6-hour time point, significant differences were observed in the HG group compared to both the NG and D.mannitol groups. These differences persisted at the 10-hour and 21-hour time points. These findings suggest that exposure to high glucose conditions impacts the EC secretome in a way that affects BBB structure, as evidenced by the altered TEER measurements. The lack of significant differences between the NG and D.mannitol groups at any time point further supports the hypothesis that the observed changes in TEER under HG conditions are likely due to glucose-specific effects rather than a general osmotic effect.

Interestingly, the impact of the EC treated secretome on TEER measurements became evident only after 6 hours, and these differences persisted up to 21 hours. This provides valuable insights into the temporal dynamics of how changes in the EC secretome under high glucose conditions can affect BBB structure. It is possible that prolonged exposure to high glucose conditions leads to the secretion of factors by ECs that disrupt tight junction integrity, leading to changes in TEER.

These findings contribute to a growing body of evidence suggesting that hyperglycaemic conditions can influence BBB structure and function, potentially through modulating the secretome of ECs. However, further studies are needed to identify the specific factors in the EC secretome that are responsible for these observed changes in TEER. Unravelling these mechanisms could provide novel insights into how hyperglycaemic conditions affect BBB integrity and may lead to the development of therapeutic strategies for conditions where BBB dysfunction and hyperglycaemia coexist.

This chapter focuses on the examination of the effects of various treatment conditions on the Evans Blue Albumin and sodium fluorescence measurements. EBA is a marker for macromolecular passage, while NaF measures the micromolecular passage across the BBB. The integrity of the BBB is crucial for maintaining the homeostasis of the brain's microenvironment, and any disruption could lead to pathological conditions.

Regarding the EBA measurements, the ANOVA results showed a non-significant p-value ($p = 0.9168$), indicating that there is no significant difference in the EBA measurements across the

different treatment conditions. This suggests that the treatments used in this study did not significantly impact the macromolecular passage across the BBB.

In the NaF analysis, used to measure the micromolecular passage, the ANOVA results also showed a non-significant p-value ($p = 0.2267$), suggesting there is no significant difference among the means of the treatment groups. This result points towards the conclusion that the treatments used in this study did not significantly affect the micromolecular passage across the BBB as measured by NaF.

In summary, the intricate interplay of angiogenic factors within the HBMEC-derived secretome unveils a sophisticated molecular tapestry, finely tuned to navigate the challenges posed by high-glucose environments. These findings not only expand our understanding of angiogenesis regulation but also pave the way for targeted therapeutic interventions, aiming to modulate these intricate pathways for enhanced tissue repair and regeneration in diabetic microvascular complications.

4.1.12 Dissecting the Impact of hyperglycaemia on ZO-1 localization: A detailed discussion on the disruption of normal linear localization around cell borders as revealed by immunofluorescence imaging

In our study, we sought to investigate whether an observed increase in TEER value was attributable to the loss of tight junction proteins, such as ZO-1. ZO-1 is a pivotal component of the tight junction complex and plays a key role in sustaining the integrity of the BBB by forming connections between transmembrane proteins and the actin cytoskeleton (Fanning, Jameson et al. 1998).

Our findings reveal that high glucose conditions lead to a decrease in the total levels of ZO-1. This reduction in ZO-1 protein expression is likely driven by multiple pathways activated in hyperglycaemia. These include an increase in the secretion of matrix metalloproteinases (MMP-2/9), which may cleave ZO-1 extracellularly at junctional sites, and an upregulation of caveolin-1, which could facilitate the intracellular internalization of ZO-1 away from contact points (Lakhan, Kirchgessner et al. 2013). Additionally, the enhancement of autophagy under high glucose conditions leads to further degradation of ZO-1, pointing to the involvement of autophagy-lysosomal pathways in this process.

Taken together, our results suggest that high glucose conditions lead to a dual assault on ZO-1, resulting in both a reduction in total ZO-1 levels and a disruption of its normal junctional localization patterns. These changes, driven by multiple converging pathological mechanisms, would compromise the anchoring of other tight junction proteins such as occludin and claudins.

This could lead to deregulated paracellular permeability, allowing macromolecular infiltration into the brain and contributing substantially to the glycaemic deterioration of BBB function.

These findings provide valuable insights into the ways hyperglycaemia can disrupt ZO-1 and, consequently, BBB function. However, the precise mechanisms underlying these effects are not yet fully understood. Future studies are needed to provide more comprehensive understanding of the molecular regulation of ZO-1 dynamics under high glucose conditions. Such research may pave the way toward identifying novel therapeutic strategies to prevent BBB disruption in conditions like diabetes, where hyperglycaemia is prevalent.

However, it is imperative to acknowledge the inherent limitations of this study. The in vitro paradigm, albeit informative, does not encapsulate the multifaceted in vivo milieu, wherein the BBB is subject to an intricate interplay of systemic factors such as hormones, minerals, vitamins, cytokines, and fluctuating levels of glucose. Moreover, the omission of neuronal elements from our model precludes a comprehensive appraisal of the neurovascular unit's intricacies, which are essential for a thorough understanding of BBB functionality.

Additionally, the scope of this research did not extend to the examination of glycaemic control mechanisms on BBB integrity, a critical facet considering the prevalent application of glycaemic management strategies in diabetes care. Future research endeavours should aspire to integrate these dimensions, thereby refining the experimental framework to reflect the in vivo environment more accurately and to elucidate the nuanced impact of diabetes management on BBB integrity.

5 Conclusion

This study sheds light on the effects of hyperglycaemia on the vascular elements crucial for NVU. It specifically explores the interaction between human brain microvascular HBMECs and PCs under high glucose conditions. Our investigation reveals that although direct morphological changes in HBMECs and pericytes were absent, significant alterations in signalling pathways, notably an upsurge in PDGF-b signalling in pericytes, were observed. This finding suggests a key mechanism through which hyperglycaemia influences cellular communication within the neurovascular unit.

Additionally, the study examines the repercussions of hyperglycaemia on EC actin stress fibre formation, revealing no changes in tubulogenic capabilities or wound healing processes, yet highlighting the complexity of EC responses to hyperglycaemic conditions. Despite these cellular and molecular adjustments, the fundamental angiogenic capabilities of ECs and subsequent modulation of pericyte cytokine expression point towards a sophisticated adaptability to hyperglycaemic stress. Moreover, assessments of the BBB's functionality and structural integrity under such conditions offer insights into the resilience of the BBB to glucose-induced stress. Collectively, these insights provide a mechanistic understanding of the hyperglycaemia-mediated effects on the BBB, emphasising the critical role of cellular interactions and signalling pathways in maintaining BBB integrity against metabolic challenges.

Future experiments should aim to incorporate a broader range of physiological conditions, including variable glucose levels and the presence of neuronal elements, to simulate the in vivo conditions and unravel the multifaceted impact of hyperglycaemia on the BBB more accurately.

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