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**The role of outgrowth endothelial  
cells as therapeutics for ischaemic  
injury-mediated cerebrovascular  
damage: impact of NOX2 inhibitor**

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Thesis submitted to the University of Nottingham for the degree of  
Doctor of Philosophy

October 2021

## **Acknowledgements**

I would like to take this opportunity to thank everyone who has provided me with help during my PhD study. First, I would like to express my deepest gratitude to my supervisor Dr Ulvi Bayraktutan for accepting me to be part of his research team, for his supervision, for his patience, for his direction and guidance throughout the entire period of my study, for his generous support for which I am very grateful. I would like to thank my second supervisor Dr Nikola Sprigg for her support, assistance, and advice during my study.

I would like to thank Dr Ulvi lab' members including Dr Kamini, Dr Rais, and Dr Othman. I would like to thank Dr Kamini for providing me with assistance, help, and training including tissue culture and other experimental procedures for which I am very grateful. I would like to thank Dr Rais for being supportive and friendly during the entire period of my study. I would like to thank Dr Othman for his support to my research.

I want to thank every member of Stroke Medicine Department at Nottingham University Hospitals NHS Trust for their assistance, the periodic meetings, the seminars, and their undeniable support to my research. I would like to thank all other departments especially the Respiratory Medicine Department and Rheumatology Medicine Department for using some of their equipment, for their help, and for their support to my research.

Finally, I would like to provide my sincere thanks to all my family members who stepped in during a difficult time and provided me with living expenses throughout the entire period of my PhD study.



## Publications, posters, and presentations

- 1) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Treatment with outgrowth endothelial cells protects cerebral barrier against ischaemic injury. *Cytotherapy November 2021*.
- 2) **Mansour Alwjwaj**, Rais Kadir, Ulvi Bayraktutan. The secretome of endothelial progenitor cells: a potential therapeutic strategy for ischaemic stroke. *Neural Regeneration Research August 2021*.
- 3) Rais Kadir, **Mansour Alwjwaj**, Zoe McCarthy, Ulvi Bayraktutan. Therapeutic hypothermia augments the restorative effects of PKC- $\beta$  and NOX2 inhibition on an *in vitro* model of human blood-brain barrier. *Metabolic Brain Disease July 2021*.
- 4) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. MicroRNA: an emerging predictive, diagnostic, prognostic, and therapeutic strategy in ischaemic stroke. *Cellular Molecular Neurobiology December 2020*.
- 5) Rais Kadir, **Mansour Alwjwaj**, Othman Ahmed, Kamini Rakkar, Ulvi Bayraktutan. Outgrowth endothelial cells form a functional cerebral barrier and restore its integrity after damage. *Neural Regeneration Research September 2019*.
- 6) **Mansour Alwjwaj**, Rais Kadir, Ulvi Bayraktutan. NADPH oxidase is essential for endothelial progenitor cell migration, proliferation, and vascular repair. *European Stroke Organisation Conference September 2021*. [Poster].
- 7) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Outgrowth endothelial cells-derived conditioned medium: a potential cell-free strategy to repair

- neuroinflammatory cerebral barrier damage. *European Stroke Organisation Conference September 2021*. [Presentation].
- 8) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Administration of outgrowth endothelial cells repair cerebral barrier function following ischaemic stroke injury. *European Stroke Organisation Conference September 2021*. [Presentation].
  - 9) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Long-term expansion of outgrowth endothelial cells evokes replicative senescence and attenuate their cerebral barrier-forming capacity. *European Stroke Organisation Conference September 2021*. [Presentation].
  - 10) **Mansour Alwjwaj**, Rais Kadir, Ulvi Bayraktutan. Senescence attenuates the ability of endothelial progenitor cells to form blood-brain barrier and repair the damage. *Joint European Stroke Organisation and World Stroke Organisation Conference ESO-WSO 2020*. [Poster].
  - 11) **Mansour Alwjwaj**, Rais Kadir, Ulvi Bayraktutan. Can inhibition of PKC- $\beta$  or NOX2 augment the *in vitro* blood-brain barrier forming activity of endothelial progenitor cells. *Joint European Stroke Organisation and World Stroke Organisation Conference ESO-WSO 2020*. [Poster].
  - 12) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Outgrowth endothelial cells: a cell-based approach to repair ischaemia-evoked cerebral barrier damage. *Joint European Stroke Organisation and World Stroke Organisation Conference ESO-WSO 2020*. [Poster].
  - 13) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Can inhibition of PKC- $\beta$  or NOX2 augment the restorative impact of hypothermia on blood-

brain barrier. *Joint European Stroke Organisation and World Stroke Organisation Conference ESO-WSO 2020*. [Poster].

- 14) **Mansour Alwjwaj**, Rais Kadir, Ulvi Bayraktutan. Comparative analyses of outgrowth endothelial cells and microvascular endothelial cells in forming an *in vitro* model of human Blood-Brain Barrier. *UK Preclinical Stroke Symposium June 2019*. [Presentation].
- 15) Rais Kadir, **Mansour Alwjwaj**, Ulvi Bayraktutan. Comparative assessment of outgrowth endothelial cells and brain microvascular endothelial cells in forming an *in vitro* model of human blood-brain barrier: role of PKC- $\beta$ . *UK Preclinical Stroke Symposium June 2019*. [Presentation].
- 16) **Mansour Alwjwaj**, Ulvi Bayraktutan. Gp91ds-tat, a selective NOX2 peptide inhibitor, protects *in vitro* blood-brain barrier from ischaemic injury. *European Stroke Organisation Conference May 2019*. [Poster].
- 17) **Mansour Alwjwaj**, Ulvi Bayraktutan. Role of outgrowth endothelial cells as therapeutics for ischaemic injury-mediated cerebrovascular damage. *Medicine & Health Science University of Nottingham Faculty Postgraduate Research Forum June 2018*. [presentation].

## List of Abbreviations

|                         |                                                       |
|-------------------------|-------------------------------------------------------|
| <b>AD</b>               | Anoxic depolarization                                 |
| <b>dRP</b>              | 2-deoxy-D-ribose-1-phosphate                          |
| <b>DAPI</b>             | 4',6-diamindino-2-phenylindole                        |
| <b>ASIC</b>             | Acid sensing ion channels                             |
| <b>ATP</b>              | Adenosine triphosphate                                |
| <b>CCL18</b>            | Alternative macrophage markers C-C motif chemokine 18 |
| <b>Ang2</b>             | Angiopoietin-2                                        |
| <b>AGS</b>              | Astrocyte cell growth supplement                      |
| <b>AM</b>               | Astrocyte medium                                      |
| <b>BCA</b>              | Bicinchoninic acid                                    |
| <b>BBB</b>              | Blood-brain barrier                                   |
| <b>BSA</b>              | Bovine serum albumin                                  |
| <b>CO<sub>2</sub></b>   | Carbon dioxide                                        |
| <b>CNS</b>              | Central nervous system                                |
| <b>CBF</b>              | Cerebral blood flow                                   |
| <b>CSF</b>              | Cerebrospinal fluid                                   |
| <b>CCL2</b>             | Chemokine (C-C motif) ligand 2                        |
| <b>CV</b>               | Cleared volume                                        |
| <b>CD</b>               | Cluster of differentiation                            |
| <b>CM</b>               | Conditioned media                                     |
| <b>CABG</b>             | Coronary artery bypass grafting                       |
| <b>CYTL1</b>            | Cytokine-like 1                                       |
| <b>DNA</b>              | Deoxyribonucleic acid                                 |
| <b>Dil-AcLDL</b>        | Dil-labelled acetylated-low density lipo- protein     |
| <b>DMSO</b>             | Dimethyl sulphoxide                                   |
| <b>DMF</b>              | Dimethylformamide                                     |
| <b>ddH<sub>2</sub>O</b> | Double distilled water                                |
| <b>EBM2</b>             | Endothelial cell basal medium-2                       |
| <b>ECGS</b>             | Endothelial cell growth supplement                    |
| <b>ECM</b>              | Endothelial cell media                                |
| <b>ECs</b>              | Endothelial cells                                     |
| <b>ECFCs</b>            | Endothelial colony-forming cells                      |

|                                   |                                                                 |
|-----------------------------------|-----------------------------------------------------------------|
| <b>eNOS</b>                       | Endothelial nitric oxide synthase                               |
| <b>EPCs</b>                       | Endothelial progenitor cells                                    |
| <b>EPC-CM</b>                     | Endothelial progenitor cells conditioned media                  |
| <b>EVOM</b>                       | Epithelial Volt/Ohm                                             |
| <b>EGTA</b>                       | Ethylene glycol tetra acetic acid                               |
| <b>FITC-UEA-1</b>                 | Fluorescein isothiocyanate-conjugated ulex europaeus agglutinin |
| <b>FBS</b>                        | Foetal bovine serum                                             |
| <b>FoxF1</b>                      | Forkhead box f1, protein coding gene                            |
| <b>G-CSF</b>                      | Granulocyte colony-stimulating factor                           |
| <b>GM-CSF</b>                     | Granulocyte-macrophage colony-stimulating factor                |
| <b>HBSS</b>                       | Hank's balanced salt solution                                   |
| <b>HGF</b>                        | Hepatocyte growth factor                                        |
| <b>HAs</b>                        | Human astrocytes                                                |
| <b>HBMECs</b>                     | Human brain microvascular endothelial cells                     |
| <b>HLAs</b>                       | Human leukocyte antigens                                        |
| <b>HPs</b>                        | Human pericytes                                                 |
| <b>H<sub>2</sub>O<sub>2</sub></b> | Hydrogen peroxide                                               |
| <b>IGF-1</b>                      | Insulin-like growth factor 1                                    |
| <b>IL</b>                         | Interleukins                                                    |
| <b>JAMs</b>                       | Junction adhesion molecules                                     |
| <b>KDR</b>                        | Kinase insert domain receptor                                   |
| <b>MIF</b>                        | Macrophage migration inhibitory factor                          |
| <b>MMP9</b>                       | Matrix metalloproteinase 9                                      |
| <b>mKitL</b>                      | Membrane-bound Kit ligand                                       |
| <b>Mn-SOD</b>                     | Mn-containing superoxide dismutase                              |
| <b>MCP-1</b>                      | Monocyte chemoattractant protein 1                              |
| <b>NMDA</b>                       | N-methyl-D-aspartate                                            |
| <b>NOX</b>                        | NADPH oxidase                                                   |
| <b>NRP-2</b>                      | Neuropilin 2                                                    |
| <b>NVU</b>                        | Neurovascular unit                                              |
| <b>NADPH</b>                      | Nicotinamide adenine dinucleotide phosphate                     |
| <b>NO</b>                         | Nitric oxide                                                    |
| <b>N<sub>2</sub></b>              | Nitrogen                                                        |

|                                  |                                                              |
|----------------------------------|--------------------------------------------------------------|
| <b>IKB</b>                       | Nuclear factor of kappa light chain gene enhancer in B-cells |
| <b>OEC-CM</b>                    | OEC-conditioned media                                        |
| <b>OECs</b>                      | Outgrowth endothelial cells                                  |
| <b>O<sub>2</sub></b>             | Oxygen                                                       |
| <b>OGD</b>                       | Oxygen-glucose deprivation                                   |
| <b>OGD±R</b>                     | Oxygen-glucose deprivation with/out reperfusion              |
| <b>PGS</b>                       | Pericyte cell growth supplement                              |
| <b>PM</b>                        | Pericyte medium                                              |
| <b>ONOO<sup>-</sup></b>          | Peroxynitrite                                                |
| <b>PBS</b>                       | Phosphate buffered saline                                    |
| <b>PDGF-BB</b>                   | Platelet derived growth factor B                             |
| <b>PDGF</b>                      | Platelet-derived growth factor                               |
| <b>PBEF</b>                      | Pre-B cell-enhancing factor                                  |
| <b>ROS</b>                       | Reactive oxygen species                                      |
| <b>rtPA</b>                      | Recombinant tissue plasminogen activator                     |
| <b>RNA</b>                       | Ribonucleic acid                                             |
| <b>NaF</b>                       | Sodium fluorescein                                           |
| <b>sKitL</b>                     | Soluble Kit ligand                                           |
| <b>SDF-1</b>                     | Stromal-derived factor                                       |
| <b>Shh</b>                       | Astrocyte-secreted Sonic Hedgehog                            |
| <b>O<sub>2</sub><sup>-</sup></b> | Superoxide anion                                             |
| <b>SEM</b>                       | Standard error mean                                          |
| <b>SOD</b>                       | Superoxide dismutase                                         |
| <b>THBS1</b>                     | Thrombospondin 1                                             |
| <b>TLRs</b>                      | Toll-like receptors                                          |
| <b>TEER</b>                      | Transendothelial electrical resistance                       |
| <b>TGFB1</b>                     | Transforming growth factor beta 1                            |
| <b>TGFBR1</b>                    | Transforming growth factor beta receptor 1                   |
| <b>TNF-α</b>                     | Tumour necrosis factor alpha                                 |
| <b>TNFAIP2</b>                   | Tumour necrosis factor alpha-induced protein 2               |
| <b>TIE2</b>                      | Tyrosine kinase                                              |
| <b>VEGF-C</b>                    | Vascular endothelial growth factor C                         |
| <b>VEGF</b>                      | Vascular endothelial growth factors                          |

|                       |                             |
|-----------------------|-----------------------------|
| <b>H<sub>2</sub>O</b> | Water                       |
| <b>WHO</b>            | World Health Organisation   |
| <b>WST-1</b>          | Water-soluble tetrazolium 1 |
| <b>ZO-1</b>           | Zonula occludens protein 1  |

## **Abstract**

**Background:** Ischaemic stroke is a major cause of mortality worldwide. Disruption of human blood-brain barrier (BBB), established mainly by human brain microvascular endothelial cells (HBMECs), constitutes the major cause of death after ischaemic stroke. Hence, discovery of new agents that can effectively replace dead/dying endothelial cells is of paramount importance for stroke patients. Outgrowth endothelial cells (OECs), a subtype of endothelial progenitor cells (EPCs), which can proliferate, migrate, and differentiate may be one of such agents. However, EPCs scarcity in peripheral circulation requires their isolation from peripheral blood and *ex vivo* expansion in order to generate adequate amounts of OECs for therapeutic purposes. In addition, the OECs secrete a vast range of trophic substances (OEC-secretome) that may restore cerebral barrier function following damage. Furthermore, oxidative stress has a major role in BBB-evoked ischaemic damage. NOX2 represents the major source of oxidative stress in vasculature. Also, tumour necrosis factor alpha (TNF- $\alpha$ ) is pro-inflammatory cytokine that affects the integrity and function of BBB during ischaemic stroke. In addition, hyperglycaemia is another factor that aggravates BBB-evoked oxidative stress damage. Thus, first, we investigated whether OECs can form BBB and restore its function following damage. Second, we explored whether NOX2 inhibitor can protect BBB against oxidative stress damage, and its impact on OECs. Third, we studied whether combining NOX2 inhibitor with therapeutic hypothermia can protect BBB against hyperglycaemic damage. Fourth, we investigated the impact of senescence on the functional characteristics and reparative capacities of



OECs. Finally, we studied the effect of OEC-secretome on TNF- $\alpha$ -evoked BBB damage.

**Methods:** The EPCs were isolated from human peripheral blood and cultured to generate OECs. The characteristics of OECs and HBMECs were studied. BBB models were constructed by co-culture of HBMECs or OECs with human pericytes (HPs) and human astrocytes (HAs) before exposure to oxygen-glucose deprivation (OGD) alone or followed by reperfusion (OGD+R) in the presence or absence of specific NOX2 inhibitor, gp91ds-tat. In some experiments, BBB models were exposed to hyperglycaemic conditions and were treated with therapeutic hypothermia with/out gp91ds-tat. In other experiments, BBB models were constructed with late passage OECs with HPs and HAs. In some experiments, OECs were cultured for 48 hours under hypoxic conditions to collect OEC-conditioned media (OEC-CM) which was used as therapeutics for TNF- $\alpha$ -evoked BBB damage. The function and integrity of BBB were assessed by measurements of paracellular flux of sodium fluorescein and transendothelial electrical resistance, respectively. NADPH oxidase activity (lucigenin chemiluminescence assay), superoxide anion ( $O_2^{\bullet-}$ ) production (cytochrome C assay), total antioxidant capacity (ELISA), proliferation rate (wound scratch assay), tube formation assays (Matrigel assay), and cytoskeletal organisation (actin filament staining) were assessed using the specific assays indicated in brackets. Exogenous OECs used as therapeutics following physical endothelial damage of the BBB model (wound scratch assay) and following OGD-induced BBB damage.

**Results:** Our study revealed that like the HBMECs, the OECs displayed endothelial and angiogenic characteristics, formed effective tight junctions, and

established functional BBB. Unlike the HBMECs, OECs show higher proliferative and migratory capacity. We revealed that exogenous OECs effectively repaired and restored BBB function following damage. Compared to HBMECs, OECs expressed lower NADPH oxidase activity,  $O_2^{\bullet-}$  levels, formed less stress fibres, and had higher total antioxidant capacity during OGD±R. Gp91ds-tat prevented disruption of actin cytoskeletal formation and restored the function of BBB established by HBMECs. In contrast, treatment with gp91ds-tat increased OGD-evoked stress fibre formation in OECs and aggravated ischaemic injury-induced BBB damage constructed by OECs. The deleterious impact of hyperglycaemia on BBB was more effectively reversed using therapeutic hypothermia with gp91ds-tat. Late passage OECs showed significant changes in morphology, failed to proliferate, and repair damage, and were not able to form functional BBB. OEC-CM appears to induce cellular proliferation, migration, adhesion, wound repair, preservation of cellular architecture, and protect BBB function against TNF- $\alpha$ -evoked BBB damage.

**Conclusion:** Our study reveals that OECs can effectively repair BBB following damage and may therefore serve as a novel cell-based therapeutic option for ischaemic stroke. While selective inhibition of NOX2 appears to protect BBB against ischaemic damage, specific targeting of NOX2 while using OECs as cellular therapy requires close attention. Co-application of NOX2 inhibitor with therapeutic hypothermia, on the other hand, appears to be an efficacious therapeutic possibility for ischaemic stroke patients with diabetes. Despite its tumour-suppressive benefits, senescence adversely affects OECs' reparative capacity. In this context, OEC secretome negate ischaemic injury-evoked inflammatory responses and emerge as a novel cell-free therapeutic strategy.

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### **3 OUTGROWTH ENDOTHELIAL CELLS FORM FUNCTIONAL CEREBRAL BARRIER AND RESTORE ITS INTEGRITY AFTER DAMAGE.**

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# **1 INTRODUCTION**



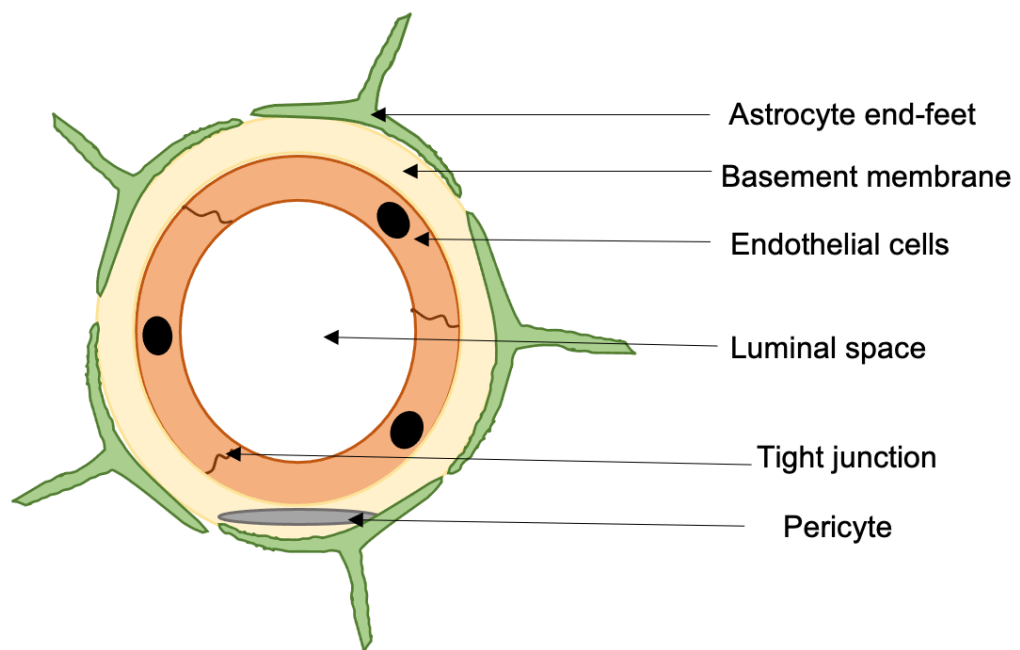
According to the World Health Organisation (WHO), each year stroke affects about 15 million people worldwide. Sadly, while one-third of these cases die, another one-third live with long-term disabilities caused by this disease (Ma et al., 2015). Hence, stroke continues to be one of the main causes of mortality and morbidity globally (Liao et al., 2017a, Hisham and Bayraktutan, 2013). Ischaemic strokes, stemming from an interruption of blood supply to the brain, account for nearly 80-85% of all strokes. Despite being the major cause of human brain damage, systemic thrombolysis with recombinant tissue plasminogen activator (rtPA) or endovascular procedure remain the only pharmacological and interventional therapeutic options for this condition (Reis et al., 2017, Allen and Bayraktutan, 2009b). However, due to short treatment window of 4.5 hours of a symptom onset and strict eligibility criteria, every year less than 1% of stroke patients globally receive either therapy (Bayraktutan, 2019b, Hacke et al., 2008), necessitating the discovery of alternative therapeutic agents or strategies that can be safely and effectively used for ischaemic stroke. Although over the last years many treatments targeting a single mechanism associated with ischaemic stroke, such as excitotoxicity, have shown favourable results in experimental settings, subsequent clinical trials have failed to generate similar outcomes. Whilst complexity of human neurovascular unit and concurrent appearance of a vast range of specific mechanisms like oxidative stress, inflammation, and apoptosis after an ischaemic injury have been suggested to explain this apparent dichotomy, consideration of new therapeutic agents or approaches has become of paramount importance (Deb et al., 2010). In this regard, cell-based or cell-free approaches, particularly treatments with outgrowth endothelial cells (OECs), a

subtype of endothelial progenitor cells (EPCs), may repair the damaged neuro-vasculature through either autocrine or/and paracrine manners (Condon et al., 2004, Di Santo et al., 2009). OECs are equipped with an inherent capacity to migrate, proliferate, and differentiate into other cells, particularly mature endothelial cells that shield the whole inner surface of all blood vessels and help conserve vascular homeostasis through control of inflammation, coagulation, and vascular tone. Endothelial cells also constitute the major cellular element of human blood-brain barrier (BBB), a fence between the blood and the brain tissue. Hence, any pathology capable of influencing endothelial cell characteristics or viability can affect the integrity and function of this barrier and promote brain oedema, the main cause of death within the early days following an ischaemic stroke (Heo et al., 2005). Naturally, mobilisation of EPCs that able to find and replace dead or dying endothelial cells to restore cerebral barrier function and integrity may be of considerable therapeutic value for patients with ischaemic stroke. EPCs are a subtype of mononuclear cells found in peripheral blood and were first isolated in 1997 by using magnetic microbeads coated with CD34 antibodies (Asahara et al., 1997). There are two subtypes of EPCs: early EPCs and late EPCs or OECs. The latter also known as endothelial colony-forming cells (ECFCs). In experimental studies, OECs show higher proliferative ability, display dedication to endothelial lineage, and have specific vascular capabilities (Moubarik et al., 2011, Medina et al., 2010b).

### **1.1 Blood-brain barrier structure and function**

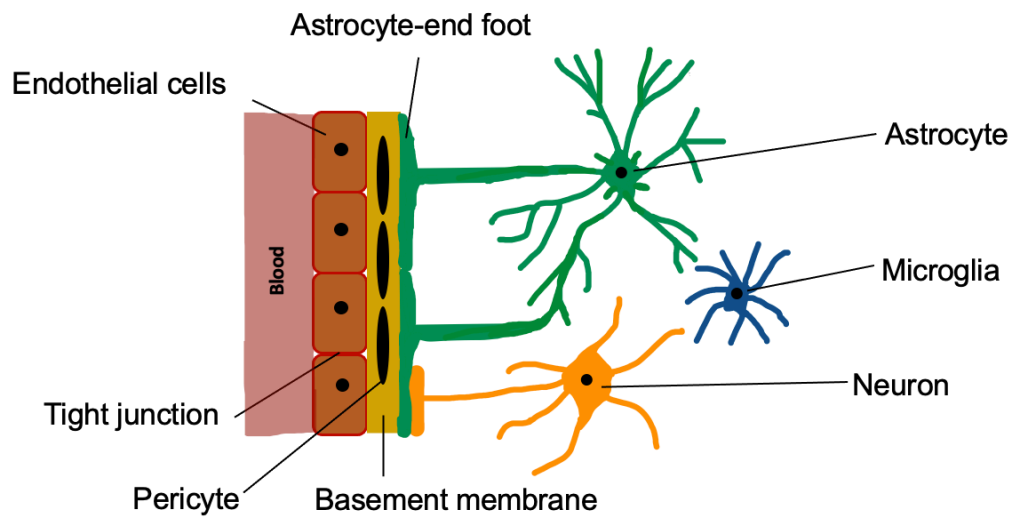
The BBB is a complex structure that act as a fence between the blood and the central nervous system, and it regulates substances and nutrients exchange

between the two compartments, thus it works as an essential protector for the brain against toxic substances and microorganisms (Cardoso et al., 2010). The BBB was first observed, in 1885, by Paul Ehrlich when he noticed that the brain and spinal cord remained white after injecting vital dyes intravenously while the peripheral organs were stained (Dyrna et al., 2013). Few years later, Edwin Goldmann, observed the opposite phenomenon when he injected trypan blue directly into the cerebrospinal fluid (CSF), this stained the central nervous system (CNS) but not the peripheral organs, indicating the presence of a barrier between blood and the brain (Serlin et al., 2015). The BBB plays key roles including: regulation of molecular traffic, keeping the toxins out, preserving connectivity between neurons, maintaining the protein at lower levels in the CNS, permits immune response with less cell injury, and assists ion homeostasis (Abbott, 2013). Due to the established BBB, the circulating molecules in the blood can enter the interstitial fluid of the brain by one of the ways whether via the free diffusion process (lipid-mediated transport of micro molecules) or through the catalysed transport process of both the micro and macromolecules (Vorbrodt and Dobrogowska, 2003). The structure of the BBB includes human brain microvascular endothelial cells layer (HBMECs) which are joined together by tight junction proteins, capillary basement membrane, human astrocytes (HAs) end-feet enclosing the blood vessels, and human pericytes (HPs) which are embedded in a basement membrane (Figure 1.1 ) (Ballabh et al., 2004). The HBMECs, neurons, HAs, HPs, and microglia act collectively together to perform the physiological functions, and they are refereed as the neurovascular unit (NVU) (Figure 1.2) (Zehendner et al., 2009, Alwjwaj et al., 2021).



**Figure 1-1 schematic representation of BBB structure.**

Figure adapted from (Ballabh et al., 2004).



**Figure 1- 2 schematic representation of neurovascular unit (NVU) structure.**  
(Alwjwaj et al., 2021).

### **1.1.1 Human brain microvascular endothelial cells**

One of the first systems to be developed during the embryonic state is the vascular system. The interior surfaces of this system are covered by a thin layer of endothelial cells which form the endothelium that plays an array of biological functions including glomerular filtration of blood in the kidney, haemostasis, neutrophil transportation, thrombosis prevention, delivering blood-borne nutrients, providing necessary oxygen levels to tissues and organs, and control of blood vessels tone (Pelosi et al., 2014, Yoder, 2010). The HBMECs constitute an important component of the BBB, have specific phenotype, and are different from the endothelial cells of the other systems with characters of intracellular tight junctions, lack of fenestration, higher number of mitochondria, less levels of pinocytosis, and many specific transport systems that actively transport the nutrients from the blood to the brain or take out toxic substances from the brain into the blood circulation (Weiss et al., 2009, Petty and Lo, 2002). HBMECs express specific markers including alkaline phosphatase,  $\gamma$ -glutamyltranspeptidase (GGT), and monoamine oxidase (MAO). In contrast, they express leukocyte adhesion molecules at very low levels, which make it almost impossible for immune cells to cross BBB. Besides, HBMECs do not express thrombomodulin, a protein commonly expressed in other endothelial cells (Goncharov et al., 2017). HBMECs have plasma membrane transport protein and receptors that allow specific passage of entry for glucose, ions, and some macromolecules, and provide routes of exit for the toxic metabolites (Paolinelli et al., 2011). There are two major types of transporters expressed by HBMECs: efflux transporters and nutrient transporters. Efflux transporters include P-glycoprotein, breast cancer resistance protein (BCRP), and multidrug

resistance proteins (MRPs). Nutrient transporters include various solute carrier (slc) class transporters, notably slc2a1 (glucose transporter protein), slc7a1 (cationic amino acids transporters), slc7a5 (neutral amino acids and L-DOPA transporters), and slc16a1 (lactate and pyruvate transporters) (Daneman and Prat, 2015). HBMECs are manifested by expression of ATP-binding cassette transporters which face the vascular space, lining the luminal plasma membrane of the endothelial cells, playing a role as a shelter for the brain against the toxic molecules and the three members of these transporters, the B, C and D, all together limit the entry of lipophilic xenobiotics (Miller, 2010).

### **1.1.2 Basement membrane**

One of the key structures of the BBB is the basement membrane. It encloses the HBMECs, contains HPs, plays an anchoring function, making connections with the HAs end-feet, and may contribute to formation of the tight junctions between the endothelial cells (Carvey et al., 2009). The basement membrane is constituted by three apposed layers, for which the HBMECs, HPs and HAs, contribute together to form these layers. They contain laminin 1, -2, -4, and -5, type IV collagen, proteoglycans, and glycoproteins (Weiss et al., 2009, Perlmutter and Helena Chang, 1990, Obermeier et al., 2013).

### **1.1.3 Human astrocytes**

The HAs are star shaped cellular components of the neuroglia with many processes and end-feet that are scaffolding and opposing the outer surfaces of the HBMECs, they express the intermediate filaments vimentin and glial fibrillary acidic protein (Lawther et al., 2011, Alexander, 2018). About 90% of

the abluminal side of the HBMECs of the cerebral capillaries is enclosed by the HAs end-feet (Correale and Villa, 2009). The HAs generate the basement membrane laminin-111 and -211 which play an essential role in maintaining the integrity of the BBB by blocking the HPs differentiation from resting stage to contractile stage, and by maintaining the tight junction protein expression in the HBMECs (Yao et al., 2014). The transendothelial electrical resistance (TEER) of cultured HBMECs without HAs is about  $90 \Omega/\text{cm}^2$  and co-culturing of HBMECs with HAs to establish the *in vitro* model BBB, boosts the TEER by about 77 %, at around  $153 \Omega/\text{cm}^2$  (Krause et al., 1991, Kondo et al., 1996, Rakkar and Bayraktutan, 2016, Abdullah and Bayraktutan, 2016a).

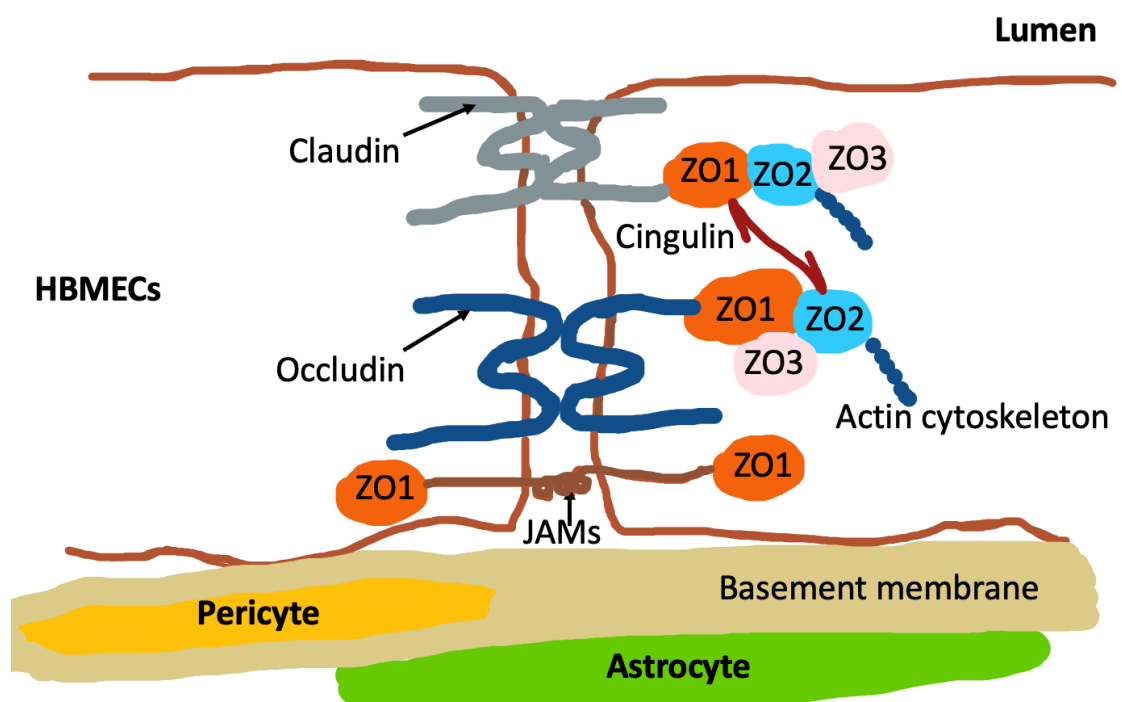
#### **1.1.4 Human pericytes**

The HPs are flat broaden cells with slender projections, have a prominent round nucleus, extended longitudinally parallel to the long axis of the capillaries, located between the HBMECs and the HAs (Nag, 2011). The HPs make direct contact with the HBMECs, enclose the blood vessels walls, and modulate the capillary diameter by tightening the vascular walls (Peppiatt et al., 2006), an action which might obstruct the blood flow during ischaemia (Bell et al., 2010, Vates et al., 2010). They play key roles in preserving BBB integrity, facilitating angiogenesis and microvascular stability, making direct synaptic-like peg-socket connections with HBMECs via N-cadherin and connexins, and promoting exchanges of metabolic substances, ions, and ribonucleic acids (Zhao et al., 2015). HPs regulate the traffic of vesicles and immune cells, assist blood flow, maintain BBB, and have a role in vascular maturation (Jiang et al., 2018a, Daneman et al., 2010).



### **1.1.5 Tight junctions**

Tight junctions which bind HBMECs together are complex anastomotic ridges that controls the exchanges of substances and water between circulation and the brain interstitial fluids (Bradbury, 1993). Using transmission electron microscopy, the tight junctions appear with penta-laminar character which are formed when the outer leaflets of neighbouring HBMECs' plasma membranes fuse together at intervals throughout the inter-endothelial space (Nag, 2003). These junctions force most of the molecular traffic away from passing through the paracellular routes but to take the transcellular routes instead (Abbott et al., 2006, Wolburg and Lippoldt, 2002). The role of these junctions in limitations of passive molecular diffusion across the BBB into the brain leads to significantly high reading in vascular TEER (Lippmann et al., 2012). The inter-endothelial cell tight junctions consist of three major membrane proteins, namely, claudin, occludin, and junction adhesion molecules (JAMs) and several cytoplasmic proteins including zonula occludens-1, -2, -3 (ZO-1, ZO-2, ZO-3), and cingulin (Kniesel and Wolburg, 2000). Claudins constitute the main structural units of tight junctions and are tasked with the prevention of passage of small molecules via the paracellular route. Claudins form this so-called primary seal by binding to claudins of adjacent cells and to cytoplasmic proteins, ZO-1, ZO-2, and ZO-3 (Kadry et al., 2020). Other tight junction proteins such as occludin play mostly functional role and some structural roles (Haseloff et al., 2015). (Figure 1.1.3). The formation of paracellular pores may be made by claudin-2, -10 and -16, and some proteins such as claudin-6, -9, and -13 may play roles in the maturation of paracellular permeability (Krause et al., 2008).



**Figure 1- 1- 3 schematic representation of tight junctional structure.**

Figure adapted from (Kadry et al., 2020).

### **1.1.6 Significance of stress fibers in BBB function**

The permeability of BBB is mainly controlled by adherence and tight junctions. While adherence junctions connect endothelial cells together, tight junctions prevent paracellular diffusion. Under pathological conditions, HBMECs become activated and as a result their actin cytoskeleton become re-arranged. In resting state, the actin of endothelial cells is present as a monomeric globular actin (G-actin). Once cells are activated, G-actin transforms into filamentous actin (F-actin). The change from G-actin to F-actin leads to formation of contractile actin bundles known as stress fibers. These fibers attach to adherence junctions of adjacent endothelial cells. When stress fibers start to contract under stress, this will result in the formation of intracellular gaps between neighboring endothelial cells, causing an increase in BBB permeability and consequently cerebral oedema (Millán et al., 2010, Manaenko et al., 2016).

### **1.1.7 Significance of cell migration in BBB function**

Endothelial dysfunction is the first step in BBB dysfunction following ischaemic stroke. It is manifested by loss of endothelial integrity and leakage of plasma contents into brain tissue. Restoration of endothelial integrity and cerebral barrier function might be very effective therapeutic approach for ischaemic stroke. Endothelial integrity can be maintained by cellular proliferation and lateral migration of neighboring endothelial cells. OECs, also, are thought to participate in this reparative process either by incorporating into the site of damage or by secreting trophic substances such as VEGF which play role in inducing proliferation, migration, and differentiation of endothelial cells (Bayraktutan, 2019b, Alwjaj et al., 2021).

### **1.1.8 Cell to cell interaction in the neurovascular unit**

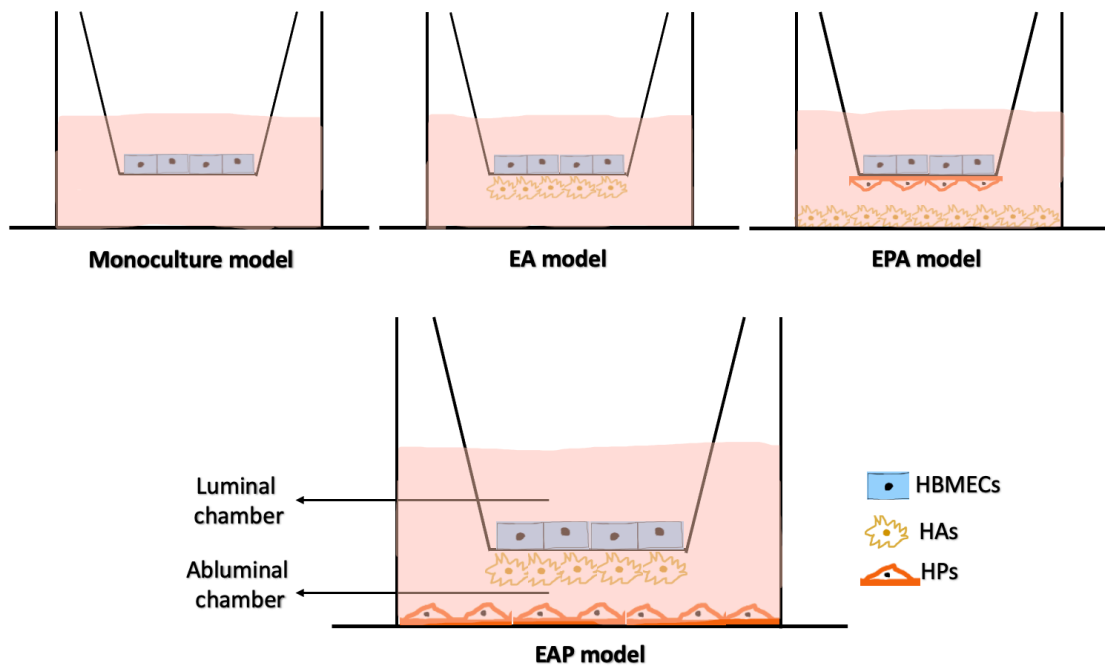
In the brain, both vascular and neuronal cells establish a physiologically integrated network, where vascular dynamics and neuronal activities are tightly linked. As HAs are in close contact with cerebral microvessels and neuronal synapses, they are in a unique position to couple vascular growth with neuronal activity. There is a cross talk amongst HA, HP, and HBMEC functions to maintain BBB integrity, mainly through expression and regulation of tight junctions. HAs release pro-angiogenic factors such as VEGF and vasoactive mediators which collectively control vascular tone and cerebral blood flow. In addition, HAs express molecules that regulate the characteristics of BBB integrity in HBMECs. For example, Sonic Hedgehog (Shh) protein secreted by HAs induces the expression of various tight junction components e.g. claudin-5 and occludin and simultaneously decrease expression of cell adhesion molecules and chemokines. HPs, also, contribute to BBB integrity and maintenance by regulating capillary diameter in response to neuronal activity. They regulate the expression and alignment of tight junction proteins, control angiogenesis, microvascular stability, and BBB permeability (Andreone et al., 2015, Lind et al., 2013, Sweeney et al., 2016).

### **1.2 Different types of in vitro BBB models**

The BBB formed mainly of HBMECs, HAs end-feet, and HPs. It is a barrier that regulates the exchange of molecules between the blood and the brain. Given that disruption of BBB leads to brain oedema, haemorrhagic complications, and thus augments morbidity and mortality among ischaemic stroke patients, a

great deal of studies have recently been conducted to identify the cellular and molecular mechanisms involved in BBB (dys)function during or following an ischaemic stroke. It is predicted that determination of such mechanisms will give rise to development of new, and possibly more efficacious, treatment options for ischaemic stroke (Jiang et al., 2018b, Li et al., 2019). Hence, stroke research community have been trying hard over the course of last decade or so to establish different *in vitro* and *in vivo* models mimicking human cerebral ischaemic damage. Even though *in vivo* models, particularly in larger species, better mirror human physiology, *in vitro* models have many benefits (Canazza et al., 2014, Stone et al., 2019). First, *in vitro* models are uncomplicated, more straightforward, and cost effective to investigate the impact of ischaemia/reperfusion injury. Second, the participation of every cell type and the complexity of molecular mechanisms caused by ischaemic stroke damage can be easily and extensively studied. Third, these models enable extensive investigations of compounds to explore their cellular and molecular signalling impacts during and after ischaemic damage (Sivandzade and Cucullo, 2018). Furthermore, regarding the variations in immune reactions among different species, the use of human brain cells in *in vitro* models may somewhat replicate the inflammatory response in humans (Holloway and Gavins, 2016). The long-established *in vitro* BBB models are constructed by co-culture of different types of human brain cells in Transwell insert system. Having considered that HAs enclose 99% of brain microvessels, and the HPs is substantially abundant in the brain as opposed to the peripheral blood vessels, co-culture models formed of HBMECs, HAs, and HPs, so-called EAP models, are physiologically and anatomically closer to human BBB (Rakkar and Bayraktutan, 2016, Abdullah

and Bayraktutan, 2016b). Moreover, extremely close proximity between HBMECs and HAs in this model produce a substantially tighter barrier integrity compared to endothelial cell monoculture, EA, and EPA models (Shao and Bayraktutan, 2013b, García-Salvador et al., 2020). Even though EAP model does not accommodate mechanical factors, such as shear stress, as shown in dynamic BBB models, its exposure to 4 hours oxygen-glucose deprivation (OGD) alone or followed by 20 hours reperfusion (OGD+R) has been shown to closely replicate the ischaemic stroke damage observed in clinical practice (Allen and Bayraktutan, 2009a, Abdulkadir et al., 2020a). HPs in *in vitro* BBB models are known to regulate expression of adherence and tight junction proteins, work as macrophages to clear cell debris, and may also contribute to communication of inflammatory signals in the BBB (Sweeney et al., 2016). In light of the above, the EAP triple cell culture model has been used for all the relevant studies outlined in this thesis. Figure 1.1.4 illustrates different types of *in vitro* BBB models.



**Figure 1- 1- 4 Different types of in vitro BBB models**

### 1.3 Ischaemic stroke

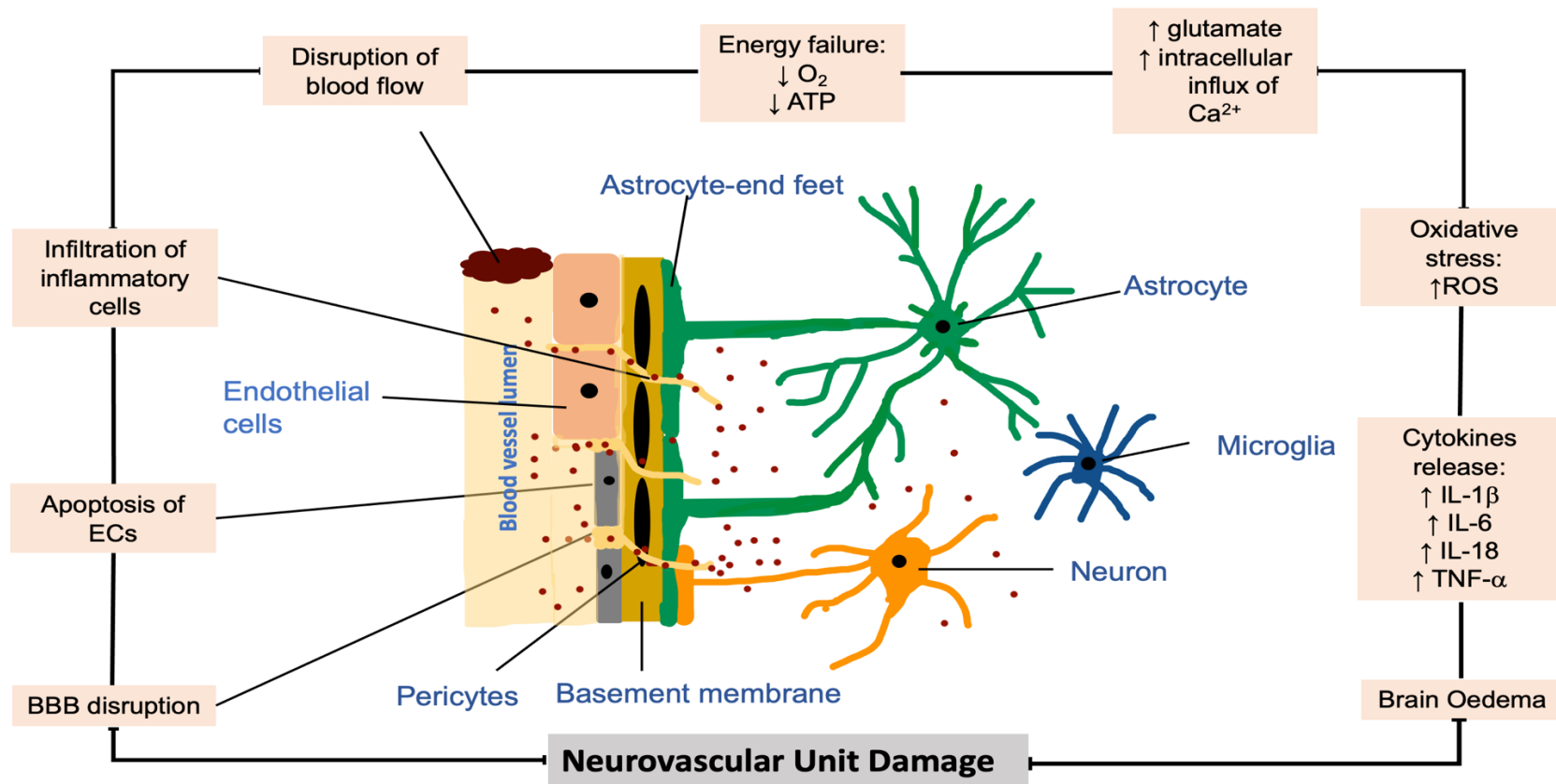
Stroke is defined as a neurological deficit due to abrupt focal damage of the central nervous system, which is provoked by a vascular source, this includes cerebral infarction, intracerebral haemorrhage, and subarachnoid haemorrhage (Sacco et al., 2013). Stroke is divided into two major types: haemorrhagic and ischaemic. In the United Kingdom alone, for example, stroke causes about 60,000 deaths annually and costs the economy around £9 billion each year (Hisham and Bayraktutan, 2013). Of all strokes, ischaemic stroke accounts for about 87% of the cases (Benjamin et al., 2018). Ischaemic stroke characterised by heterogenous pathogenesis and is caused mainly by occlusion of large arteries whether by emboli or thrombus, also can be caused by small arteries or venous occlusion, severe anaemia, arterial dissections, and arteritis (Montaner et al., 2008, Meschia and Brott, 2018).

### **1.3.1 Pathophysiology**

The cause of ischaemic stroke can be manifested in the following forms: embolic stroke, large or small vessel thrombotic stroke, and venous thrombosis or systemic hypoperfusion. Regardless of the aetiology, disruption of blood supply to the brain tissues is the first event in most acute ischaemic strokes (Deb et al., 2010). Acute disruption of blood flow produces different areas of ischaemia in the affected blood vessel region. The affected areas with cerebral blood flow of lower than 10ml/100 gram of tissue per minute are known as the core, where cells in this zone are expected to die within minutes. Areas of perfusion of less than 25ml/100 gram of tissue per minute are called ischaemic penumbra, where cells can survive for many hours because of peripheral tissue perfusion (Latchaw et al., 2003). The pathophysiology of ischaemic stroke is complex and includes several processes, involving energy depletion, excitotoxicity process, inflammatory pathways, oxidative stress damage, ionic imbalance, acidosis, increased intracellular calcium levels, apoptosis, BBB disruption, leukocytes infiltrations, glial cells activation, and neuroprotective mechanisms (Deb et al., 2010, Woodruff et al., 2011). Excitotoxicity harms NVU through increases in sodium and calcium influx triggered by accumulation of extracellular excitatory amino acids glutamate and aspartate (Deb et al., 2010, Williams-Karnesky and Stenzel-Poore, 2009, Fann et al., 2013, Woodruff et al., 2011, Martin and Wang, 2010a, Xing et al., 2012, Lipton and Rosenberg, 1994, Brennan-Minnella et al., 2015). Oxidative stress and post-ischaemic inflammation, characterised by excessive availabilities of reactive oxygen species (ROS) and inflammatory cytokines such as IL-1 $\beta$ , IL-18, IL-6, and TNF-



$\alpha$ , which harm NVU by disrupting structural integrity of the tight junctions (Fann et al., 2013, Doyle et al., 2008, del Zoppo and Hallenbeck, 2000, Allan and Rothwell, 2001, Prakash and Carmichael, 2015). Figure 1.3 shows the impact ischaemic stroke mechanisms on NVU.



**Figure 1-5 schematic representation of the impact of ischaemic stroke on neurovascular unit.**

Abrupt disruption in blood flow to the brain which result in reduction in oxygen and energy supply provokes oxidative stress and compromises the integrities of BBB and tight junctions. Excitotoxicity and excessive production of inflammatory cytokines induce further damage to the neurons and BBB leading to formation of cerebral oedema. ROS: reactive oxygen species, ATP: adenosine triphosphate, ECs: endothelial cells, BBB: blood-brain barrier (Alwjaj et al., 2021).

#### **1.3.1.1 Energy failure**

Disruption of blood supply to a certain area in the brain will lead to critical reduction of oxygen and glucose supply to the cells which will result in a drop in the levels of adenosine triphosphate (ATP) productions by mitochondria (Deb et al., 2010, Williams-Karnesky and Stenzel-Poore, 2009). Failure of ATP production will result in functional disruption of all ATP-related ion pumps, which will make glial cells and neurons highly vulnerable for cerebral ischaemia (Fann et al., 2013).

#### **1.3.1.2 Excitotoxicity mechanisms**

One of the main mechanisms that leads to ischaemia-induced cerebral damage is caused by uncontrolled excessive accumulation of extracellular excitatory amino acids, glutamate, and aspartate, which will raise the amount of intracellular calcium (Woodruff et al., 2011, Martin and Wang, 2010b). Glutamate is essential excitatory neurotransmitter in the brain and its stimulation to special membrane receptors is important for several neurological functions involving memory, cognition, sensation, and movement (Lipton and Rosenberg, 1994). This amino acid is produced through intermediate metabolite glutamine in the HAs, and it acts on four receptors including: N-methyl-D-aspartate (NMDA), kainite,  $\alpha$ -amino-3-hydroxy-5-methyl-4-propionat (AMPA), and metabotropic receptors. The NMDA, AMPA, and kainite receptors are linked to membrane ion channels (Lipton and Rosenberg, 1994). Excessive activation and continuous action of glutamate at NMDA type glutamate receptors during stroke lead to excitotoxicity and neuronal injury or death

(Brennan-Minnella et al., 2015, Lipton and Rosenberg, 1994). The release of glutamate happens when blood supply to neurons drops below specific levels (blood flow of less than 20ml per 100 gram per minute) (Kostandy, 2012, Shimada et al., 1989). Then glutamate bind to NMDA and AMPA receptors which results in increases in sodium and calcium influx (Xing et al., 2012, Deb et al., 2010, Lipton and Rosenberg, 1994). The elevated intracellular calcium will cause serious effects on neuronal membrane such as activation of many of enzymes namely proteases, nucleases, and lipases which can lead to damage of cell membrane and genomic DNA then to ultimate cell death (Mehta et al., 2007, Woodruff et al., 2011).

#### **1.3.1.3 Inflammatory pathways**

Inflammatory response is a type of innate immunity that happen due to tissue damage or infections which work to protect host from harm. However in ischaemic stroke patient, this response causes a significant brain damage (Fann et al., 2013). In the brain, microglia are major resident cells like immunological cells which can be activated within few minutes in response to brain ischaemia and this activation can last for several weeks after ischaemic injury. However, the precise roles of microglia in ischaemic stroke are unclear (Jin et al., 2013). Microglia can play dual function contributing to cell injury and death or playing neuroprotective role through production of transforming growth factor beta (TGF- $\beta$ 1) (Lai and Todd, 2006). During ischaemic stroke, pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, IL-18, and TNF- $\alpha$  are produced by triggered cells involving endothelial cells, HAs, microglia, and neurons. This leads to glial cells death during cerebral ischaemia (Fann et al., 2013, Allan and

Rothwell, 2001). TNF- $\alpha$  and IL-1 stimulate the endothelium of blood vessels to release nitric oxide (NO), can cause damage to BBB, and can induce cerebral vasogenic oedema (Allan and Rothwell, 2001, Bonmann et al., 1997, Wong and Dorovini-Zis, 1992).

#### **1.3.1.4 Reactive oxygen species release**

ROS in the brain is produced primarily by the mitochondrial respiratory chain and the cellular enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), xanthine oxidase and lipoxygenases (Chen et al., 2011b, Shirley et al., 2014). In addition to ATP generation, mitochondria have an essential role in production of ROS, whether mainly by enzymes through mitochondrial electron transport chain (the respiratory chain) or by other mitochondrial enzymes such as NOX4. However, the production of ROS by the later enzymes is remarkably lower than respiratory chain contribution (Kalogeris et al., 2014, Chen et al., 2011b). In the electronic transport chain, the mitochondrial enzymes including complex I (NADH dehydrogenase), complex III (coenzyme Q), and cytochrome C oxidoreductase convert the oxygen molecule into  $O_2^{\bullet-}$  which result in hydrogen peroxide ( $H_2O_2$ ). This can, also, produce hydroxyl radical (Kalogeris et al., 2014, Navarro and Boveris, 2007). In addition,  $O_2^{\bullet-}$  reaction with NO can produce peroxynitrite ( $ONOO^-$ ) (Kalogeris et al., 2014). In physiological state, low levels of ROS are important for cellular proliferation, growth, differentiation, and apoptosis. However, ROS production increases during cerebral ischaemia and further after reperfusion. When ROS reaches toxic levels, leading to an imbalance between production and neutralization by antioxidant enzymes, they cause cellular injury

through DNA damage, protein oxidation, and lipid peroxidation (Olmez and Ozyurt, 2012). The elevated levels of intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , during cerebral ischaemia, stimulate the mitochondria to generate high levels of ROS (Doyle et al., 2008). The major source of ROS generation after cerebral ischaemia and specially during reperfusion injury is NADPH oxidases (Shirley et al., 2014, Chen et al., 2009). Inhibition of NADPH oxidases may decrease or stop the ongoing damage of brain cells during or following ischaemic stroke.

#### **1.3.1.5 Ionic imbalance**

Loss of oxygen and glucose supply to neurons in cerebral ischaemia causes anoxic depolarization (AD), which result from immense physiological stress on the neurons due to imbalance of ionic changes and failure of ATP production that preserve electrochemical gradients (Weilinger et al., 2013). When cerebral blood flow drops under 10%, the membrane function of neural and glial cells become immensely depolarized within two minutes which leads to AD generation even without glutamate activation (Anderson et al., 2005). Physiological state of some ion transport proteins starts to be harmful during brain ischaemia such as NMDA receptors, volume-regulated anion channels, aquaporins water channels, hemichannels, and acid sensing ion channels (ASIC) (Khanna et al., 2014). Continuous stimulation of non-NMDA receptors, such as  $\text{Na}^+/\text{H}^+$  exchanger isoform one and transient receptor potential melastatin 7, leads to uncontrolled influx of  $\text{Zn}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Ca}^{2+}$  which will result in ischaemic reperfusion injury (Leng et al., 2014).

#### **1.3.1.6 Apoptosis**

Apoptosis is defined as a programmed cell death which happen physiologically and can be activated and become pathological (Cohen et al., 1992). It has been found that several neurons in the ischaemic penumbra might experience apoptosis following many hours or days of ischaemic stroke. Thus, ischaemic penumbra is highly salvageable after ischaemic stroke onset (Broughton et al., 2009). There are several essential mediators play essential roles in the neuronal apoptosis including: generation of oxyradicals, dysfunction of mitochondria, disrupted calcium homeostasis, and cysteine proteases activation (Caspases) (Mattson et al., 2000). Ischaemic stroke activates the apoptosis pathway by several signals including generation of free radicals, growth factor deficiency, DNA damage, release of cytochrome C from mitochondria, and deficiency of neurotrophins (MacManus and Linnik, 1997). Apoptosis is featured by defined morphological changes of the cells which will be removed quickly by phagocytosis without triggering inflammatory reactions. This is opposite to necrosis which leads to cell membrane rupture and release of cellular contents causing inflammatory response (Love, 2003).

#### **1.3.1.7 Impact of ischaemia on blood-brain barrier**

The proteins of BBB's tight junctions regarded as the first defence against barrier disruption (Yang and Rosenberg, 2011). BBB dysfunction starts by the effect of oxidative stress and free radical generation following ischaemic event. This will disrupt the tight junction proteins and the opening of the barrier junctions (Prakash and Carmichael, 2015). Losing tight junctions during ischaemia results in transmission of fluid and plasma components with

molecular weights more than 360 kDa (del Zoppo and Hallenbeck, 2000). An *In vitro* study, by Koto et al, shows that claudin-5 was decreased in plasma membranes during conditions of hypoxia. Also, an *in vivo* study by the same group shows that the expression of claudin-5 was dropped in retinal blood vessels of mice when exposed to air with low oxygen concentration (Koto et al., 2007).

#### **1.3.1.8 Oedema formation**

One of the major pathological complications of ischaemic stroke is oedema formation, which is defined as accumulation of fluid in the extracellular or intracellular spaces (O'Brien, 1979). The brain is surrounded by firm skull and small increase in its volume can elevate the intracerebral pressure leading to neurological damage, and this is one of the main causes of high morbidity and mortality rates after ischaemic stroke (Kahle et al., 2009). Ischaemic brain oedema starts by failure of ionic pumps of cell membrane, cell swelling, disruption of the cell membrane by proteases and ROS generation; leading to irreversible damage (Rosenberg, 1999). Reduction of ATP supply affect the cells causing metabolic cellular dysfunction leading to cellular swelling and cytotoxic oedema which appear to be severe in HAs. In addition, disruption of BBB function leads to leakage of fluid from blood to cerebral parenchyma causing vasogenic oedema which increase the volume of the brain and usually leads to death (Heo et al., 2005).



### **1.3.2 Impact of hyperglycaemia on BBB function**

Diabetes is a major risk factor for ischaemic stroke. It has been proven that patients with hyperglycaemia have worse outcomes following ischaemic stroke. Compared to non-diabetics with ischaemic stroke, diabetic stroke patients have higher rates of hospitalisation (Kaarisalo et al., 2005). It is well-documented that the permeability of BBB increases in patients with diabetes due to the suppressive effects of hyperglycaemia on the expression of tight junction proteins, occludin, claudin-5, and ZO-1. In addition to tight junctions, hyperglycaemia also damages the communications between gap junctions and major cellular elements of NVU, especially with the HAs. Indeed, swollen astrocyte-end feet at the BBB interface are noted in patients with type two diabetes. Furthermore, rat models of hyperglycaemia show enhanced formation of superoxide anion levels by NADPH oxidase in brain tissue and blood vessels following reperfusion, which may exacerbate BBB permeability (Jiang et al., 2018b). Experimental studies have revealed that hyperglycaemia induces ROS production after ischaemia/reperfusion damage (Muranyi and Li, 2006, Kamada et al., 2007). Targeting the deleterious impact of oxidative stress caused by hyperglycaemia through inhibition of NOX2 is not fully explored under experimental settings and may be important therapeutic target for diabetic patients affected by ischaemic stroke.

### **1.3.3 Effect of hyperthermia on BBB function**

The brain cells are especially sensitive to the rise in temperature, where irreversible structural and functional changes can occur at ~40 °C. Extreme hyperthermia shown to have a profound effect on BBB permeability by inducing

cellular damage and increasing tissue oedema (Kiyatkin and Sharma, 2016, Kiyatkin, 2019). In rabbit models, hyperthermia at 42.5 °C shown to induce permeability to Trypan blue (Patel et al., 2020). *In vitro and in vivo* studies show that heat stress could induce BBB dysfunction by decreasing expression of claudin-5 and induce the expression of P-glycoprotein, which play role in the efflux of several chemical mediators to the brain parenchyma (Yamaguchi et al., 2019).

#### **1.3.4 Risk factors**

Risk factors are generally nonmodifiable, including age, gender, low birth weight, ethnicity, and genetic factors, or modifiable risk factors including diabetes, high blood pressure, dyslipidaemia, atrial fibrillation, cardiogenic embolism, asymptomatic carotid stenosis, sickle cell disease, therapy containing conjugated equine oestrogens, high salt intake, increased alcohol consumption, and high body weight (Goldstein et al., 2011).

#### **1.3.5 Current treatments**

Therapeutic approach for ischaemic stroke management has two aims: blood flow restoration and decreasing the effects of ischemia on brain (Deb et al., 2010). For all patients with suspected diagnosis of acute stroke, non-contrast CT scan imaging should be done because it will reveal important information related to choosing the right management approach, and also it detects the acute intracerebral haemorrhage and help to avoid the use of thrombolytics in these patients (Powers et al., 2018). For blood flow restoration, rt-PA is the most

widely used fibrinolytics for treatment of acute ischaemic stroke in selected patients, and it may resolute the neurological deficits (Adams et al., 2007). Previously, this thrombolytic drug was administered within 3 hours of acute ischemic stroke symptom onset then this therapeutic window was only expanded to 4.5 hours of symptom onset (Hacke et al., 2008, Del Zoppo et al., 2009). Mechanical thrombectomy is used alternatively for acute ischemic stroke patients when thrombolytic drug is contraindicated or inefficient. According to two important trials the window of this intervention has been extended up to 24 hours in highly eligible patients (Nogueira et al., 2018, Albers et al., 2018, Etherton et al., 2018). Unfortunately, only less than 1 % of ischaemic stroke patients worldwide get benefits from these therapeutic strategies, due it increased risk of bleeding, short treatment window, strict eligibility criteria, and requirement for highly skilled professionals for those who need thrombectomy (Bayraktutan, 2019b). Thus, finding new therapeutic options are with paramount importance for ischaemic stroke patients.

#### **1.4 Role of oxidative stress in ischaemic stroke**

The brain is highly sensitive organ to oxidative stress injury because it consumes high levels of oxygen during the normal conditions and has high amounts of lipids and high concentrations of iron which play pro-oxidant role during oxidative stress (Uttara et al., 2009). Oxidative stress, manifested by imbalance between oxidants and antioxidants in favour of oxidants, plays a major role in ischaemic stroke pathogenesis and it causes potential damage to proteins, lipids, and nucleic acid (Chehaibi et al., 2016, Zitnanova et al., 2016). Ischaemic stroke insult will lead to activation of NADPH oxidase and uncoupling

of endothelial nitric oxide synthase (eNOS). This will result in overproduction of ROS which have a major role in dysfunction of the cerebral endothelium of BBB (Sierra et al., 2011). In animal models, ROS leads to opening of potassium channels, alteration of vascular reactivity, and focal damage to BBB (Allen and Bayraktutan, 2009b). In normal conditions, mitochondria produce ATP using oxygen throughout oxidative phosphorylation process and this result in generation of low concentrations of  $O_2^{\bullet-}$  in the matrix of the mitochondria (Manzanero et al., 2013). The forms of superoxide dismutase enzyme, both in the mitochondria and in the cytoplasm, convert two molecules of  $O_2^{\bullet-}$  to  $H_2O_2$  which regulates the intracellular neural activity, function, and growth of brain cells (Rice, 2011). In the condition of oxidative stress, NOS which form NO become uncouple and generate ROS instead (Nash et al., 2018). ROS are produced after ischaemic stroke, including hydroxyl radical,  $H_2O_2$ , and  $O_2^{\bullet-}$ . Also, reactions between NO and  $O_2^{\bullet-}$  generate  $ONOO^-$ , potent oxidative radical that leads to protein damage (Chen et al., 2011a).

### **1.5 Antioxidants and NADPH oxidase inhibitor**

Glutathione peroxidase, catalase, and superoxide dismutase (SOD) are endogenous antioxidants which control ROS levels, however their action impaired during oxidative stress (Nanetti et al., 2007). Cherubini et al, found that acute ischaemic stroke patients with lower levels of vitamin C and erythrocyte SOD, and higher levels of vitamin A and uric acid, experienced the worst early outcome compared to controls, which may be because of increased oxidative stress (Cherubini et al., 2000). Another study showed that total antioxidant capacity and serum levels of vitamin C were reduced in ischaemic

stroke patients in comparison to controls (Gariballa et al., 2002). Antioxidants, as therapeutics for ischaemic stroke, may be used to block ROS generation. These include NADPH oxidase inhibitors, xanthine oxidase inhibitors, and cyclooxygenase inhibitors (Margaill et al., 2005). Indeed, the main source of ROS, which play major role in oxidative stress ischaemic damage, is recognized to be NADPH oxidases. These are discovered to be 7 isoforms and many of their inhibitors are proven to be non-specific (Cifuentes-Pagano et al., 2012). NADPH oxidases are multiunit enzymes proteins which use NADPH as an electron donor in catalysing the oxygen reduction and they generate  $O_2^{\bullet-}$  by reduction of single electron (Brown and Griendling, 2009). The family of NADPH oxidases (NOX) constitutes seven members including NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase-1 and dual oxidase 2. The main member associated with cerebrovascular disease is NOX2 (Kahles and Brandes, 2013). Vascular endothelial cells are important source of  $O_2^{\bullet-}$  and other ROS which are mainly produced by NOX2 (gp91<sup>phox</sup>). Inhibition of NADPH oxidase with apocynin, for example, protected the BBB from damage and was effective in maintaining its function (Kahles et al., 2007). Brait et al found that during post ischaemia-reperfusion of male mice, the salvation of brain tissue was limited due to generation of NOX2-derived  $O_2^{\bullet-}$  from the T lymphocytes which infiltrate the ischaemic inflamed area in the brain (Brait et al., 2010). The most effective and specific NOX2 inhibitor is the NOX2ds-tat (gp91ds-tat), which are formed of 18 amino acids (Cifuentes-Pagano et al., 2012). It has been found to inhibit specifically NADPH oxidase and significantly blocked the increase of ROS level which result from angiotensin II (Liu et al., 2003). Csanyi et al demonstrated that NOX2ds selectively inhibited the NOX2-oxidase in cell free systems

(Csanyi et al., 2011). The tat portion of this peptide is belonging to the sequence of 9 amino acids of the human immunodeficiency virus-tat (HIV-tat) transport region. This enable the peptide to be delivered inside the cells (Fawell et al., 1994). This portion was linked to NOX2ds part which is, also, formed of 9 amino acids, that designed to prevent the p47<sup>phox</sup> to associate with gp91<sup>phox</sup> in NADPH oxidase which can block O<sub>2</sub><sup>•-</sup> generation i.e., the NOX2ds sequence (docking portion) block the assembly of p47<sup>phox</sup> (the cytosolic subunit) to the gp91<sup>phox</sup> (membrane subunit). As a result, this inhibits the formation and activation of NOX2 (Rey et al., 2001, Chen et al., 2016b). In a recent study, gp91ds-tat selectively inhibited the NOX2 and showed significant decrease in the size of atherosclerotic lesions in the aorta of mice (Quesada et al., 2015). Gp91ds-tat prevented the increase in ROS and reversed the cerebrovascular impairment of 12-month-old mice (Park et al., 2007). Also, it blocked the attenuation of physiological hyperaemia which is essential for maintaining the homeostasis of the brain (Kazama et al., 2004). In addition, it has been demonstrated that infusion of gp91ds-tat in rats with injured common carotid artery (experimental model of neointimal hyperplasia) significantly reduced the vascular production of O<sub>2</sub><sup>•-</sup> and neointimal size (Jacobson et al., 2003). The effect of NOX2 inhibitor on *in vitro* BBB model subjected to ischaemia/reperfusion injury still not fully understood.

## **1.6 Endothelial progenitor cells**

The number of circulating EPCs in normal peripheral blood are extremely limited, ranging between 0.01% and 0.0001% of peripheral mononuclear cells (Khan et al., 2005). Therefore, any plan to use these cells as therapeutics would

necessitate their isolation and ex-vivo expansion. There are two sub-types of the EPCs: early EPCs which appear after 3-5 days in culture, and their number increases for two weeks. However, in vitro, they start to disappear gradually in 4 weeks after culture. The second sub-type is late EPCs or OECs which appear firmly attached in the plate within 2-4 weeks (Hur et al., 2004). The OECs have endothelial characteristics and are fully committed to the endothelial lineage (Medina et al., 2010b).

### **1.6.1 Outgrowth endothelial cells**

#### **1.6.1.1 Origin**

The source of the OECs is still debateable. While some researchers claim that the OECs are not originally derived from the bone marrow (Tura et al., 2013), other investigators believe that the OECs are generated from the bone marrow multipotent adult progenitor cells, which then they can migrate and circulate in peripheral blood (Reyes et al., 2002, Wagner and Myrup, 2005). Researchers showed that the origin of OECs, which were isolated from peripheral blood samples of subjects who received bone marrow transplantation from a gender-mismatched persons, is mostly appeared to be from the transplanted marrow-derived cells (Lin et al., 2000). This supported by a study by Shi et al, who used canine bone marrow transplantation model, in which there were genetic differences between the marrow cells of the recipient and donor. After implantation of a Dacron graft in the descending aorta of dogs, they found that a subset of CD34<sup>+</sup> cells which are from the donor's bone marrow cells can be mobilized to the peripheral blood circulation and can colonise the flow surfaces of vascular grafts (Shi et al., 1998).

### **1.6.1.2 Characteristics**

In phenotype, OECs are like vascular endothelial cells. They have cobblestone morphological appearance in culture, have endothelial cell-specific Weibel-Palade bodies, and express endothelial cell markers. However, early EPCs are elongated, have spindle-shaped character, and appear in clusters (Martin-Ramirez et al., 2012, Hur et al., 2004). OECs produce more NO and are phenotypically different from early EPCs. The marker profile for the OECs is the cluster of differentiations (CD) CD31<sup>+</sup>, CD34<sup>+</sup>, CD105<sup>+</sup>, CD146<sup>+</sup>, VE-cadherin<sup>+</sup>, Flt-1<sup>+</sup> (VEGF receptor 2), CD14<sup>-</sup>, CD45<sup>-</sup> and CD133<sup>-</sup>. Also, they express kinase insert domain receptor (KDR) and eNOS angiogenic genes (Minami et al., 2015, Hur et al., 2004, Timmermans et al., 2007, Fuchs et al., 2006). Early stages of OECs express higher levels of pro-angiogenic genes such as inhibitor of DNA binding 3 (ID3), transforming growth factor beta receptor 1 (TGFB $\beta$ R1), matrix metalloprotease 9 (MMP9), chemokine (C-C motif) ligand 2 (CCL2), insulin-like growth factor 1 (IGF-1), tumour necrosis factor alpha-induced protein 2 (TNFAIP2), and transforming growth factor beta 1 (TGF $\beta$ 1). However, mature OECs overexpress vascular endothelial growth factor C (VEGE-C), neuropilin 2 (NRP-2) and thrombospondin 1 (THBS1) genes compared to HBMECs (Navarro-Sobrino et al., 2013).

### **1.6.1.3 Migration and homing**

EPCs can migrate and move to the peripheral blood and differentiate into mature cells. This is complex process beginning with MMP-9 activation which transform the membrane-bound Kit ligand (mKitL) to soluble Kit ligand (sKitL)



followed by movement of progenitor cells into the vascular space of the bone marrow before they proliferate into early EPCs and OECs in blood circulation (Hristov et al., 2003, Heissig et al., 2002). There are many factors that control the homing process of OECs such as macrophage migration inhibitory factor (MIF), pro-inflammatory factor. MIF is stored inside the cells and can be released into the circulation in response to many stimuli such as infarction or ischaemia (Emontzpohl et al., 2017). In addition, growth factors including VEGF, stem cell factors, granulocyte colony-stimulating factor (G-CSF), and stromal-derived factor (SDF-1) play major roles as signalling factors to mediate the mobilization process of the OECs (Bogoslovsky et al., 2011). Furthermore, many physiological factors mobilise the EPCs from the bone marrow including the exercise, trauma, age, peripheral tissue hypoxia, and oestrogen. These factors induce generation and release of EPCs activation factors. Also, the oestrogen mediates EPCs mobilisation via stimulation of beta and alpha oestrogenic receptors through eNOS and MMP-9 (Du et al., 2012). A previous study found that eNOS of the bone marrow stromal cells stimulates EPCs migration (Aicher et al., 2003). Another study, demonstrated that tissue ischaemia (endogenous factor) and therapeutic cytokine (exogenous factor) in rabbits and mice induced EPCs mobilisation to the circulation (Takahashi et al., 1999). Furthermore, angiopoietin-2 (Ang2), expressed by endothelial cells as a soluble ligand, significantly stimulate EPCs mobilisation and activate adhesion between EPCs and endothelial cells (Gill and Brindle, 2005). Migration of EPCs were studied in two groups of patients who had vascular injury secondary to coronary artery bypass grafting (CABG) or burns. In both groups there were dramatic continuous increase in the OECs numbers in the peripheral blood to a

nearly 50 folds within the first 6-12 hrs following injury, suggesting that vascular damage may stimulate chemokines release which induce quick movement of OECs to the circulation (Gill et al., 2001).

#### **1.6.1.4 Function**

Beside their presence in the peripheral circulation of the blood, the EPCs which contains CD34<sup>+</sup> and express VEGFR2 and TIE2, have been reported that they may exist in a specific zone between adventitial layer and smooth muscle of the vascular wall and able differentiate into mature endothelial cells (Zengin et al., 2006). Researchers genetically changed OECs to over-express human eNOS, to investigate whether they can be used as a gene therapy for rat model of pulmonary hypertension. They found that OECs may be useful as carrier cells for genetic therapies against pulmonary hypertension (Somani et al., 2019). In a study by Hristov et al, EPCs contributed to the recovery of endothelial injury in mice (Hristov et al., 2007). Ex vivo expanded human OECs, significantly improved the blood flow and increased capillary density of ischaemic hindlimb mice (Kalka et al., 2000). Others found that OECs have a potent inhibitory activity against platelet aggregation suggesting that OECs may be used as part of a therapeutic strategy targetting high thrombotic risk (Chantzichristos et al., 2018). In mice model of arterial injury, intravenous transfusion of *in vitro* differentiated EPCs, improved reendothelialization and reduced neointima formation of an injured carotid artery (Werner et al., 2003). Researchers showed that seeding an expanded ex vivo EPCs, which were isolated from sheep blood, on decellularized porcine iliac vessels kept the seeded grafts open for more than 4 months in comparison to non-seeded grafts which were closed

in just about 2 weeks, also the seeded grafts showed functional activities, such as contraction and vascular relaxation, similar to normal carotid arteries (Kaushal et al., 2001). Nevertheless, OECs proven to have the capacity to form well-established tubules on Matrigel, a reliable marker for angiogenesis (Bayraktutan, 2019a). Also, eNOS, an important OECs regulatory protein, play a role in tube formation (Gulati et al., 2003). In a different study, while OECs have been proven to form tubular structures both in *in vivo* and *in vitro*, early EPCs were not able to construct these tubules (Mukai et al., 2008). Several *in vivo* studies have shown that OECs possess vascular reparative effect in disease models (Tasev et al., 2016). This is due to their ability to directly incorporate at the sites of vascular injury and generate new blood vessels and/or also to their paracrine effects. OECs have been shown to induce angiogenesis in *in vivo* models of hind limb ischemia and myocardial infarction. The work performed with OECs in models of traumatic brain injury suggest that they also improve tissue viability and function by promoting angiogenesis and neurogenesis (Paschalaki and Randi, 2018). Others, also, showed that OECs have a significant proliferation potential and were able to construct blood vessels in *in vivo* (Yoder et al., 2007). Experimental study reveals that OECs express bone morphogenetic proteins 2 and 4 which shown to enhance angiogenesis in *in vitro*. Also, infusion of OECs in *in vivo* model of ischemic limb mice, increased collateral blood vessels formation (Smadja et al., 2008).

#### **1.6.1.5 Enhancing bioactivity**

The possibility of using OECs as therapeutics in clinical settings is limited because the low quantity and quality of patient derived circulating EPCs. To

enhance bioactivities of EPCs, preconditioning or short-term priming might be an effective strategy. The three priming small molecules (Tauroursodeoxycholic acid, fucoidan and oleuropein) significantly induced the differentiation potential of CD34<sup>+</sup> stem cells into OECs lineage cells and increase cells survival ratio. Also, increased the proliferation potential and enhanced migration, invasion and tube-forming capacity of OECs (Kim et al., 2018). It might be challenging to get the required dose of OECs after expansion in culture because they may show continuous qualitative and quantitative decline during the life span. Glycosaminoglycans mimetic shown to enhance the colony forming efficiency, amplification, and migration of human OECs (Chevalier et al., 2014). Eicosapentaenoic acid and docosahexaenoic acid, important polyunsaturated fatty acid of the n-3 series available in fish and fish oil, enhance viability, decrease TNF- $\alpha$  and MCP-1 gene expression, and improve the capacity of tube formation on Matrigel of both early EPCs and OECs (Spigoni et al., 2014). Other researchers show that OECs cobblestone colonies appear since day 10 and they were classified according to their time of emergence in culture into three subpopulations including moderate OECs (appear on days 10-16), late OECs (appear on days 17-23), and very late OECs (appear on days 24-30). Late OECs showed the best angiogenic potential, and significantly recovered blood flow in mice limb ischemia model (Minami et al., 2015).

#### **1.6.1.6 Factors affect the EPCs number and function**

People with risk factors for cardiovascular disease have less number and more rapidly senescent EPCs than those with low-risk factors (Hill et al., 2003). Also,

researchers show that reduction in EPCs counts is associated with worse prognosis after ischaemic stroke (Sobrino et al., 2007), higher incidence of cardiovascular events, and can be an independent predictor for atherosclerosis progression (Schmidt-Lucke et al., 2005). In patients with stable coronary artery disease, statin therapy was associated with an increase in the number and the functional activity of EPCs (Vasa et al., 2001). The number of EPCs in peripheral blood and the proliferative capacity of OECs decrease by obesity (Tobler et al., 2010). The number of EPCs increases in patients with atherothrombotic stroke but not at 30 days after stroke event (Regueiro et al., 2015). The number and the functional ability of EPCs are low in patients with migraine (Lee et al., 2008). In acute ischaemic stroke EPCs number decreases in comparison to patients with chronic stroke and healthy people (Chu et al., 2008). Others found that strenuous physical activity boosts the number of OECs colonies that were isolated from peripheral blood of healthy subjects. Also, the levels of serum VEGF and neutrophilic granulocytes were correlated to the OECs numbers (Thorell et al., 2009). Indeed, as OECs preparation for vasoreparative therapeutics requires ex vivo expansion, the impacts of such expansion on OECs functions are poorly understood. In light of this, senescence, which defined as a state of growth arrest, is important factor that may influence the functional characteristics and reparative capacity of OECs. It might impair the ability of OECs to proliferate, migrate, survive, and generate new blood vessels (Medina et al., 2013, Williamson et al., 2012).

#### **1.6.1.7 Experimental and clinical studies**

The number of circulating EPCs may be used as biological marker for cardiovascular risks and vascular functions. It has been found that the level of circulating EPCs may anticipate the occurrence of cardiovascular incidents and may assist in identifying whom at high risks of cardiovascular events (Werner et al., 2005). A recent study shows that infusion of ex vivo expanded OECs in 18 patients with acute cerebral infarction is safe and efficient, and that autologous transfusion of OECs improved long-term safety compared to control groups (Fang et al., 2018). Deng et al found that patients with acute ischaemic stroke have significantly lower EPCs numbers compared to control, but the numbers of circulating EPCs and the concentration of SDF-1 increased on day 7, peaked on day 14 and dropped on day 21 (Deng et al., 2018). Researchers studied the use of OECs in a mouse model of ischaemic retinopathy and show that OECs dramatically decreased the size of the avascular area and boosted the normal vascular areas (Medina et al., 2010a). EPCs mobilisation and angiogenic capacities were assessed by other researchers, who studied 45 ischaemic stroke patients by isolating EPCs from peripheral bloods at 24hrs and 7 days post-stroke events. After assessing cerebral blood flow (CBF) and collateral flow, they found that the ability of EPCs to migrate and involve into angiogenesis is linked with increased collateral flow during the acute state and CBF at day 7 (Sargento-Freitas et al., 2018). Nevertheless, the ability for OECs to establish effective and functional *in vitro* BBB model and repair its damage following ischaemic injury is not fully understood.

### **1.6.2 The secretome of outgrowth endothelial cells**

While direct physical incorporation of OECs at the site of endothelial damage is crucial for repairing damaged cerebral barrier, OECs also contribute to angiogenesis by secreting a vast range of trophic substances which may enhance cell proliferation, angiogenesis, and migration (Urbich et al., 2005a, Kalka et al., 2000, Urbich et al., 2003, Yamaguchi et al., 2003, Gallina et al., 2015, Di Santo et al., 2009, Felice et al., 2018). As application of OEC-secretome may eliminate the risks linked with transplantation of living cells including infection, emboli, and immune response, OEC-free approach may be an alternative therapeutic strategy for ischaemic stroke. Table 1.1 shows the main advantages associated with treatments with OEC-secretome which might be available as an off-the-shelf therapy that may open new avenues in the field of regenerative medicine and should be studied as potential therapeutics for ischaemic stroke (Vizoso et al., 2017, Rosell et al., 2013, Seminatore et al., 2010, Lodi et al., 2011, Wyler von Ballmoos et al., 2010).

**Table 1-1 Comparison between OECs and OEC-secretome (Alwjwaj et al., 2021)**

|                                            | OECs therapy | OEC-secretome therapy |
|--------------------------------------------|--------------|-----------------------|
| Risks of immune incompatibility            | + + +        | +                     |
| Emboli formation                           | + +          | +                     |
| Risk of infection                          | + + +        | +                     |
| Need for toxic agents for cryopreservation | + +          | +                     |
| Preparation time                           | + +          | +                     |
| Cost                                       | + +          | +                     |

#### **1.6.2.1 Analysis of OEC-secretome**

The secretome is as set of molecules, including of growth factors, lipids, cytokines, free nucleic acids, chemokines, and extracellular vesicles released or shed from living cells. The secretome can be classified into apoptotic bodies, microparticles, and exosomes (Makridakis and Vlahou, 2010, Makridakis et al., 2013, Pavlou and Diamandis, 2010, Tjalsma et al., 2000, Skalnikova et al., 2011, Beer et al., 2017). The exosomes have much of the attention by many researchers due their capabilities to regulate cell to cell communications by assisting the transfer of proteins, RNAs, and lipids to the target cells. Exosomes are tiny vesicles that have size range between 40-150 nm (De Jong et al., 2014, Li et al., 2016a). OEC-exosomes are surrounded by two layers of lipids and have biconcave or cup morphology which shield them from enzymatic degradation and allow them to work as transportation cargoes in the blood circulations (Kong et al., 2018, Yellon and Davidson, 2014, Vicencio et al., 2015). By enhancing the production of vascular VEGF and eNOS, OEC-exosome shown to induce the proliferation, angiogenesis , and survival of endothelial cells (Zhang et al., 2019) while blocking blood vessels leakage in



kidneys and lungs (Zhou et al., 2018). Furthermore, OEC-exosome appears to prevent neointima formation and induce vasculogenesis *in vitro* and *in vivo* (Kong et al., 2018, Sahoo et al., 2011). Recent study reveals that like OEC-derived exosome, OEC-derived mitochondria also protect neurovascular unit from ischaemic injury by incorporating into endothelial cells, increasing tight junctional tightness, and boosting intracellular ATP levels (Hayakawa et al., 2018, Borlongan, 2019).

Like endothelial cells, OECs are also able to secrete a vast array of trophic substances with different mechanisms (Table 1.2). Researchers used difference gel electrophoresis studies and shotgun proteomics to study the content of the human OEC-secretome. They have found that conditioned media of OECs (OEC-CM) contains about 82 proteins. These include different members of cathepsins, various substances with proangiogenic activity like haemoglobin scavenger receptor CD163, chemokine ligand 18, and thymidine phosphorylase and other antioxidants such as hemoxygenase-1 and mitochondrial superoxide dismutase (Urbich et al., 2011). 71 proteins of these in OEC-secretome appeared to be different from those of human umbilical veins endothelial cells and CD14<sup>+</sup> monocytes and (Urbich et al., 2011). Other researchers found that OECs that was cultured in growth factor-free medium over a period of 72 h secretes varieties of angiogenic growth factors such as VEGF, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and hepatocyte growth factor (HGF) (Rehman et al., 2003). Other studies have revealed that human EPC-CM also contains thrombo-inflammatory mediators, chemokines, and adhesion

molecules. Although early EPCs release thrombo-inflammatory mediators such as tissue factors, OECs secrete factors regulate infiltration and migration of macrophages or monocytes for example MCP-1 (Zhang et al., 2009). These support the findings of other studies which revealed that OECs and early EPCs represent distinct EPCs populations. However, while early EPCs express various genes related to inflammation and immunity including human leukocyte antigens (HLAs), CD14, and toll-like receptors (TLRs) (Medina et al., 2010b), OECs express senescence markers and secretes higher levels of cytokines. Inflammatory elements such as IL-6, IL-8, IL-1 $\alpha$ , IL-1 $\beta$  have been identified as the major inducers of OECs senescence (Medina et al., 2013). Considering this, the pre-existing inflammatory response in the ischaemic microenvironment should be considered when using early or late EPC-secretome as free-cell therapeutics.

**Table 1-2 Factors secreted by EPCs (Alwjwaj et al., 2021)**

| <b>Factors</b>                                | <b>Function</b>                              | <b>Reference</b>                                                 |
|-----------------------------------------------|----------------------------------------------|------------------------------------------------------------------|
| Angiogenin                                    | Angiogenesis, migration, proliferation       | (Di Santo et al., 2009, Maki et al., 2018)                       |
| Hepatocyte growth factor (HGF)                | Angiogenesis, motility, morphogenesis        | (Di Santo et al., 2009, Rehman et al., 2003)                     |
| Interleukin-8 (IL-8)                          | Chemoattraction, proliferation, angiogenesis | (Di Santo et al., 2009, Pula et al., 2009, Urbich et al., 2005a) |
| Platelet derived growth factor B (PDGF-BB)    | Angiogenesis, proliferation, recruitment     | (Di Santo et al., 2009)                                          |
| Stromal derived factor-1 (SDF-1)              | Angiogenesis, antiapoptotic, migration       | (Di Santo et al., 2009, Maki et al., 2018, Paneni et al., 2016)  |
| Vascular endothelial growth factor (VEGF)     | Migration, proliferation, angiogenesis       | (Rehman et al., 2003)                                            |
| Vascular endothelial growth factor A (VEGF-A) | Migration, mitogenesis, angiogenesis         | (Di Santo et al., 2009, Urbich et al., 2005a)                    |
| Pre-B cell-enhancing factor (PBEF)            | Angiogenesis, antiapoptotic                  | (Pula et al., 2009)                                              |
| Matrix metalloproteinase-9 (MMP-9)            | Angiogenesis, migration, proliferation       | (Pula et al., 2009, Maki et al., 2018)                           |
| Macrophage migration inhibitory factor (MIF)  | Angiogenesis, migration, recruitment         | (Pula et al., 2009)                                              |
| Cathepsins                                    | Invasion, angiogenesis                       | (Pula et al., 2009, Urbich et al., 2011, Urbich et al., 2005b)   |
| Protease inhibitors                           | Angiogenesis, migration, invasion            | (Pula et al., 2009)                                              |
| S100 proteins                                 | Angiogenesis, proliferation, invasion        | (Pula et al., 2009)                                              |
| Granulocyte colony-stimulating factor (G-CSF) | Proliferation, differentiation, angiogenesis | (De Jong et al., 2014, Rehman et al., 2003)                      |

| <b>Factors</b>                                                | <b>Function</b>                            | <b>Reference</b>                              |
|---------------------------------------------------------------|--------------------------------------------|-----------------------------------------------|
| Vascular endothelial growth factor B (VEGF-B)                 | Angiogenesis, migration                    | Maki et al., 2018, Urbich et al., 2005a)      |
| 2-deoxy-D-ribose-1-phosphate (dRP)                            | Migration, angiogenesis                    | (Pula et al., 2009)                           |
| Mitochondrial superoxide dismutase                            | Antioxidants, antiapoptotic                | (Urbich et al., 2011, Dernbach et al., 2004a) |
| Homoxxygenase-1                                               | Antioxidant, antiapoptotic                 | (Urbich et al., 2011, Dernbach et al., 2004a) |
| Alternative macrophage markers C-C motif chemokine 18 (CCL18) | Healing, migration                         | (Urbich et al., 2011)                         |
| Haemoglobin scavenger receptor CD163                          | Adhesion, anti-inflammatory                | (Urbich et al., 2011)                         |
| Cytokine-like 1 (CYTL1)                                       | Angiogenesis, migration                    | (Schneller et al., 2019)                      |
| Platelet-derived growth factor (PDGFAA/AB/BB)                 | Angiogenesis, proliferation, recruitment   | (Maki et al., 2018, Urbich et al., 2005a)     |
| Granulocyte-macrophage colony-stimulating factor (GM-CSF)     | Angiogenesis, proliferation, antiapoptotic | (Rehman et al., 2003)                         |

### **1.6.2.2 Mechanism of action of OEC-secretome**

#### **1.6.2.2.1 Anti-inflammatory role**

Inflammation, manifested by the increased release of pro-inflammatory cytokines e.g., TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , constitutes one of the major mechanisms that result after a cerebral ischaemic damage. Stroke patients with cerebral or systemic inflammation usually have clinically poorer prognosis (Abdullah and Bayraktutan, 2016a, Fann et al., 2013, Allan and Rothwell, 2001). By modulation of different anti-inflammatory cytokines and inflammatory genes e.g., IKB, Brahma, and Foxf1, OECs might reduce the toxic and apoptotic impact of the inflammatory conditions. In this regard, co-culture of human OECs with endothelial cells has been proven to substantially reduce the increases of the pro-inflammatory genes during experimental ischaemic stroke. Interestingly, injection of OEC-secretome into a rat model of spinal cord damage has been shown to enhance functional recovery where inhibition of M1 macrophage activation, IL-6 release, apoptosis, and enhancement of angiogenesis supported this recovery (Wang et al., 2018). Likewise, administration of OEC-exosome into the trachea of a mice model following acute lung damage has substantially decreased cytokine, protein, and chemokine accumulations in the bronchoalveolar lavage fluid, aiding the anti-inflammatory and tissue-protective benefits of OEC-secretome (Zhou et al., 2019). Other researchers found that infusion of a specific factor, known as thymosin B4 which released by embryonic EPCs, into pigs subjected to myocardial ischaemia reduced the size of infarct areas and induced post-hypoxia cardiomyocyte survival by regulating the post-ischaemic inflammatory

reactions (Hinkel et al., 2008). The effect of OEC-secretome on BBB models exposed to ischaemic damage is not explored.

#### **1.6.2.2.2 Tissue repair and angiogenic regulation**

Finding that OEC-CM contain an excess amount of highly mitogenic cytokines, including angiogenin and IL-8, strongly suggests that OEC-secretome can enhance the proliferative, chemotactic, tubulogenic capacity of endothelial cells (He et al., 2005). Administration of OEC-secretome following an ischaemic stroke may help facilitate vascular repair and inhibit neointima formation by stimulating proliferation and migration of the local endothelial cells at the site of endovascular injury (Paneni et al., 2016). While the proliferation, migration, and activation of endothelial cells are important steps of angiogenesis process, it is worth noting that cytokines and chemokines also play an essential role in this process (Gnecchi et al., 2008). It has been proven that OEC-secretome, which contains many chemokines and cytokines, enhanced the angiogenic capacity of brain microvascular endothelial cells in a mouse model subjected to cerebral hypoperfusion (Di Santo et al., 2014b). Moreover, increased amounts of various growth factors, such as VEGF and IGF-1, has also been revealed to enhance angio-neurogenesis in the injured area of rat models subjected to ischaemic stroke following treatment with OECs (Urbich et al., 2005b, Schneller et al., 2019, Moubarik et al., 2011).

#### **1.6.2.2.3 Antioxidant activity**

Oxidative stress, resulting from an imbalance between anti- and pro-oxidants, is another main process that can worsen cerebrovascular damage following an

ischaemic stroke (Allen and Bayraktutan, 2009b). Compared to endothelial cells, OECs shown to has significantly higher total antioxidant capacity. Indeed, higher expression of antioxidant enzymes such as Mn-containing superoxide dismutase (Mn-SOD), glutathione peroxidase, and catalase allows OECs to readily tolerate a certain degree of oxidative stress (Dernbach et al., 2004a). This may potentiate their vasoreparative and angiogenic functions under ischaemic conditions. For example, administration with OEC-secretome shown to reduce the deleterious effects of oxidative stress damage in human umbilical vein endothelial cells by enhancing the expression of antioxidants, CuZn-SOD and Mn-SOD (Yang et al., 2010).

#### **1.6.2.2.4 Tissue invasion and migration**

In addition to the angiogenic function, OECs also control cellular chemotaxis by regulating the secretion of pro-angiogenic factors like IGF-1, HGF, VEGF, and SDF-1. Both SDF-1 and VEGF enhance recruitment and adhesion OECs to the site of ischaemic damage (Anderson et al., 2015). Also, OEC-secretome has been reported to facilitate mobilisation, migration, and proliferation of different progenitor and resident vascular cells such as cardiac progenitor cells and endothelial cells, in turn. Enhanced migration of endothelial cells to the site ischemic damage in rat models subjected to limb ischemia and following treatment with human OEC-CM support this notion (Urbich et al., 2005a).

Apart from growth factors and cytokines, OECs also produce various lysosomal peptidases such as cathepsin D, cathepsin L, cathepsin O and cathepsin H. Cathepsin L inhibition has been shown to diminish the incorporation, invasion,

and function of OECs in a model of hindlimb ischaemia (Urbich et al., 2005b). OEC-secretome that contain various cathepsin such as cathepsin L might significantly enhance homing, incorporation, adhesion of circulating OECs to the site of ischaemic injury.

### **1.6.2.3 OEC-secretome modification**

Hypoxia is the major factor that may enhance the migratory, proliferative, and angiogenic capacities of cells (Wei et al., 2012, Moranco et al., 2013). Exposure of OECs to hypoxic conditions has been proven to stimulate secretion of a various of factors such as basic fibroblast growth factor, VEGF, PDGF-BB, epidermal growth factor, thrombopoietin, angiogenin, and leptin (Di Santo et al., 2014b). Thus, it is likely that treatment with CM obtained from OECs exposed to hypoxia may enhance repair and restore *in vitro* cerebral barrier models following ischaemic damage. Indeed, such treatments shown to enhance migration of OECs from bone marrow and results in enhancement of hindlimb blood flow, increased capillary density, and blocked apoptosis of endothelial cells (Di Santo et al., 2009). Moreover, treatment with hypoxic OEC-CM entrapped in polymer-based nanoparticles, cargos for controlled release of OEC-secretome, proven to enhance blood flow and vasculogenesis in ischaemic limb model (Felice et al., 2018). EPCs subjected hypoxia have been revealed to release about 647 proteins. Of these, 83 proteins have been shown to be differently regulated compared to the cells grown under normal conditions. While the release of 17 of these 83, which mainly have angiogenic functions including protease inhibitors, apolipoprotein E, and protein S100 family, are upregulated by hypoxia, the secretion of 12 proteins, such as cystatin-C,



dermcidin, calcium-binding protein 39, trypsin-1, are downregulated (Felice et al., 2018).

Inflammatory mechanism can also affect the number and functional capacity of OECs. Inflammatory factors including SDF-1 and GM-CSF stimulate mobilisation of OECs from bone marrow (Takahashi et al., 1999, Tousoulis et al., 2008). For instance, the rises shown in the number of functional OECs reflected rises in plasma levels of IL-8, VEGF, and SDF-1 in patients suffered burns within the first 24 hours of hospitalization (Rignault-Clerc et al., 2013). Different study focusing on the migration of EPCs in two various groups of patients with acute vascular damage emerging in coronary artery bypass grafting or burns shown significant increases in the numbers of circulating EPCs associated with significant rises in plasma cytokine levels and VEGF (Gill et al., 2001). These findings suggest that enhancement of OEC-secretome by exposure to inflammatory stimuli such as hypoxia in *in vitro* settings may enhance its therapeutic effect when given following ischaemic injury.

#### **1.6.2.4 Therapeutic potential of OEC-secretome**

In light of that factors released by OECs into conditioned media in *in vitro* conditions mimicking an ischaemic damage are more likely to be mirroring of those found in blood circulation following an ischaemic stroke, their accurate identification might be of profound diagnostic, therapeutic, and prognostic values (Stastna and Van Eyk, 2012). Considering the studies revealing those post-ischaemic increases in circulating numbers of OECs, it is possible that similar increases in the number of the components that comprise the OEC-secretome may also increase which in turn support their diagnostic and

prognostic value (Sobrino et al., 2007). Indeed, experimental studies exploring the effect of OEC-secretome in *in vitro* settings are limited, to our knowledge there is currently no studies that has explored the potential therapeutic role of OEC-secretome in *in vitro* models of BBB exposed to ischaemic injury. In fact, the beneficial effect of OEC-secretome on HBMECs architecture, cellular adhesion, cell repair, and oxidative stress not fully explored. However, in clinical settings, clinical trials of allogenic mesenchymal stem cells transplantation for patients suffered ischaemic stroke (given >6 months of stroke onset) have been shown to be safe and resulted in a significant behavioural improvement over a year of follow up (Levy et al., 2019). As the microenvironment surrounding the NVU is under continuous change following ischaemic stroke (Liao et al., 2017b), while designing experimental, transitional, and future clinical trials, the timing of treatments with OEC-secretome is an essential factor to be considered. Transplantation of OECs or OEC-secretome rich in VEGF, PDGF-BB, and fibroblast growth factor into a mouse model of ischaemic stroke, within 30-32 hours of ischaemic stroke, shown to enhance capillary density in the peri-infarct areas and improved functional strength of forelimbs. The improvements in functional aspects remained noticeable at two weeks following ischaemic insult and were largely attributed to an appearance of additional blood vessels in the peri-infarct areas (Rosell et al., 2013). Administration of OECs into a rodent model of ischaemic stroke 24 hours following middle cerebral artery occlusion resulted in substantial functional recovery. Given that the direct incorporation of OECs into rat NVU could not be detected in this study, functional recovery noticed may be due to the paracrine effects of OEC-secretome (Moubarik et al., 2011). Considering these findings and to support this conclusion, experimental

studies are required to investigate the potential benefits of OEC-secretome on *in vitro* BBB model exposed to damage. A study investigated the angiogenic capacity of early (within 1 hour of stroke) and late (48 hour after stroke) administration of VEGF, a key factor of OEC-secretome, to a rodent model of ischaemic stroke revealed that while the former approach caused haemorrhagic transformation in the focal ischaemic lesions, the latter approach induced angiogenesis in the ischaemic penumbra and substantially enhanced functional recovery. Considering these results imply that prospective therapeutic approaches considering treatment with OEC-secretome should consider the timing of therapy administration (Zhang et al., 2000). An *in vitro* study has revealed that OEC-derived factors protect cultured cortical neuronal progenitor cells from a metabolic injury induced by glucose and serum deprivation (Santo et al., 2020). Furthermore, administration of OECs to a mice model of traumatic brain injury enhanced neovasculogenesis and neurological recovery. In this study, activation of axonal outgrowth of neurons along with enhanced proliferation, survival, and recruitment of circulating OECs to the site of ischaemic brain damage by main components of OEC-secretome, including SDF1, VEGF, and proangiogenic cytokines, were thought to have a major role (Zhang et al., 2013). OEC secretome-mediated inhibition of inflammatory response with the regulation of functional recovery, angiogenesis, and axonal regeneration resulted in neuroprotection in a rat model of spinal cord injury (Wang et al., 2018). Regardless of these findings, the restorative impact of OECs or OEC-secretome on BBB models exposed to ischaemic damage is not fully understood and need further investigation before OECs (cell therapy) or

OEC-secretome (cell-free therapy) become therapeutic possibilities for ischaemic stroke patients.

## **1.7 Summary**

Ischaemic stroke is a common cause of morbidity and mortality worldwide. It is characterised by occlusion of a blood vessel leading to the brain. Sudden decreases in cerebral blood flow during an ischaemic stroke cause HBMEC damage, the main cellular component of cerebral barrier. This leads to disruption of BBB integrity and function through involvement of series of mechanisms such as oxidative stress and inflammation. NOX2, one of the members of NADPH oxidases, represents the major source of oxidative stress in vasculature. Inflammation, associated with excessive release of pro-inflammatory cytokines, notably TNF- $\alpha$  also affects the integrity and function of BBB during ischaemic stroke. Hyperglycaemia, a major modifiable cardiovascular risk factor, also aggravates ischaemic injury-mediated oxidative stress and BBB damage. Despite its anticipated vasculoprotective benefits, whether treatments with OECs, a functional subtype of endothelial progenitor cells, can replace dead or dying HBMECs to restore BBB integrity and function following an ischaemic injury remain unknown. Besides, the possible BBB-protective role of NOX2 inhibition in hyperglycaemic settings is poorly understood. Moreover, the ability of OEC-secretome, as a cell free therapy, to protect BBB integrity and function against TNF- $\alpha$  damage is unknown.

## **1.8 Hypothesis and aims**

### **1.8.1 Hypothesis**

We hypothesized that bone marrow derived OECs may establish a functional *in vitro* human BBB model and restore its function following ischaemic damage. Furthermore, gp91ds-tat, selective NOX2 inhibitor, may protect cerebral barrier against ischaemic damage and might augment therapeutic effect of hypothermia against hyperglycaemic damage. In addition, cellular senescence may affect the proliferative, migratory, and reparative capacities of OECs. Finally, OEC-secretome may protect cerebral barrier against damage, induce repair, and maintain cellular architecture against ischaemic injury.

### **1.8.2 Aims**

In view of this hypothesis, this project has investigated the following specific points:

- 1- Determine the characteristics of OECs by measuring cell proliferation, tube formation, and migration capacities. Also, by investigating the reparative capacity of these cells in BBB model by using wound scratch assay and by exposure to ischaemic insult.
- 2- Determine the effects of NOX2 inhibitor (gp91ds-tat) during OGD with and without reperfusion in *in vitro* human BBB models and explore the impact of this in *in vitro* BBB models formed with OECs.
- 3- Determine the effects of therapeutic combination of gp91ds-tat with hypothermia in *in vitro* BBB model exposed to hyperglycaemia.

- 4- Identify whether OECs with lower passage and higher passage number act in different ways in terms of reparation in *in vitro* human BBB models settings.
- 5- Determine whether OEC-secretome, as cell-free therapeutics, can protect *in vitro* human BBB models against TNF- $\alpha$  damage.

To investigate these aims the following points have been considered throughout the process:

- 1- OECs have been isolated from human peripheral blood.
- 2- The integrity of *in vitro* human BBB has been assessed using TEER and paracellular flux (NaF).
- 3- F-actin staining has been used to study stress fibres formation and the impact on cellular architecture.
- 4- NADPH oxidase activity has been measured by lucigenin chemiluminescence assay, and O<sub>2</sub><sup>•-</sup> production has been measured by cytochrome C assay.

## **2 MATERIAL AND METHODS**

**Table 2-1 Sources of key materials**

|                                            |                                 |
|--------------------------------------------|---------------------------------|
| Allopurinol                                | Sigma-Aldrich                   |
| Anti-gamma H2AX antibody                   | Abcam                           |
| Aprotinin                                  | Sigma-Aldrich                   |
| Astrocyte medium                           | ScienCell Research Laboratories |
| Beta galactosidase staining kit            | Abcam                           |
| Bicinchoninic acid (BCA) protein assay kit | ThermoFisher Scientific         |
| Bovine serum albumin                       | Sigma-Aldrich                   |
| Cytochrome C                               | Sigma-Aldrich                   |
| DAPI                                       | Sigma-Aldrich                   |
| Dil-AcLDL                                  | Invitrogen                      |
| DMSO                                       | Sigma-Aldrich                   |
| EDTA                                       | Sigma-Aldrich                   |
| EGTA                                       | Sigma-Aldrich                   |
| Endothelial cell growth medium 2           | Fisher Scientific               |
| Endothelial cell medium                    | ScienCell Research Laboratories |
| Fibronectin                                | Sigma-Aldrich                   |
| FITC-UEA-1                                 | Sigma-Aldrich                   |
| Foetal bovine serum                        | Sigma-Aldrich                   |
| gp91ds-tat                                 | 2BScientific Limited            |
| Growth factor reduced Matrigel             | BD Biosciences                  |
| Hank's balanced salt solution              | Sigma-Aldrich                   |
| Human astrocytes                           | ScienCell Research Laboratories |
|                                            | TCS CellWorks Ltd UK            |



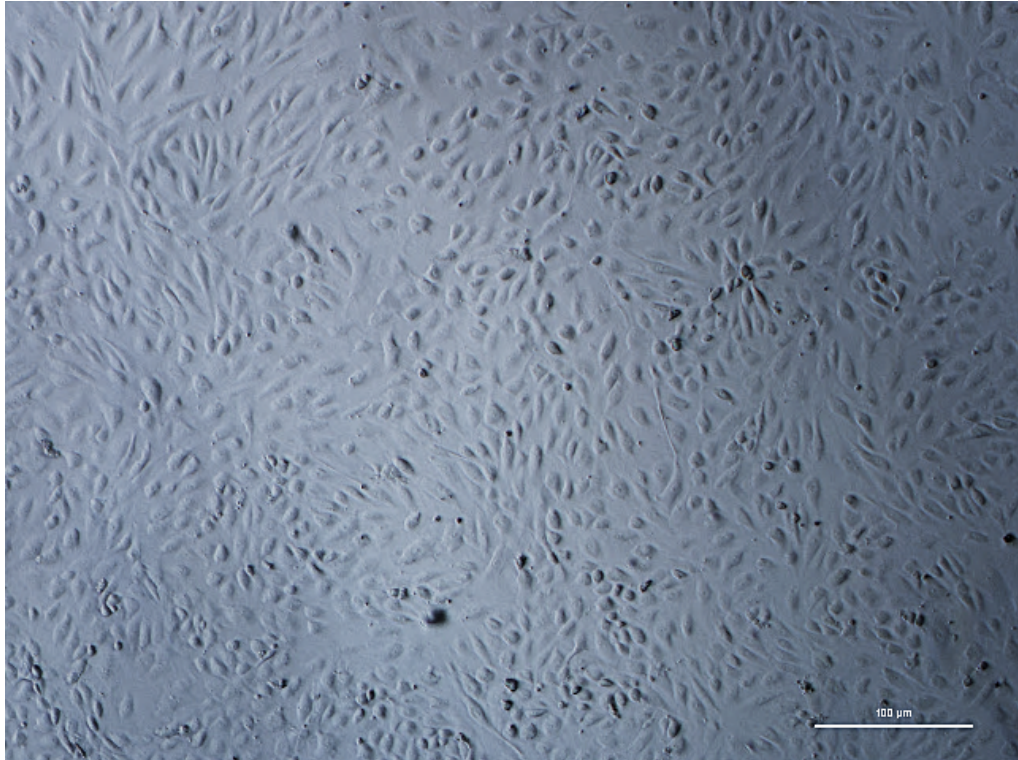
|                                             |                                 |
|---------------------------------------------|---------------------------------|
| Human brain microvascular endothelial cells | ScienCell Research Laboratories |
|                                             | TCS CellWorks Ltd               |
|                                             | Neuromics                       |
| Human pericytes                             | ScienCell Research Laboratories |
|                                             | TCS CellWorks Ltd UK            |
| Lucigenin                                   | Sigma-Aldrich                   |
| Mannitol                                    | Sigma-Aldrich                   |
| Monobasic potassium phosphate               | Sigma-Aldrich                   |
| Mounting medium                             | Vector Laboratories             |
| NADPH                                       | Sigma-Aldrich                   |
| Nitric oxide assay kit                      | Abcam                           |
| Paraformaldehyde                            | Sigma-Aldrich                   |
| Penicillin/Streptomycin solution            | Sigma-Aldrich                   |
| Pericyte cell medium                        | ScienCell Research Laboratories |
| Phosphate buffered saline                   | Sigma-Aldrich                   |
| Protease cocktail inhibitor                 | Sigma-Aldrich                   |
| Rhodamine phalloidin                        | Abcam                           |
| RPMI 1640 media                             | ThermoFisher Scientific         |
| Sodium fluorescein                          | Sigma-Aldrich                   |
| Sucrose                                     | Sigma-Aldrich                   |
| Total antioxidant capacity assay kit        | Abcam                           |
| Triton X-100                                | Sigma-Aldrich                   |
| Trypan blue                                 | Invitrogen                      |
| Trypsin                                     | Sigma-Aldrich                   |

|               |               |
|---------------|---------------|
| Tween-20      | Sigma-Aldrich |
| ZO-1 antibody | Abcam         |

## **2.1.1 Cell culture**

### **2.1.1.1 Human brain microvascular endothelial cells**

HBMECs, kept in liquid nitrogen, were sub-cultured according to the manufacturer's instructions and used between passages 7 and 9. These cells were obtained from human donors and provided by ScienCell Research Laboratories, TCS CellWorks Ltd, and Neuromics. Frozen cells vials were collected from liquid nitrogen and thawed in water bath at 37°C before they were added in the flasks for culture. The HBMECs were cultured in endothelial cell media (ECM) which contain 5% foetal bovine serum (FBS), buffered bicarbonate, and has pH of 7.4. Each 500 ml of endothelial cell media consists of 25 ml FBS, 5 ml endothelial cell growth supplement (ECGS), 5 ml penicillin/streptomycin solution. This media was stored in -20°C and protected from light. The cells were incubated and cultured in humidified conditions with an atmosphere of 5% CO<sub>2</sub>, 95%, 21% O<sub>2</sub>, 74% N<sub>2</sub>, and at 37°C. First media change was done under sterile conditions in the second day to remove the dimethyl sulphoxide (DMSO), then every other day until cells become confluent. Figure 2.1 shows the morphology of HBMECs under light microscopy.



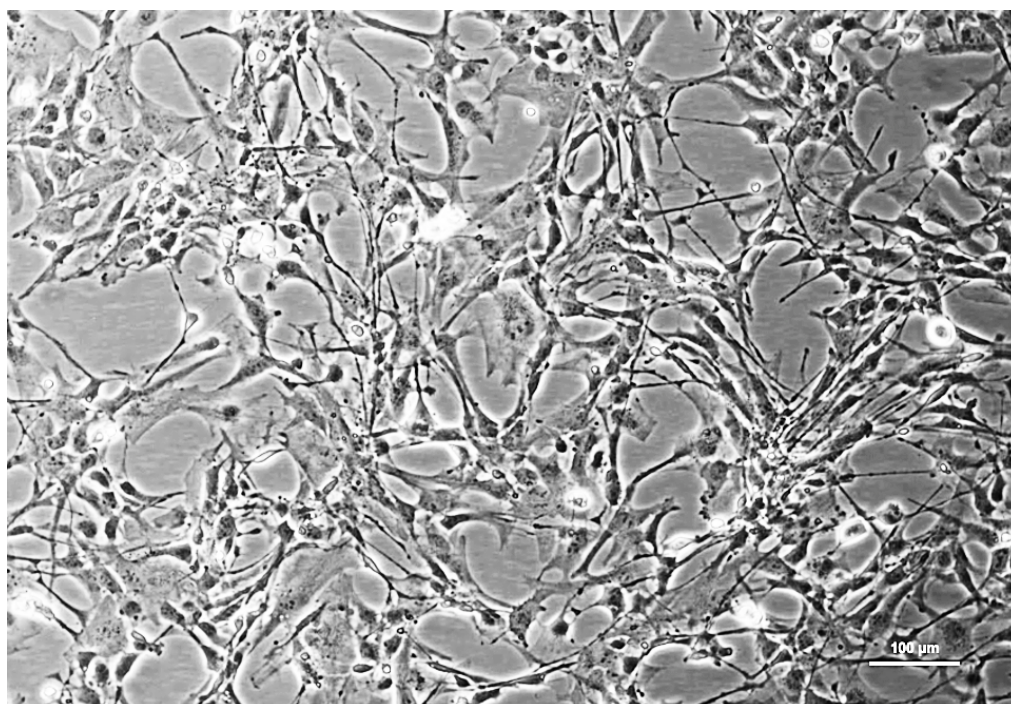
**Figure 2-1 Human brain microvascular endothelial cells.**

HBMECs were observed on a light microscope. Scale bar: 100  $\mu\text{m}$ .

#### **2.1.1.2 Human astrocytes**

HAs, kept in liquid nitrogen, were sub-cultured according to the manufacturer's instructions and used between passages 7 and 9. Also, these cells were obtained from human donors and provided by ScienCell Research Laboratories and TCS CellWorks Ltd. Frozen cells vials were collected from liquid nitrogen and thawed in water bath at 37°C before they were added in the flasks for culture. The HAs were cultured in astrocyte medium (AM) which contain 2% FBS, buffered bicarbonate, and has pH of 7.4. Each 500 ml of HAs media consists of 10 ml FBS, 5 ml astrocyte cell growth supplement (AGS), 5 ml penicillin/streptomycin solution. The media was stored in -20°C and protected from light. The cells were cultured in humidified conditions 95% with an atmosphere of 5% CO<sub>2</sub>, 21% O<sub>2</sub>, 74 % N<sub>2</sub>, and at 37°C. First media change was

done under sterile conditions in the second day to remove the DMSO, then every other day until they become confluent. Figure 2.2 shows the morphology of HAs under light microscopy.



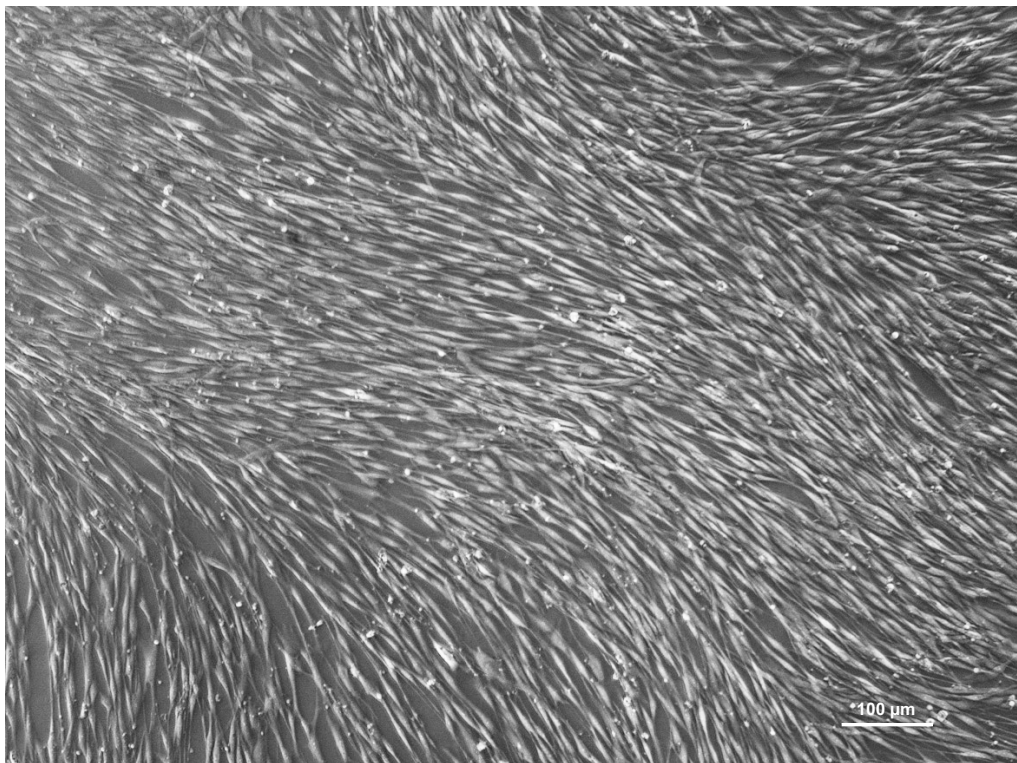
**Figure 2-2 Human astrocytes.**

HAs were observed on a light microscope. Scale bar: 100  $\mu\text{m}$ .

#### **2.1.1.3 Human pericytes**

HPs, stored in liquid nitrogen, were sub-cultured according to the manufacturer's instructions, and used between passages 7 and 9. The cells were purchased from ScienCell Research Laboratories and TCS CellWorks Ltd. Frozen cells vials were collected from liquid nitrogen and thawed in water bath at 37°C before they were added in the flasks for culture. The pericytes were cultured in pericyte medium (PM) which contain 2% FBS, buffered bicarbonate and has pH of 7.4. Each 500 ml of pericyte cell media contains 10 ml FBS, 5 ml

pericyte cell growth supplement (PGS), 5 ml penicillin/streptomycin solution. The media was stored in -20°C and protected from light. Pericytes were cultured in humidified conditions 95% with an atmosphere of 5% CO<sub>2</sub>, 21% O<sub>2</sub>, 74% N<sub>2</sub>, and at 37°C. First media change was done under sterile conditions in the second day to remove the DMSO, then every other day until cells become confluent. Figure 2.3 shows the morphology of HPs under light microscopy.



**Figure 2-3 Human pericytes.**

HPs were observed on a light microscope. Scale bar: 100 μm.

#### **2.1.1.4 Cell passaging**

When the cells (HBMECs, HAs, and HPs) become confluent at 90%, trypsin was used in the passaging step to detach them from the 75 cm<sup>2</sup> flasks. Firstly, media was removed from flasks and phosphate buffered saline (PBS) was used to wash the cells two times. Then 2.5 g/l diluted trypsin in PBS 1:4 was added

over the cells making sure it covers the surface of the flask and then incubated for about 5 minutes. The flask, then, was seen under the microscope and tapped gently to make sure that cells were detached from the surface. Next, 1:1 volume of each cell specific media was added to neutralize the trypsin and this mixture was added to 50 ml Falcon tube. More media was used to wash the flask and collect the remaining cells and added them to the 50 ml tube. This suspension was centrifuged at 250 g for 5 minutes and supernatants was removed. Then the pellet of the cells was resuspended in specific freezing cell media for the freezing step or in specific cell media (ECM, AM, or PM) for the re-seeding step.

#### **2.1.1.5 Cell freezing**

Specific freezing media was prepared for every cell type. Nine ml of either (ECM, AM, or PM), 9 ml of FBS, and 2 ml of DMSO were added in 50 ml Falcon tube at percentages of 9:9:2. About 1 ml to 5 ml (according to the pellet volume) of the specific freezing media were used to dilute the pellet of the centrifuged cells from the previous process. Then, 1 ml of this suspension was added in 1 ml vials to maintain the cells stock. These vials then collected in containers and stored at  $-80^{\circ}\text{C}$  freezer for one day then transferred to the liquid nitrogen.

#### **2.1.1.6 Cell counting**

Cells were counted under a light microscope using haemocytometer. This and its coverslip were cleaned using lens tissue and 70% propanol then the coverslip was located on the top of the haemocytometer. Twenty  $\mu\text{l}$  of the aliquot cells volume were mixed with same volume of 0.1 % trypan blue (1:1)

for 5 minutes. Then, 10 µl of this was put in the haemocytometer. Next, the cells were counted under the microscope in each of 5 large squares (girds). The following formulas were used to obtain the total cell count (cells per ml).

Total cells/ml = average cells number per gird × dilution factor × 10<sup>4</sup>

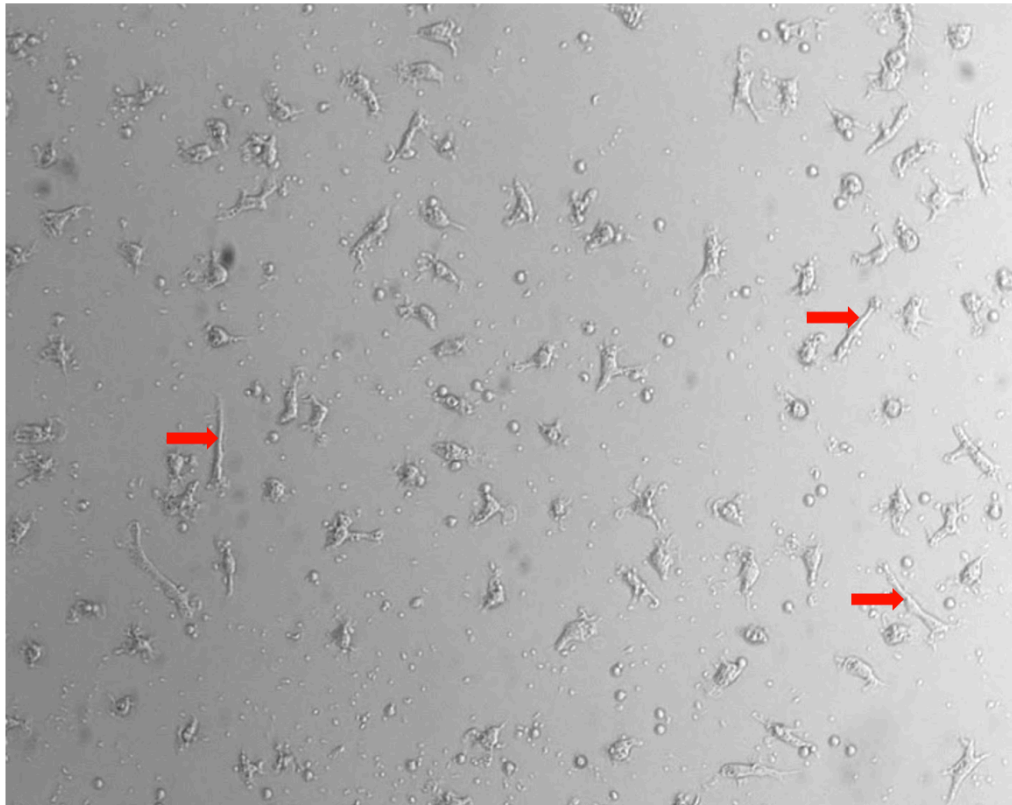
Dilution factor = total volume of cell suspension in trypan blue / original cell suspension volume.

### **2.1.2 Isolation and expansion of outgrowth endothelial cells**

In this study we processed blood samples from healthy volunteers who had been recruited as part of a large observational study, the Bayraktutan Dunhill Medical Trust EPC study. Ethical approval had been obtained from West Midlands – Coventry & Warwickshire Research Ethics Committee (REC number: 16/WM/0304), and written informed consent was obtained from all volunteers. The Bayraktutan Dunhill Medical Trust EPC study is registered in clinicaltrials.gov (NCT02980354) on November 15, 2016. The study investigated whether circulating EPCs in peripheral blood of ischaemic stroke patients can be used as a prognostic or diagnostic marker. For this study, blood samples were obtained from 10 healthy volunteers (6 males and 4 females) aged between 18 and 65 (Rakkar et al., 2020). Thirty ml or 60 ml of human blood was withdrawn from the volunteers. Then this was transferred into 50 ml Falcon tubes (each tube would have 15 ml of blood). This was then diluted 1:1 with PBS so each tube had 30 ml of diluted blood in total. Each tube was mixed gently to avoid red blood cells (RBCs) damage. Next, the blood in each tube was poured very slowly over a new tilted 50 ml Falcon tube contains 15ml of histopaque i.e., tilting the tube and adding the diluted blood very slowly over the

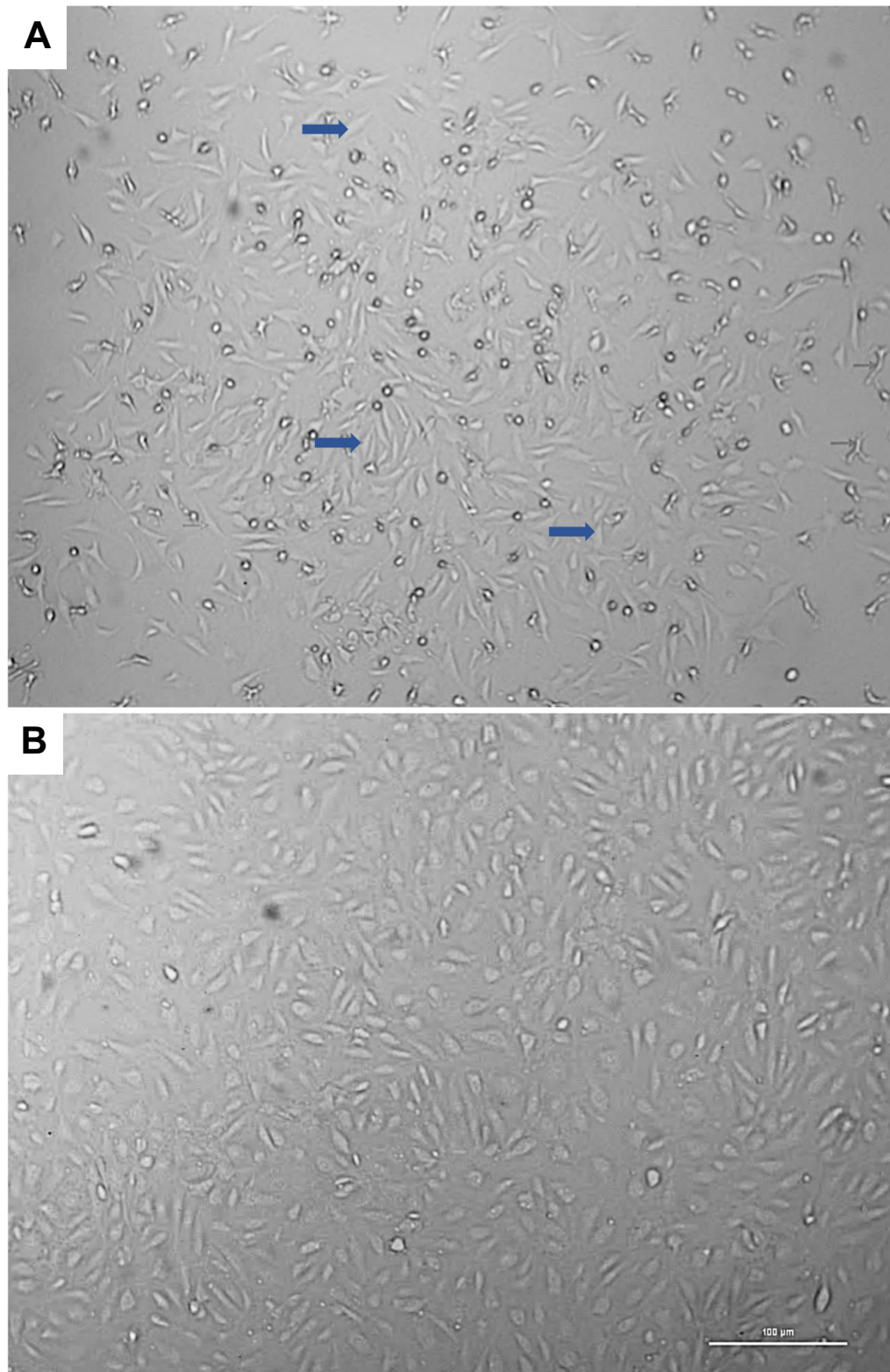


histopaque, will generate a tighter buffy coat and increase the yield. Then this centrifuged at 400 g for 30 minutes at room temperature with break off. Then buffy coat was collected after discarding the plasma layer and diluted with PBS 1:3, then centrifuged again at 300 g for 10-15 minutes with break on. Next, the supernatant was discarded, and pellet was resuspended in 1ml of endothelial cell basal medium-2 (EBM2). Then this suspension was diluted in 10 ml EBM2 again and centrifuged at 250 g for 10-15 minutes at room temperature with break on. The supernatant was discarded, and the pallet was resuspended in 1 ml EBM2 and diluted further in 1 ml EBM2. This was plated in a single well in 12 well plate which was coated with fibronectin (i.e., 35  $\mu$ l of fibronectin of 1 mg/ml were diluted in 1 ml PBS and this was sterile filtered, then 500  $\mu$ l of this was added to one well in 12 well plate and incubated for one hour then this added solution was removed before plating the pallet of mononuclear cells). Next, this plate was incubated at 37°C in a humidified condition with 5% CO<sub>2</sub>. First media change was done after 48 hours of plating time and thereafter every other day. The well was monitored for EPCs colonies which usually appear in spindle shape character in the first 2 weeks and was watched (Figure 2.4). OECs colonies usually seen with classic cobblestone morphology and appear from 14 days up to 35 days after plating time (Figure 2.5 A and B). Then cells were sub-cultured and used between passages 7 and 9 for experimental studies, and passage 14 was used to study the impact of senescence on OECs.



**Figure 2-4 Early endothelial progenitor cells.**

Early EPCs show spindle shape morphology. They appeared in culture within one week of seeding of the blood-derived mononuclear cells (indicated by red arrows).



**Figure 2-5 Outgrowth endothelial cells.**

OEC colonies appeared in culture within 2 weeks of seeding of the blood-derived mononuclear cells (indicated by blue arrows) (A). OECs show cobblestone morphology (B). Scale bar: 100 µm.

### **2.1.3 Characterization of outgrowth endothelial cells**

#### **2.1.3.1 Immunocytochemistry study**

The HBMECs and OECs ( $5 \times 10^4$ ) were cultured on coverslips coated with fibronectin (35  $\mu$ l of fibronectin of 1 mg/ml of stock solution was diluted in 1 ml PBS) using EBM2 medium which contains VEGF (5  $\mu$ l/ml of 20  $\mu$ g/ml of stock solution, Fisher Scientific, UK). Once both cells reached a confluence of about 95%, they were incubated in Dil-labelled acetylated-low density lipo- protein (Dil-AcLDL, 1  $\mu$ l of 1 mg/ml of stock solution was added to each 50  $\mu$ l of media, Invitrogen, UK) for a period of 4 hr before staining them with FITC-conjugated Ulex europaeus agglutinin (FITC-UEA-1, 7.5  $\mu$ l of 1 mg/ml stock solution was added to each 250  $\mu$ l of media, Sigma) for a period of 2 hr. Next, 4% formaldehyde was used to fix the cells before they were mounted on glass slides using mounting medium (Vector Laboratories, UK). Then both cells were seen under fluorescence microscope. (Abdulkadir et al., 2020b).

#### **2.1.3.2 Matrigel tube formation assay**

HBMECs and OECs were grown in 48-well plates ( $9 \times 10^4$  cells/well) pre-coated with growth factor-reduced Matrigel (BD Biosciences) and incubated for a period of 8 hr to establish whether cells able to form *in vitro* tubules, i.e., to assess the angiogenic activity of the cells. Tube formation was defined as the appearance of a circle-like structure and can be assessed by a light microscope. Images of tubes were obtained from five random fields per sample. ImageJ software was used to calculate the number of tubes per image (Zhao et al., 2018, Abdulkadir et al., 2020b).

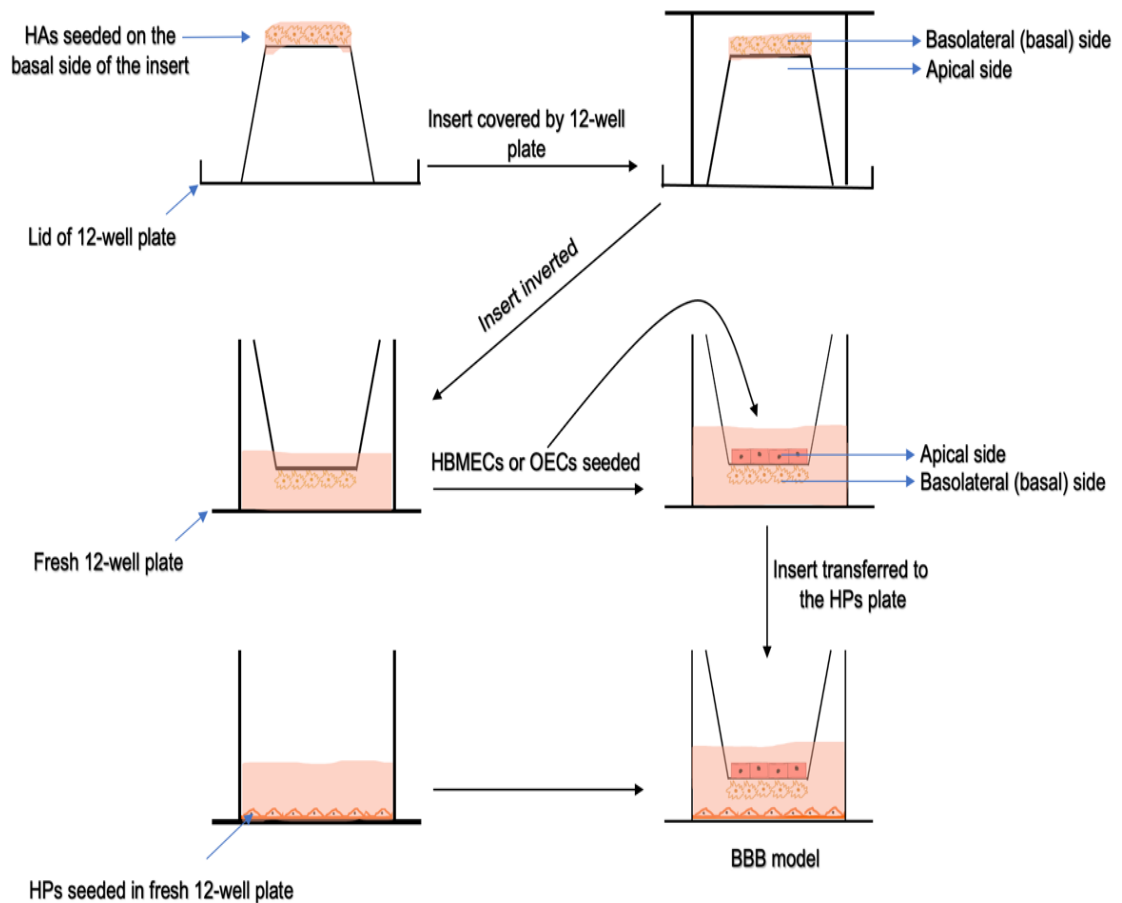
#### **2.1.4 Preparation of OEC-conditioned medium**

To generate human OEC-conditioned medium (OEC-CM), which contain OEC-secretome, the OECs were cultured in 75-flask until they reach a confluence of 90%. Then, cells were washed with sterile PBS twice before growth factors and serum free EBM2 was added to the flask. Next, cells were incubated under hypoxic conditions (5% CO<sub>2</sub>, 1.5% O<sub>2</sub>, and 93.5% N<sub>2</sub>) for 48 hours. Next, the conditioned medium was collected from the flasks and then centrifuged at 4000 g for 30 minutes at 4°C. Then, this collection was filtered using a 0.4 µm filter. This concentrated OEC-CM was stored at – 80°C for experimental use. Growth factors and serum free EBM-2 medium was used as control medium as well in this experiment (Wang et al., 2018, Yang et al., 2010). To study the effect of OEC-CM on cellular cytoskeletal organisation, cells were cultured on coverslips in their respective media until they reach a confluence of ~ 80%, then exposed to highly purified recombinant human TNF- $\alpha$  (10 ng/ml) in presence/absence of OEC-CM for 6 hours.

#### **2.1.5 Construction of human blood-brain barrier model**

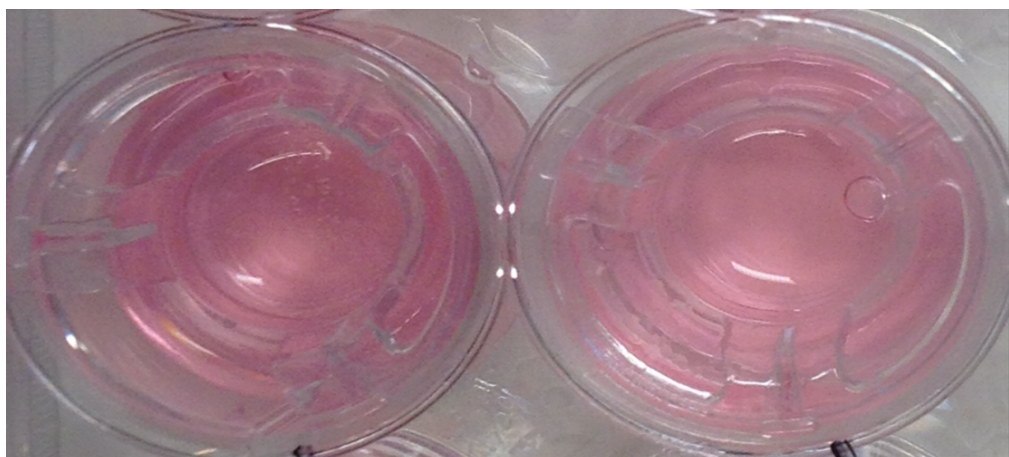
Firstly, about 200,000 HAs were resuspend in 400 µl AM then were seeded into the outer surfaces of 12 mm inserts (0.4 µm polyester membrane) which were placed in 6-well plate. Another 6 well plate was used to cover the inserts to prevent this suspension from dripping down in the plate or sticking into the upper surface of the normal cover. Next, these covered inserts in the plate were incubated for one day in humidified conditions (95%) with an atmosphere of 5% CO<sub>2</sub>, 21% O<sub>2</sub>, 74% N<sub>2</sub>, and at 37°C. On the following day, these inserts were

turned upside down and placed in 12-well plates and 1 ml AM was added to the well of each insert and then seen under the microscope and returned to the incubator. Media was changed every day until HAs reach a confluence of 90%. Secondly, at the same time of the previous step, about 75,000 HPs were seeded in 12-well plates using 1 ml of PM for each well and incubated until reach a confluence of 90%. Then, media changed every other day. Thirdly, about 50,000 of HBMECs or OECs (in some experiments a mixture of HBMECs and OECs, at 2:1) were seeded in the inner surfaces of the inserts which contain the cultured HAs in the other surfaces from the first step. Then, 800 µl of ECM or EBM2 were added to the seeded HBMECs or OECs in the 12-well plate before were returned to the incubator. Finally, on the following day, the media was removed from the inserts before transferred to the 12-plates that have the cultured HPs. Media was added to the cells as following: 800 µl AM and 800 µl PM for the lower surface of the 12-well plate (the abluminal chamber) and 800 µl ECM or EBM2 for the inner surfaces of the inserts (the luminal chamber). These BBB models were placed into the incubator and were seen under the microscope. Figure 2.6 shows schematic presentation of *in vitro* human BBB model construction steps. The media were changed every day until cells become fully confluent and ready for experiments. Figure 2.7 shows *in vitro* human BBB models.



**Figure 2-6 schematic representation of the construction process of *in vitro* human Blood-Brain Barrier model.**

Co-culture of HAs, HPs, with HBMECs or OECs.



**Figure 2-7 *In vitro* model of human Blood-Brain Barrier BBB.**

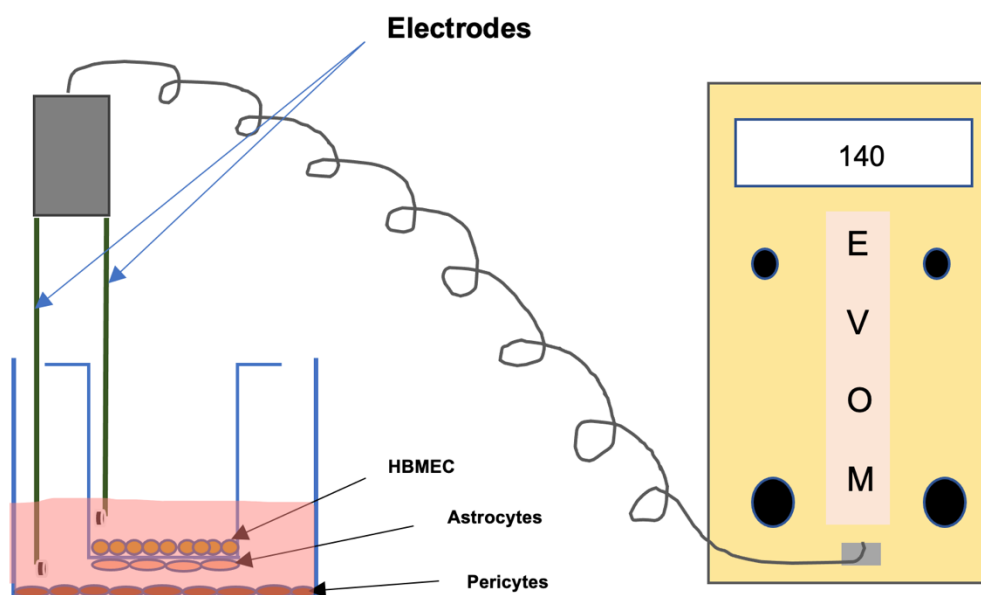
Co-culture of HBMECs, HAs and HPs cells.

### 2.1.5.1 Assessing BBB integrity and function

#### 2.1.5.1.1 Measurement of transendothelial electrical resistance

Similar to previously reported studies, the integrity of BBB models was measured using an EVOM resistance meter with STX electrodes (World Precision Instruments, Sarasota, USA) (Abdullah and Bayraktutan, 2016a, Rakkar and Bayraktutan, 2016, Mathur and Bayraktutan, 2017, Kadir et al., 2021a). This assessment was done in normal state before exposure to any pathological conditions, after exposure, and following treatment (Srivastava et al., 2013b). Figure 2.8 shows schematic representation of the use of the EVOM resistance meter to detect TEER. The expression of the resistance was in unit area resistance  $\Omega\text{cm}^2$  and according to the equation below.

$$\Omega\text{cm}^2 = \text{EVOM reading} \times \text{insert membrane surface area.}$$



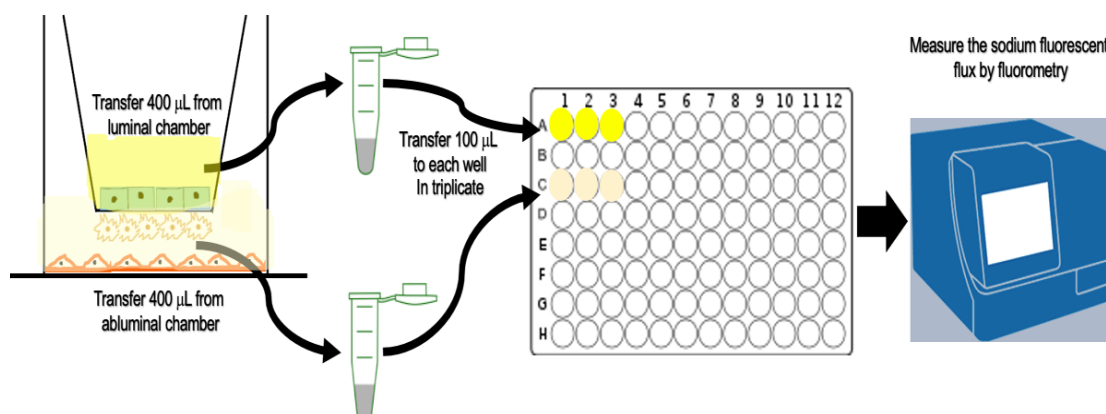
**Figure 2-8 Schematic representation of measurement of blood-brain barrier integrity with EVOM resistance meter.**



#### **2.1.5.1.2 Measurement of paracellular flux of sodium fluorescein**

The function of the BBB models was assessed by measurement of sodium fluorescein flux, a low molecular weight permeability marker (NaF, 376 Da), across the barriers. After the experiments and measuring the TEER, the media was removed from the BBB models and the inserts were transferred to a new 12-well plate containing 2 ml of Hank's Buffered Salt Solution (HBSS) in the abluminal side of each well. In the luminal side of the inserts, 500 µl of NaF (10 µg/ml in HBSS, labelled with 0.1% bovine serum albumin). The plate, then, was incubated for an hour which followed by taking 400 µl from both the abluminal and luminal sides. 300 µl of each collection were added on 3 wells of 96 well plates (each well contain 100 µl). The concentration of NaF in the samples were measured using OMEGA plate reader. Figure 2.9 shows schematic representation of NaF measurement. The excitation and emission for NaF were at 440 nm and 525 nm, respectively. The flux of NaF marker was calculated as cleared volume (CV) in µl using the following formula:

$$CV (\mu l) = \frac{\text{concentration of abluminal reading} \times \text{volume abluminal}}{\text{concentration of luminal reading}}$$



**Figure 2-9 Schematic representation of sodium fluorescence measurement.**

### 2.1.6 BBB damage models

Different types of BBB damage models have been used in this thesis. Firstly, to mimic ischaemic stroke in clinical settings, *in vitro* human BBB model has been exposed to OGD alone or followed by reperfusion (OGD+R). In this model, we studied the effect of oxidative stress on BBB integrity and function and investigated whether NOX2 inhibitor or exogenous OECs can restore BBB integrity and function. Secondly, the endothelial layer of BBB models has been damaged physically using wound scratch assay. This type of damage model allowed us to study whether exogenous OECs can incorporate directly to the site of endothelial damage and restore BBB integrity and function as a result. Thirdly, some BBB models have been exposed to hyperglycaemia which is known to disrupt BBB integrity and function, in part, through elevated oxidative stress. Through this model, we investigated whether NOX2 inhibitor could block hyperglycaemia-induced oxidative stress and restore BBB integrity and function. Finally, some BBB models were exposed to TNF- $\alpha$  to assess whether

OEC-CM (OEC-secretome) can protect BBB against damage evoked by TNF- $\alpha$ , main pro-inflammatory cytokine involved in post-stroke neuroinflammation.

### **2.1.7 Experimental ischaemic stroke**

To study the effect of ischaemia reperfusion injury and mimic this in *in vitro* BBB models, OGD method was used. Experiments of exposing human BBB models to OGD alone for 4 hours or OGD followed by reperfusion for 20 hours (OGD+R), were carried out. After the BBB models were confluent, the normal media was changed using glucose-free media (RPMI-1640 media without glucose and sodium bicarbonate) and the plates containing BBB were put in an incubator for 4 hours under hypoxic conditions (94.95% N<sub>2</sub>, 0.05% O<sub>2</sub>, 5% CO<sub>2</sub>, and at 37°C). During OGD+R, normal media was used in the reperfusion step. Co-cultured BBB under normal conditions throughout the experiments were used to serve as controls. During the experiments, BBB integrity and function were assessed as by measuring TEER and NaF flux through the barrier.

### **2.1.8 Treatment with NOX2 inhibitor**

Following through review of the literature and data availability from company, we found that gp91ds-tat (also known as NOX2ds-tat) is the most efficacious and specific NOX2 inhibitor. It is a polypeptide formed of 18 amino acids, which has two portions: the NOX2ds portion which made to block the interaction between p47<sup>phox</sup> and NOX2, and the tat portion which enable the peptide to enter the cell. However, 50  $\mu$ M of gp91ds-tat proven to inhibit the activity of NADPH oxidase and O<sub>2</sub><sup>•-</sup> levels *in vitro* and *in vivo*. (Al-Shabrawey et al., 2005,

Cifuentes-Pagano et al., 2012, Liu et al., 2003, Rey et al., 2001). The inhibitor stock solution was made in ultra-pure H<sub>2</sub>O according to the manufacturer instructions (2BScientific Limited, UK). This was diluted to working solution according to the experiments either in endothelial media or RPMI 1640 media for the OGD experiments. Before carrying out different experiments and to check the efficacy of this inhibitor, HBMECs were cultured until reached a confluence of about 80%. Then cells were exposed to either oxygen-glucose deprivation for 4 hours (OGD), OGD with gp91ds-tat (50 µM), OGD followed by reperfusion for 20 hours (OGD+R), OGD+R with gp91ds-tat given at the beginning of the reperfusion. Then, NADPH oxidase activity and O<sub>2</sub><sup>•-</sup> production were assessed by lucigenin chemiluminescence and cytochrome C reduction assays, respectively as described in this chapter (Abdullah and Bayraktutan, 2014, Shao and Bayraktutan, 2013a, Ulker et al., 2004).

#### **2.1.9 Pyrogallol experiment**

In some experiments, HBMECs or OECs were cultured in their specialised media on coverslips or in 6-well plates until they reach a confluence of about 80% followed by exposure to pyrogallol (O<sub>2</sub><sup>•-</sup> generator, 100 µM) in presence/absence of gp91ds-tat (50 µM) for 20 hours. The architecture of stress fibres of different cell types was studied by staining of F-actin filaments as described in this chapter.

#### **2.1.10 Exposure of human blood-brain barrier to hyperglycaemia**

Human BBB models were established as described before. HBMECs, HAs, and HPs, were co-cultured using their specialized media using 12 mm inserts (0.4

µm polyester membrane) until they reach a confluence of about 95%. Throughout the experiment, BBB models were incubated in humidified conditions with 5% CO<sub>2</sub>, 74% N<sub>2</sub>, 21% O<sub>2</sub>, and at 37°C. Then, BBB models were exposed to hyperglycaemia HG (25 mM D-glucose) for 120 hours in absence or presence of hypothermia, gp91ds-tat (50 µM), or hypothermia with gp91ds-tat. During the experiments, BBB integrity and function were measured as stated in this chapter.

#### **2.1.11 Cellular architecture study**

The architecture of stress fibres of different cell types was studied by staining of F-actin filaments. Under normal or experimental conditions, cells were cultured over coverslips until they reach a confluence of about 80%. Then, cells were washed twice with PBS then fixed in 4% of paraformaldehyde/PBS for 20 minutes before washed again twice with PBS. Next, the cells were permeabilised for 15 minutes in 0.1% Triton X-100/PBS before washed twice in PBS. Then cells were blocked with 1% BSA/PBS before washed twice with PBS. In the subsequent step, cells were stained with 1× rhodamine phalloidin for 60 minutes (Abcam, UK) and then washed once with PBS. Next, the coverslips were mounted on glass slides before seen with fluorescence microscopy (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK). The number of stress fibres on the cells was calculated manually using ImageJ software.

#### **2.1.12 Senescence-associated β-galactosidase study**

To study cellular senescence, we used beta Galactosidase Staining Kit (Abcam, UK). Cells were cultured in 12-well plates until they reach a confluence of about

80%, under normal or different experimental conditions. The culture media was removed, and cells were washed once with 1X PBS solution. Next, cells were fixed with 0.5 ml of fixative solution for 15 minutes at room temperature. Staining Solution was prepared as per manufacturer protocol by using polypropylene plastic tubes only. In this step, a mixture of 500 µl was prepared for each well including 470 µl of staining solution, 5 µl of staining supplement, and 25 µl of 20 mg/ml x-gal in DMF. Following fixation step, cells were washed twice with 1X PBS. Next, 500 µl of the prepared staining solution mixture was added to each well. Then, the plates were covered and incubated at 37°C overnight. Then plates were observed under light microscope to identify senescence-associated-β-galactosidase positive cells (Saheera et al., 2019).

#### **2.1.13 Senescence-associated γH2AX study**

To study cellular senescence, anti-gamma H2AX (phospho S139) antibody was used (Abcam, UK). Cells were grown on coverslips precoated with poly-L-lysine until they reach a confluence of about 80% under normal or different experimental conditions. Then coverslips were rinsed with sterile H<sub>2</sub>O for three times, 1 hr every time. Then coverslips were rinsed with 1X PBS. Next, cells were fixed in 4% paraformaldehyde in PBS with pH of 7.4 for 10 minutes at room temperature. Then cells were washed three times with ice-cold PBS. Next, cells were permeabilized using 0.1% triton X-100, then washed three times in PBS. To block unspecific binding of the antibodies, cells were incubated overnight in the diluted antibody in 1% BSA in PBST (PBS+ 0.1% Tween 20) at 4 °C. Then the solution was removed, and cells were washed three times in PBS for 5 minutes for each wash. Next, cells were incubated in secondary

antibody (goat anti-rabbit IgG H&L) in 1% BSA for 1 hour in dark at room temperature. Then, solution was removed, and cells washed three times in PBS for 5 minutes for each wash in dark. Cells were incubated also in DAPI (DNA stain) for one minute then rinsed with PBS. Then, coverslips were mounted on a glass slide with mounting media before visualisation and counting cells positive for  $\gamma$ H2AX (Polonis et al., 2020).

#### **2.1.14 Assessment of tight junctions**

To study cellular ability to form tight junctions, tight junction protein ZO-1 antibody was used (Abcam, UK). Cells were grown on coverslips until they reach a confluence of about 80% under normal or different experimental conditions. Then coverslips were rinsed with sterile H<sub>2</sub>O for three times, 1 hr for every time. Then coverslips were rinsed with 1X PBS. Next, cells were fixed in 4% paraformaldehyde in PBS with pH of 7.4 for 10 minutes at room temperature. Then cells were washed three times with ice-cold PBS. Next, cells were permeabilized using 0.1% triton X-100, then washed three times in PBS. To block unspecific binding of the antibodies, cells were incubated overnight in the diluted antibody in 1% BSA in PBST (PBS+ 0.1% tween 20) at 4°C. Then the solution was removed, and cells were washed three times in PBS for 5 minutes for each wash. Next, cells were incubated in secondary antibody (goat anti-rabbit IgG H&L) in 1% BSA for 1 hour in dark at room temperature. Then, solution was removed, and cells washed three times in PBS for 5 minutes for each wash in dark. Cells were incubated also in DAPI (DNA stain) for one minute then rinsed with PBS. Then, coverslips were mounted with mounting media before seen under fluorescence microscope.

#### **2.1.15 Proliferation assays**

Cells were cultured in T-25 flasks until reach a confluence of nearly 85%. For this experiment, starvation medium was prepared by adding 25 ml of cell medium, which contain supplements but without FBS, to 25 mg of BSA. A plate shaker was used to dissolve this mixture which then sterile filtered. Then, cultured cells were washed twice with 3 ml of PBS and once with 3 ml of starvation medium. Then, cells were incubated in 6 ml of starvation medium for 48 hr. Subsequently, medium removed, and cells were detached from the flasks using accutase before been counted using trypan blue. Four thousand cells of these were seeded in 200  $\mu$ l in normal medium specific for each cell type in each well of 96-well plate. Specific plates were used for each time points at 6, 24, and 48 hr. Next, plates were incubated until they completed their time of incubation. Then, medium was removed from wells and 100  $\mu$ l of fresh normal medium was added for each well. Then, 10  $\mu$ l of WST-1 reagent was added to each well before scanning using a FLOUstar Omega Plate Reader at 0.5 and 2 hr. The wavelength range between 400-660 nm with resolution at 5 nm. With 22 flashes per well and double shaking at 200 rpm for one minute before plate reading.

#### **2.1.16 Adhesion assay**

Cells (10,000 cells per well) were placed and incubated for 30 minutes on 24-well plates pre-coated overnight with 5  $\mu$ g/ml purified human fibronectin or 10  $\mu$ g/ml human collagen. Then, plates were gently washed with PBS three times to remove non-adherent cells. Finally, adherent cells observed under light



microscope and counted manually in five random microscopic fields (20X) (Chang et al., 2016, Zhang et al., 2020).

#### **2.1.17 Wound scratch assay**

Same passage number of HBMECs and OECs were seeded in 6-well plates ( $1.5 \times 10^4$  cells/well) and cultured in their specialised media until they become fully confluent. A scratch was then made manually down the centre of the well with a p1000 micropipette tip in one swift motion and the cells were maintained under normoxic conditions at 37°C. Pictures of wound closure were taken at 6-, 12-, 36-, and 48-hours post-scratch period. Wound closure was quantified as the percentage of difference in scratch area between the initial image (0-hour image) and the images obtained at different time points using ImageJ software (version 1.52k, NIH, Maryland, USA) (Abdulkadir et al., 2020b, Alwjwaj et al., 2021).

#### **2.1.18 Wound scratch assay on blood-brain barrier.**

In some experiments, a scratch was made manually down the centre of the endothelial layer with a p10 micropipette tip in one swift motion. Endothelial cell media was then replaced with serum free media containing OECs ( $3.5 \times 10^4$ ). 48 hours after the induction of scratch, the characteristics of the BBB were assessed as described in this chapter. Due to difficulties regarding clear photography of endothelial monolayer in the BBB model, in additional experiments HBMECs were seeded in 48-well plates (that provided closer growth area to inserts used;  $0.95 \text{ cm}^2$  *versus*  $1.12 \text{ cm}^2$ , respectively) and cultured to confluence in endothelial cell media. Wounds were created by

scratching the monolayer with a p10 micropipette tip before replacing endothelial cell media with serum-free media containing OECs ( $3.5 \times 10^4$ ). Images were taken immediately after scratch and 8 hours after addition of OECs (Abdulkadir et al., 2020b, Alwjaj et al., 2021).

#### **2.1.19 Protein isolation and quantification assay (BCA)**

Protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, UK). According to the manufacturer instructions, the bovine serum albumin standards dilutions was prepared with concentration ranged from 0 µg/ml to 2000 µg/ml and the working reagent BCA was prepared by mixing one part of reagent B with 50 parts of reagent A. Then, 10 µl of the samples and the standards were placed in duplicate into a flat bottomed 96-well plate. To each of these wells, 200 µl of the working reagent was added. Then, plate was incubated for 30 minutes at 37 °C. In the subsequent step, plate was measured in a FLUOstar Omega plate reader (BMG Labtech, UK) at absorbance of 562 nm. By using Omega software, the standard curve was used to calculate the concentration of proteins in the samples.

#### **2.1.20 Measurement of NADPH oxidase activity**

The activity of NADPH oxidase was detected by lucigenin chemiluminescence assay (Abdullah and Bayraktutan, 2014, Shao and Bayraktutan, 2013a, Ulker et al., 2004). Cell pellets were resuspended in 200 µl of ice-cold lysis buffer containing monobasic potassium phosphate (20 mM, pH 7.0), ethylene glycol tetra acetic acid (EGTA, 1 mM), leupeptin (0.5 µg/ml), aprotinin (10 µg/ml), PMSF (0.5 mM) and protease inhibitor (5 µl/ml).

To measure NADPH oxidase activity, an equal amounts of homogenate (100 µg) were incubated in assay buffer containing lucigenin (5 µM), sucrose (150 mM), EGTA (1 mM) and potassium phosphate buffer (50 mM) at 37 °C. Specific inhibitors for other ROS-generating enzymes, namely L-nitro-arginine methyl ester (100 µM, nitric oxide synthase), rotenone (50 µM, mitochondrial respiratory chain complex 1), allopurinol (100 µM, xanthine oxidase) and indomethacin (50 µM, cyclooxygenase) were then added to assay buffer for 15 min. NADPH (100µM) was then added to initiate the reaction. The luminescence was monitored for 2 hours using FLUOstar Omega plate reader (BMG Labtech, UK). Buffer blank readings were subtracted from the experimental readings before the final rates were obtained per mg protein by calculating the reaction rate using MARS data analysis software and displayed as LU/min/mg. Protein concentrations were measured as described in this chapter.

Reaction rate = (maximum luminescence / time reach maximum) / mg protein.

#### **2.1.21 Measurement of total superoxide levels**

Cytochrome reduction assay was used to measure total O<sub>2</sub><sup>•-</sup> levels. Following exposure of cells to different experimental settings, ice cold PBS was used to wash the cells. Then, cells were scrapped into a Falcon tube in cold PBS. Next, the cells pellet was centrifuged for 10 minutes at 4°C and speed of 2000 g. Then, 300 µl of cold lysis buffer was added to cell pellet before sonication for three times (15 seconds bursts with 30 seconds rests on ice in-between) at 20 KHz and amplitude of 50%. Lysis buffers containing monobasic potassium

phosphate (20 mM, pH 7.0), ethylene glycol tetra acetic acid (EGTA, 1mM), leupeptin (0.5 µg/ml), aprotinin (10 µg/ml), PMSF (0.5 mM), and protease inhibitor (5µl/ml). Next, equal amounts of protein 100 µg were incubated for one hour with 50 µM cytochrome C at 37°C before absorbance measurement at 570 nm on FLUOstar Omega plate reader (BMG Labtech, UK). Buffer blanks were also subtracted to obtain the final readings. Protein concentrations were measured as described in this chapter and data was displayed as unit/mg.

#### **2.1.22 Measurement of Nitric Oxide**

Nitric oxide assay kit (Abcam, UK) was used to quantify nitric oxide (NO) production. As the NO is rapidly converted to nitrate and nitrite, the total concentration of nitrate and nitrite was used to measure NO production in two steps as per manufacturer protocol. In step one, by using nitrate reductase, the nitrate is converted into nitrite. In step two, nitrite make reaction with fluorescent probe DNA (2,3 diaminonaphthalene). The intensity of fluorescent indicates the total NO release. To begin, cells were cultured until they become confluent. Then washed with cold PBS twice and detached from the flask before resuspension in 500 µl of assay buffer on ice. Next, this suspension was homogenized by pipetting up and down for a few times. Then, the homogenate was centrifuged for about 5 minutes at 4°C at speed of 2000 g. Then, the supernatant was collected and kept into ice. According to the manufacturer instructions, the standards dilutions were prepared with concentration ranged from 0 pmol/well to 1000 pmol/well by adding nitrate or nitrite into the assay buffer in a microplate in duplicate. A fresh set of standards was use for every experiment within 4 hours of preparation. To set up reaction wells, 75 µl of

assay buffer was added in duplicate to background wells and 75 µl standard dilutions was added in duplicate to standard wells. Then, 5 µl of enzyme cofactor solution was added to all wells. Then 5 µl of nitrate reductase was added to samples and standards. Next, the microplates were incubated for 4 hours at room temperature, the step of nitrate to nitrite conversion. Then, 5 µl of enhancer was added to every well followed by incubation for 30 minutes at room temperature. Next, 5 µl of DNA Probe was added to every well followed by incubation for 10 minutes at room temperature. Next, 5 µl of NaOH was added to each well followed by incubation for 10 minutes at room temperature. Then, microplates were read on FLUOstar Omega plate reader (BMG Labtech, UK), at excitation of 360 nm and emission of 450 nm. Data was displayed as total NO in µM.

#### **2.1.23 Measurement of total antioxidant capacity**

A total antioxidant capacity assay kit (Abcam, UK) was used to quantify the antioxidant capacity of both HBMECs and OECs. The assay was carried out according to the manufacturer's instructions. Cells were cultured until reach a confluence of about 90% before exposure to different experimental conditions. Then cells were washed with ice-cold PBS and resuspended in double distilled water (ddH<sub>2</sub>O). The mixture was then homogenised by pipetting up and down for few times. The homogenate was then incubated for 10 minutes on ice before centrifugation for 4 minutes at 4°C. The supernatant was then transferred to a new tube kept on ice. Working solution was prepared by adding 1 part of the Cu<sup>2+</sup> reagent to 49 parts of the assay diluent. Also, 1 mM of Trolox standard solution was used to prepare standard curve dilution as described by the

manufacturer. Then, 100  $\mu$ l of working solution was added to all standard and sample wells before being incubated at room temperature for about 90 minutes. Then plates measured in microplate reader at absorbance of OD 570 nm.

#### **2.1.24 Statistical analysis**

Data are displayed as mean  $\pm$  SEM from a minimum of three independent experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc analysis or Student's *t*-test, where appropriate. GraphPad Prism 8.0 statistical software package (GraphPad Software Inc., La Jolla, Ca, USA) was used to perform these analyses.  $P < 0.05$  was considered as significant.

**3 OUTGROWTH ENDOTHELIAL CELLS FORM  
FUNCTIONAL CEREBRAL BARRIER AND  
RESTORE ITS INTEGRITY AFTER DAMAGE.**

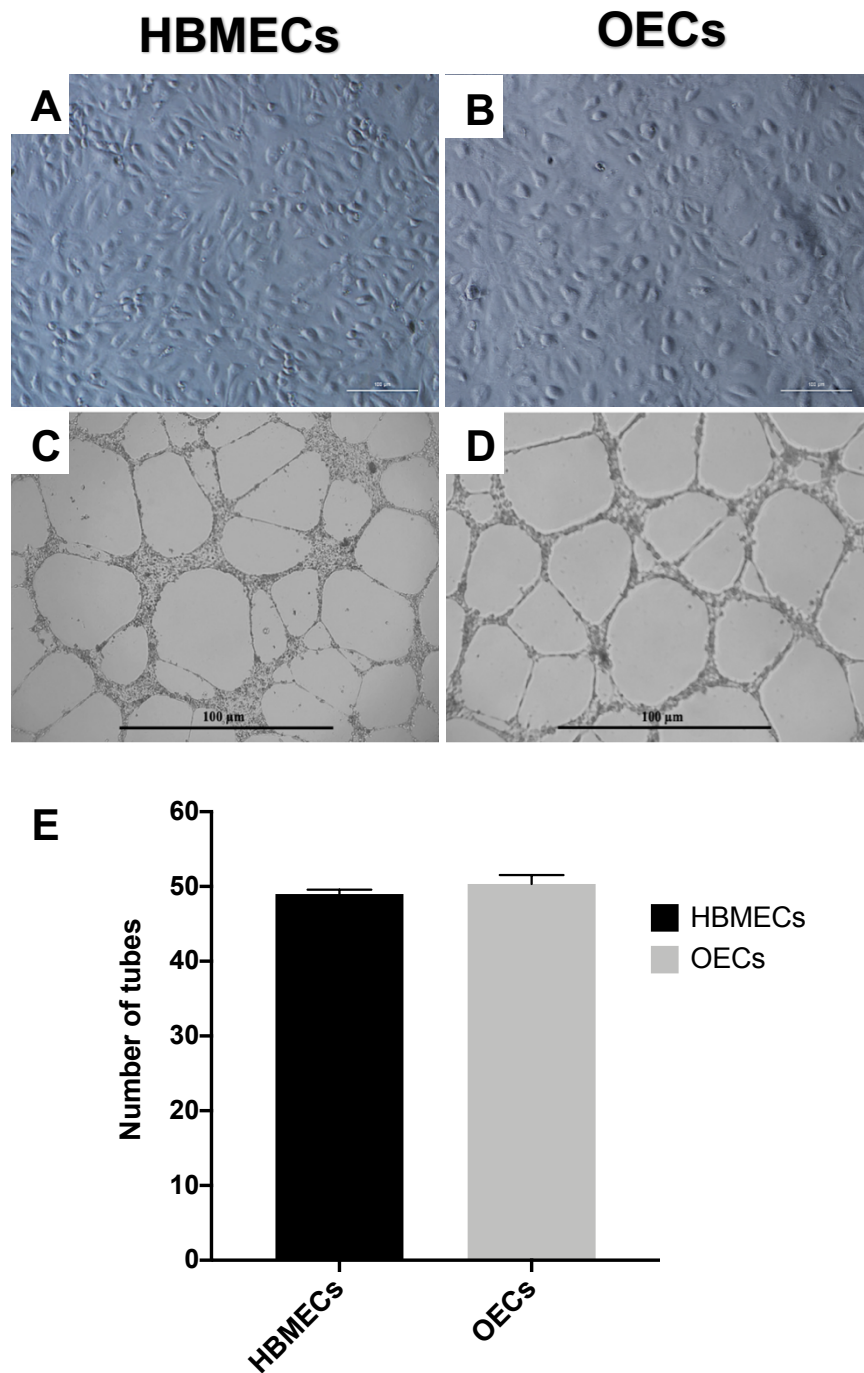
### **3.1.1 Introduction and aims**

It is evident that EPCs has the capacity to differentiate into endothelial cells to assist repair stroke-evoked neurovascular damage. However, it is impossible to isolate EPCs in sufficient numbers to be used as cell therapy for diseases such as ischaemic stroke. This is because their scarcity in bone marrow or circulatory system. This obviously entails *ex vivo* expansion of few isolated cells to produce substantial quantities of clinically suitable homogenous cells. It is evident that EPCs in culture generate two different types of cells, early EPCs that have limited proliferation rate and no capacity to form tubules and late EPCs (OECs), which exhibit progenitor cell markers and have strong proliferative and tubulogenic capacity. Collectively, the special characteristics of OECs may constitute the EPCs subtype that can crucially participate in new blood vessels formation and neurovascular repair. Considering the above, this thesis has comparatively investigated whether OECs has true endothelial characteristics of HBMECs. Next, we examined whether OECs could expand in a directional manner and successfully repair endothelial damage. If this is the case, would the findings be comparable to those acquired with HBMECs, the main cellular element of the BBB? Then, using a triple-culture model of human BBB consisting of HBMECs or OECs with HAs and HPs, the study explored whether OECs can form equally tight and functional BBB compared to the HBMECs. If this is so, would these barriers respond to experimental ischaemic insult in alike manner and can OECs repair endothelial damage in a model formed with HBMECs and restore its function?



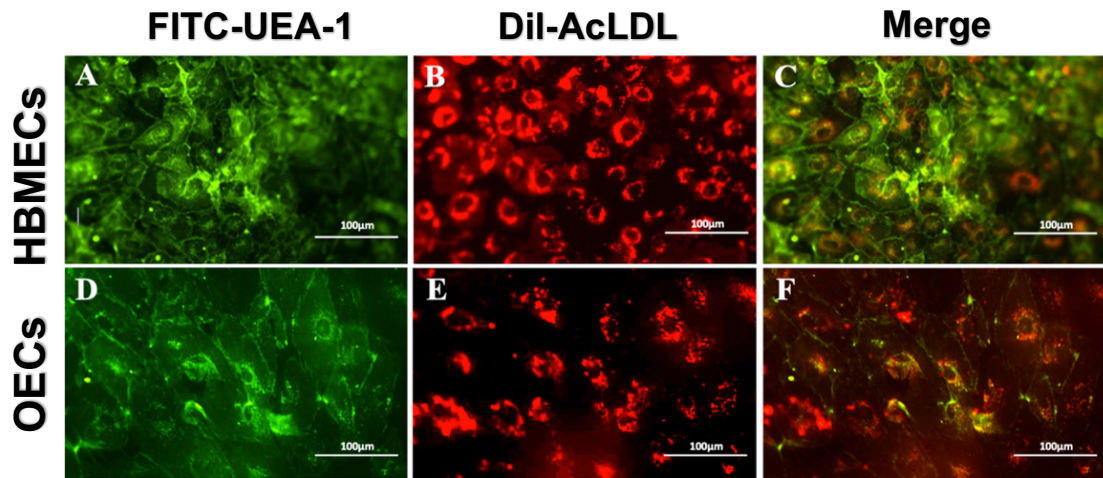
### **3.1.2 Outgrowth endothelial cells display true endothelial characteristics**

Like HBMECs, OECs displayed cobblestone morphology, the classical endothelial phenotype. In addition, like HBMECs, OECs formed well-established tubules on Matrigel, a marker of *in vivo* angiogenesis. OECs formed nearly the similar number of tubes like HBMECs (Figure 3.1). Moreover, both HBMECs and OECs endocytosed Dil-AcLDL and bound ulex europaeus agglutinin (Figure 3.2). Others, also, assessed the characterisation of OECs (Abdulkadir et al., 2020a, Bayraktutan, 2019a).



**Figure 3-1 Morphological and tubulogenic features of HBMECs and OECs.**

Light microscopy imaging show that both HBMECs and OECs exhibit typical cobblestone morphology (A and B). HBMECs and OECs, grown in 48-well plates pre-coated with growth factor-reduced Matrigel, establish similar number of tubules (C-E). Each data point represents mean  $\pm$  SEM from three independent experiments (Unpaired t test). Scale bars: 100  $\mu$ m.

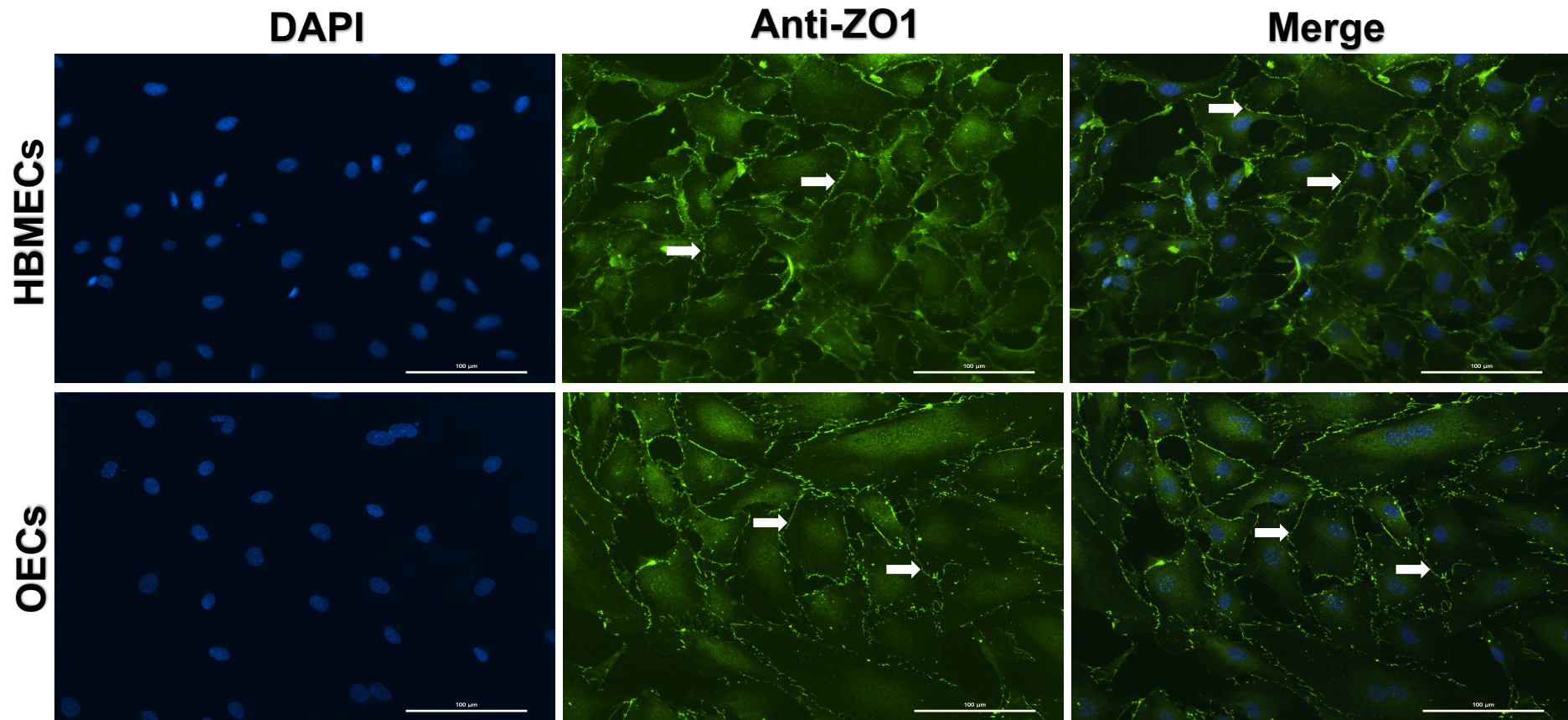


**Figure 3-2 Immunocytochemical analyses, endothelial characteristics of HBMECs and OECs.**

HBMECs and OECs were cultured to about 95% confluence on coverslips. Then, cells were first incubated in Dil-labelled acetylated-low density lipo- protein (Dil-AcLDL) (B and E) before staining further with FITC-conjugated Ulex europaeus agglutinin (FITC-UEA-1) (A and D). The cells were then fixed, using 4% of formaldehyde and visualised under fluorescence microscope. Merged images reveal the pattern of staining in both cell types (C and F). Scale bars: 100 µm.

### **3.1.3 Outgrowth endothelial cells form tight junctions**

To serve as therapeutics and to be able to restore cerebral barrier integrity and function following/during ischaemic stroke, OECs should have the capacity to form intracellular tight junctions. Considering this, immunostaining for intracellular tight junctions was performed. Similar passages (P7) of OECs and HBMECs were cultured and exposed to tight junction protein ZO-1 antibody as described in detail in chapter two. Compared to the HBMECs, OECs jointly interacted together by establishing tight junctions, as illustrated by the uniform expression of ZO-1 throughout the entire cell monolayer (Figure 3.3).

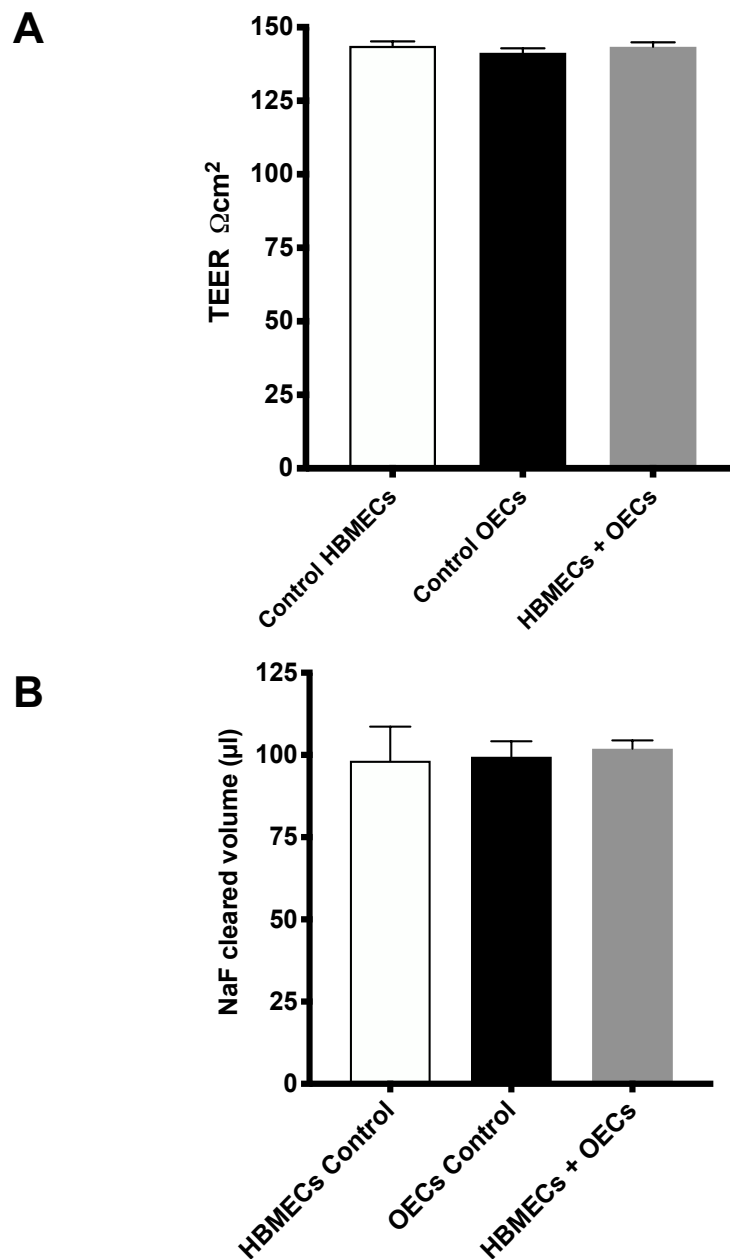


**Figure 3-3 Immunofluorescence labelling of ZO-1**

HBMECs and OECs were cultured to about 80% confluence. Then, fixed in 4% paraformaldehyde. Then, incubated in primary antibody (anti ZO-1) in 1% BSA in PBST (PBS+ 0.1% tween 20). Then, incubated in secondary antibody (goat anti-rabbit IgG H&L) in 1% BSA. Next, cells were seen under fluorescence microscope. White arrows show a uniform expression of ZO-1 throughout the entire cell monolayer. Scale bars: 100 µm.

### **3.1.4 OECs able to form functional cerebral barrier alone and with HBMECs**

We established *in vitro* models of human BBB by co-culture of HAs and HPs with either HBMECs or OECs alone or the combination of both cell types, as described in chapter two. Then, the integrity and function of these models were assessed by measurements of TEER and paracellular flux of NaF, respectively. Our findings show that, similar to HBMECs, OECs form equally tight and functional BBB with HAs and HPs. Furthermore, our studies show that OECs can readily interact with HBMECs and form fully functional endothelial monolayer for the BBB models as evidenced by similar TEER and NaF flux readings as the BBB models set up with HBMECs or OECs alone (Figure 3.4). This supports the notion, that OECs have the capacity to establish tight and functional cerebral barrier alone and in combination with resident HBMECs, *in vivo*.



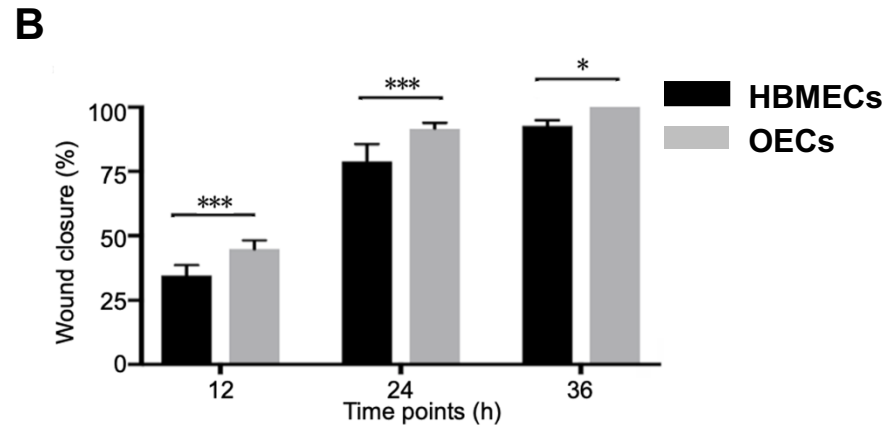
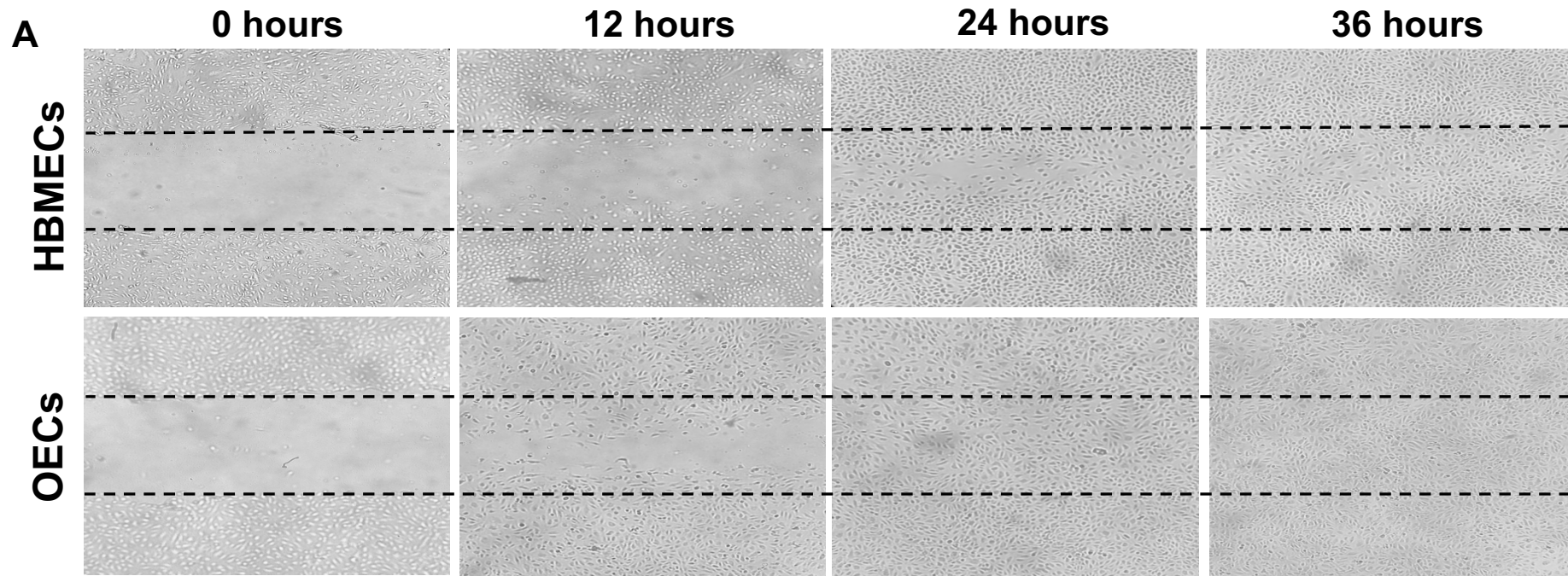
**Figure 3-4 Assessment of the integrity and function of the BBB established with HBMECs or OECs or a mixture of both cells.**

Triple cell culture models of human BBB were established by co-culture of HAs and HPs with either HBMECs or OECs alone or the combination of both cell types. The integrity and function of the models were assessed by measurements of TEER (A) and paracellular flux of NaF (B). Each data point represents mean  $\pm$  SEM from three independent experiments. (one-way analysis of variance followed by Tukey's post hoc analysis).

### **3.1.5 OECs repair endothelial damage faster than HBMECs**

HBMECs and OECs were cultured to full confluence before a scratch was made manually down the centre of the well. The cells were then incubated in their specialised media. Images of wound closure were captured at different time points and wound closure was calculated as described in chapter two. The assessment of cellular proliferative and migratory capacities through wound scratch assays shows that both OECs and HBMECs repair endothelial layer damage in time-dependent manner. These studies showed that OECs were more effective at covering the wound area at 12, 24, and 36 hours after induction of wound thereby attributing a higher proliferative and migratory capacity to them (Figure 3.5). These findings indicate that OECs have the capacity to induce robust endothelial coverage, organise wound bed, accelerate angiogenesis, and prevent wound contraction faster than HBMECs (Paschalaki and Randi, 2018).





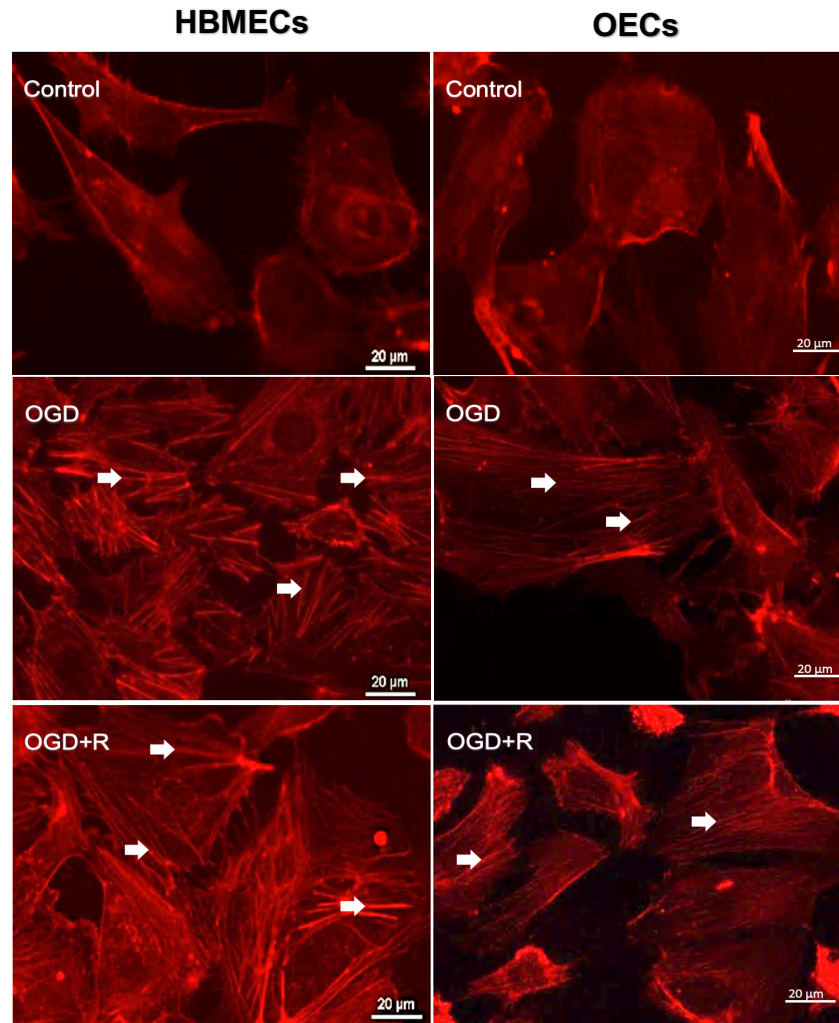
**Figure 3-5 Wound scratch assay**

HBMECs and OECs were cultured in 6-well plates to full confluence. By using a p1000 micropipette tip, a scratch was made manually down the centre of the well in one swift motion. Images of wound closure were captured at 0, 12-, 24-, and 36-hour time points post-scratch. Data are presented as mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$  compared with HBMECs (one-way analysis of variance followed by Tukey's post hoc analysis).

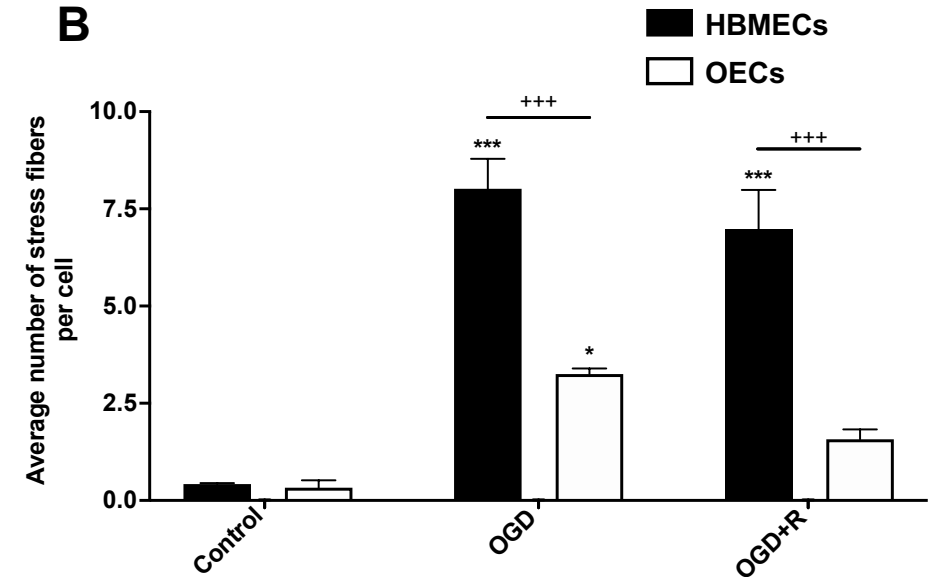
### **3.1.6 Effects of OGD and reperfusion on HBMECs and OECs architecture**

Scrutiny of cytoskeletal organisation in both HBMECs and OECs maintained under normoxic conditions revealed a cortical actin staining. Exposure of HBMECs and OECs to oxygen-glucose deprivation (OGD, 4 hours) alone or followed by reperfusion (20 hours, OGD+R) promoted appearance of thick actin stress fibres in both cell types (Figure 3.6 A). Compared to HBMECs, the number of stress fibres appear to be significantly lower in OECs subjected to OGD±R. The number of stress fibres in OECs appears to be not significant during OGD+R compared to control (Figure 3.6 B).

**A**



**B**

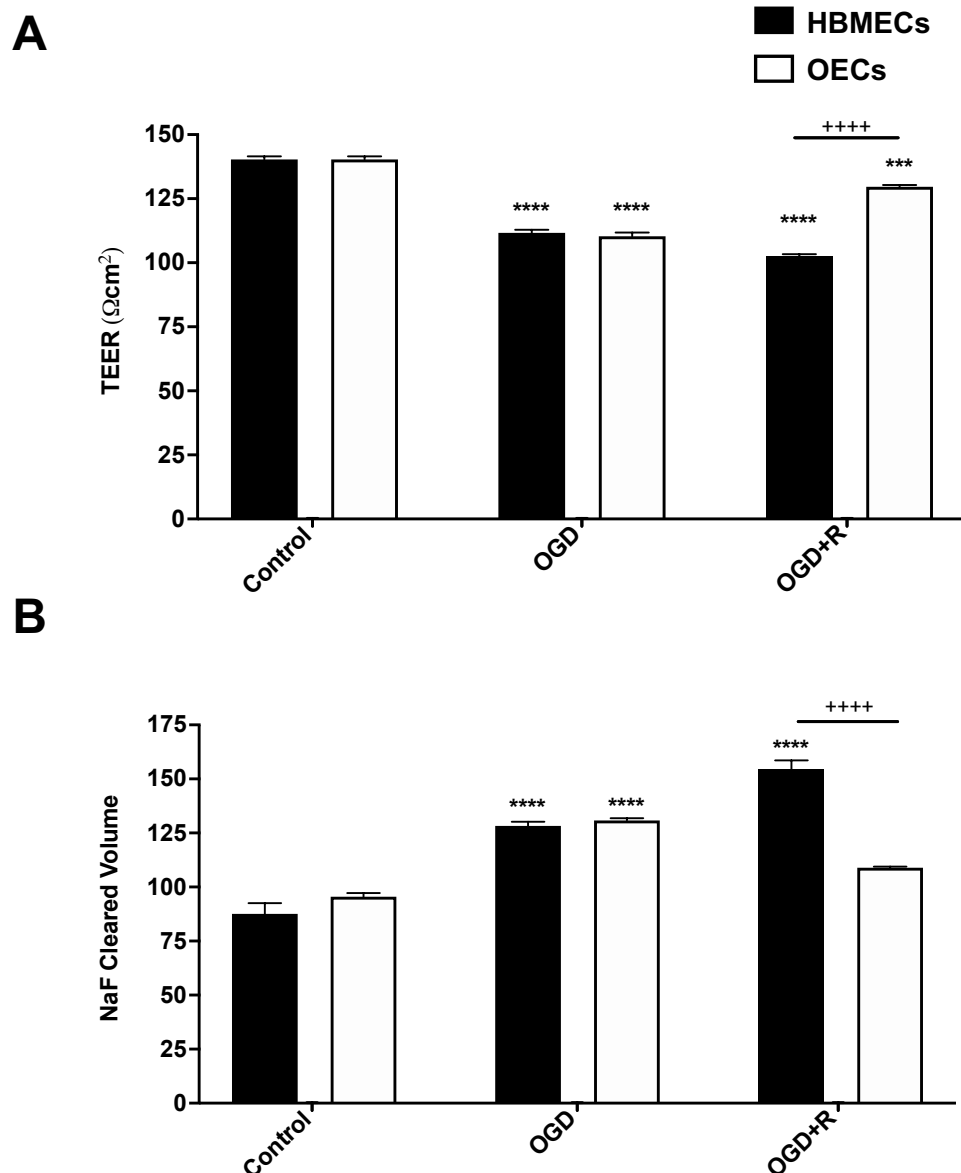


**Figure 3-6 Staining of actin microfilaments in HBMECs and OECs.**

HBMECs and OECs were cultured on coverslips to 80% confluence. Cells were fixed in 4% paraformaldehyde/PBS for 20 min and then permeabilised in 0.1% Triton X-100/PBS for 15 min prior to staining for 60 min in 1x Rhodamine Phalloidin. Then, cells were viewed under fluorescence microscope. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\* $P$ <0.001 compared to HBMECs control. \* $P$ <0.05 compared OECs control. +++ $P$ <0.001 compared to HBMECs subjected to OGD $\pm$ R. (one-way analysis of variance followed by Tukey's post hoc analysis). Scale bars: 20  $\mu$ m.

### **3.1.7 Effects of experimental ischaemia on BBB integrity and function**

In line with our previous experiments, when cultured with HAs and HPs, both HBMECs and OECs established an equally tight and functional human BBB models, suggesting that both endothelial cell types were able to establish tight junctional complexes. Exposure of either model to OGD alone significantly disturbed BBB integrity and function as evidenced by similar decreases in TEER and increases in paracellular flux of NaF. OGD+R appears to aggravate BBB damage in models formed with HBMECs. This was evidenced by significant decreases in TEER and increases in NaF flux readings compared to those of controls. Interestingly, models established with OECs appear to regain their integrity and function following 20 hours of reperfusion, as evidenced by rises in TEER and decreases in NaF flux readings compared to models formed by HBMECs (Figure 3.7).

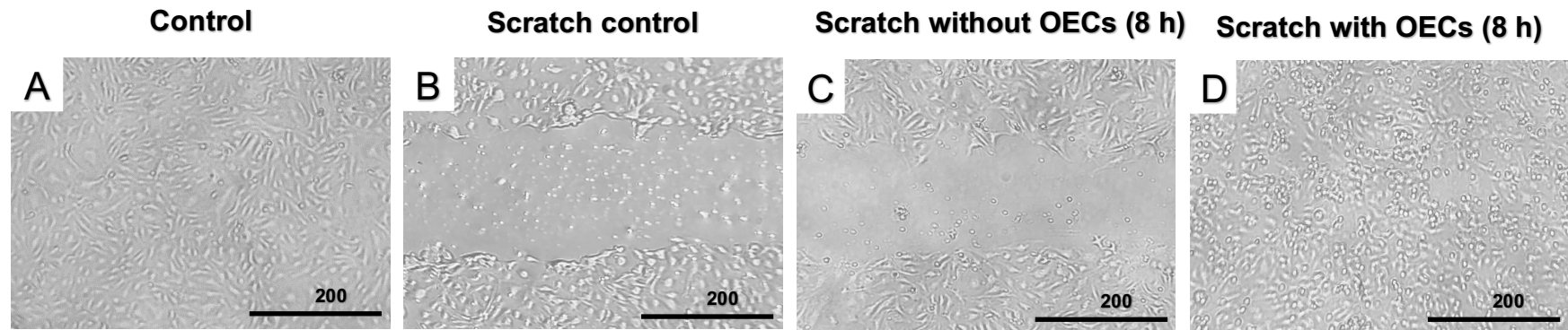


**Figure 3-7 Assessment of the integrity and function of the BBB models exposed to experimental ischaemia.**

BBB models were established by co-culture of HBMECs or OECs with HAs and HPs then exposed to oxygen-glucose deprivation alone (OGD, 4 hours) or followed by reperfusion (20 hours, OGD+R). BBB integrity and function were assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF, B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\* $P < 0.0002$  and \*\*\*\* $P < 0.0001$  compared to control. \*\*\*\* $P < 0.0001$  compared HBMECs subjected to OGD+R (one-way analysis of variance followed by Tukey's post hoc analysis).

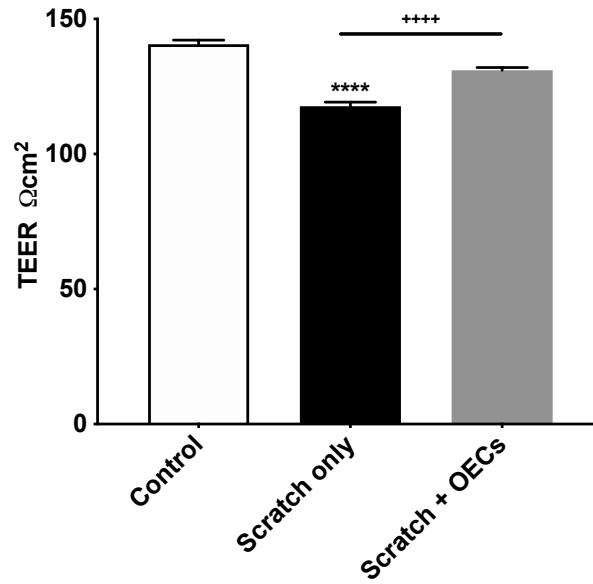
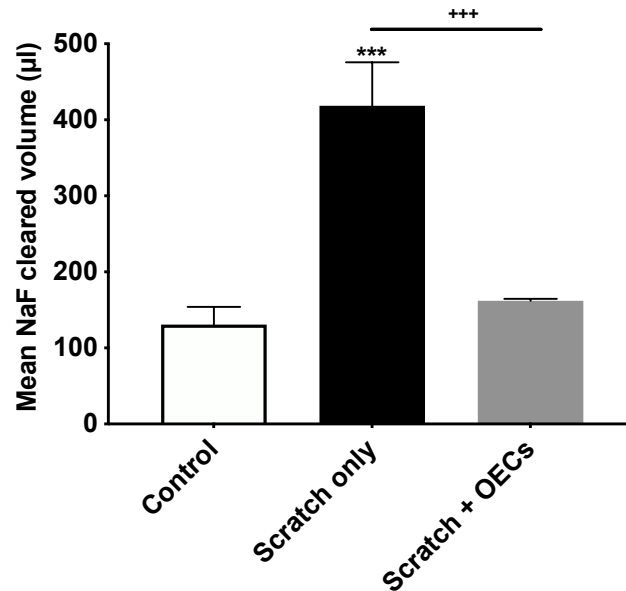
### **3.1.8 Outgrowth endothelial cells repair endothelial damage**

Our previous wound scratch assays showed that, compared to HBMECs, OECs repaired endothelial damage in a significantly shorter duration suggesting the ability of OECs to proliferate and migrate at faster rates. However, the capacity of OECs to repair endothelial damage in a triple culture model of human BBB is unknown. Our investigations of endothelium-reparative feature of OECs in a triple culture model of human BBB show that OECs can effectively home into the scratch area, repair endothelial damage (Figure 3.8). Exogenous OECs appear to restore barrier integrity and function as evidenced by significant increases in TEER and decreases in paracellular flux of NaF measurements (Figure 3.9).



**Figure 3-8 Restorative endothelium function of OECs with wound scratch assays**

HBMECs were cultured in 48-well plates until reaching full confluence. Then, wounds were induced by scratching the centre of the endothelial monolayer in one swift motion by using a p10 micropipette tip. Then, media was replaced with serum-free media containing OECs ( $3.5 \times 10^4$ ). The investigations shows that exogenous OECs can effectively home into the scratch area and repair the wound scratch induced on HBMECs maintained in serum-free conditions. Scale bars: 200  $\mu\text{m}$ .

**A****B**

**Figure 3-9 Assessment of the reparative function of OECs on an *in vitro* model of human BBB**

Human BBB model was established by co-culture of HBMECs, HAs, and HPs. Wound was induced in the endothelial monolayer of the model before replacing endothelial media with serum-free media containing OECs ( $3.5 \times 10^4$ ). BBB integrity and function were assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF, B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\* $P < 0.0001$  and \*\*\* $P < 0.0002$  compared to control. \*\*\*\* $P < 0.0001$  and \*\*\* $P < 0.0003$  compared to BBB models subjected to scratch wound (one-way analysis of variance followed by Tukey's post hoc analysis).



### **3.1.9 Discussion**

Preserving endothelial integrity and capacity for neovascularisation help protect the brain against ischaemic damage (Gibson et al., 2014). Therefore, re-endothelialisation of blood vessels of brain parenchyma by lateral migration and proliferation of resident brain microvascular endothelial cells and/or by the complex function of circulating EPCs or exogenous OECs may considerably attenuate the risks of cerebral ischaemia and also reduce the notorious complications of ischaemic stroke (Liman and Endres, 2012). Certainly, by releasing several growth factors and evolving into mature endothelial cells, OECs are expected to repair endothelial damage and induce formation of new blood vessels, angiogenesis (Hur et al., 2004). Accordingly, regional, and systemic therapeutic strategies that can enhance OECs migration and recruitment, e.g., by SDF-1 and GM-CSF, might substantially promote regenerative functions of OEPCs (Sprigg et al., 2006, Bogoslovsky et al., 2011, Cheng et al., 2017). Though comparatively efficacious, or at least safe, in some transitional studies or clinical stroke trials, these strategies necessitate long period, may be days or even weeks, to induce the numbers of EPCs in blood circulation to therapeutic levels that can have positive outcomes, indicating that allogenic or autologous exogenous administration of OECs in adequate numbers would be a superior therapeutic approach (Lee et al., 2005, Sprigg et al., 2006, Kawada et al., 2006). Studies scrutinizing this perception have shown various outcomes, neurovasculo-protective as opposed to neutral, and linked this inconsistency broadly to the diversified nature of EPCs and the dissimilarity of methodologies used to isolate these cells. Indeed, one of most widely used methodologies to detect EPCs in whole blood is via flow cytometry which work

based on multiple analysis of single cells in a mixed types of cell population by concurrent identification of various cell surface markers. However, due to the remarkable overlap of surface markers expressed on EPCs and haematopoietic, it is challenging to accurately detect EPCs by this method (Peichev et al., 2000, Fadini et al., 2006).

Expansion of cells *in vitro* by using specific adhesive substrates, such as collagen or fibrinogen, before seeding the cells in their endothelial specific culture media supplemented with all the required growth factors, foetal bovine serum, and hormones is thought to be the most convenient methodology to produce a substantial amount of homogeneous OECs (Reinisch et al., 2009). This method produces two functionally and morphologically different cells subtypes, early EPCs and OECs (Hur et al., 2004). Differentiation of morphological and functional features that define endothelial cells in culture has shown that, like HBMECs, only OECs exhibit cobblestone morphology, bind to specified lectins, endocytose Dil-AcLDL, form well-established tubules on Matrigel, and intracellular tight junctions throughout the entire cell monolayer (Medina et al., 2010b). Accordingly, these results reveal that OECs has most, if not all, the distinctive characteristics necessary to successfully replace dead/dying mature endothelial cells in vasculature to preserve blood vessels functions and tone and show that OECs can effectively enhance angiogenesis when activated by a specific stimulant. In fact, being an essential component of neural stem cell niche, endothelial cells have been proven to induce endogenous neurogenesis (Shen et al., 2004, Ohab et al., 2006). In addition, OECs and different trophic factors secreted by them shown to participate in

neurogenesis following stroke in particular migration and proliferations of neuroblast appear to play essential role in the recovery of cellular functions of the nerves in the penumbra (Zhao et al., 2013). These beside a steady cellular phenotype and determinate growth potential coupled with a substantially diminished risk of tumorigenicity together with their compliance to genetic moderation and simplicity of isolation from human peripheral blood for therapy put forward OECs as effective cellular therapeutics (Medina et al., 2010a, Medina et al., 2013).

Our study has, for the first time, investigated the therapeutic capacity of OECs to repair well-established *in vitro* human BBB model following damage induced by using a wound scratch assay (Allen and Bayraktutan, 2009a, Shao and Bayraktutan, 2013a, Abdulkadir et al., 2020b). In these settings, the first wound scratch assays carried out on endothelial cell monolayers revealed that both the HBMECs and OECs have the capacity to proliferate and migrate in directional manner where the speed of healing was significantly faster with OECs. Succeeding investigations exploring the reparative capacity of OECs on the endothelial layer of the *in vitro* human BBB model proven that exogenous OECs therapy fully restored BBB integrity and function as evidenced by considerable improvements in TEER and paracellular flux of NaF readings, respectively. Following endothelial injury, restoration of BBB integrity and function is likely to be due to both incorporation of OECs to the site of damage as well as to proliferation and lateral migration of resident endothelial cells. OECs are also thought to participate in this reparative process by secreting trophic substances such as VEGF. Exposing HBMECs or OECs to OGD

induced formation of stress fibers in both cells but significantly more so in HBMECs. During OGD+R, the number of stress fibers in OECs was significantly lower than those in HBMECs. Upon exposure to ischaemic insult, G-actin transforms into filamentous actin (F-actin). The change from G-actin to F-actin leads to formation of contractile actin bundles also called as stress fibers. These fibers attach to adherence junctions of adjacent endothelial cells. When stress fibers start to contract under stress, intracellular gaps between neighboring endothelial cells form and therefore BBB permeability increase. While other study showed that the effect of OGD+R on BBB model established by HBMECs or OECs was similar (Abdulkadir et al., 2020b), this study reveals that BBB model formed in part by OECs has restored its integrity and function during reperfusion. This could be due to their capacity to proliferate faster and resist oxidative stress environment by secreting more antioxidants than HBMECs (Alwjaj et al., 2021, Dernbach et al., 2004b). Indeed, removing the serum from the culture media used for these studies proves that the shown healing process can only be related to the capacity of OECs to effectively home to site of injury and differentiate to endothelial cells rather than HBMECs proliferation. Again, formation of similarly tight and functional *in vitro* models of human BBB with OECs and HBMECs, in this study, additionally proves the capacity of OECs to establish functional tight junctions and differentiate into mature endothelial cells. Indeed, taken into the account that effective and rapid restoration of BBB integrity and function is of paramount importance in the prevention of brain oedema, the major cause of death within the first few days of ischaemic stroke, our findings are of certain significance (Qureshi et al., 2003, Dostovic et al., 2016). Moreover, the alleviation of endothelial dysfunction by therapeutic

administration of OECs through replacement of dying or dead HBMECs cells might be also playing a role in inhibiting cerebral microvascular atherosclerosis and neurovascular unit anatomical or physiological dysfunctions. Considering this assumption, inhibiting the endothelial dysfunction by exogenous administration of OECs has been proven to decrease infarct volume, neointima formation, and neurological deficits in translational studies of models exposed to ischaemic stroke (Ohta et al., 2006, Liu et al., 2011). However, others propose that the reparative capacity of OECs in *in vivo* studies might be due to their ability to activate resident endothelial cells by releasing various trophic substances and growth factors such as exosomes and VEGF (Rehman et al., 2003, Li et al., 2016b, Alwjaj et al., 2021).

Following a particular number of replication in culture OECs lose their functional capacities and begin to show certain signs of cellular senescence, particularly telomere shortening, substantial transcriptome alterations, less telomerase activity, and DNA damage (Medina et al., 2013). While senescence is the most useful test for tendency to generate tumour cells in *in vitro* studies, it is feasible to presume that therapeutic administration of OECs in clinical practices would not cause such complication. Regardless, identification of molecular processes related to senescence of OECs is of paramount importance to generate adequate numbers of distinctly functional OECs *in vitro* and to control the degree and extent of activity *in vivo*. Between different elements nominated, the inflammatory chemokines and cytokines, namely IL-6, IL-8, IL-1B, and IL-1 $\alpha$  shown to play a major role in OEC senescence (Xu et al., 2000, Hayashi et al., 2008, Medina et al., 2013). However, their separate or joint purpose to OECs'

therapeutic capacity in ischemic stroke settings needed to be investigated. In addition to matters related to safety, survival, homing, tracking, time window and effective dose of OECs also required to be studied. It is anticipated that, as shown with mesenchymal stem cells, the number of OECs may regulate their immunological condition, immune-evading vs immune-privileged (Ankrum et al., 2014).

### **3.1.10 Summary**

In conclusion, this study reveals that in *in vitro* settings, OECs can effectively repair and restore BBB function following damage, supporting the notion that treatment with these oligopotent cells may be a feasible therapeutic approach to heal the damage of neurovascular unit after an ischaemic stroke.

## **4 ROLE OF GP91DS-TAT ON CEREBRAL BARRIER DAMAGE: THE IMPACT ON OUTGROWTH ENDOTHELIAL CELLS**

#### 4.1.1 Introduction and aims

In the previous chapter we have assessed the reparative effect of OECs on endothelial layer of a well-established *in vitro* model of human BBB, composed of HBMECs, HAs, and HPs. We have shown for the first time that exogenous OECs can restore integrity and function of BBB by physically incorporating into the site of endothelial injury. In addition, this study has also revealed that, like the HBMECs, when co-cultured with HAs and HPs, the OECs can also form a functional BBB (Abdulkadir et al., 2020b, Alwjwaj et al., 2021). Since endothelial cells constitute the major cellular element of human BBB, any pathology capable of altering their phenotype or viability can also affect the function and integrity of this distinctive barrier (Heo et al., 2005, Rosenberg, 1999, Kahle et al., 2009).

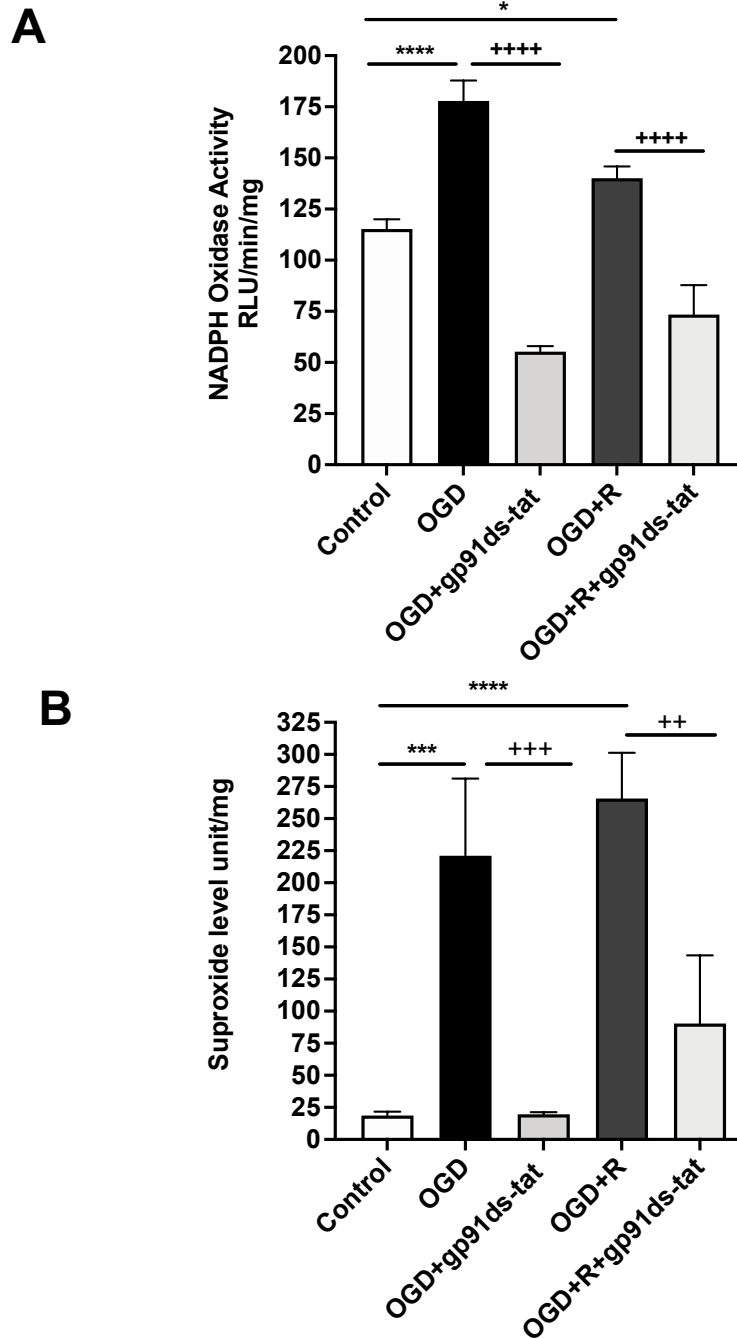
Oxidative stress, associated with overproduction of ROS such as  $H_2O_2$  and  $O_2^{\bullet}$ , is considered as one of the main mechanisms that adversely affect cerebral barrier after an ischaemic stroke (Allen and Bayraktutan, 2009b). ROS not only influence the intracellular redox environment but also play a major role in the regulation of cellular proliferation, differentiation, self-renewal, senescence, and apoptosis (Case et al., 2008, Adler et al., 1999, Yoshida et al., 2003). NADPH oxidase constitutes the major enzymatic source of ROS in vasculature, with many studies reporting that NOX2 (previously known as gp91<sup>phox</sup>) is the main element required for ROS generation (Case et al., 2008, Cifuentes-Pagano et al., 2012, Görlach et al., 2000, Fürst et al., 2005). Compared to mature endothelial cells, OECs shown to express higher levels of antioxidant enzymes such as catalase, Mn-SOD, and glutathione peroxidase (Dernbach et al., 2004a). This naturally potentiate their vasoreparative and angiogenic capacity



in ischaemic (micro)environments, suggesting that OECs may be efficacious therapeutics following ischaemic stroke. Using a triple culture model of human BBB, composed of HAs and HPs with HBMECs, the study of this chapter has explored whether inhibition of oxidative stress by specifically targeting NOX2, a member of NADPH oxidases family, can preserve the barrier integrity and function in experimental settings of ischaemic injury. This study also assessed whether such suppression of NADPH oxidase activity in OECs, that possess inherently greater antioxidant capacity, regulate barrier characteristics in a different manner. To assess the precise impact of NADPH oxidase-mediated oxidative stress on barrier-forming capacity of OECs, these cells were used on their own rather than in mixture with HBMECs.

#### **4.1.2 The effect of NOX2 inhibitor on HBMECs**

To check the effectiveness of gp91ds-tat on NADPH oxidase, HBMECs were cultured to about 80% confluence before exposing to OGD for 4 hours in the absence or presence of gp91ds-tat (50  $\mu$ M) or OGD+R in the absence or presence of gp91ds-tat which was given at the beginning of the reperfusion. Then, NADPH oxidase activity and  $O_2^{\bullet-}$  production were measured as described using the specific assays detailed in chapter two. As evidenced by significant increases in NADPH oxidase activity and  $O_2^{\bullet-}$  production, OGD induced oxidative stress. The levels of parameters remained significantly elevated following 20 hours of reperfusion compared to controls. Treatments with 50  $\mu$ M of gp91ds-tat significantly decreased the activity of NADPH oxidase activity and  $O_2^{\bullet-}$  release in HBMECs exposed to OGD and OGD+R compared to respective untreated counterparts (Figure 4.1).

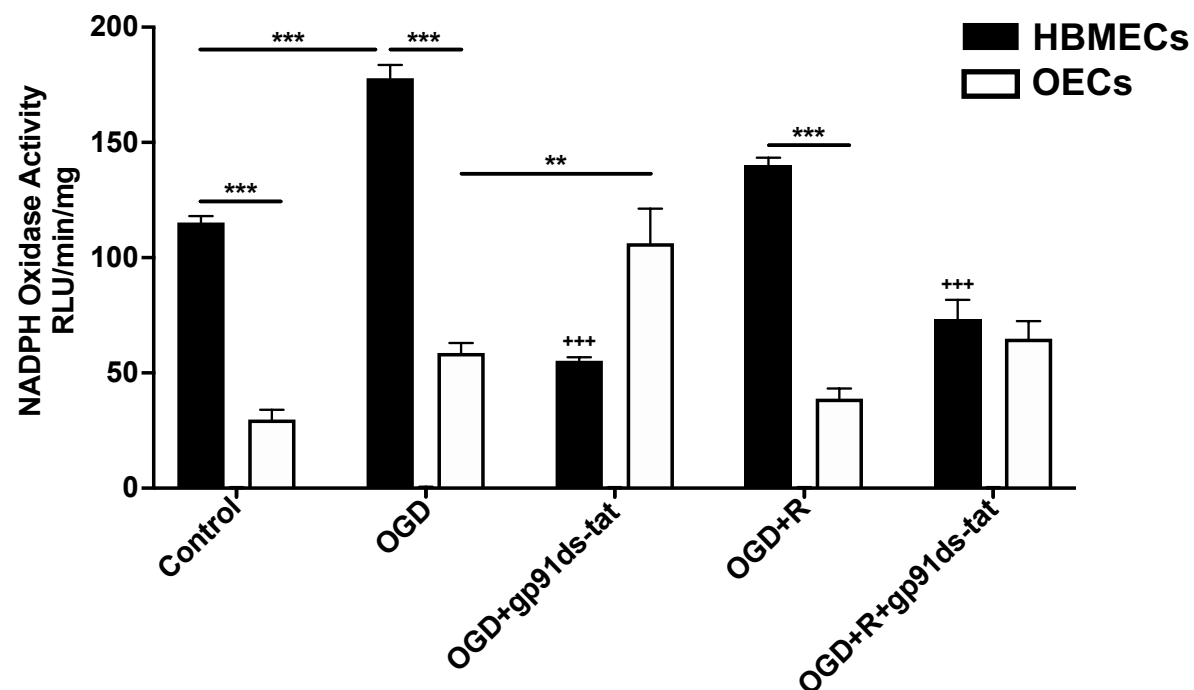


**Figure 4-1 Effect of gp91ds-tat on NADPH oxidase activity and superoxide level.**

HBMECs were exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). The activity of NADPH oxidase and superoxide levels were measured by lucigenin chemiluminescence and cytochrome reduction assays, respectively. Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.0006$ , \*\*\*\* $P < 0.0001$  compared to OGD $\pm$ R. ++ $P < 0.002$ , +++ $P < 0.0007$ , \*\*\*\* $P < 0.0001$  compared to OGD+gp91ds-tat or OGD+R+gp91ds-tat. (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **4.1.3 Effects of gp91ds-tat on NADPH oxidase activity in OECs compared to HBMECs**

HBMECs and OECs were cultured to about 80% confluence. They were then exposed to OGD (4 hours) alone or followed by reperfusion (OGD+R, 20 hours) in the absence or presence of gp91ds-tat (50  $\mu$ M). In OGD+R treatment group, gp91ds-tat was introduced to culture at the beginning of the reperfusion. NADPH oxidase activity was measured using the lucigenin chemiluminescence assay detailed in chapter two. Under normoxic conditions, OECs had significantly lower levels of NADPH oxidase activity compared to HBMECs. While OGD significantly increased NADPH oxidase activity in both HBMECs and OECs, the level of activity in OECs remained well below those seen HBMECs. Reperfusion diminished OGD-mediated increases in NADPH oxidase activity in both cells. Again, the oxidase activity was substantially lower in OECs compared to HBMECs. Interestingly, while treatments with gp91ds-tat substantially diminished the effects of OGD $\pm$ R on NADPH oxidase activity in HBMECs, it increased the enzymatic activity in OECs subjected to OGD and OGD+R, albeit less so in the latter group (Figure 4.2).

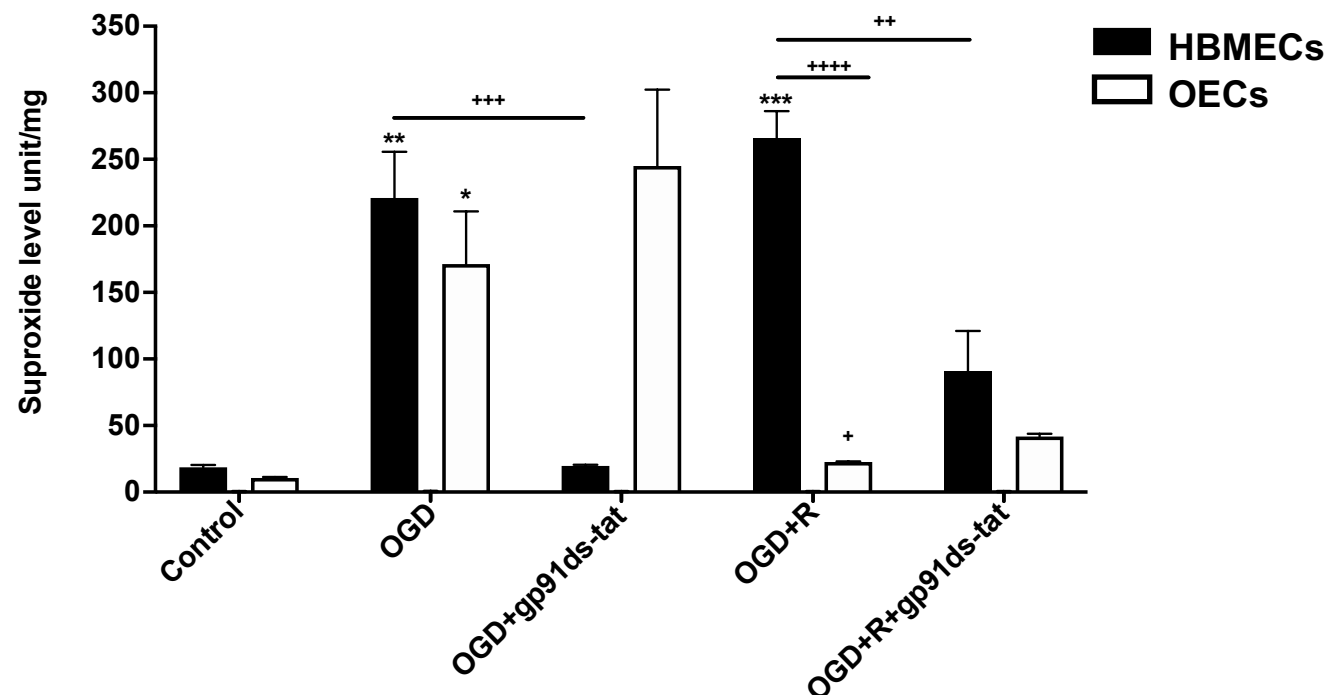


**Figure 4-2 Effect of gp91ds-tat on NADPH oxidase activity on HBMECs vs OECs.**

HBMECs or OECs were exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). The activity of NADPH oxidase was detected by lucigenin chemiluminescence assay. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\* $P < 0.0001$  compared to HBMECs (control or OGD or OGD+R). +++ $P < 0.0001$  compared to HBMECs (OGD or OGD+R). \*\* $P < 0.003$  compared to OECs OGD (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **4.1.4 Effects of gp91ds-tat on superoxide levels in OECs compared to HBMECs**

HBMECs and OECs were cultured to about 80% confluence. They were then exposed to OGD (4 hours) alone or followed by reperfusion (OGD+R, 20 hours) in the absence or presence of gp91ds-tat (50  $\mu$ M). In OGD+R treatment group, gp91ds-tat was introduced to the culture at the beginning of the reperfusion. The levels of  $O_2^{\bullet-}$  production were measured by cytochrome C reduction assay as described in chapter two. Under normoxic conditions, OECs expressed lower levels of  $O_2^{\bullet-}$  compared to HBMECs. Although exposure to OGD significantly increased  $O_2^{\bullet-}$  levels in both cells, the levels remained slightly lower in OECs compared to HBMECs. Reperfusion significantly decreased  $O_2^{\bullet-}$  levels in OECs compared to OGD. Also, during reperfusion, the levels of  $O_2^{\bullet-}$  were substantially lower in OECs compared to HBMECs. While treatments with gp91ds-tat significantly reduced the effects of OGD $\pm$ R on  $O_2^{\bullet-}$  release in HBMECs, it evoked an increase in  $O_2^{\bullet-}$  release in OECs in both settings (Figure 4.3).



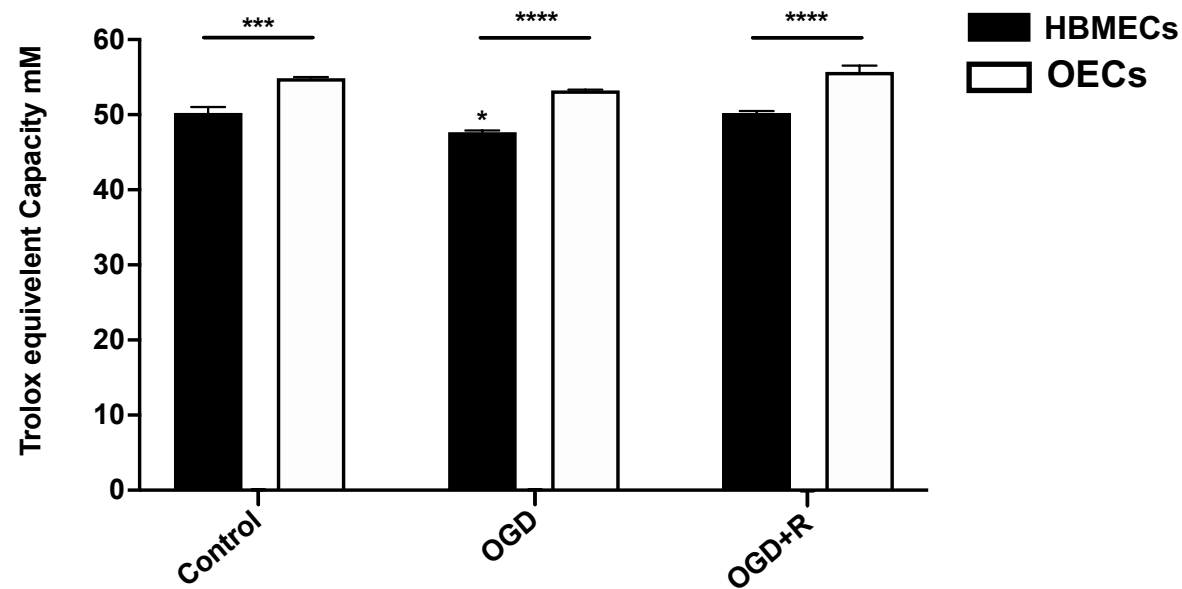
**Figure 4-3 Effect of gp91ds-tat on superoxide release in HBMECs vs OECs.**

HBMECs or OECs were exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cytochrome reduction assay was used to measure total  $O_2^{\cdot -}$  levels. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*P<0.002, \*\*\*P<0.0001 compared to HBMECs control. \*P<0.02 compared to OECs control. +P<0.02 compared to OECs OGD. \*\*\*P<0.002 compared to HBMECs OGD. \*\*P<0.006, +++P<0.0001 compared to HBMECs OGD+R. (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **4.1.5 Effect of oxygen-glucose deprivation on total antioxidant capacity in HBMECs and OECs**

Compared to HBMECs, the OECs possess significantly higher basal levels of total antioxidant capacity. Although, total antioxidant capacity was significantly decreased in HBMECs during OGD, reperfusion following OGD normalised these levels. In contrast, the impact of OGD on total antioxidant capacity of OECs was statistically insignificant and reperfusion following OGD appeared to significantly increase its levels. Exposure to OGD±R shows that total antioxidant capacity remained significantly higher in OECs compared to HBMECs (Figure 4.3). This indicates that OECs may resist oxidative stress and work in ischaemic (micro)environments.





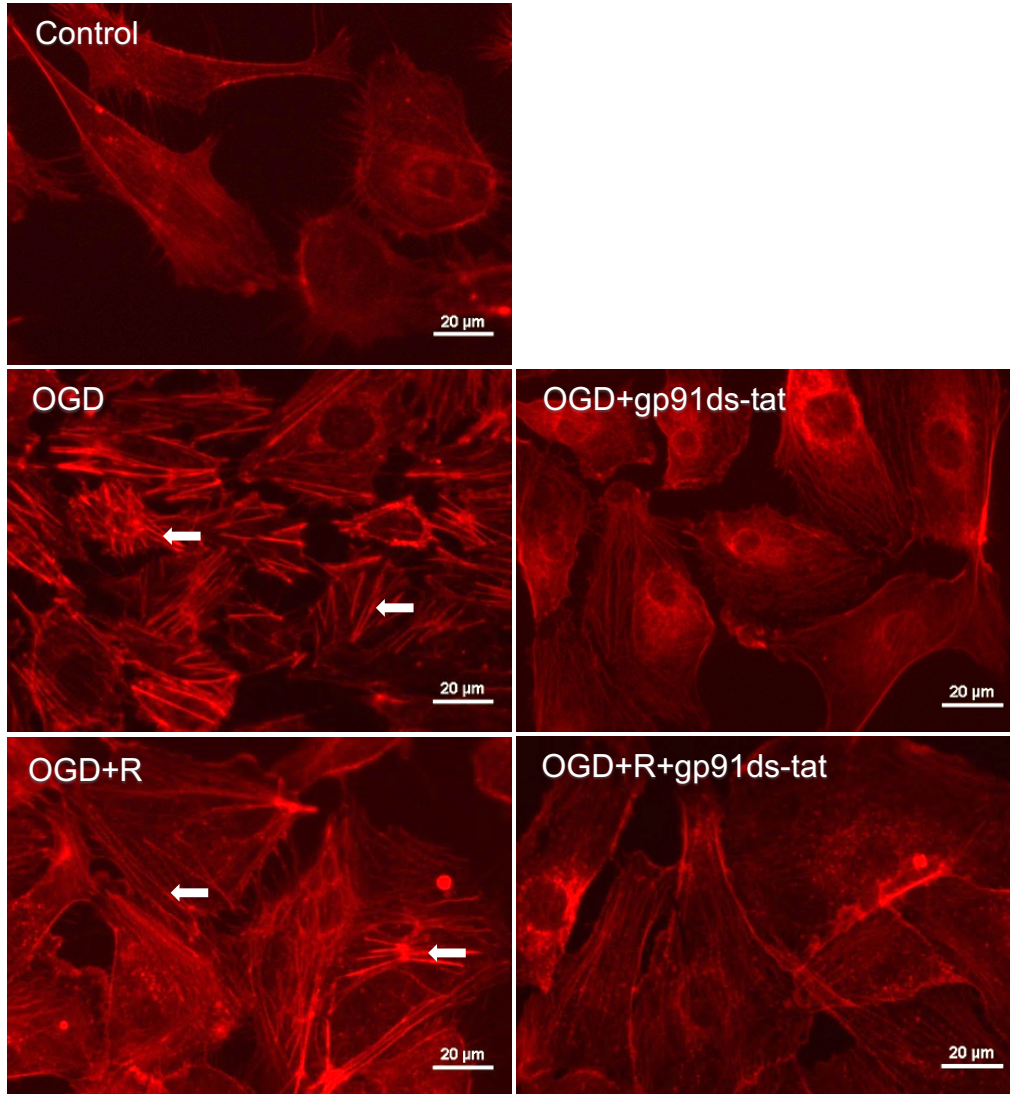
**Figure 4-4 Effect of oxygen-glucose deprivation on total antioxidant capacity in HBMECs and OECs**

Confluent HBMECs or OECs were exposed to 4 h of OGD alone or OGD followed by 20 h of reperfusion (OGD+R). The total antioxidant capacity was measured in both cell types. Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.03$  compared to HBMECs control. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  compared to HBMECs under corresponding condition (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **4.1.6 Effects of gp91ds-tat on OGD-induced stress fiber formation**

Scrutiny of cytoskeletal organisation under normoxic conditions revealed cortical actin staining in HBMECs, OECs, HAs, and HPs. However, exposure of HBMECs and OECs to OGD or OGD+R promoted the appearance of thick actin stress fibres in both HBMECs and OECs which were more prominent in HBMECs. Compared to HBMECs, the number of stress fibres appeared to be significantly lower in OECs subjected to OGD±R. While treatment with gp91ds-tat decreased the numbers of stress fibres in HBMECs, it increased the number of stress fibres in OECs during OGD±R (Figures 4.5, 4.6, 4.7). While exposure of HAs and HPs to OGD or OGD+R significantly increased the numbers of stress fibres formation, selective inhibition of NADPH oxidase, by gp91ds-tat, significantly diminished the numbers of these fibres and in both cells under these conditions (Figures 4.8, 4.9, 4.10, 4.11)

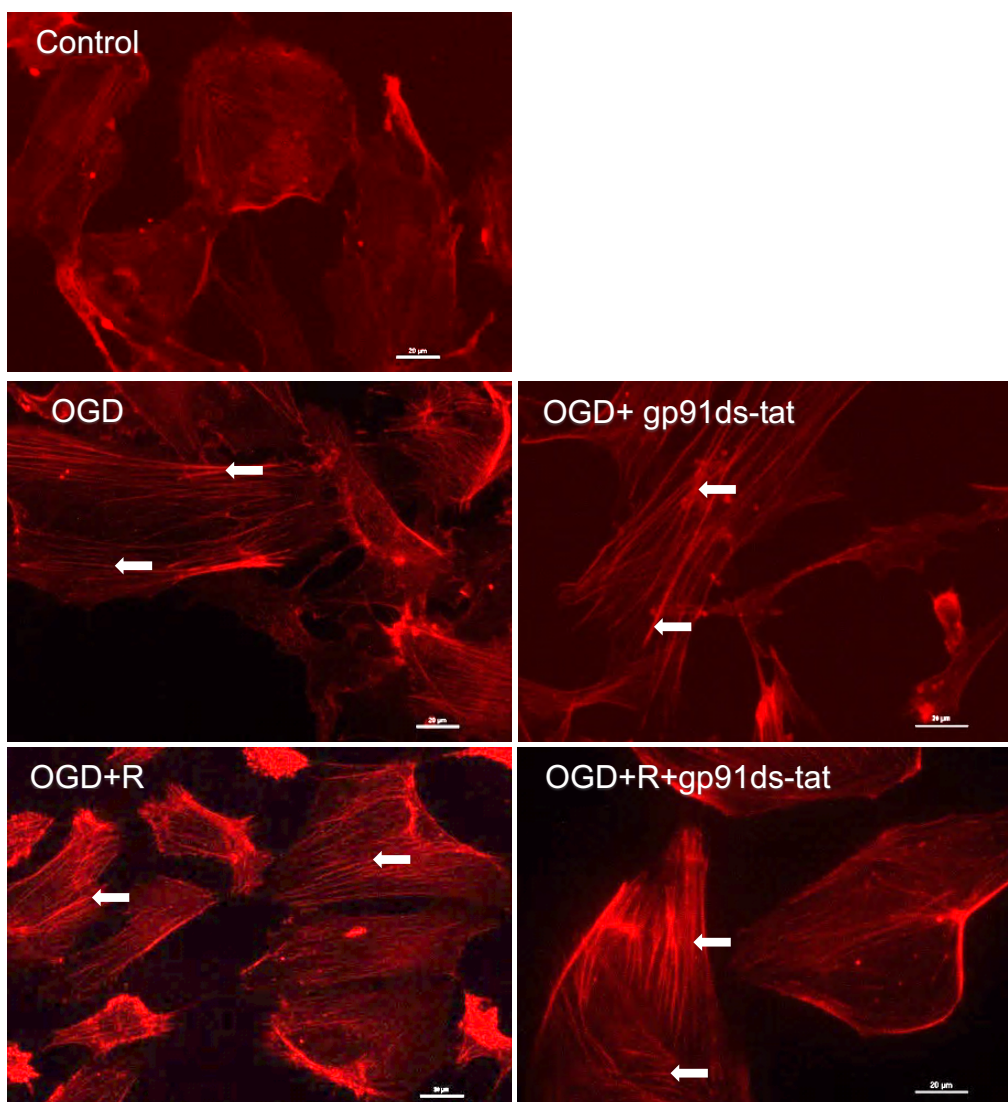
### **HBMECs**



**Figure 4-5 F-actin staining in HBMECs.**

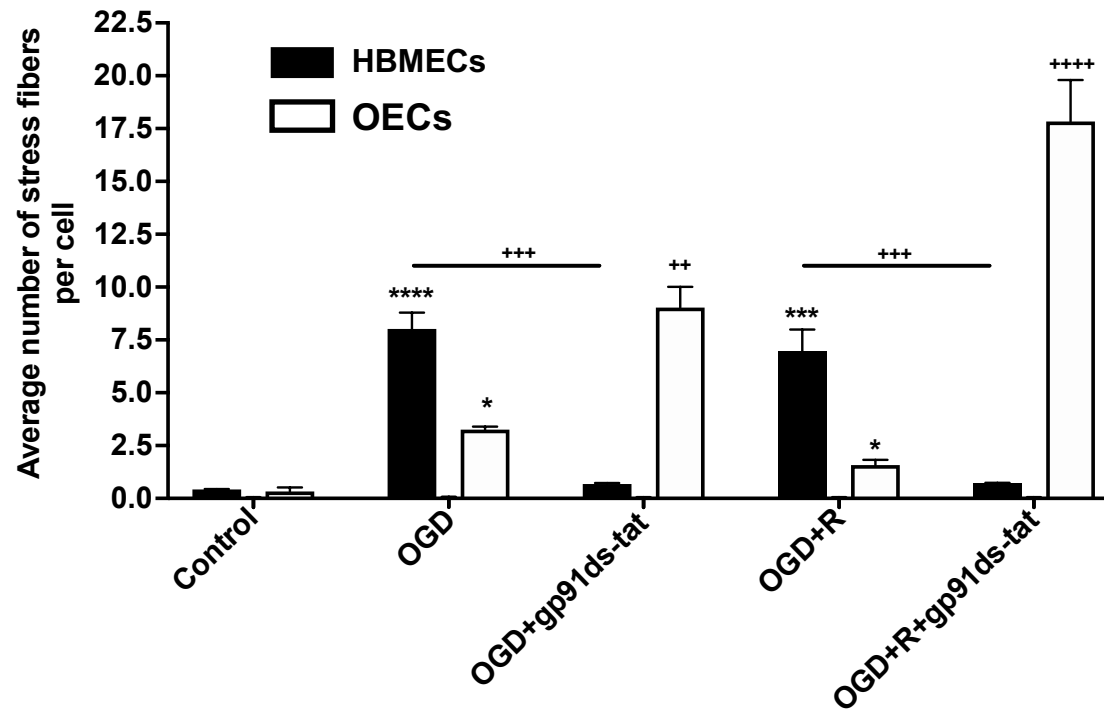
HBMECs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows. Scale bars = 20μm.

## OECs



**Figure 4-6 F-actin staining in OECs.**

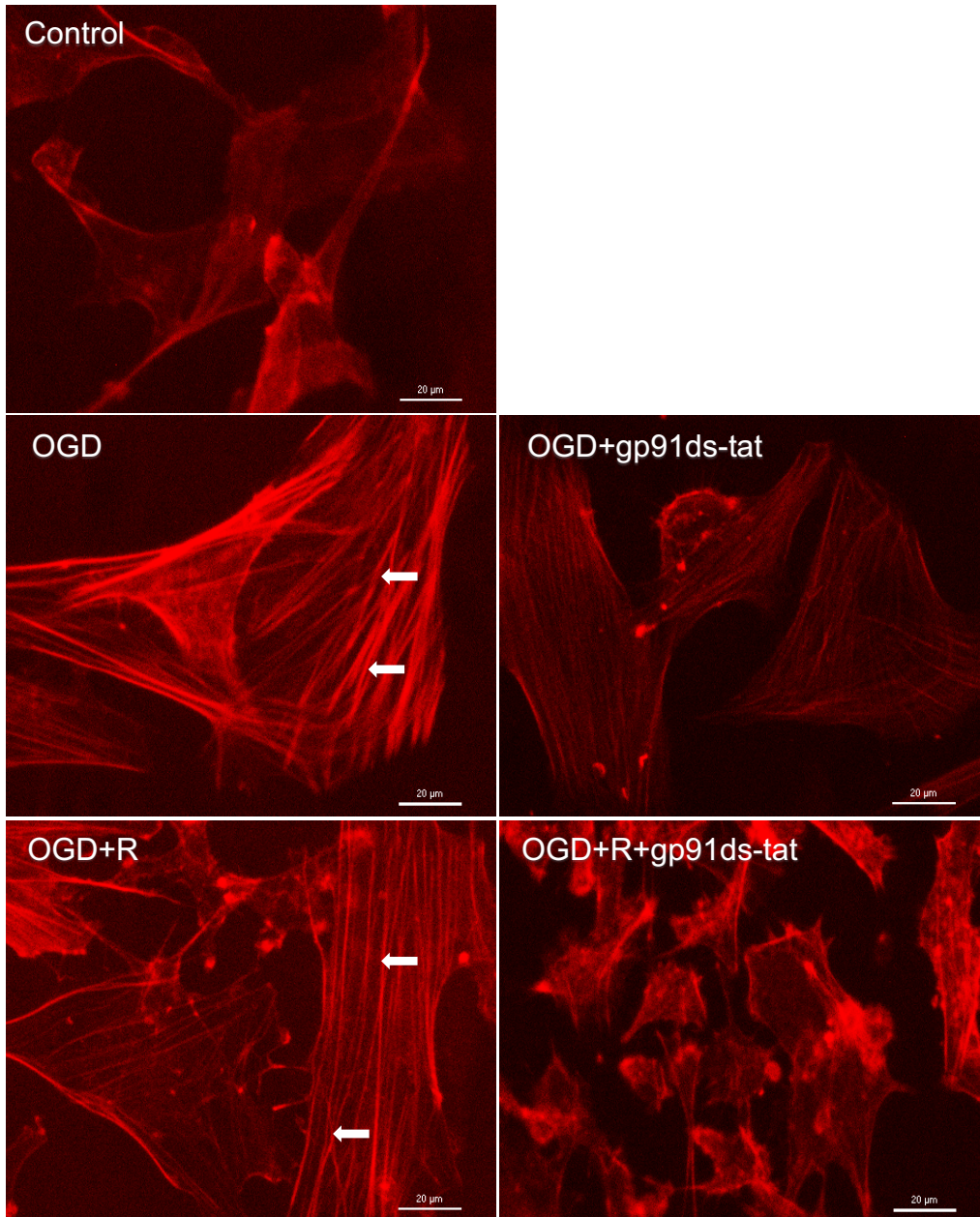
OECs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows. Scale bars = 20μm.



**Figure 4-7 The effect of OGD±R on the number of stress fibres in HBMECs compared to OECs**

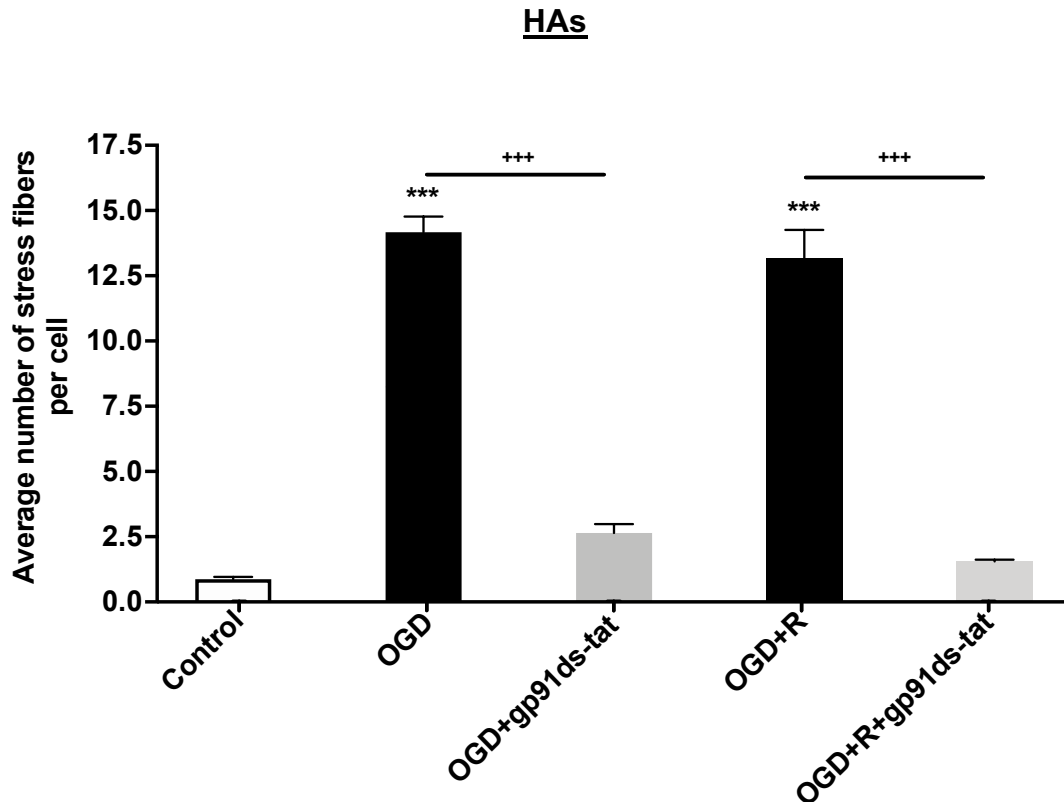
HBMECs or OECs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001, \*\*\*P<0.0001 compared to HBMECs control. \*P<0.05 compared HBMECs subjected to OGD±R. \*\*P<0.01 compared to OECs subjected to OGD. \*\*\*P<0.01 compared to HBMECs subjected to OGD±R. \*\*\*\*P<0.01 compared to OECs subjected to OGD+R (one-way analysis of variance followed by Tukey's post hoc analysis).

## HAs



**Figure 4-8 F-actin staining in HAs.**

HAs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows. Scale bars = 20μm.

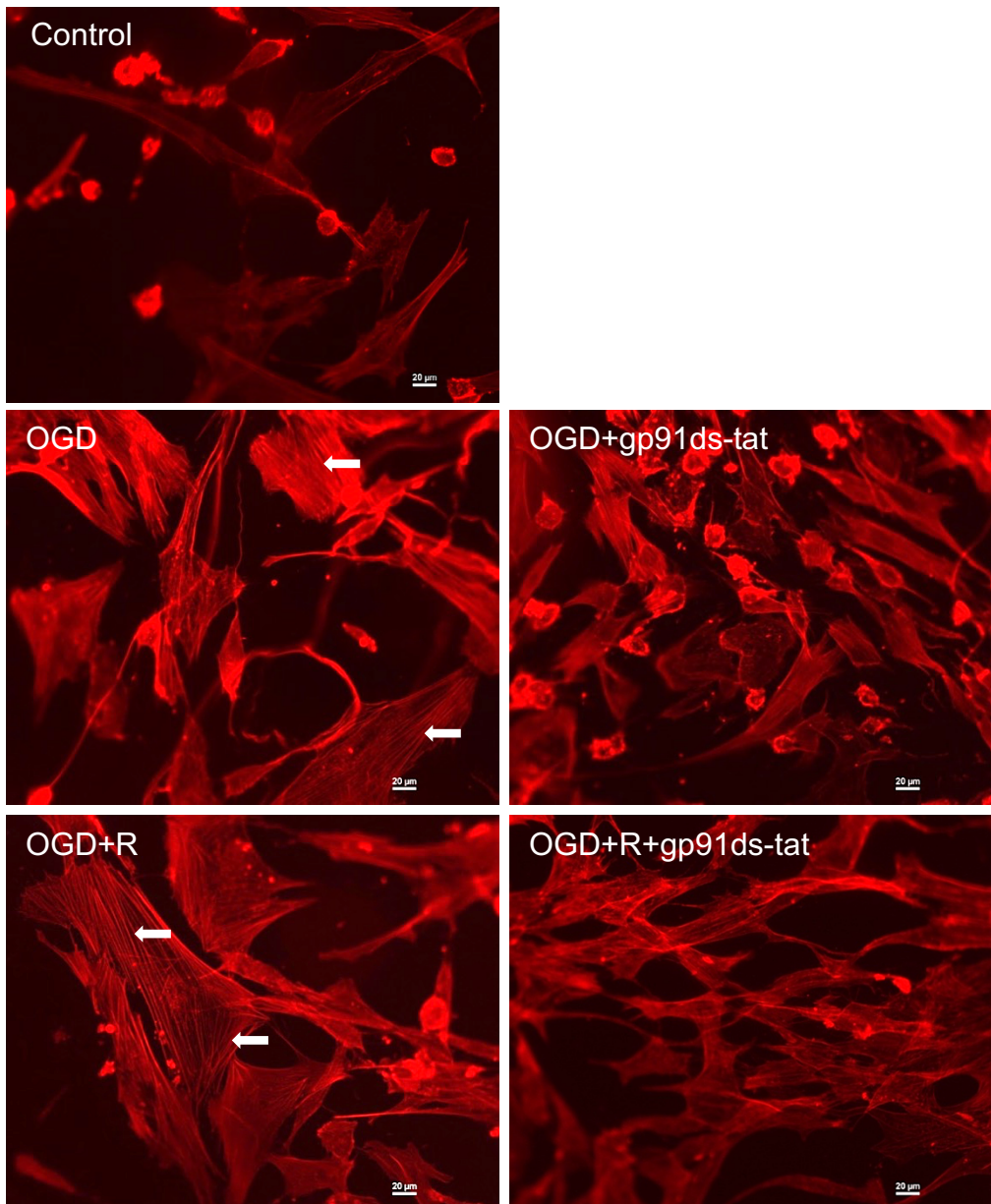


**Figure 4-9 The effect of OGD±R on the number of stress fibres in HAs.**

HAs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*P<0001 compared to control. \*\*\*P<0.05 compared HAs subjected to OGD±R. (one-way analysis of variance followed by Tukey's post hoc analysis).



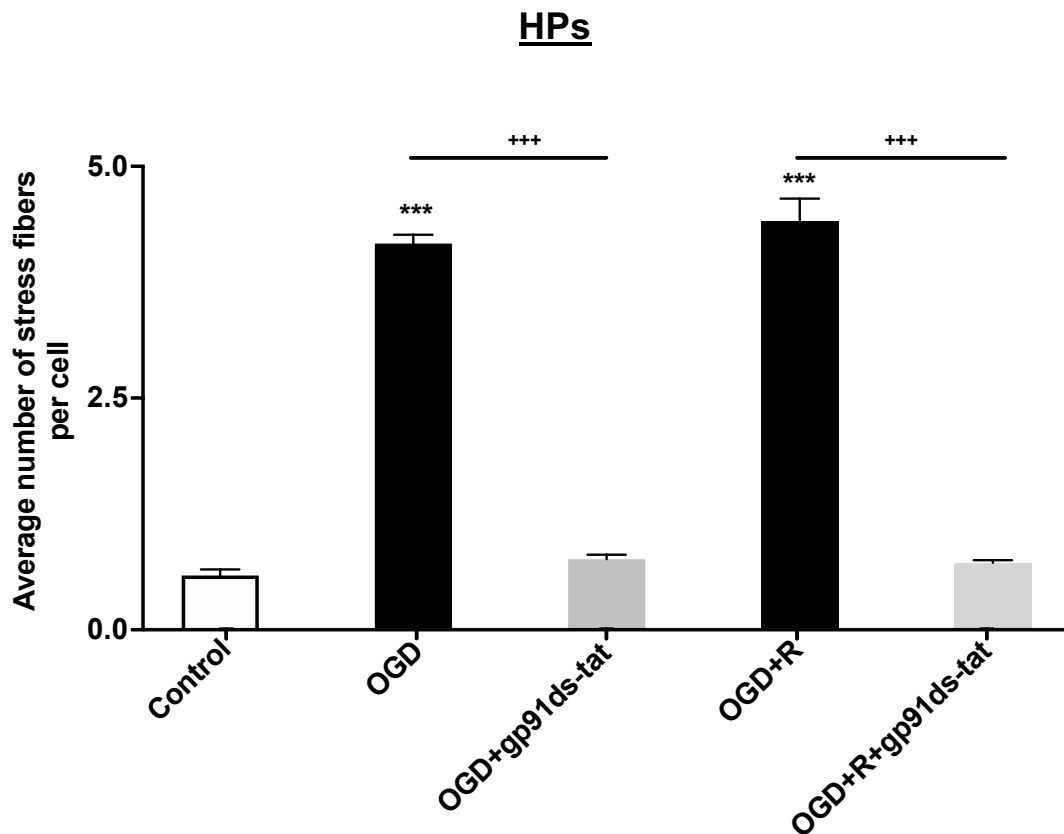
## HPs



**Figure 4-10 F-actin staining in HPs.**

HPs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows. Scale bars = 20μm.



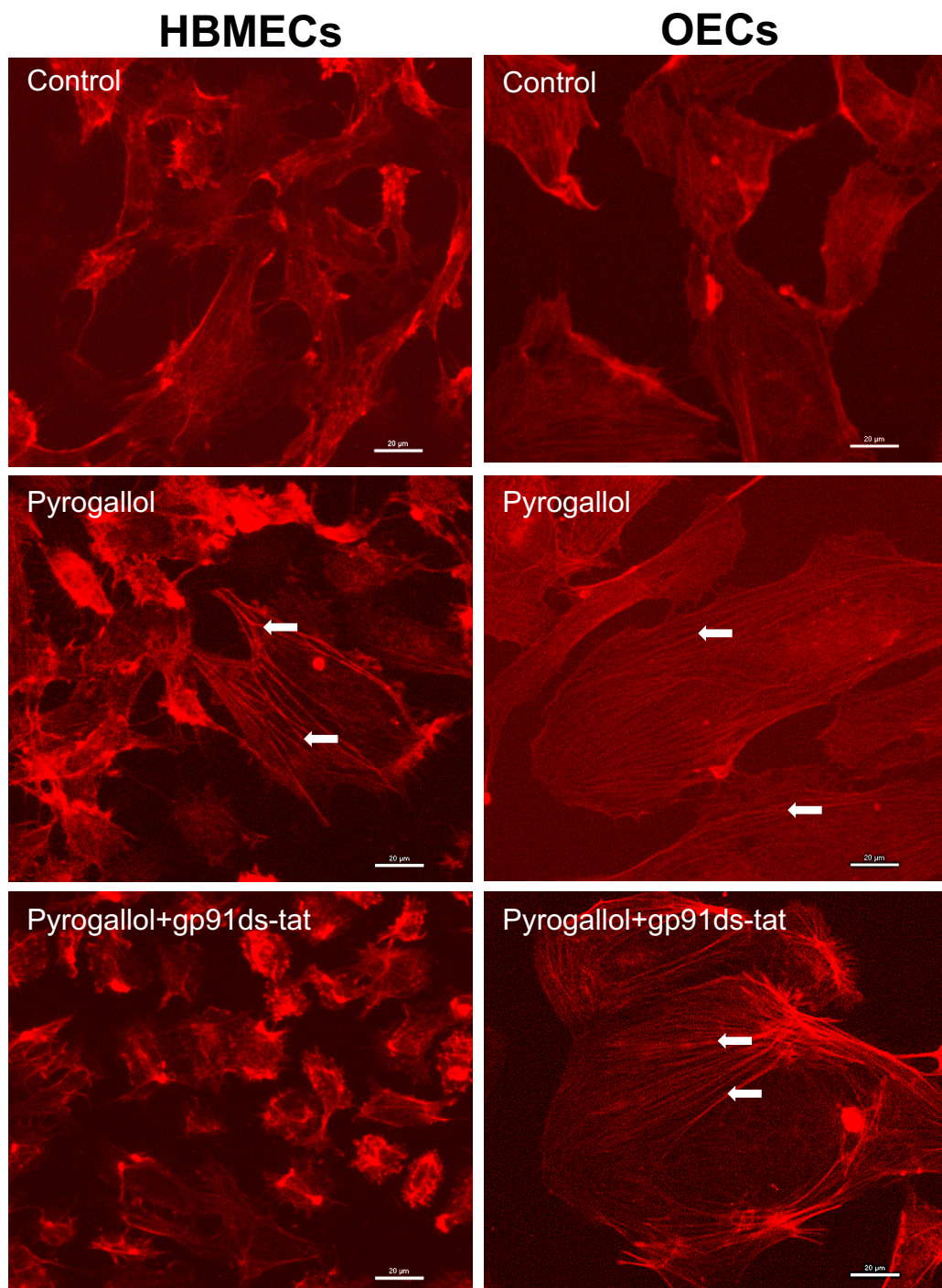


**Figure 4-11 The effect of OGD±R on the number of stress fibres in HPs.**

HPs were cultured to about 80% confluence on coverslips. Then, exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat (given at the beginning of reperfusion). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*P<0001 compared to control. \*\*\*P<0.05 compared HPs subjected to OGD±R. (one-way analysis of variance followed by Tukey's post hoc analysis).

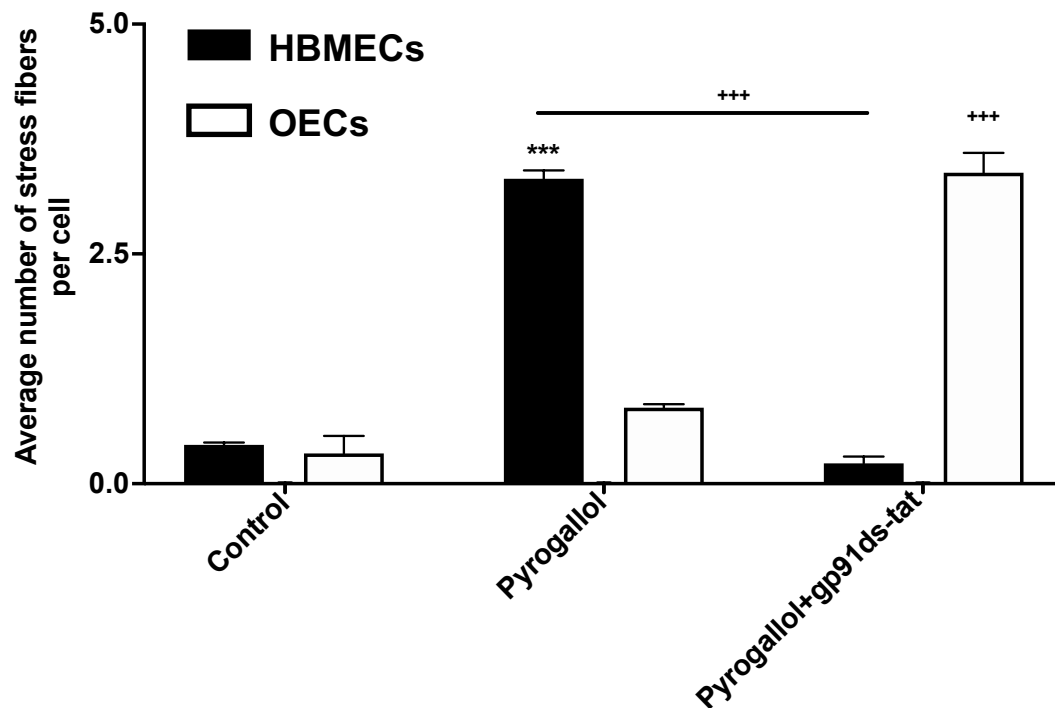
#### **4.1.7 Effects of pyrogallol on stress fibers formation in HBMECs and OECs.**

HBMECs or OECs were exposed to pyrogallol, an  $O_2^{\bullet-}$  generator, in the absence or presence of gp91ds-tat (50  $\mu$ M) for 20 hours. The architecture of stress fibres of both cells was studied as described in chapter two. Pyrogallol induced stress fibres formation in both HBMECs and OECs which were more prominent in HBMECs. This was evidenced by significant increases in the numbers of stress fibres in HBMECs compared to OECs. However, co-treatments with gp91ds-tat, an NOX2 inhibitor, significantly decreased the numbers of stress fibres in HBMECs while significantly increasing their formation in OECs (Figures 4.12 and 4.13).



**Figure 4-12 F-actin staining in HBMECs and OECs after exposure to pyrogallol.**

HBMECs or OECs were cultured to about 80% confluence on coverslips. Then, exposed to pyrogallol in the absence or presence of gp91ds-tat (50 μM) for 20 hours. Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows. Scale bars = 20μm.

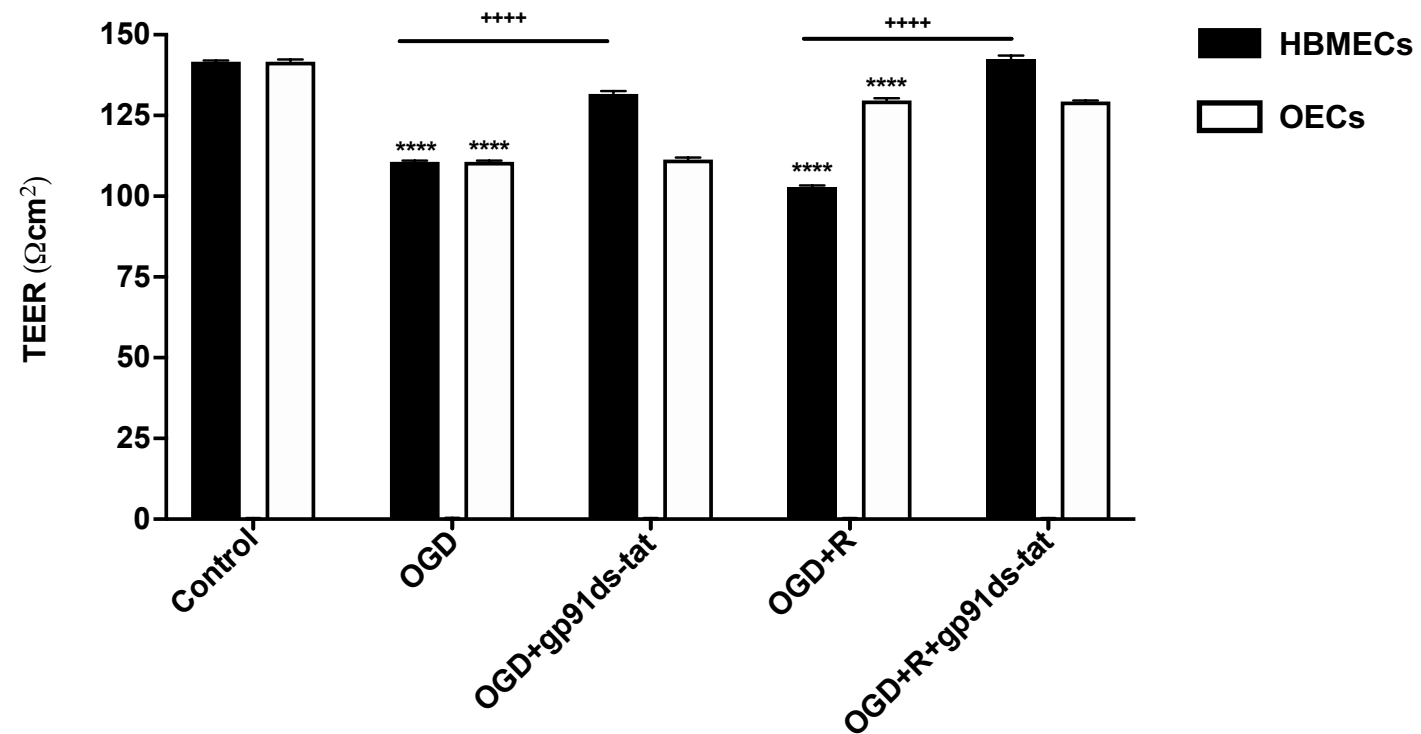


**Figure 4-13 The effect of pyrogallol on the number of stress fibres in HBMECs and OECs.**

HBMECs or OECs were cultured to about 80% confluence on coverslips. Then, exposed to pyrogallol in the absence or presence of gp91ds-tat (50  $\mu$ M) for 20 hours. Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*P<0.0001 compared to HBMECs control. \*\*\*P<0.0001 compared HBMECs or OECs subjected to pyrogallol. (one-way analysis of variance followed by Tukey's post hoc analysis).

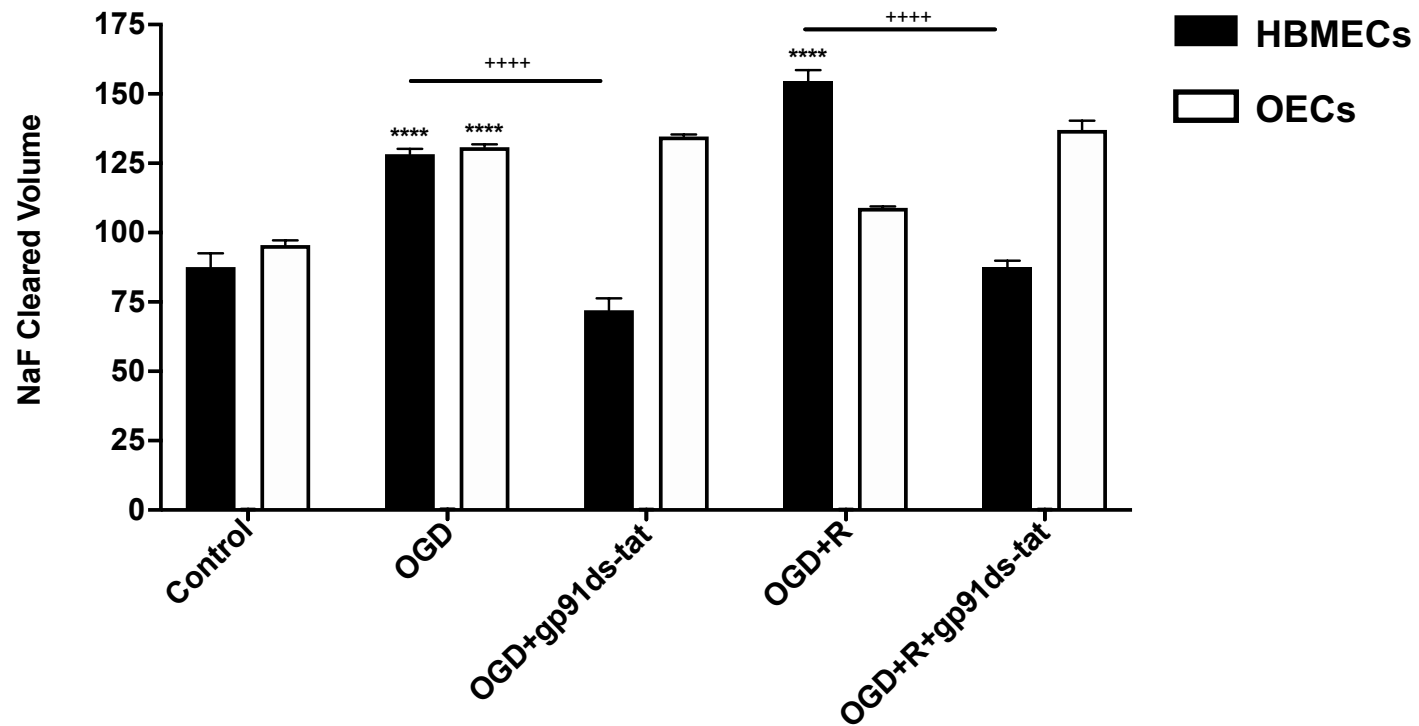
#### **4.1.8 Effects of gp91ds-tat on OGD-evoked BBB damage – model set up using HBMEC and OEC co-cultures**

Similar to the triple cell culture model of human BBB consisting of HBMECs, HAs and HPs, another model set up by co-culture of OECs with HAs and HPs also led to formation of an equally tight and functional *in vitro* model of human BBB, indicating establishment of tight junction complexes between adjacent OECs. Exposure to OGD significantly impaired the BBB integrity and function in both models as evidenced by decreases in TEER and increases in paracellular flux of NaF. Despite significantly improving barrier integrity and function, restoration of normal oxygen and glucose levels (reperfusion for 20 hours) failed to normalise these parameters in either model. Inhibition of NADPH oxidase by gp91ds-tat further improved the integrity and function of BBB model established with HBMECs in both OGD and OGD+R conditions as evidenced by significant improvements in TEER and NaF flux readings compared OGD±R alone groups. However, gp91ds-tat did not improve these readings in the BBB model established with OECs (Figures 4.14 and 4.15).



**Figure 4-14 Effect of NOX2 inhibitor on BBB integrity subjected to OGD damage**

BBB models were established by co-culture of HAs and HPs with HBMECs or OECs before exposure to OGD (4 h) alone or followed by reperfusion (20 h) in the absence or presence of gp91ds-tat which were given at the beginning of reperfusion in OGD+R studies. BBB integrity was assessed by measurement of transendothelial electrical resistance (TEER). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to control. \*\*\*\*P<0.0001 compared HBMECs subjected to OGD $\pm$ R (one-way analysis of variance followed by Tukey's post hoc analysis).



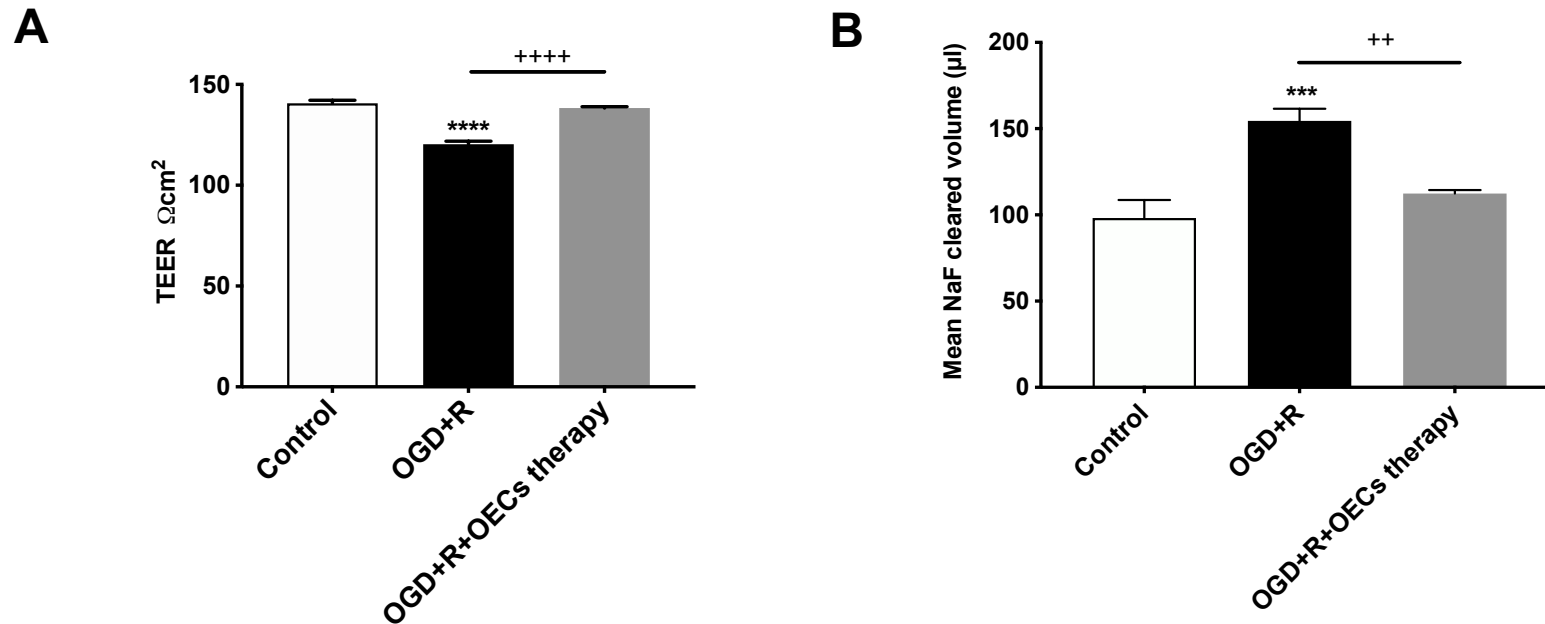
**Figure 4-15 Effect of NOX2 inhibitor on BBB function subjected to OGD damage**

BBB models were established by co-culture of HAs and HPs with HBMECs or OECs before exposure to OGD (4 h) alone or followed by reperfusion (20 h) in the absence or presence of gp91ds-tat which were given at the beginning of reperfusion in OGD+R studies. BBB function was assessed by measurements of paracellular flux of sodium fluorescein (NaF). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to control. ++++P<0.0001 compared HBMECs subjected to OGD±R (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **4.1.9 OECs restore BBB integrity and function following OGD**

In the previous chapter, we had shown that exogenously added OECs can repair wound scratch-induced damage to endothelial monolayers and endothelial layer of a triple culture model of BBB composed of HAs, HPs and HBMECs. The present study shows that OECs added to the abovementioned triple culture model during OGD+R injury also neutralises the deleterious effect of reperfusion injury on barrier integrity and function as ascertained by significant improvements in TEER and NaF paracellular flux readings (figure 4.15).





**Figure 4-16 Effects OECs therapy on ischaemic injury-evoked BBB damage.**

A triple culture model of human BBB composed of HAs, HPs and HBMECs, were exposed to 4 h of OGD followed by 20 h of reperfusion (OGD+R) with and without exogenously added OECs (10,000 cells). BBB integrity and function were assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF, B), respectively. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\* $P < 0.0001$  and \*\*\* $P < 0.0002$  compared to control. +++++ $P < 0.0001$  and ++ $P < 0.001$  compared to OGD+R (one-way analysis of variance followed by Tukey's post hoc analysis).

#### 4.1.10 Discussion

Oxidative stress, characterised by excessive availability of ROS, represents one of the key mechanisms involved in the pathophysiology of BBB damage following an ischaemic stroke (Fann et al., 2013, Doyle et al., 2008, del Zoppo and Hallenbeck, 2000, Allan and Rothwell, 2001, Prakash and Carmichael, 2015, Kadir et al., 2021b, Kadir et al., 2020). As the breakdown of BBB and ensuing formation of cerebral oedema constitute the main cause of death within the first week after an ischaemic stroke (Bayraktutan, 2019b), targeting specific mechanisms or elements implicated in the pathogenesis or exacerbation of oxidative stress may be of pivotal importance to reduce mortality as well as the risk and severity of post-ischaemic cerebral events. Given that endothelial cell dysfunction and opening of the inter-endothelial junctional complexes account for much of the early BBB damage, restoration and preservation of vascular endothelium are thought to be of significant therapeutic potential. Re-endothelialisation of cerebral barrier either by proliferation and lateral migration of resident endothelial cells or by incorporation of circulating progenitor cells (OECs) into endothelial layer play a pivotal role in this context. By releasing trophic factors and differentiating into mature endothelial cells, OECs induce angiogenesis following ischaemic damage and help restore normal endothelial function (Alwjaj et al., 2021). In this regard, our recent study has demonstrated that OECs can readily home into the site of endothelial injury and restore *in vitro* BBB integrity and function. Indeed, in accordance with our previous studies, the present study also shows that, similar to mature endothelial cells like HBMECs, OECs also display cobblestone morphology, bind to specific lectins, endocytose

Dil-AcLDL and form tube like structures on Matrigel (Abdulkadir et al., 2020b, Medina et al., 2010b).

Through employment of an *in vitro* model of human BBB in previous studies, we have successfully identified various signalling pathways involved in ischaemic injury-mediated breakdown of the BBB such as protein kinase C- $\beta$  and NADPH oxidase (Abdulkadir et al., 2020b, Abdullah and Bayraktutan, 2014, Srivastava et al., 2013b, Kadir et al., 2020). As continuation of these studies, the current study has explored whether OECs function differently in experimental settings of ischaemia-reperfusion injury, mimicked by oxygen-glucose deprivation alone or followed by reperfusion (OGD $\pm$ R) and effectively restore BBB integrity and function. In addition, this study has explored whether targeting NOX2 activity, a key member of NADPH oxidase family which mainly responsible for vascular oxidative stress, may augment their barrier-forming capacity. In these studies, OECs, rather than OEC-HBMECs mixture, were used to establish endothelial monolayer to scrutinise the precise impact of NADPH oxidase-derived oxidative stress on barrier-forming characteristics of OECs. In this context, the current study has shown that, like HBMECs, OECs can form an equally functional *in vitro* model of human BBB when co-cultured with human astrocytes and pericytes. The study has also shown that exposure to OGD (4 hours of OGD alone) significantly compromises the integrity and function of BBB established with either OECs or HBMECs (Abdulkadir et al., 2020b). While OGD+R (20 hours of reperfusion following OGD) has aggravated OGD-induced damage of BBB established with HBMECs, the BBB models established with OECs have shown a significant improvement in integrity and

function following reperfusion. Intriguingly, while suppression of oxidative stress by an NOX2 inhibitor, i.e., gp91ds-tat (50  $\mu$ M), attenuated the deleterious effects of OGD $\pm$ R on BBB established with HBMECs, it augmented the deleterious effects of both phenomena; OGD and OGD+R on BBB established with OECs. These results imply that a therapeutic approach targeting NOX2 while employing OECs as regenerative or restorative compounds may be counterproductive rather than vascular protective. Studies demonstrating that NOX2 is required for the regulation of neovascularisation, VEGF and SDF-1 $\alpha$ -mediated angiogenesis, migratory and vasculoreparative functions of OECs add further weight to this hypothesis (Case et al., 2008, Al-Shabrawey et al., 2005, Tojo et al., 2005, Schroder et al., 2009). Furthermore, ROS released by NADPH oxidase have been shown to be a prerequisite for lineage commitment of EPCs (Hahner et al., 2020). However, reports showing that inhibition of NADPH oxidase can negate hypoxia-induced EPCs dysfunction also exist (Liu et al., 2017). The present study shows that compared to mature endothelial cells, OECs possess significantly lower basal levels of O<sub>2</sub><sup>-</sup> and NADPH oxidase activity and higher total antioxidant capacity under normal conditions. In line with these findings, OECs have previously been shown to possess significantly lower basal levels of ROS and higher levels of antioxidant manganese superoxide dismutase (He et al., 2004), glutathione peroxidase, and catalase activities compared to endothelial cells (Dernbach et al., 2004a, Sattler et al., 1999). In addition, the current study shows that, compared to mature endothelial cells, OECs also express significantly higher total antioxidant capacity and lower NADPH oxidase activity and O<sub>2</sub><sup>-</sup> production during ischaemic insult. These suggest that OECs can handle oxidative stress better in ischaemic

settings than mature endothelial cells, a prerequisite for their endothelium-reparative and cerebral barrier-restorative function. However, suppression of NOX2 in OECs by gp91ds-tat exacerbated the detrimental effect of OGD±R on integrity and function of the BBB where sharp increases in NADPH oxidase activity and  $O_2^{\cdot-}$  availability appeared to play a crucial role. This might happen due to several reasons. Firstly, under normal and ischaemic conditions OECs express low levels of  $O_2^{\cdot-}$  compared to HBMECs, so it is possible that inhibition of NADPH oxidase may cause complete neutralisation of this pro-oxidant enzyme activity and ensuing  $O_2^{\cdot-}$  generation and as a result trigger a feedback mechanism to compensate these changes in OECs. Secondly, NADPH oxidase constitute the main source of reactive oxygen species and associated oxidative stress in HBMECs (Chen et al., 2011b, Shirley et al., 2014), different pathways, in part regulated by NADPH oxidase, may be active in OECs. It is probable that inhibition of NADPH oxidase may trigger these pathways. Further studies scrutinising these pathways in the OECs could dis/prove this notion. Sensitivity to agents manipulating NADPH oxidase activity has also been reported in haematopoietic stem cells which contain various self-fuelling mechanisms to maintain NADPH oxidase in an assembled state to continuously generate low levels of  $O_2^{\cdot-}$ . As redox signalling may promote the differentiation of stem cells, it requires close consideration (Piccoli et al., 2005, Piccoli et al., 2007).

Changes in cellular architecture, accompanied by stress fibres formation, during and after ischaemic insult also contribute to BBB damage. Upon exposure to ischaemic insult, G-actin transforms into filamentous actin (F-actin). The change from G-actin to F-actin leads to formation of contractile actin bundles

also called as stress fibers. These fibers attach to adherence junctions of adjacent endothelial cells. When stress fibers start to contract under stress, intracellular gaps between neighboring endothelial cells form and therefore BBB permeability increase. Compared to HBMECs, OECs have shown significantly lower numbers of stress fibres during OGD±R, another prerequisite that can be added to the OECs endothelium-reparative and cerebral barrier-restorative function. Co-application of gp91ds-tat with OGD±R attenuates stress fibre formation and restores cortical actin staining in HBMECs but greatly influenced the intensity and localisation of stress fibres in OECs subjected to OGD±R. Hypoxia-induced increases in the expression of NOX4 and NOX2 have previously been associated with the diminished proliferative, migratory, tubulogenic and adherence capability of OECs (Liu et al., 2017). Further evidence show that hypoxia induces OECs migration and re-endothelialisation selectively in wild type, but not NOX2 knock out, mouse model of carotid artery damage (Schroder et al., 2009). Others also show that inhibition of NOX4, a member of NADPH oxidases family, results in decreased proliferation, migration, and reduced survival of OECs (Hakami et al., 2017). Collectively these data support the notion that a certain degree of  $O_2^{\cdot-}$ , derived from NOX2, is required for appropriate OEC function. Our study demonstrates that compared to HBMECs, OECs have higher antioxidant capacity, lower basal levels of NADPH oxidase activity and  $O_2^{\cdot-}$  generation, and perform better during the reperfusion, supporting the idea that progenitor cells can work effectively in ischaemic environments and may therefore be reliable therapeutics to repair neurovascular damage even during subacute phase. It also demonstrates that inhibition of NOX2 in microvascular endothelial cells, that constitute the main

cellular element of cerebrovascular barrier *in vivo*, protects the integrity and function of an *in vitro* model of human BBB against ischaemic damage. Thirdly, inhibition of NOX2 aggravates the detrimental impact of ischaemic injury on BBB established with OECs by promoting cytoskeletal reorganisation characterised by actin stress fibre formation and augmenting oxidative stress. These findings indicate that targeting oxidative stress through inhibition of NOX2 requires close attention while OECs are being used as reparative cellular therapeutics.

There are some limitations to this study. Considering the studies revealing that OECs or targeting NOX2 are both beneficial in restoring *in vitro* BBB evoked-ischaemic damage, it might have been good idea to assess whether attenuation of NOX2 during OGD and subsequent addition of OECs as therapeutics during reperfusion to same group of BBB model established with HBMECs, has any subsequent protective effect, i.e., OGD+gp91ds-tat followed by 20 hours of reperfusion with OECs therapy. Future studies are needed to explore this point and are required to assess whether therapeutic approach targeting NOX2 may be useful in an animal model of ischaemic stroke.

#### **4.1.11 Summary**

In conclusion, this study reveals that OECs have higher antioxidant capacity and exert better barrier-protective effect during reperfusion phase, supporting the notion that progenitor cells can work effectively in ischaemic environments and may therefore be reliable therapeutics for repair of neurovascular damage. In addition, it shows that NOX2 inhibitor protects the integrity and function of

human BBB against ischaemic damage. It also reveals that targeting oxidative stress through inhibition of NOX2 requires close attention while OECs are being used as reparative cellular therapeutics.



## **5 ROLE OF GP91DS-TAT ON HYPERGLYCAEMIA INDUCED CEREBRAL BARRIER DAMAGE**

### 5.1.1 Introduction and aims

Hyperglycaemia is one of the main factors that aggravates BBB disruption following ischaemic stroke among diabetic patients. Chronic effect of hyperglycaemia induces stroke-evoked BBB injury by inducing oxidative stress, apoptosis of cells forming neurovascular unit, and thickening of vascular basement membrane (Venkat et al., 2017, Srivastava et al., 2013b, Kadir et al., 2021b). In fact, diabetic patients who are affected by ischaemic stroke proven to have higher morbidity, mortality rates, and worse complications than others who are non-diabetic (Thorén et al., 2017, Yu et al., 2016). In addition, diabetes affects the therapeutic benefits of rtPA, the only proven thrombolytic drug for ischaemic stroke, and it also associated with higher risks of cerebral bleeding (Masrur et al., 2015). Unfortunately, under 1% of people who are affected by ischaemic stroke globally get treated with rtPA due to a short treatment window, 4.5 hours of symptom onset (Kleindorfer et al., 2008). In chapter 3 and 4 we proven that NADPH oxidase inhibitor and OECs can restore BBB function following ischaemic damage. In this chapter we aim to explore the role of HADPH oxidase inhibitor and hypothermia against the impact of hyperglycaemia on BBB integrity and function.

Hypothermia, core body temperature  $\leq 35^{\circ}\text{C}$ , is commonly used to decrease the complications of global hypoxia-evoked brain damage in unconscious patients affected by asystole and in cases of ischaemic brain damage among neonates (Shankaran et al., 2005, Bernard et al., 2002). Researchers have shown that combination of hypothermia with mechanical thrombectomy or rtPA is safe and feasible for patients affected by ischaemic stroke and is linked with reduction in the size of infarcted areas in the brain, and has better functional neurological

recovery in *in vivo* model of brain ischaemia (Piironen et al., 2014, Chen et al., 2016a, van der Worp et al., 2007). Nevertheless, further experimental, and clinical studies are required to explore the benefits of hypothermia on cerebral barrier affected hyperglycaemic insult.

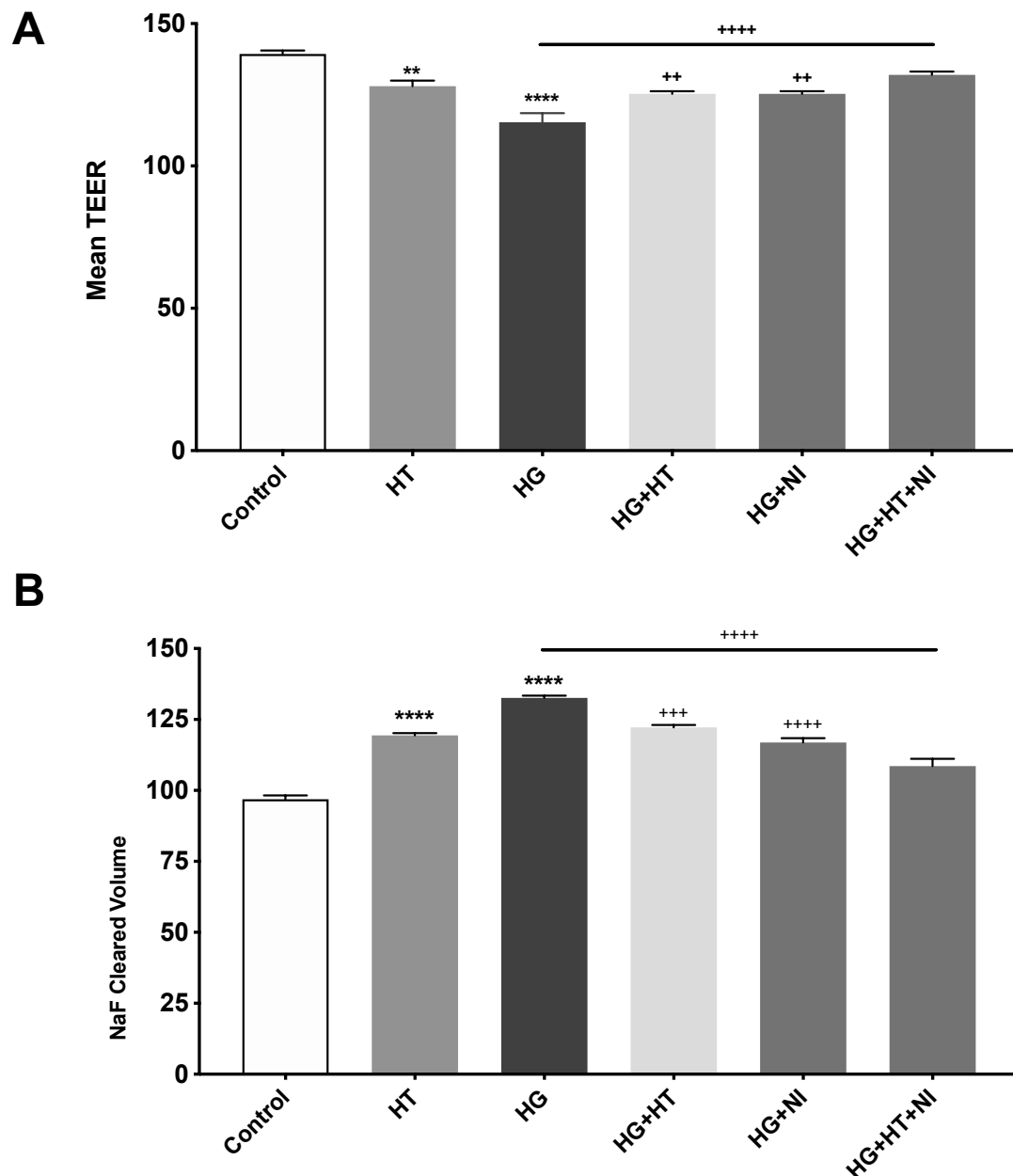
Oxidative stress, manifested by increased release of ROS, particularly  $O_2^{\bullet-}$ , is known to be associated with hyperglycaemia-evoked endothelial dysfunction and precipitation of neurovascular unit damage. Certainly, dysfunction of endothelium known to be a main cause of sudden and potentially salvageable injury following ischaemic stroke in people with history of diabetes mellitus. In this regard, stimulation of NADPH oxidase is known to have vital role (Bayraktutan, 2002, Shao and Bayraktutan, 2013a, Kadir et al., 2021b). NOX2, a member of NADPH oxidases family, is linked with the cerebrovascular damage (Kahles and Brandes, 2012). Thus, targeting NOX2 activity may protect BBB integrity and function against oxidative stress damage caused by hyperglycaemia (Kahles et al., 2007, Shao and Bayraktutan, 2013a, Chen et al., 2016b).

In this chapter we investigated whether approaches of combining hypothermia with gp91ds-tat, selective NOX2 inhibitor, may have better protective effect of an *in vitro* model of cerebral barrier against the deleterious effect of hyperglycaemia. In addition, this chapter also investigates the benefits of this combinatory strategy as opposed to the single treatment regimen for oxidative stress control, actin cytoskeletal formation, and wound repair using HBMECs and HAs, the major two elements of BBB structure. This chapter explores whether hypothermia induces the protective benefits of gp91ds-tat on BBB. In

this chapter, BBB model was exposed to hyperglycaemic insult. While both OGD and hyperglycaemia disrupt BBB integrity and function through oxidative stress damage and related pathways, using this type of damage model allows us to investigate whether NOX2 inhibitor can block oxidative stress caused by hyperglycaemia and whether this can restore BBB integrity and function.

### **5.1.2 Hypothermia induces the barrier protective effects of gp91ds-tat**

Hyperglycaemia appeared to significantly compromise BBB integrity and function, as proven by significant drops in TEER and simultaneous raises in NaF flux through the barrier, in turn. While the integrity and function of BBB models (incubated in normoglycaemia state) shown to be also impaired by the effect of hypothermia (35°C) alone, the impacts of this damage were significantly lower than those caused by hyperglycaemia. While gp91ds-tat restored the integrity and function of barrier models in a similar manner to hypothermia in hyperglycaemic conditions, the combination treatment of hypothermia with gp91ds-tat nearly fully counterbalance the harmful impact of hyperglycaemic insult on BBB integrity and function, demonstrating a synergetic impact. This was evidenced by significant improvements in TEER and NaF flux readings compared to hyperglycaemia (Figure 5.1).



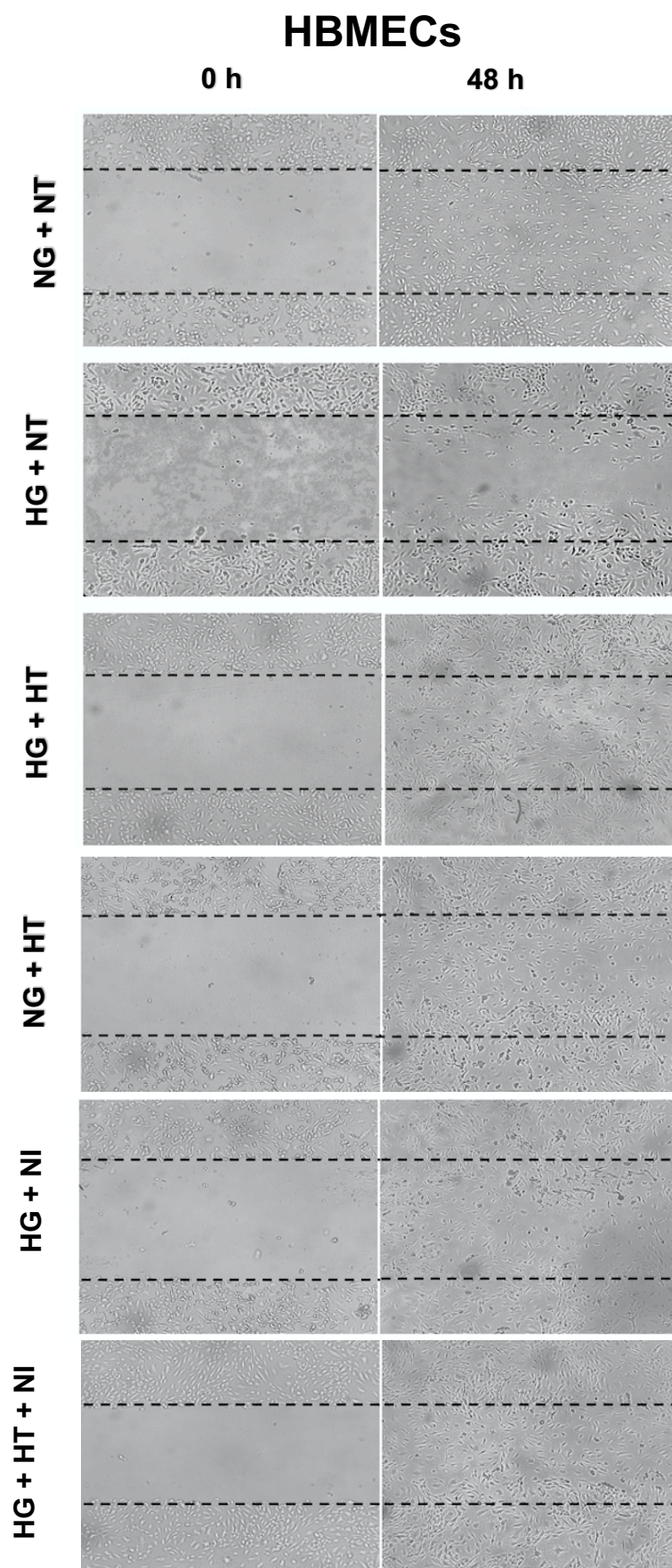
**Figure 5-1 Effects of hypothermia and gp91ds-tat on BBB**

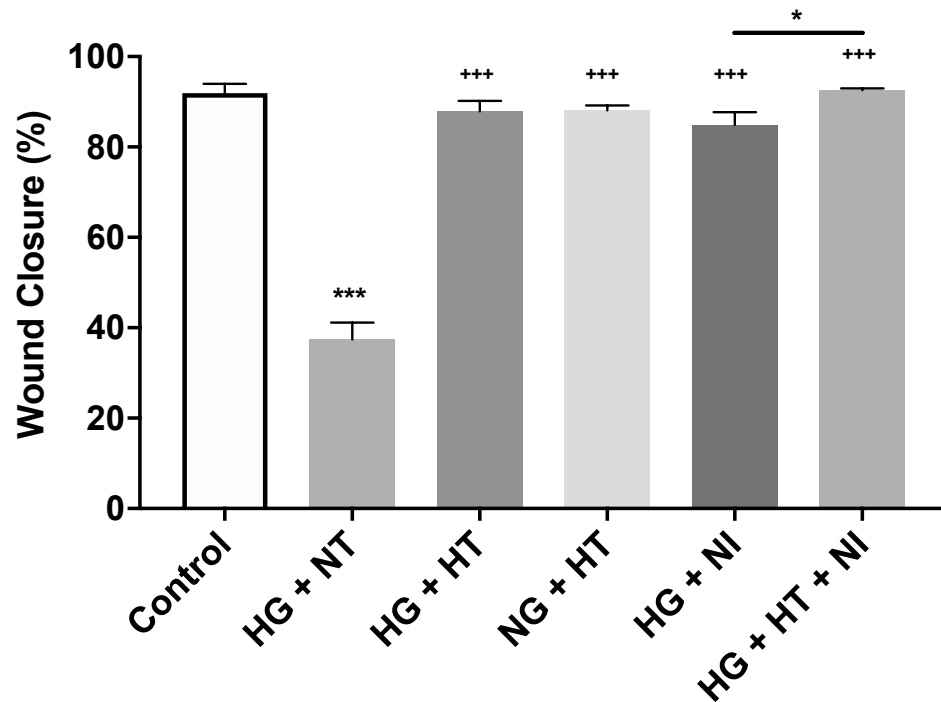
BBB models were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). BBB integrity and function were assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF, B), respectively. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\* $P$ <0.002 and \*\*\*\* $P$ <0.0001 compared to control. ++ $P$ <0.006, +++ $P$ <0.008, and \*\*\*\* $P$ <0.0001 compared to HG (one-way analysis of variance followed by Tukey's post hoc analysis).

### **5.1.3 Hypothermia augments wound repair induced by gp91ds-tat**

To establish how combining gp91ds-tat and hypothermia affects the migration and proliferation of major cellular elements of the BBB, a wound was created on HBMECs and HAs monolayers by scratch as described in chapter two. Treatment with therapeutic hypothermia or gp91ds-tat alone repaired the wound induced on HBMECs and HAs monolayers when exposed to hyperglycaemia. Therapeutic hypothermia appeared to accelerate the reparative effect of gp91ds-tat when given as a combination therapy as opposed to single treatment approach. However, exposure to hypothermia alone shown to have an adverse effect on the proliferative capacities of HBMECs and HAs under normoglycaemic settings (Figures 5.2 and 5.3).

**A**



**B****HBMECs**

**Figure 5-2 Effects of hypothermia or gp91ds-tat on HBMECs wound closure.**

HBMECs were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). By using a p1000 micropipette tip, a scratch was made manually down the centre of the well in one swift motion. Images of wound closure were captured at 0- and 48-hours' time points post-scratch. Graph representing the surface area of wound closure in all experimental groups (B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to control. \*\*\*P<0.0001 compared to HG+NT. \*P<0.02 compared to HG+NI (one-way analysis of variance followed by Tukey's post hoc analysis).



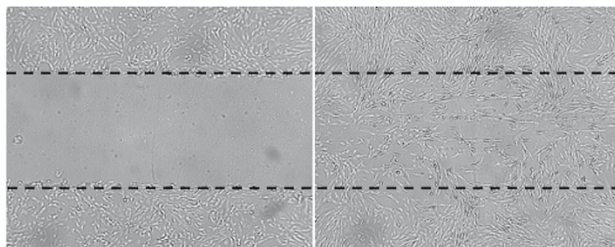
**A**

**HAs**

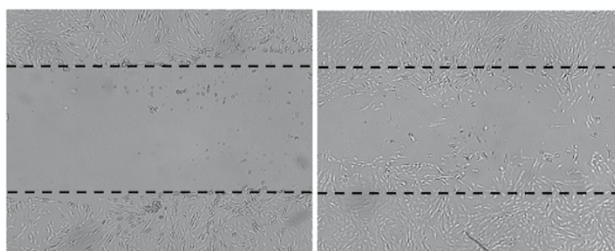
**0 h**

**48 h**

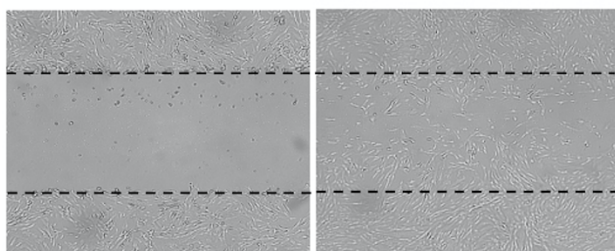
**NG + NT**



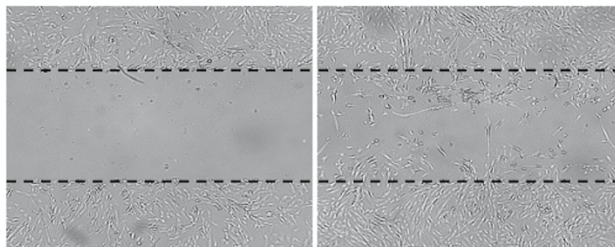
**HG + NT**



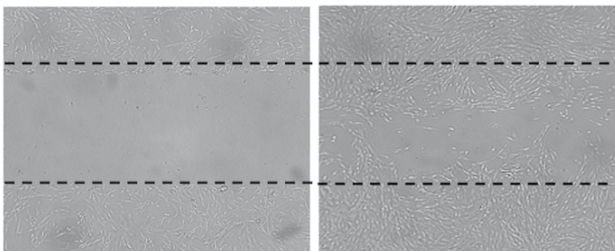
**HG + HT**



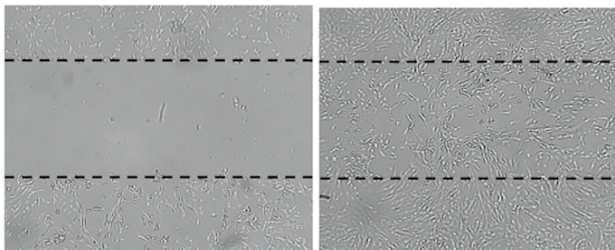
**NG + HT**

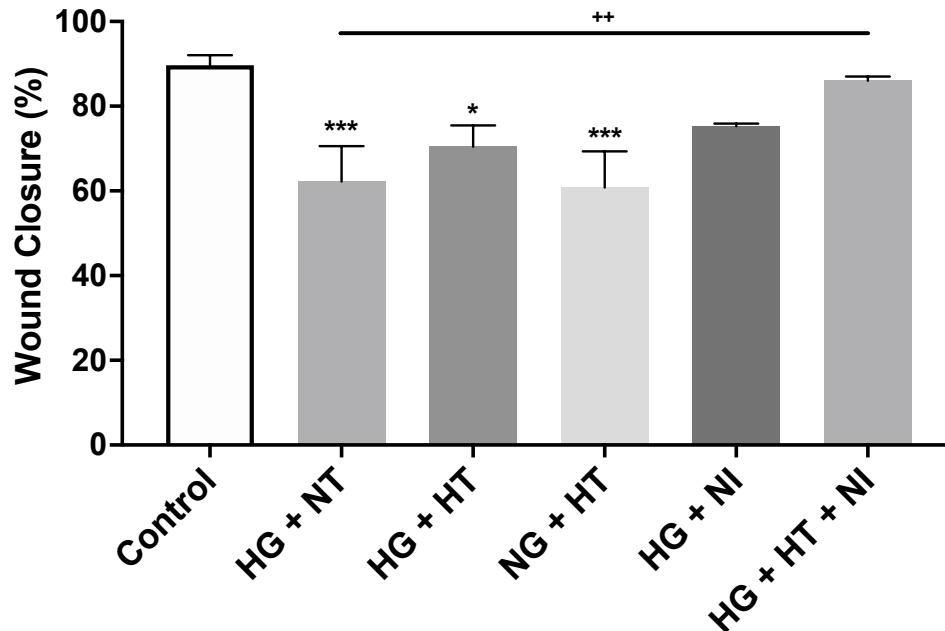


**HG + NI**



**HG + HT + NI**



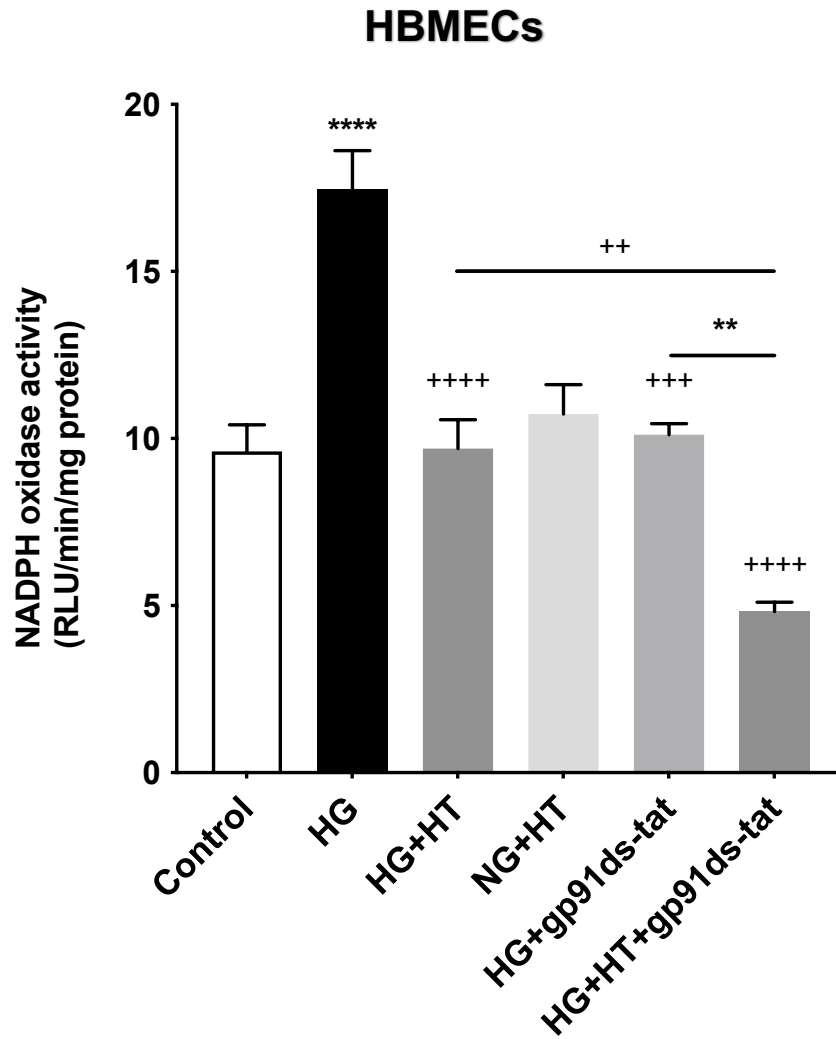
**B****HAs**

**Figure 5-3 Effects of hypothermia or gp91ds-tat on HAs wound closure.**

HAs were exposed to hyperglycaemia (HG) for 120 hours in absence or prescence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). By using a p1000 micropipette tip, a scratch was made manually down the centre of the well in one swift motion. Images of wound closure were captured at 0- and 48-hours' time points post-scratch. Graph representing the surface area of wound closure in all experimental groups (B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.02$ , \*\*\* $P < 0.001$  compared to control. \*\* $P < 0.002$  compared to HG+NT. (one-way analysis of variance followed by Tukey's post hoc analysis).

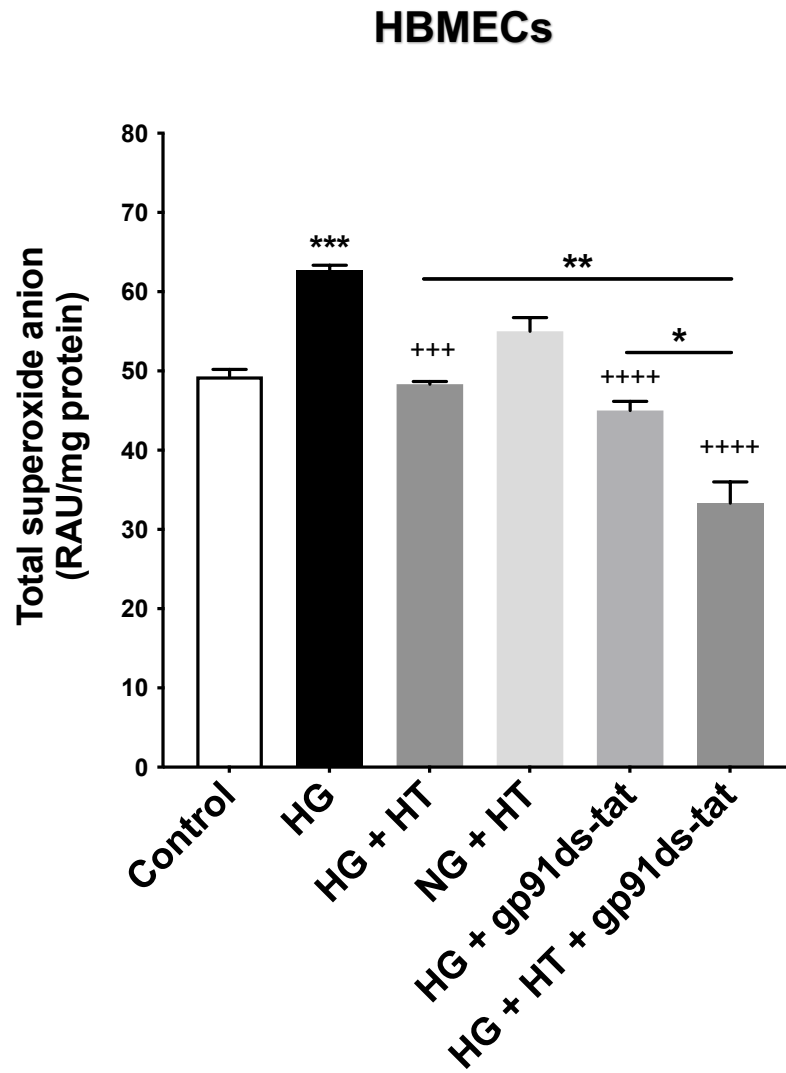
#### **5.1.4 Hypothermia augments the suppressive effect of gp91ds-tat on oxidative stress**

Hyperglycaemia induced the expression of  $O_2^{\bullet-}$  in HBMECs and HAs through an increase in NADPH oxidase activity. Treatments with either therapeutic hypothermia or NOX2 inhibitor, gp91ds-tat, significantly inhibited the increases in NADPH oxidase activity and  $O_2^{\bullet-}$  levels in both cell lines. Combination of hypothermia with gp91ds-tat significantly inhibited  $O_2^{\bullet-}$  generation and NADPH oxidase activity below the levels observed in control cells maintained in normoglycaemic and normothermic conditions. Conversely, hypothermia did not significantly affect the basal levels of  $O_2^{\bullet-}$  production and NADPH oxidase activity in either cell line cultured under normoglycaemic conditions (Figures 5.4, 5.5, 5.6, and 5.7).



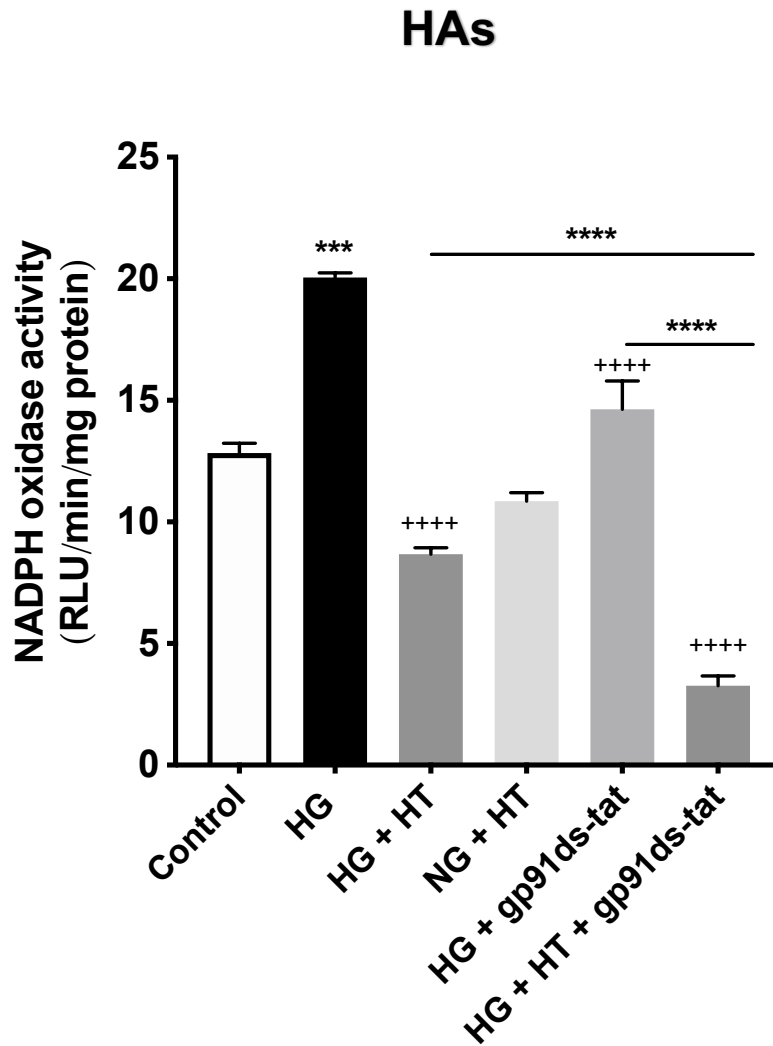
**Figure 5-4 Effects of hypothermia and gp91ds-tat on NADPH oxidase activity in HBMECs**

HBMECs were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). The activity of NADPH oxidase was detected by lucigenin chemiluminescence assays. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\* $P$ <0.0001 compared to control. \*\*\* $P$ <0.0002, +++ $P$ <0.0001, compared to HBMECs subjected to HG. ++ $P$ <0.004, \* $P$ <0.002 compared to HBMECs subjected to HG+HT+gp91ds-tat. (one-way analysis of variance followed by Tukey's post hoc analysis).



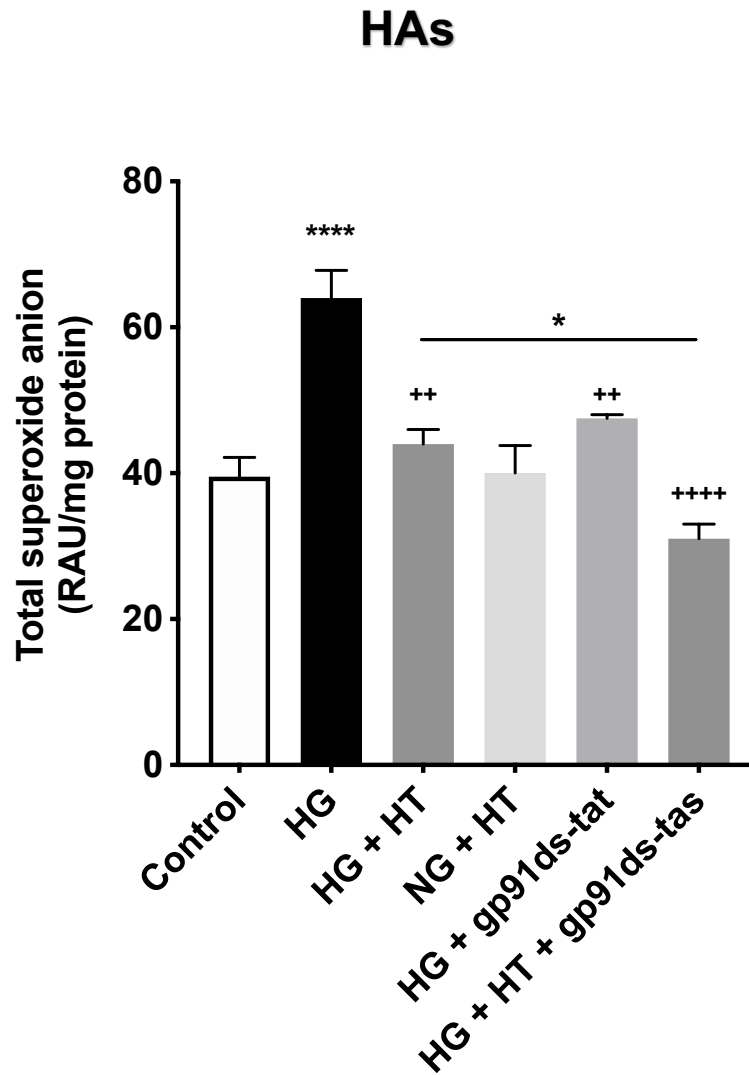
**Figure 5-5 Effects of hypothermia and gp91ds-tat on superoxide levels in HBMECs**

HBMECs were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). Cytochrome reduction assay was used to measure total  $O_2^{\bullet}$  levels. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*P<0.0003 compared to control. +++P<0.0002, ++++P<0.0001, compared to HBMECs subjected to HG. \*\*P<0.0002, \*P<0.002 compared to HBMECs subjected to HG+HT+gp91ds-tat. (one-way analysis of variance followed by Tukey's post hoc analysis).



**Figure 5-6 Effects of hypothermia and gp91ds-tat on NADPH oxidase activity in human astrocytes**

HAs were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). The activity of NADPH oxidase was detected by lucigenin chemiluminescence assays. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\* $P$ <0.0001 compared to control. \*\*\*\* $P$ <0.0001 compared to HAs subjected to HG. \*\*\*\* $P$ <0.0001 compared to HAs subjected to HG+HT+gp91ds-tat. (one-way analysis of variance followed by Tukey's post hoc analysis).



**Figure 5-7 Effects of hypothermia and gp91ds-tat on superoxide levels in human astrocytes**

HAs were exposed to hyperglycaemia (HG) for 120 hours in absence or prescence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50 $\mu$ m). Cytochrome reduction assay was used to measure total  $O_2^{\cdot-}$  levels. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to control. ++P<0.005, \*\*\*\*P<0.0001 compared to HAs subjected to HG. \*P<0.05 compared to HAs subjected to HG+HT. (one-way analysis of variance followed by Tukey's post hoc analysis).

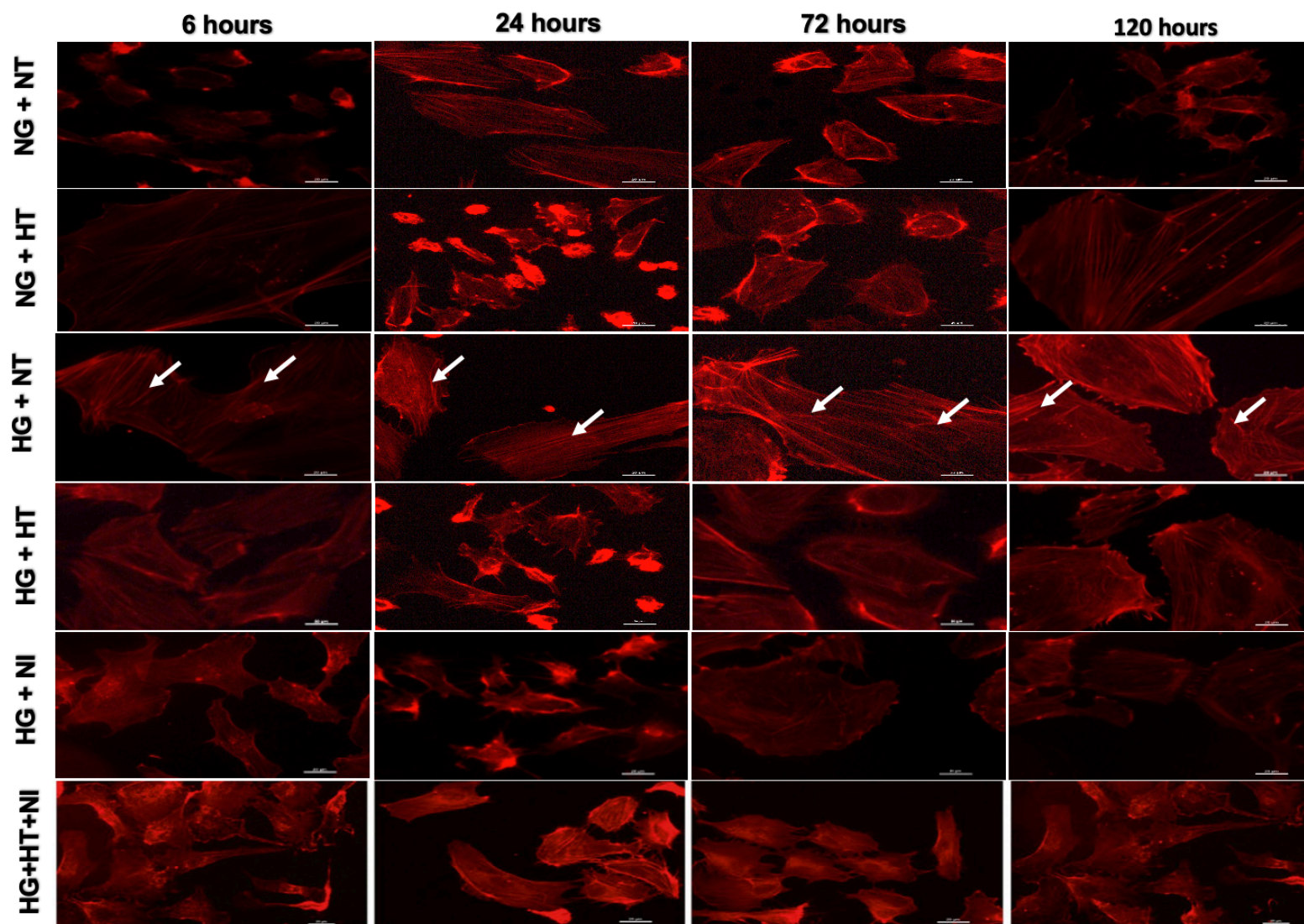
#### **5.1.5 Hypothermia induces the protective effect of gp91ds-tat on HBMECs and HAs architecture.**

The actin cytoskeleton of the cells usually affected by changes in the microenvironments which result in a shift of G-actin to F-actin and induce the formation of stress fibres, contractile actin bundles. Studying the actin cytoskeleton of HBMECs and HAs by using rhodamine-phalloidin staining showed that hyperglycaemia induces the formation of stress fibres in both cells. Exposure to hypothermia alone also induces stress fibres in both cell lines subjected to normoglycaemic conditions. While either gp91ds-tat or hypothermia decreases the amount of hyperglycaemia-evoked stress fibres formation, combination of hypothermia with gp91ds-tat shown to further decrease the effect of hyperglycaemia on actin cytoskeleton (Figure 5.6).



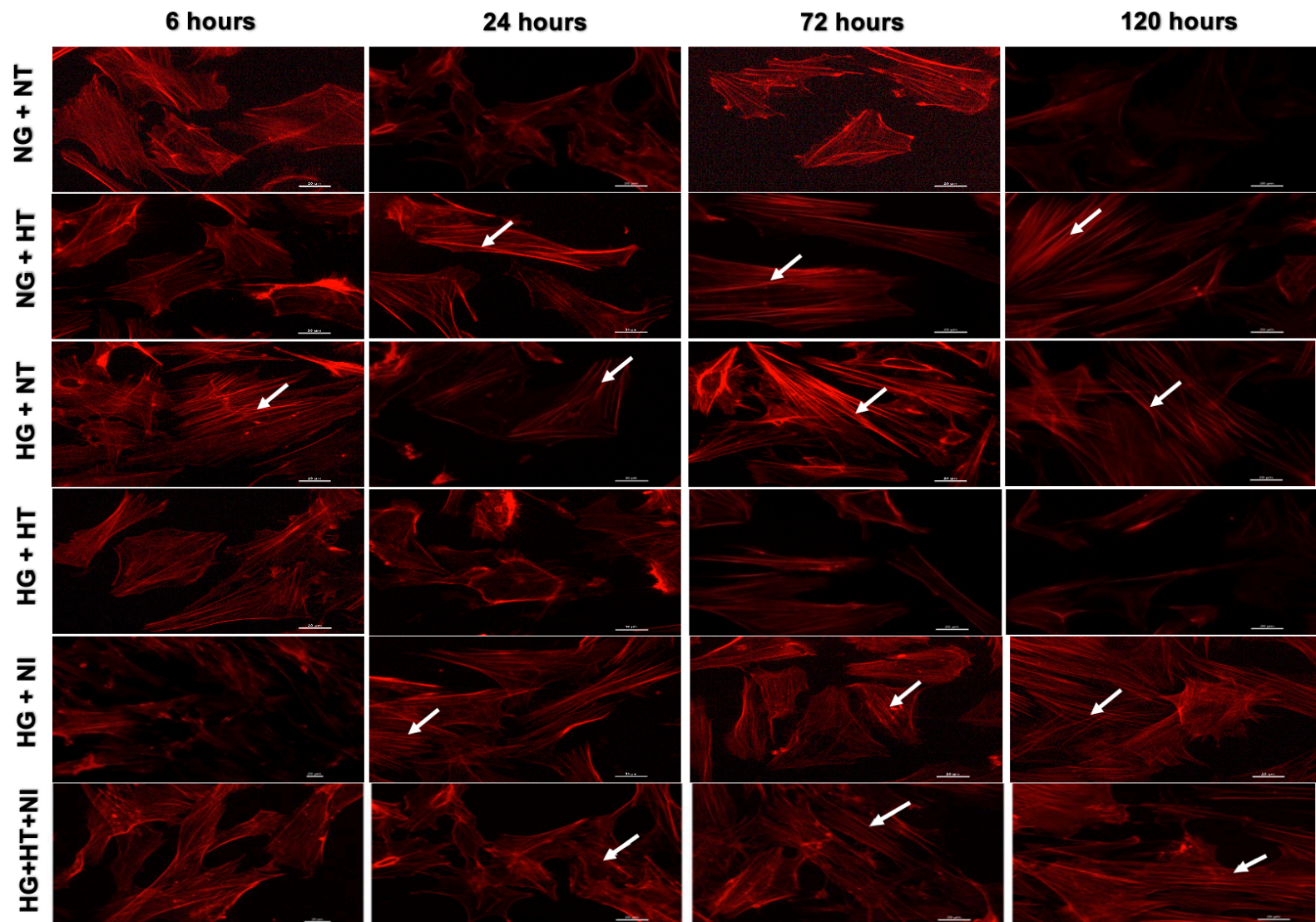
**A**

**HBMECs**



**B**

**HAS**



**Figure 5-8 Effects of hypothermia and gp91ds-tat on cytoskeletal organisation of HBMECs and HAs subjected to hyperglycaemia**

HBMECs and HAs were exposed to hyperglycaemia (HG) for 120 hours in absence or presence of hypothermia (HT), NOX2 inhibitor (NI), or HT with NI (gp91ds-tat, 50µm). Cells were then successively fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS prior to staining in 1x rhodamine phalloidin. Cells were then viewed under fluorescence microscope. Stress fibres are indicated by white arrows (A and B). Scale bars = 20µm. NG, normoglycaemia; NT, normothermia.

### 5.1.6 Discussion

The BBB is complex structure that regulates cerebral homeostasis and protects neurons from toxins and other unwanted substances. BBB dysfunction, manifested by raised interendothelial cell permeability, is considered as a major pathological process that leads to cerebral damage and subsequent neurological deficit following ischaemic stroke (Jiang et al., 2018b). Patients who are affected by chronic illnesses, in particular diabetes mellitus, is proven to further induce dysfunction of BBB following an ischaemic stroke, in that stimulation of oxidative stress, endothelial and neuronal cell apoptosis, and increased thickness of capillary basement membrane shown to play a major role (Venkat et al., 2017). In fact, diabetic patients who are affected by ischaemic stroke usually will end up with worse outcomes including severe neurological disabilities and increased mortality rates (Li et al., 2013). In addition, ischaemic stroke patients who have diabetes mellitus and treated with rtPA usually have increased risk of intracranial haemorrhage (Thorén et al., 2017, Yu et al., 2016, Masrur et al., 2015). Thus, in this chapter we explored mechanisms that may be of beneficial benefits in protecting diabetic stroke patients from these devastating outcomes.

In this regard, the ability of therapeutic hypothermia to target various pathways, including inflammatory responses, oxidative stress, apoptosis signals, and metabolic disruption may have a potential benefit in protecting BBB against ischaemic damage (Loach and Bayraktutan, 2017). In fact, therapeutic hypothermia has been used as effective therapy for patients with cardiac arrest or for neonates with hypoxic-ischaemic encephalopathy (Shankaran et al., 2005, Bernard et al., 2002). In addition, *in vivo* studies have proven that



therapeutic hypothermia reduces the size of infarction and neurological disabilities by about 30% in animal models of ischaemic stroke and restored cerebral barrier integrity and function during and after following ischaemic insult (Mathur and Bayraktutan, 2016, Mathur and Bayraktutan, 2017). Nevertheless, the effect of therapeutic hypothermia in diabetic stroke patients or in hyperglycaemic cerebral barrier damage *in vitro* still unknown.

By using a triple culture model of the human BBB formed by co-culture of HBMECs, HAs and HPs, this study has revealed that therapeutic hypothermia can protect cerebral barrier against hyperglycaemic insult. In this regard, NOX2 also appears to play a vital role in hyperglycaemic BBB disruption. Compared to single therapy approach, the combination therapy of NOX2 inhibitor and therapeutic hypothermia results in higher protection against hyperglycaemic barrier injury, as evidenced by the significant increases in TEER and decreases in NaF flux readings. Using TEER to evaluate the barrier's integrity and permeability by measuring electrical resistance across its cellular monolayers has been proven to be a very sensitive and reliable methodology. Measuring the paracellular flux of NaF across the barrier also indicates whether tight junctions between adjacent endothelial cells are leaking and whether barrier maintained its homeostasis function (Srinivasan and Kolli, 2019, Helms et al., 2016). Thus, any alterations in TEER and paracellular flux readings following exposure to hyperglycaemia, in this study, indicates a breakdown in tight junctional complexes. Studies have shown that inhibition of NOX2 decreased apoptosis of endothelial cells after exposure to hyperglycaemia and inflammatory stimuli such as TNF- $\alpha$  (Shao and Bayraktutan, 2014, Abdullah and Bayraktutan, 2016b). It is likely that treatment with therapeutic hypothermia

might protect endothelial cell against damage and block cellular apoptosis to preserve BBB integrity. Significant reductions shown in oxidative stress, as indicated by decreases in pro-oxidants NADPH oxidase activity and  $O_2^{\bullet}$  release along with rapid and efficacious repair of HBMECs and HAs damage, proven by wound scratch assay, may additionally provide better outcomes resulted from the combination therapy.

Upon exposure to oxidative stress insult, G-actin transforms into filamentous actin (F-actin). The change from G-actin to F-actin leads to formation of contractile actin bundles also called as stress fibers. These fibers attach to adherence junctions of adjacent endothelial cells. When stress fibers start to contract under stress, intracellular gaps between neighboring endothelial cells form and therefore BBB permeability increase (Chen et al., 2013, Srivastava and Bayraktutan, 2017). By studying cellular architecture, we have shown that combination therapy of NOX2 inhibitor and therapeutic hypothermia is more effective in protecting both HBMECs and HAs against formation of stress fibres. In support of these results, therapeutic hypothermia has also been proven to reduce barrier hyperpermeability, infarct size, volume of brain oedema, and neurological deficits in an *in vivo* model of diabetic stroke where inhibition of apoptosis shown to play a vital impact (Tu et al., 2019). Interestingly, despite being effective therapy in hyperglycaemic experiments, therapeutic hypothermia shown to have an adverse effect on barrier function as well as HBMECs and HAs wound repair and cellular architecture in normoglycaemic settings without influencing oxidative stress mechanism in these conditions. In support of these findings, profound reductions in proliferation and rises in cell death rates have been shown in human umbilical vein endothelial cells when

exposed to therapeutic hypothermia where changes in the levels of multiple inflammatory factors, including MCP-1, COX-2, IL-8, and IL-6, were proven to play a key role (Diestel et al., 2008). Other studies have shown that exposure of these cells to therapeutic hypothermia under normoglycaemic conditions induces inflammatory mediators release, in particular IL-6, and apoptosis rate of neuronal, microglial, and HAs (Schmitt et al., 2006, Schmitt et al., 2007). These studies support our findings that therapeutic hypothermia may induce cerebral cells dysfunction under physiological settings.

In line with the other studies (Lorenzi et al., 1985, Zhu et al., 2015, Huang et al., 2019), exposing HBMECs or HAs to hyperglycaemia, in our study, has also adversely affected the speed of wound closure of both cell lines which might be due to overproduction of ROS, particularly  $O_2^{\bullet-}$  in these conditions. It is known that certain levels of  $O_2^{\bullet-}$  have an important role in physiological functions including neurotransmitter release, immune defence system, and intracellular communication. However, overproduction of  $O_2^{\bullet-}$  in hyperglycaemic conditions is associated with significant reduction in proliferation rate of endothelial cells. Furthermore, overexpression of  $O_2^{\bullet-}$  damages cellular architecture and tight junction formation leading to disruption of BBB integrity and function (Zanetti et al., 2001, Shao and Bayraktutan, 2014, Abdullah et al., 2015). Excessive expression in NADPH oxidase, particularly NOX2, and  $O_2^{\bullet-}$  production have also been reported in HBMECs exposed to hyperglycaemia (Ulker et al., 2004, Allen and Bayraktutan, 2009a). This study explored the effect of selective NOX2 inhibition using gp91ds-tat as single therapy or in combination with therapeutic hypothermia on *in vitro* models of human BBB under hyperglycaemic conditions. Our study shows that combination therapy of gp91ds-tat with

therapeutic hypothermia has better barrier-protective effect. In support to these findings, other studies have shown that NOX2-deficient mice subjected to MCAO have less levels of oxidative stress and smaller infarct size after inhibition of NOX2 activity in neuronal cells subjected to ischaemic stroke under hyperglycaemic conditions. (Kahles et al., 2007, Zeng et al., 2018).

Evidence gathered from other studies has shown that the combinatory approach of therapeutic hypothermia with various therapies has better outcomes in restoring barrier functions after ischaemic insult. For example, combining therapeutic hypothermia with tirilazad (antioxidant) and with calcium and magnesium (antioxidant) induces the reparative effect of therapeutic hypothermia on neurovascular injury following ischaemic damage, as evidenced by significant decreases in infarct size and better neurological outcomes in rats subjected to ischaemic stroke (Zausinger et al., 2003). Better defence of BBB integrity and function by combination of gp91ds-tat with therapeutic hypothermia in the current study additionally confirm the effectiveness of the combinatory approach in hyperglycaemic settings.

#### **5.1.7 Summary**

In conclusion, this study has revealed that inhibition of NOX2 augments the BBB-reparative effects of hypothermia in hyperglycaemic conditions by decreasing oxidative stress, preserving cellular architecture, and inducing proliferation and migration of HBMECs and HAs, two major cellular components of human cerebral barrier.



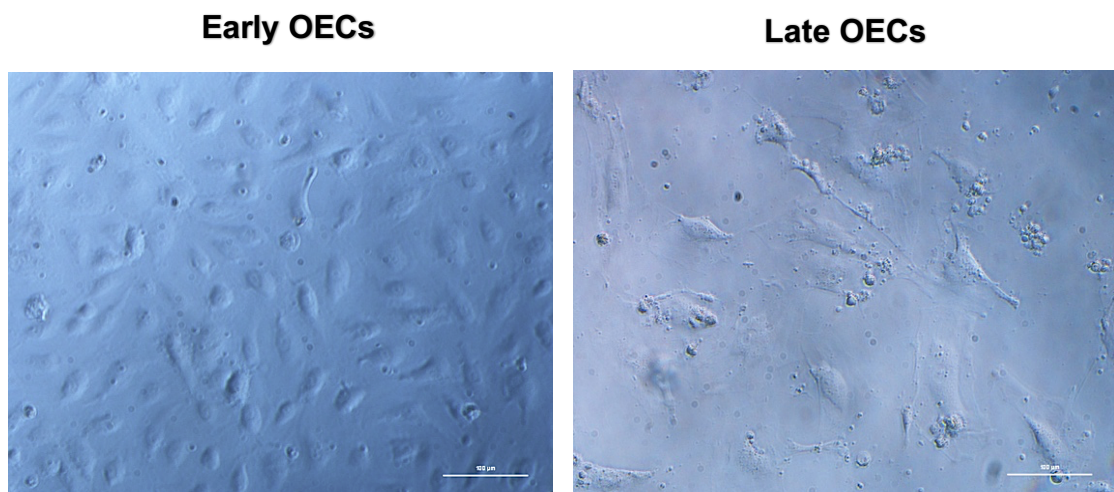
## **6 EFFECTS OF SENESENCE ON OUTGROWTH ENDOTHELIAL CELLS**

### **6.1.1 Introduction and aims**

OECs may play essential role following ischaemic damage by direct contribution to vascular repair or by inducing angiogenic function of resident endothelial cells. There is accumulating body of evidence suggests that OECs can lose their functional capacities and become prematurely senescent in patients with chronic vascular diseases (Goligorsky, 2014). In addition, preparing OECs to be used as cellular therapeutics for ischaemic stroke, requires ex vivo expansion. However, it is documented that OECs can enter a stage of cell-cycle arrest and become prematurely senescent which might cause a significant loss of their functional and reparative capacities (Medina et al., 2013). In the previous chapters we found that OECs can repair endothelial damage, form tight junctions, establish functional BBB, and repair BBB following ischaemic damage. The impact of senescence on OECs functional abilities to repair endothelial damage and establish functional BBB is poorly understood. Our aim in this chapter is to further explore the impact of senescence on the morphology and the architecture of OECs. In addition, to study the proliferative, reparative, and adherence capacities of senescent OECs. Furthermore, to investigate whether senescent OECs can form well-established tubules (angiogenesis marker), tight junctions, and functional human BBB. Finally, to investigate oxidative stress and antioxidant status of senescent OECs.

### 6.1.2 Late passage OECs display larger and flattened morphology

An equal number of early passage (P8) and late passage (P14) OECs were cultured and visualised by light microscope. While Passage 8 OECs had cobblestone morphology, typical morphological feature of endothelial cells, late passage OECs manifested a flattened morphology and bigger size (“fried egg” appearance) (Figure 6.1).

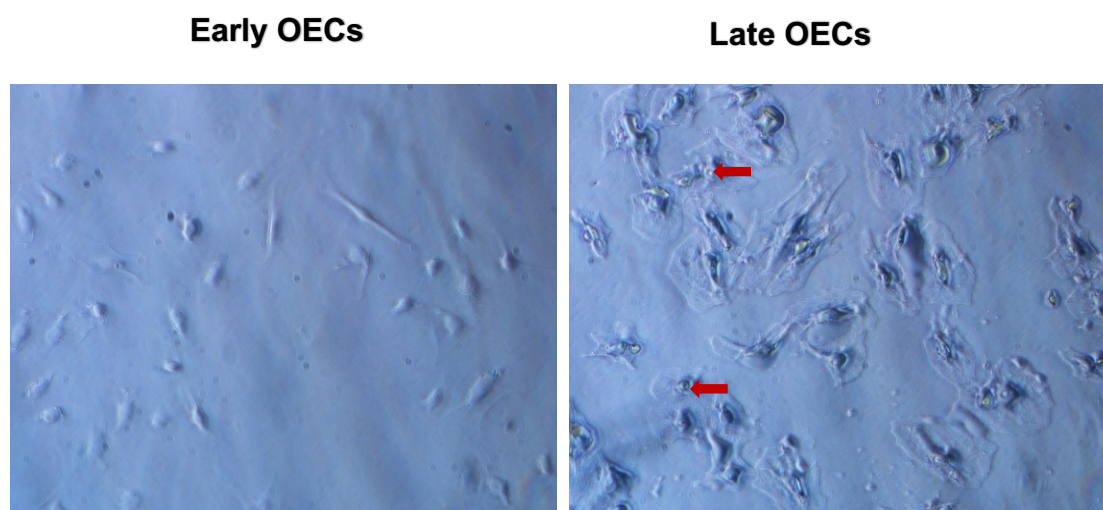


**Figure 6-1 Morphological features of early OECs compared to late OECs.**

Equal numbers of early passage (P8) and late passage (P14) OECs were cultured on coverslips and inspected by light microscopy. Early OECs show cobblestone morphology while late OECs have flattened and bigger morphology. Scale bars: 100 µm.

### 6.1.3 Late passage OECs are positive for senescence-associated $\beta$ -galactosidase.

Equal numbers of early passage (P8) and late passage (P14) OECs were cultured then stained using  $\beta$ -galactosidase staining kit as described in chapter two. Then, senescence-associated- $\beta$ -galactosidase positive cells were identified. Compared to early passage OECs, late passage OECs showed significantly higher number of positive stained cells with senescence associated  $\beta$ -galactosidase. This was evidenced by appearance of blue colour areas within the cells (Figure 6.2).

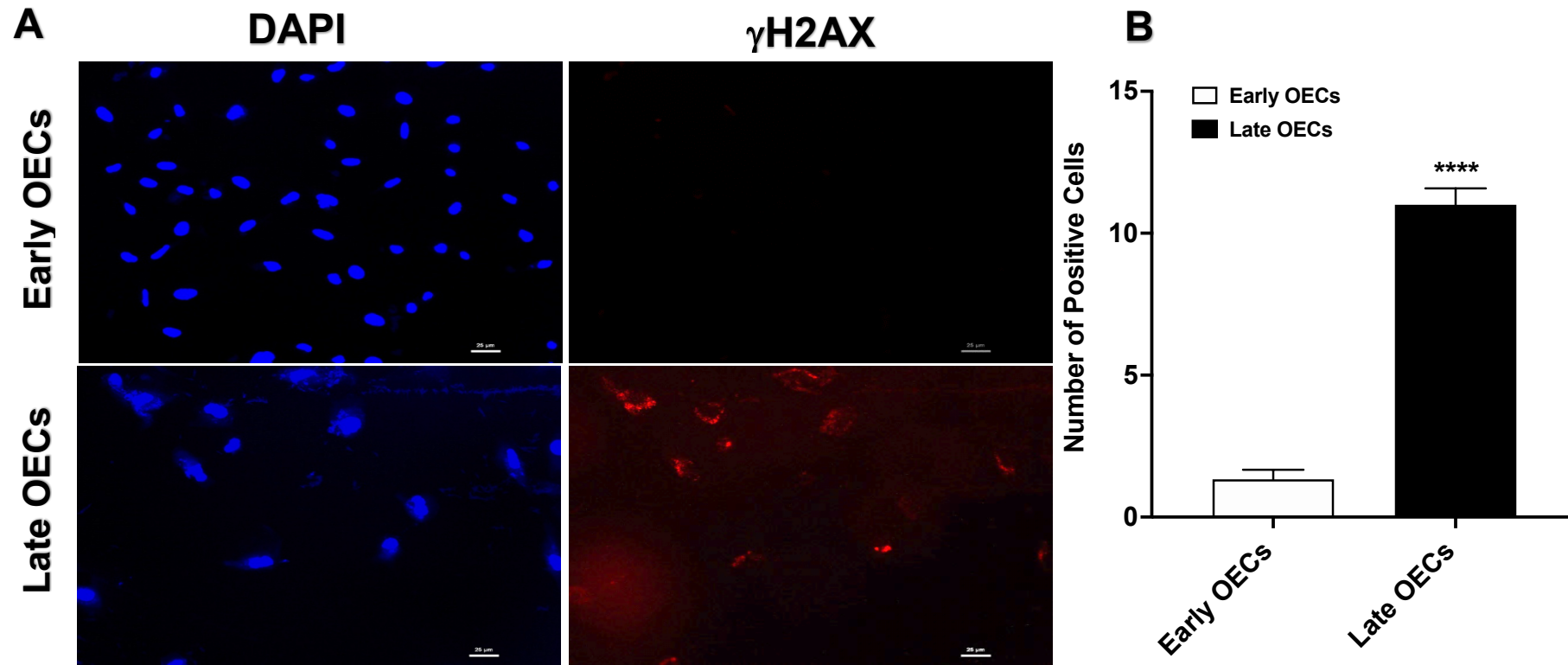


**Figure 6-2 Senescence-associated  $\beta$ -galactosidase staining in early and late passage OECs.**

Equal numbers of early passage (P8) and late passage (P14) OECs were cultured on coverslips then stained with senescence associated  $\beta$ -galactosidase stain. Positively stained cells show blue colour areas within the cells (indicated by red arrows).

#### **6.1.4 Late passage OECs are positive for senescence-associated $\gamma$ H2AX**

DNA damage constitutes one of the main characteristics of cellular senescence. To this end, the presence of  $\gamma$ H2AX, a DNA damage marker, was investigated in this study in early passage (P8) and late passage (P14) OECs. In line with senescence-associated  $\beta$ -galactosidase data, compared to early passage OECs, the number OECs positive for  $\gamma$ H2AX was significantly higher in late passage OECs. Concurrent staining of DNA in early and late OECs with DAPI (4,6-diamidino-2-phenylindole) reveals large nuclear size was more prevalent in late passage OECs than their early passage counterparts (Figure 6.3).



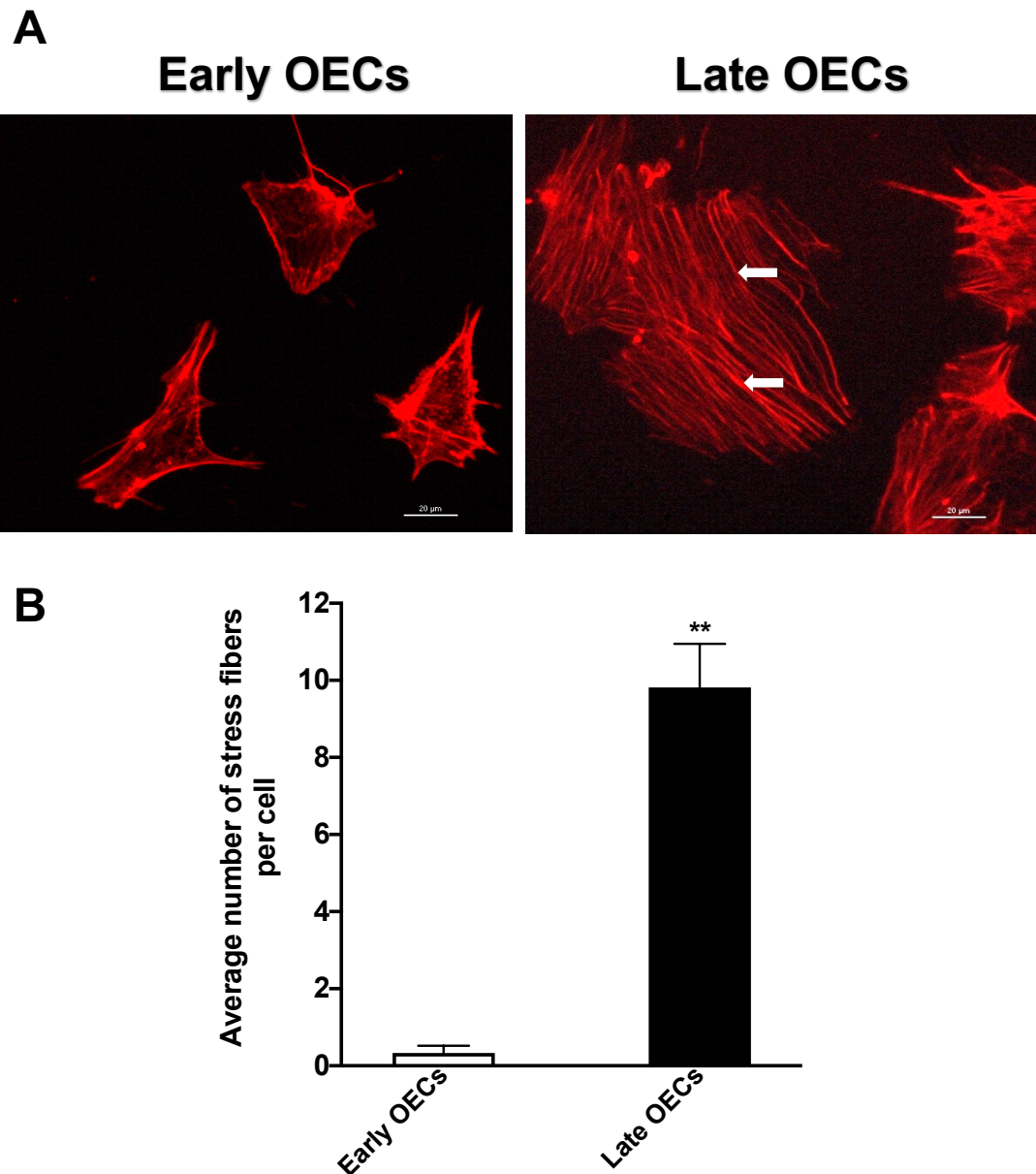
**Figure 6-3 Staining with anti-gamma H2AX antibody**

Early passage (P8) OECs and late passage (P14) OECs were cultured on coverslips to about 80% confluence before fixing and permeabilising them in 4% paraformaldehyde and 0.1% Triton X-100, respectively. Cells were then successively stained with primary antibody in 1% BSA in PBST (PBS+ 0.1% Tween 20) and then in secondary antibody (goat anti-rabbit IgG H&L) in 1% BSA. Coverslips were then visualised by fluorescent microscopy and positive cells were counted (A and B). Data are expressed as mean  $\pm$  SEM from three independent experiments.

\*\*\* $P < 0.0001$  compared to early OECs (unpaired t test). Scale bars: 25  $\mu$ m.

### **6.1.5 Senescence in OECs enhances stress fibers formation**

To study the effect of senescence on cellular cytoskeletal organisation, early passage (P8) and late passage (P14) OECs were cultured on coverslips in their respective media. F-actin filaments staining with rhodamine phalloidin was performed as described in chapter two. Compared to early passage OECs, late passage OECs appear to have significantly higher numbers and thicker stress actin stress fibres compared to early OECs. Also, late OECs shown to be bigger in size compared to early OECs (Figure 6.4).



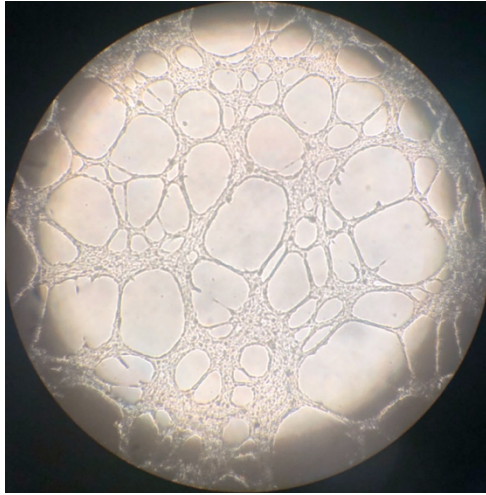
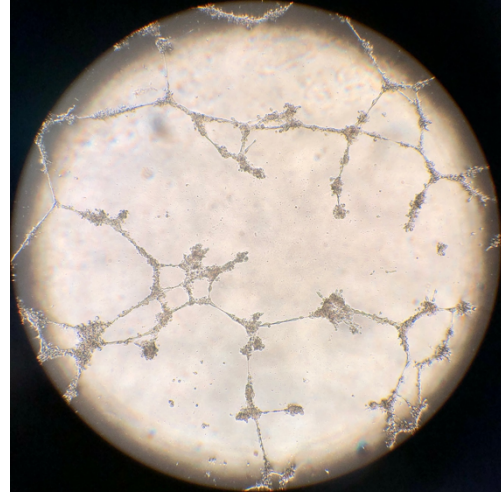
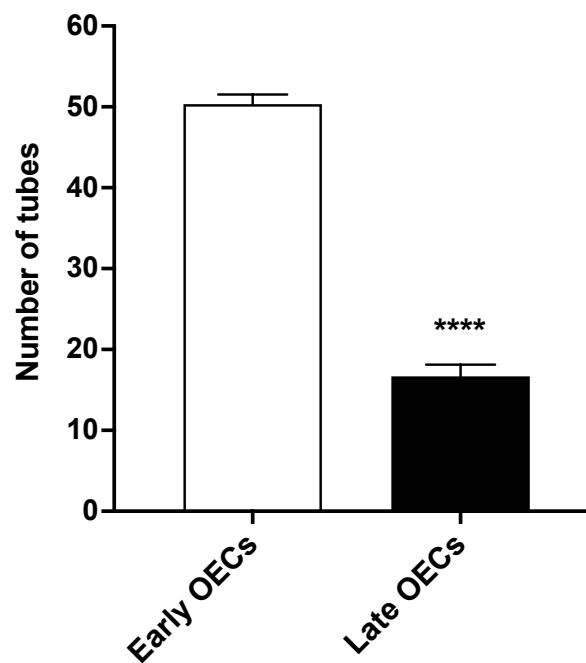
**Figure 6-4 Staining of actin microfilaments in early and late passage OECs**

Early passage (P8) OECs and late passage (P14) OECs were cultured on coverslips to about 80% confluence before fixing and permeabilising them in 4% paraformaldehyde and 0.1% Triton X-100, respectively. Cells were viewed under fluorescence microscope. Stress fibres are indicated with white arrows (A). Graph shows the average number of stress fibres per cell (B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\* $P < 0.008$  compared to early OECs. (Unpaired t test). Scale bars: 20  $\mu$ m.



#### **6.1.6 Senescent OECs fail to establish *in vitro* tubules**

To study the impact of senescence on the capacity of cells to form *in vitro* tubules, equal numbers of early passage (P8) and late passage (P14) OECs were seeded on 48-well plates that had been pre-coated with growth factor-reduced Matrigel as described in chapter two. The cells were then visualised under light microscope to examine the status of circle-like structure, as a marker of tube formation. Compared to early passage OECs, late passage OECs were not able to form well-established tubules. Besides, the number of tubes was markedly less in late passage OEC group (Figure 6.5).

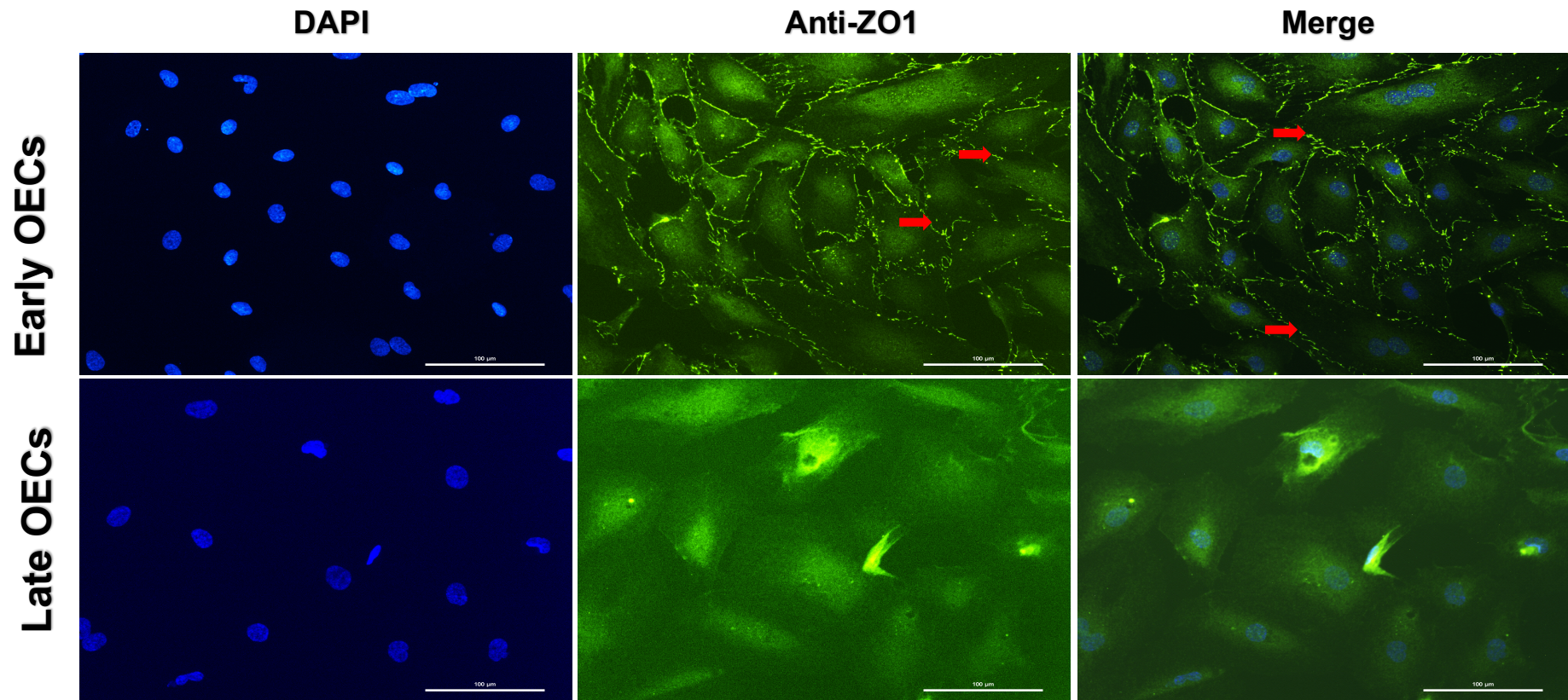
**A****Early OECs****Late OECs****B**

**Figure 6-5 Tubulogenic features of early and late passage OECs.**

An equal number of early passage (P8) and late passage (P14) OECs were cultured in 48-well plates pre-coated with growth factor-reduced Matrigel and incubated for 8 hr. The appearance of tube formation (a circle-like structure) was assessed by a light microscope. (A) Graph shows the average number of tube-like structures. (B) Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\* $P < 0.0001$  compared to early OECs (unpaired t test).

#### **6.1.7 Late passage OECs are not able to establish tight junctions**

To study the impact of senescence on cellular ability to form tight junctions, the localisation of tight junction protein ZO-1 was studied in early passage (P8) or late passage (P14) OECs. The results showed that early passage OECs could establish tight junctions. This was ascertained by the ubiquitous presence of ZO-1 throughout the entire cell monolayer. Unlike early passage OECs, cell margin or cell–cell border staining of ZO-1 appeared to be completely lost in late passage OECs, confirming the hypothesis that senescence adversely affects the ability of OECs to form tight junctions (Figure 6.6).

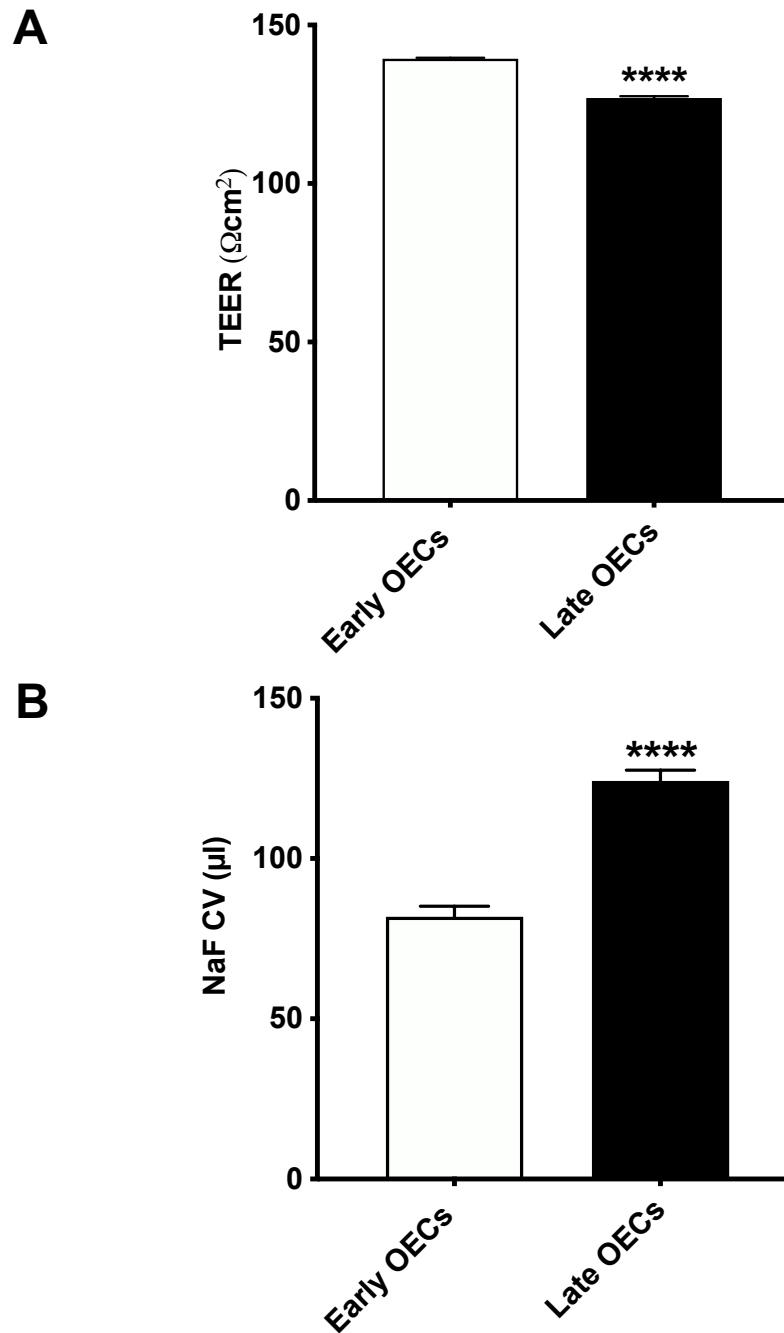


**Figure 6-6 Immunofluorescence labelling of ZO-1**

Early passage (P8) OECs and late passage (P14) OECs were cultured to about 80% confluence. Then, fixed in 4% paraformaldehyde. Then, incubated in primary antibody (anti ZO-1) in 1% BSA in PBST (PBS+ 0.1% tween 20). Then, incubated in secondary antibody (goat anti-rabbit IgG H&L) in 1% BSA. Next, cells were seen under fluorescence microscope. Red arrows show a uniform expression of ZO-1 throughout the entire cell monolayer. Scale bars: 100 µm.

#### **6.1.8 Senescent OECs fail to form BBB**

To investigate how the presence of senescent cell affect the BBB characteristics, *in vitro* models of human BBB were established by co-culture of HAs and HPs with early passage (P8), or late passage (P14) OECs as previously described (Shao and Bayraktutan, 2013a, Allen et al., 2010, Allen and Bayraktutan, 2009a, Srivastava et al., 2013a). The integrity and function of BBB were assessed by measurements of TEER and paracellular flux of NaF, respectively. While early passage OECs were able to establish tight and functional BBB. In contrast, BBB established with late passage OECs demonstrated significant decreases in TEER and increases in NaF readings (Figure 6.7).

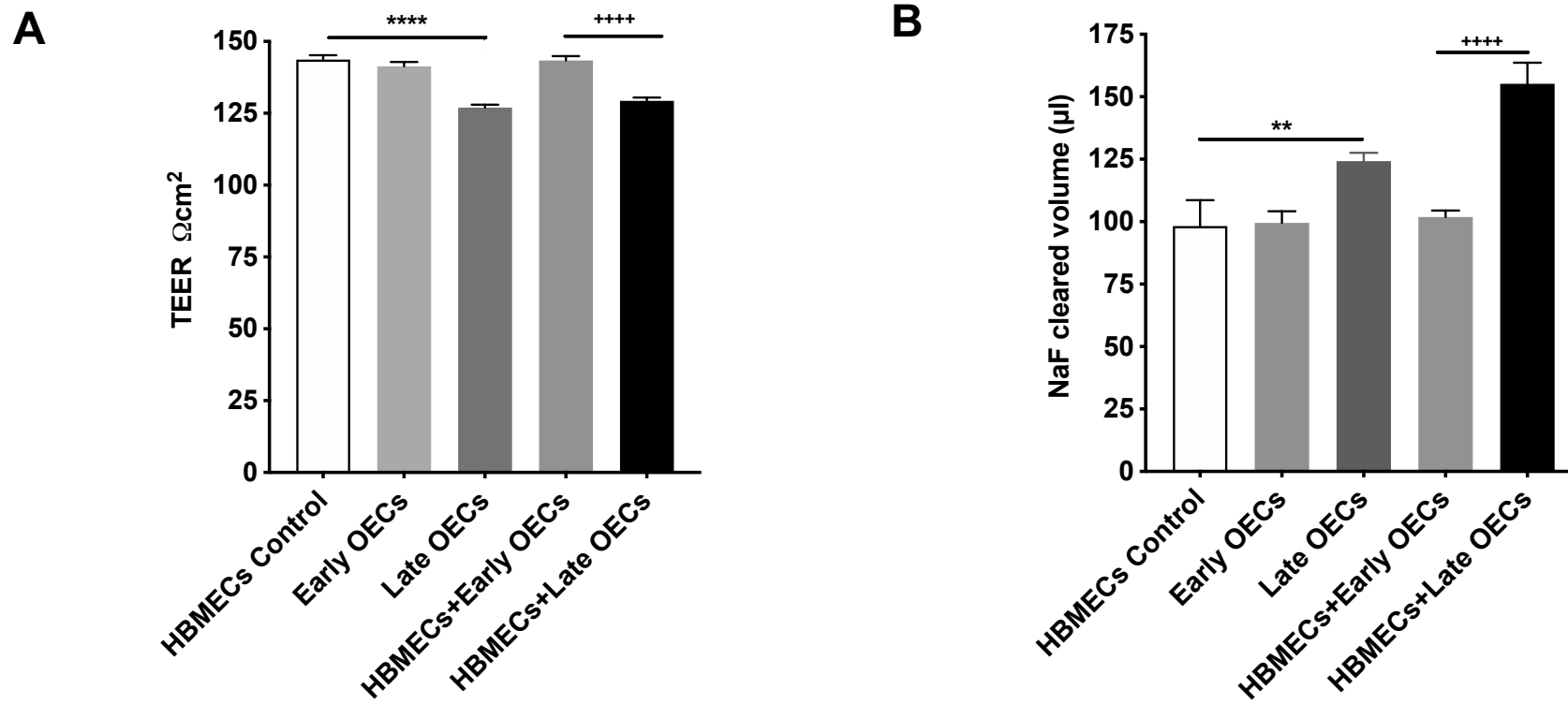


**Figure 6-7 Assessment of the integrity and function of the BBB established with early or late passage OECs**

*In vitro* models of human BBB were established by co-culture of HAs and HPs with either early passage (P8) or late passage (P14) OECs. The integrity and function of the BBB were assessed by measurements of TEER (A) and paracellular flux of NaF (B), respectively. Each data point represents mean  $\pm$  SEM from three independent experiments. \*\*\*\*  $P < 0.0001$  compared to early OECs (unpaired t test).

### **6.1.9 The presence of late passage OECs adversely affect the capacity of HBMECs to form BBB**

To investigate whether the presence of early passage (P8) or late passage (P14) OECs may affect the capacity of HBMECs to form tight and functional BBB models, we mix HAs and HPs with the combinations of HBMECs with early or late OECs. The integrity and function of the BBB were assessed by measurements of TEER and paracellular flux of NaF, respectively. The results show that when mixed with early passage OECs, HBMECs formed well-established tight and functional BBB models as evidenced by similar TEER and NaF readings compared to BBB models formed with HBMEC alone. In contrast, the presence of late passage OECs dramatically reduced the capacity of HBMECs to form tight and functional BBB models (Figure 6.8).



**Figure 6-8 Assessment of the integrity and function of the BBB established with late passage OECs and HBMECs**

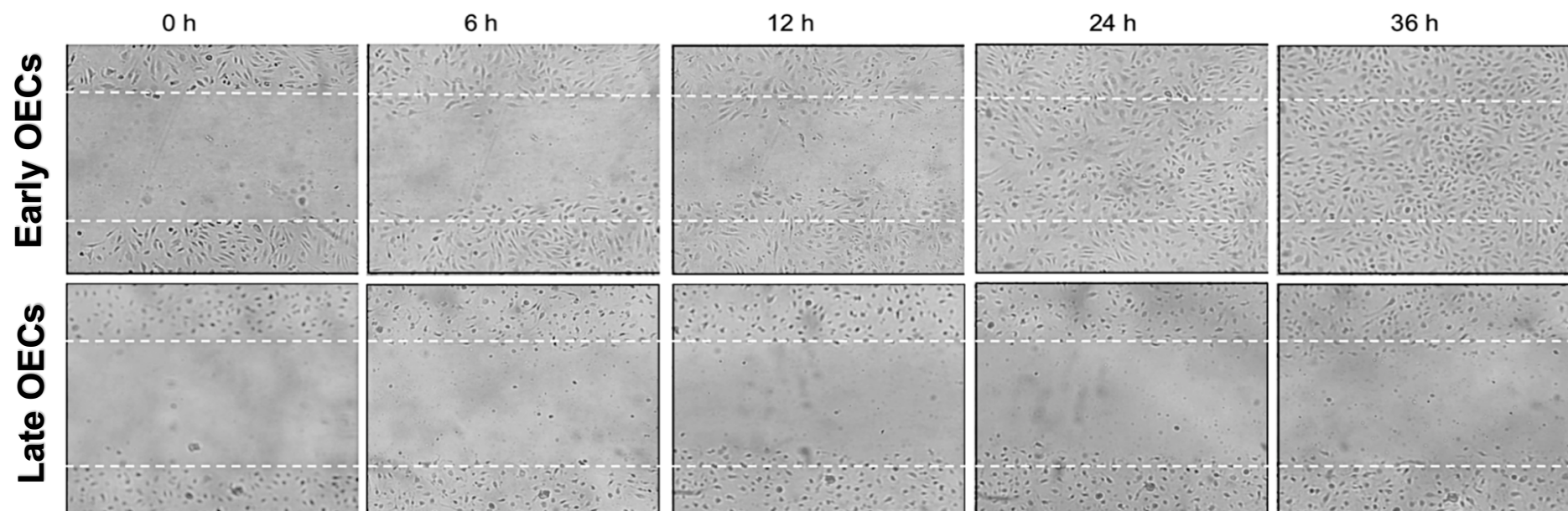
Human BBB models were established by co-culture of HAs and HPs with the combinations of HBMECs with early or late OECs. The integrity and function of the BBB were assessed by measurements of TEER (A) and paracellular flux of NaF (B), respectively. Each data point represents mean  $\pm$  SEM from three independent experiments. \*\* $P < 0.005$ , \*\*\* $P < 0.0001$  compared to HBMECs control. \*\*\*\* $P < 0.0001$  compared to HBMECs+Early OECs (one-way analysis of variance followed by Tukey's post hoc analysis).

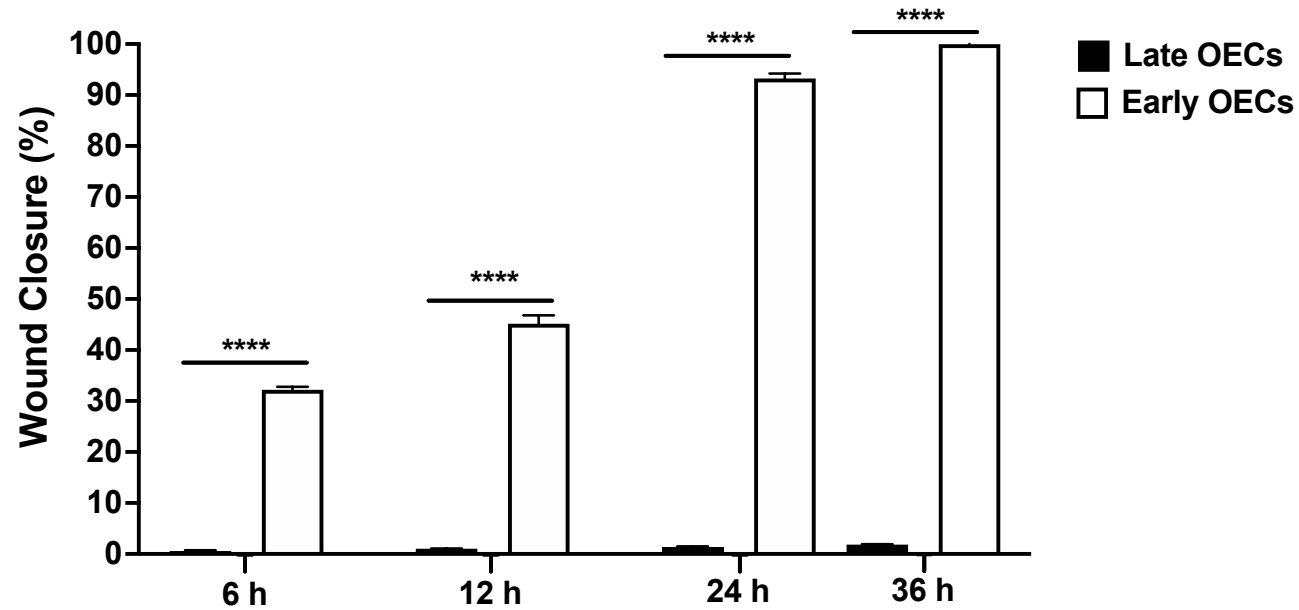


#### **6.1.10 Senescent OECs fail to proliferate and repair endothelial damage**

In brief, similar cell numbers of early passage (P8) and late passage (P14) OECs were seeded in 6-well plates and cultured until they have reached full confluence. A scratch was then made manually, and images of wound closure were taken at 0-, 6-, 12-, 24-, and 36-hours post-scratch period. The assessment of cellular proliferative and migratory capacity using wound scratch assays showed that early OECs repaired endothelial layer damage in a time-dependent manner. It also showed that the percentages of wound closure realised by early OECs, at 6-, 12-, 24-, and 36-hours, were significantly higher than those realised by late passage OECs which in fact failed to show any proliferative or migratory activity (Figure 7.9).

**A**

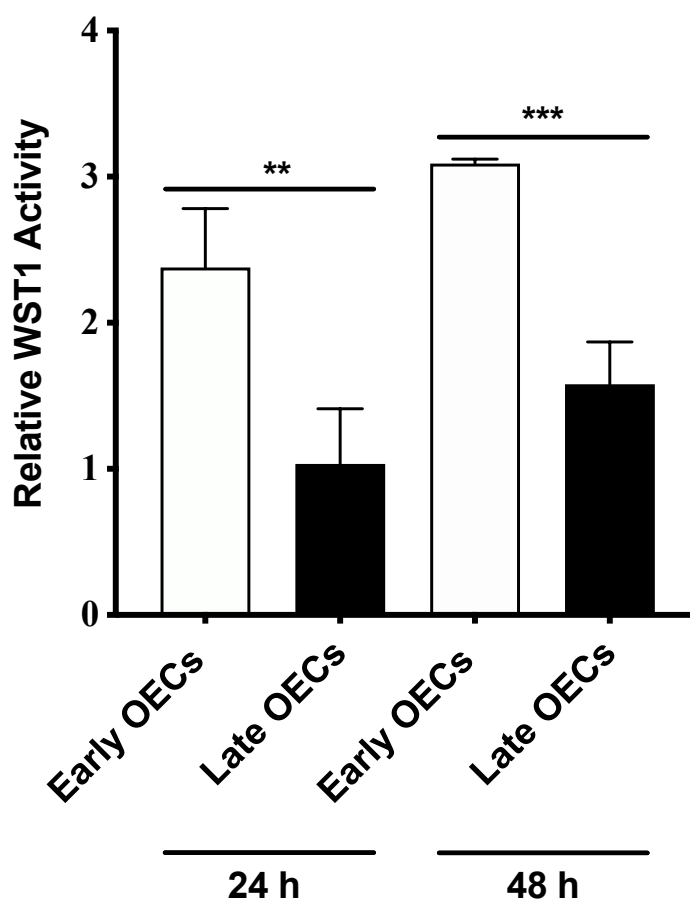


**B****Figure 6-9 Effects of senescence on OECs wound closure**

Early passage (P8) and late passage (P14) OECs were cultured to confluence. By using a p1000 micropipette tip, a scratch was made manually down the centre of the well in one swift motion. (A) images of wound closure were captured at 0, 6-, 12-, 24-, and 36-hours time points post-scratch. (B) Data are presented as mean  $\pm$  SEM from three independent experiments. \*\*\*\* $P < 0.0001$  compared with late OECs (one-way analysis of variance followed by Tukey's post hoc analysis).

### 6.1.11 Senescence affects the ability of OECs to proliferate

To further assess the proliferative capacity of late OECs, cell proliferation reagent WST-1 was used as described in chapter two. The study reveals that, compared to early passage OECs, the rate of proliferation of late passage OECs are significantly reduced and independent of time (Figure 6.10).

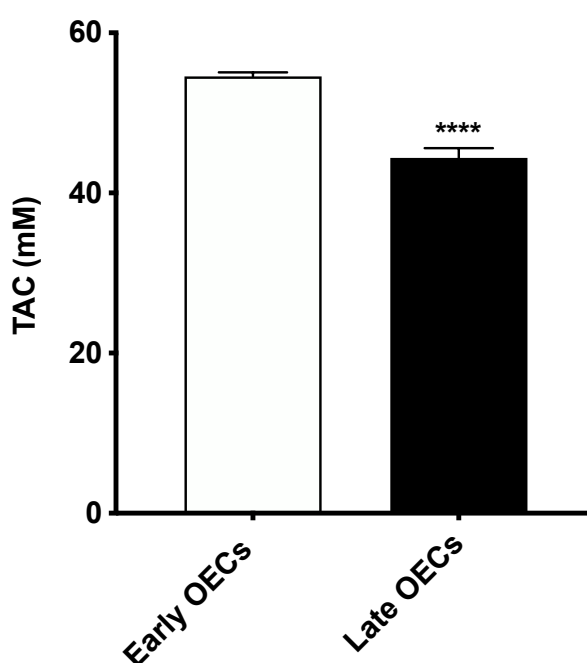


**Figure 6-10 Assessment of proliferative capacity of senescent OECs by WST-1**

Early passage (P8) and late passage (P14) OECs were cultured in T-25 flasks to 80% confluence. By using proliferation reagent WST-1, the proliferation rates of cells were assessed over a period 24 hours and 48 hours. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\* $P < 0.002$ , \*\*\* $P < 0.001$  compared to early OECs at corresponding time points (one-way analysis of variance followed by Tukey's post hoc analysis).

### 6.1.12 Late passage OECs possess lower total antioxidant levels

A total antioxidant capacity assay kit was used to quantify the antioxidant capacity of both early passage (P8) and late passage (P14) OECs. The assay was carried out as described on chapter two. The results show that late passage OECs express a significantly lower antioxidant capacity compared to early passage OECs (Figure 6.11).

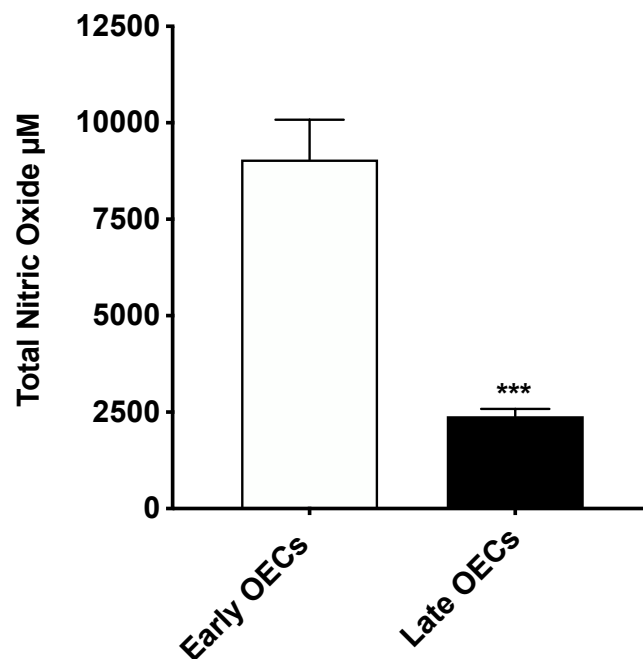


**Figure 6-11 Effect of senescence on total antioxidant capacity of OECs.**

Early passage (P8) and late passage (P14) OECs were cultured to about 90% confluence. The cells were then washed and resuspended in double distilled water. The mixture was then centrifuged. Trolox standard solution was used to prepare standard curve dilution. Then, 100  $\mu$ l of working solution was added to all wells before being incubated at room temperature. Then plates measured in microplate reader. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to early OECs (Unpaired t test).

### 6.1.13 Late passage OECs release low amounts of nitric oxide

NO derived from endothelial cells (eNOS) has a major neuroprotective function in ischemic stroke. In brain tissue, eNOS is mainly generated by microvascular endothelial cells, it has several functions including the preservation of the blood-brain barrier, the regulation of cerebral microvascular tone, the reduction of oxidative stress(Chen et al., 2017). Nitric oxide assay kit was used to quantify NO production as described in detail in chapter two. This study shows that late passage OECs express significantly lower amounts of NO compared to early passage OECs (Figure 6.12).

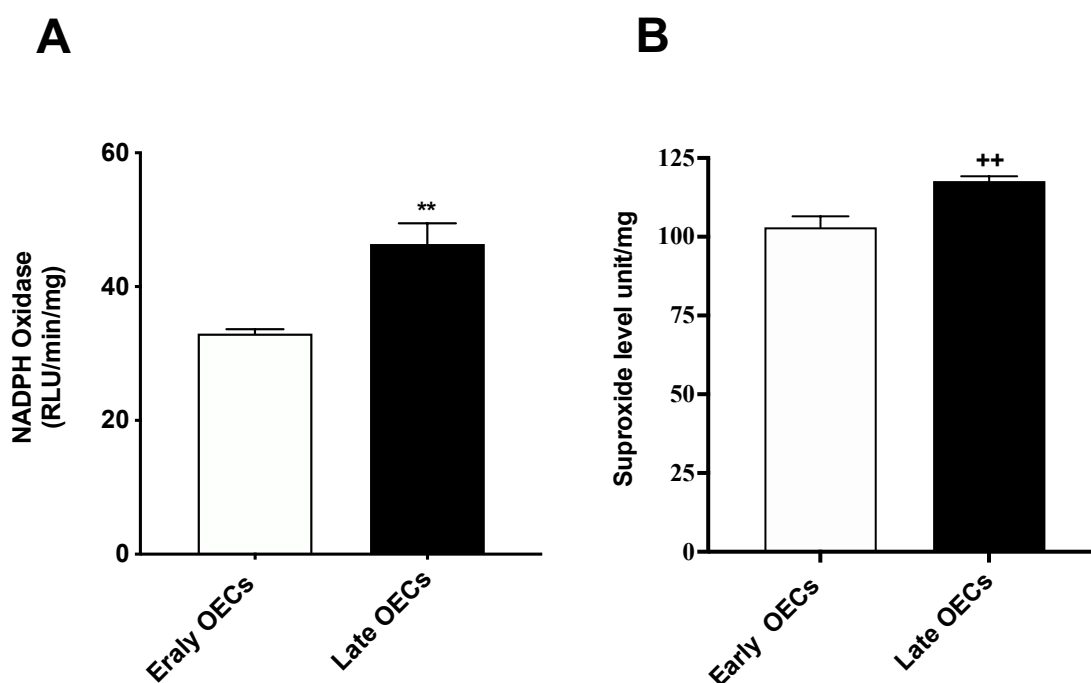


**Figure 6-12 Effects of senescence on nitric oxide expression on OECs.**

Early passage (P8) and late passage (P14) OECs were cultured to about 90% confluence. Then cells were detached and resuspended before nitric oxide assay was used to measure the amount of NO. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*P<0.001 compared to early OECs (Unpaired t test).

#### 6.1.14 Late passage OECs release high amounts of NADPH oxidase and superoxide anion

The NADPH oxidase activity and total  $O_2^{\bullet-}$  levels were measured by lucigenin chemiluminescence and cytochrome C reduction assays as described in chapter two (Abdullah and Bayraktutan, 2014, Shao and Bayraktutan, 2013a, Ulker et al., 2004). This study shows that compared to early passage OECs, late passage OECs express significantly higher NADPH oxidase activity and superoxide anions levels (Figure 6.13).



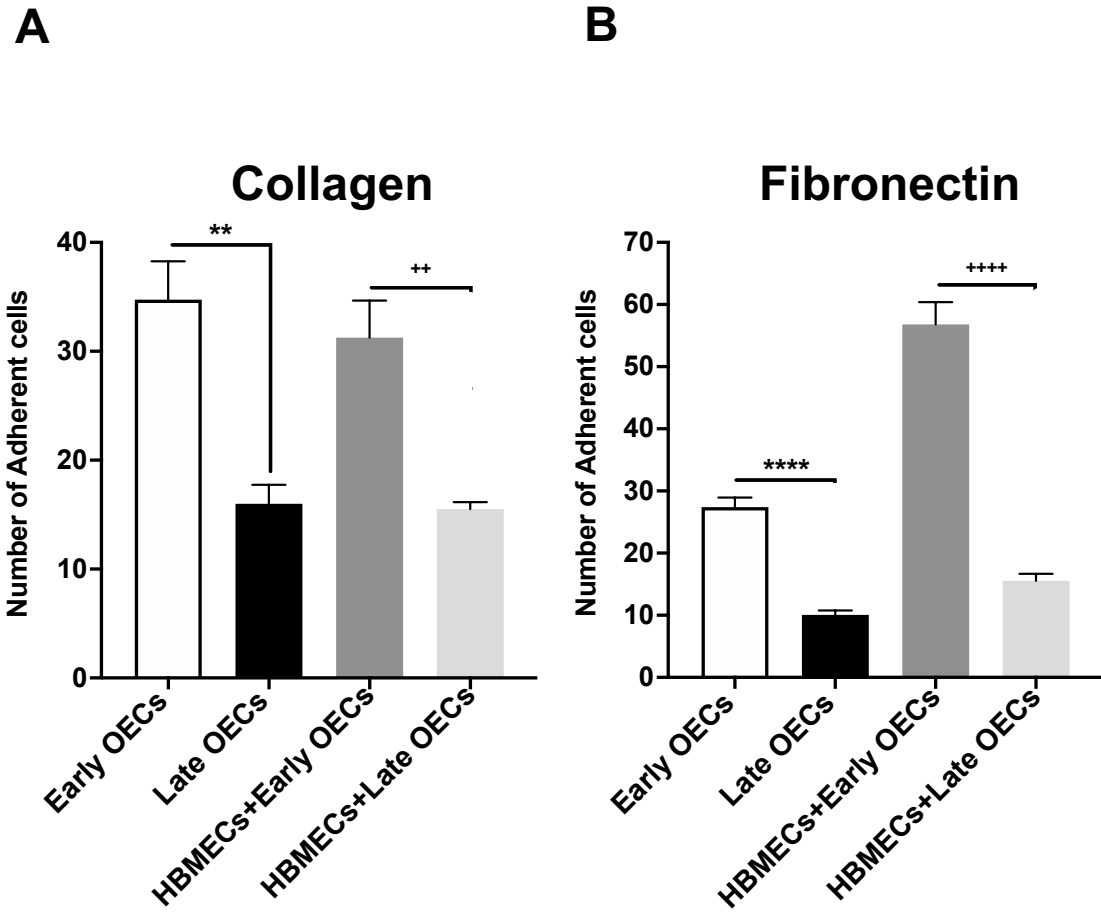
**Figure 6-13 Effects of senescence on OECs expression of NADPH oxidase and superoxide anion levels.**

Early passage (P8) and late passage (P14) OECs were cultured to about 90% confluence. The activity of NADPH oxidase was detected by lucigenin chemiluminescence assays and superoxide anion levels were measured by cytochrome reduction assays, respectively. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\* $P < 0.002$ , ++ $P < 0.003$  compared to early OECs (Unpaired t test).

#### **6.1.15 Senescence affects the adhesion ability of OECs**

The adhesion capacity of early passage or late passage OECs alone or in combination with HBMECs were studied. This study shows that late passage OECs lose their ability to adhere to collagen or fibronectin. This was evidenced by significant reductions in the numbers of adherent late OECs compared to early OECs. Similarly, combination of late passage OECs with HBMECs appears to have less adherence capacity than the mixture of early OECs and HBMECs. This was evidenced by significant reductions in the numbers of adherent late OEC and HBMECs mixture. Senescence appears to significantly impair the adherence capacity of late passage OECs to the plates pre-coated with collagen or fibronectin in presence or absence of HBMECs (Figure 6.14).





**Figure 6-14 effects of senescence on adhesion capacity of OECs**

Equal number of early passage (P8) or late passage (P14) OECs alone or in combination with HBMECs were placed for 30 minutes on 24-well plates pre-coated overnight with fibronectin or collagen. Then, adherent cells were manually counted. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\* $P < 0.002$ , \*\*\* $P < 0.0001$  compared to early OECs. \*\* $P < 0.006$ , \*\*\*\* $P < 0.0001$  compared to HBMECs + early OECs (one-way analysis of variance followed by Tukey's post hoc analysis).

### 6.1.16 Discussion

Cellular senescence was defined as a physiological mechanism which restricts the lifespan of living cells due to reduction in the intrinsic replicative cycle that commences following a certain number of cells divisions (Rurali et al., 2016, Herrera et al., 2010). Prospective use of OECs as cell therapy require large numbers of these cells for allogenic or autologous administration. It has been shown that about 30 million OECs are required for an individual with acute myocardial infarction (Dubois et al., 2010). This number is proven to be achievable for peripheral blood derived OECs (Medina et al., 2013). However, our study shows that ex vivo expanded OECs can enter senescence phase which ultimately cause a significant impact on their vasculoreparative functions. We show that senescence damage OECs phenotype and induce stress fibres formation. Stress fibers attach to adherence junctions of adjacent cells. When stress fibers start to contract under stress, this leads to formation of intracellular gaps between neighboring cells, causing an increase in BBB permeability (Millán et al., 2010, Manaenko et al., 2016). This support our findings that senescent OECs are not able to form tight junctions and establish functional BBB models. Indeed, while our study shows that OECs senescence can occur when expanded *ex vivo*, other investigators reported that it can happen *in vivo* (Satyanarayana et al., 2003, Schmitt et al., 2002). This may support the notion that people with poorer prognosis following ischaemic vascular diseases might have dysfunctional senescent OECs. We also proven that late passage OECs lose their proliferation, migration, and adhesion capacities. *In vitro* investigations of isolated OECs from older people shown that their survival, migration, and proliferation rates appear to be disturbed (Scheubel et al., 2003,

Imanishi et al., 2008). Senescent cells have been discovered in mammalian tissues which were linked to cellular stress, ageing, and age-related diseases (Erusalimsky, 2009, Campisi and d'Adda di Fagagna, 2007, Erusalimsky and Kurz, 2005). Other studies have also revealed that cells may enter senescence phase rapidly in response to different stressors such as oxidative stress, radiation, and DNA damage (Imanishi et al., 2008, Gire et al., 2004). To work as reparative agent in ischaemic (micro)environments, OECs must be equipped with defence system against oxidative stress. In chapter four, we proven that OECs have higher antioxidant capacity compared to mature endothelial cells. Interestingly, resistance to oxidative stress has been known as one of the cardinal features of stem cells (Blokhina et al., 2003). In this chapter, we show that senescence has substantially decreased the antioxidant capacity of OECs which underscore their ability to resist oxidative stress in ischaemic niches. Other studies revealed that OECs isolated from older subjects have lower antioxidants levels, in particular glutathione peroxidase-1, and higher sensitivity to oxidative stress-induced cell death compared to OECs of younger subjects (He et al., 2009). Also, it has been suggested that reduced number of circulating EPCs may be used as predictor for vascular ageing and diagnostic/prognostic for age-related degenerative vascular diseases (Balistreri et al., 2019). OECs mobilization to the site of ischaemic damage requires eNOS expression and NO generation (Bernardini et al., 2005, Altabas et al., 2016). Our investigation shows that senescent OECs have expressed lower availability of NO, and higher levels of NADPH oxidase and  $O_2^{\bullet-}$ . These can generate higher levels of ROS which known as major senescence inducer (Endtmann et al., 2011, Williamson et al., 2012). Oxidative stress known to accelerate and induce

cellular senescence by shortening telomere and limiting the number of cell divisions. Telomeres are located at the end of the chromosomes that prevent their degradation during DNA replication. During each cycle of cellular division, telomeres continuously shorten until cell reach senescence stage. Interestingly, OECs, like other stem cells, can extend their telomeres through secretion of telomerase reverse transcriptase enzyme (TERT). Oxidative stress and reduction in bioavailability of NO, which found to be major features of senescent OECs in our study, are also known to reduce the activity of TERT (Kushner et al., 2009, Herrera et al., 2010, Marín et al., 2013). Other study, assessed the risk of hypertension of postmenopausal females, has revealed that prehypertensive subjects of this population have higher numbers of senescent OECs with impaired migration and proliferation rates, and reduced levels of NO expression. This indicates that senescence of OECs could be associated with the pathophysiology of prehypertension and increased risk of vascular events (Wu et al., 2019). Our data suggest that enhancing NO availability and/or targeting oxidative stress may be potential therapeutic target to halt senescence of OECs *in vivo*, to maintain endovascular integrity and prevent vascular diseases, or *ex vivo* to enhance their expansion for cell therapy. Long exposure to risk factors of chronic vascular diseases continuously damages endothelial cells which requires their rigorous replacement. At the same time, these factors might induce early senescence of OECs and may affect their reparative and angiogenic capacity. The studies outlined in this thesis prove that senescent OECs lose the capacity to form well-established tubules *in vitro*. Other study showed that culture media of OECs isolated from diabetic patient has a feature of low angiogenic capacity and contain inhibitor that reduces tube formation *in*

*vitro* (Tepper et al., 2002, Loomans et al., 2004, Imanishi et al., 2008). It is safe to propose that inability of OECs to form tubules *in vitro* might be used as indicator for their senescence and predictor for vascular events. To be used as cell therapy for ischaemic stroke, OECs should migrate to the site of damage and repair BBB by replacing dead/dying endothelial cells. This complex process requires cells to form tight junction proteins. Our *in vitro* studies show that, unlike early OECs, late OECs are not able to form tight junctions. This explains our findings which show that senescent OECs are not able to form well-established BBB. Ex vivo expansion of OECs ultimately leads to their senescence associated with cellular dysfunction. Regulation of  $O_2^{\bullet-}$  and NADPH oxidase expression, and enhancement of NO bioavailability could be potential strategies to prevent OECs senescence to harness their ex vivo expansion and protect their reparative features required for cellular therapeutics (Reid et al., 2013).

### **6.1.17 Summary**

In conclusion, this study demonstrates that OECs expanded *ex vivo* enter senescence and this in turn adversely influences their vasculoreparative functions. Our findings have shown that senescence damages cellular architecture and phenotype and senescent OECs lose their proliferative, migratory, and adhesive capacities. They have substantially lower antioxidant capacity than their younger counterparts which underscore their inability to resist oxidative stress in ischaemic environments. Moreover, senescent OECs generate lower levels of NO, and possess higher levels of NADPH oxidase and

$O_2^{\bullet-}$ . Senescent OECs lose their angiogenesis capacity and cannot form well-established BBB.

## **7 EFFECT OF OEC-CM ON BLOOD-BRAIN BARRIER DAMAGE**

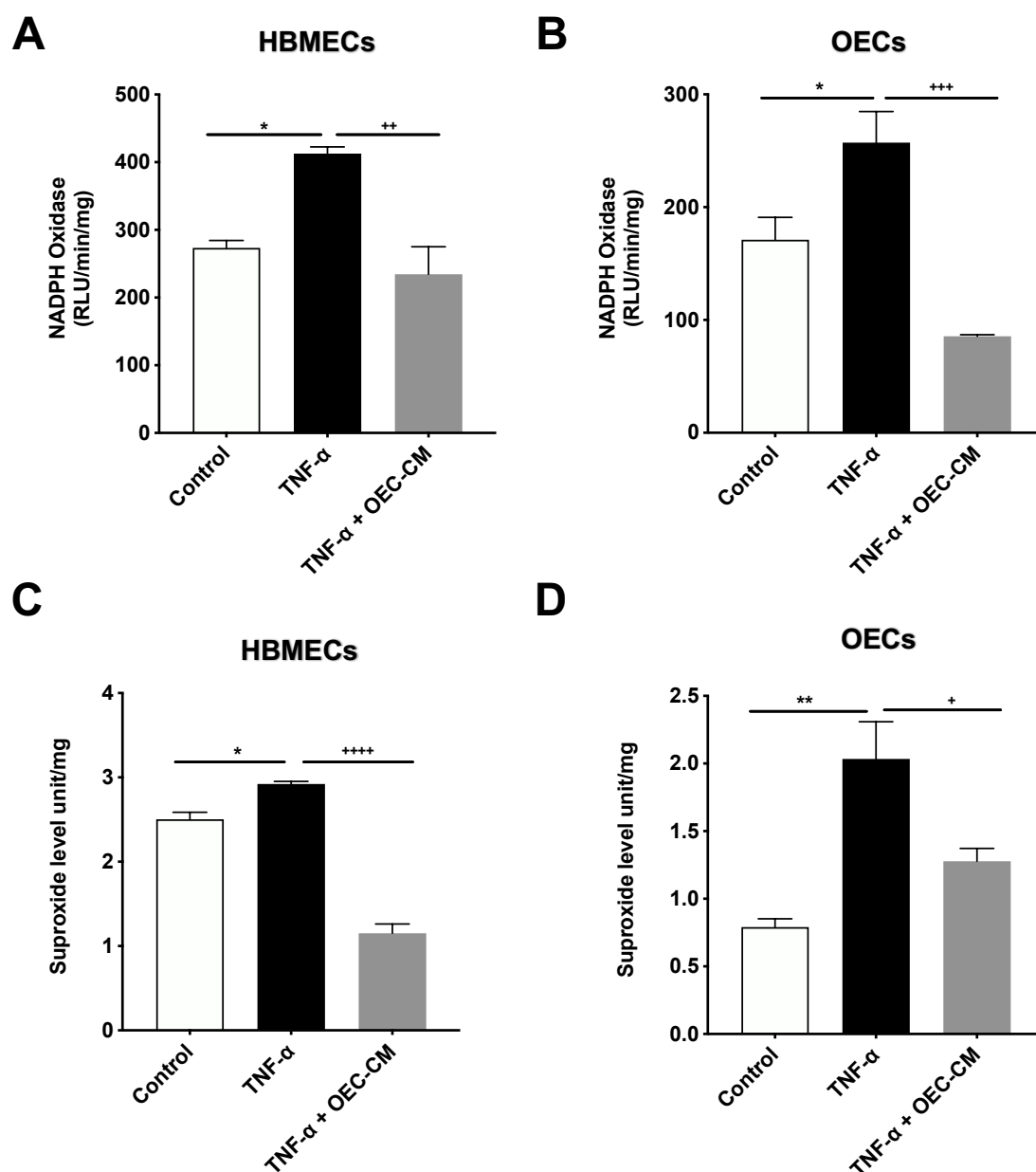
### **7.1.1 Introduction and aims**

In the previous chapters, we showed that OECs can restore BBB integrity and function by direct physical incorporation into the site of damage. TNF- $\alpha$  is one of the pro-inflammatory cytokines that severely affects the integrity and function of BBB during ischaemic stroke. As many studies have revealed that OECs secrete a vast range of substances with important vascular reparative or protective functions, in this study we investigated whether treatments with OEC-CM may somewhat negate the deleterious effects of TNF- $\alpha$  on BBB integrity and function and also attenuate its possible stimulatory role in cellular senescence. To investigate this, OEC-CM (to be used as cell-free therapy) was obtained by incubating OECs in growth factors- and serum-free EBM2 under hypoxic conditions for 48 hours.

### **7.1.2 OEC-CM inhibits TNF- $\alpha$ induced NADPH oxidase activity and superoxide release in HBMECs and OECs**

Exposure of HBMECs and OECs to highly purified recombinant human TNF- $\alpha$  in the absence or presence of OEC-CM showed that TNF- $\alpha$  increased the activity of NADPH oxidase and O<sub>2</sub><sup>•-</sup> release in both cell types which were significantly attenuated with co-treatments with OEC-CM (Figure 7.1).



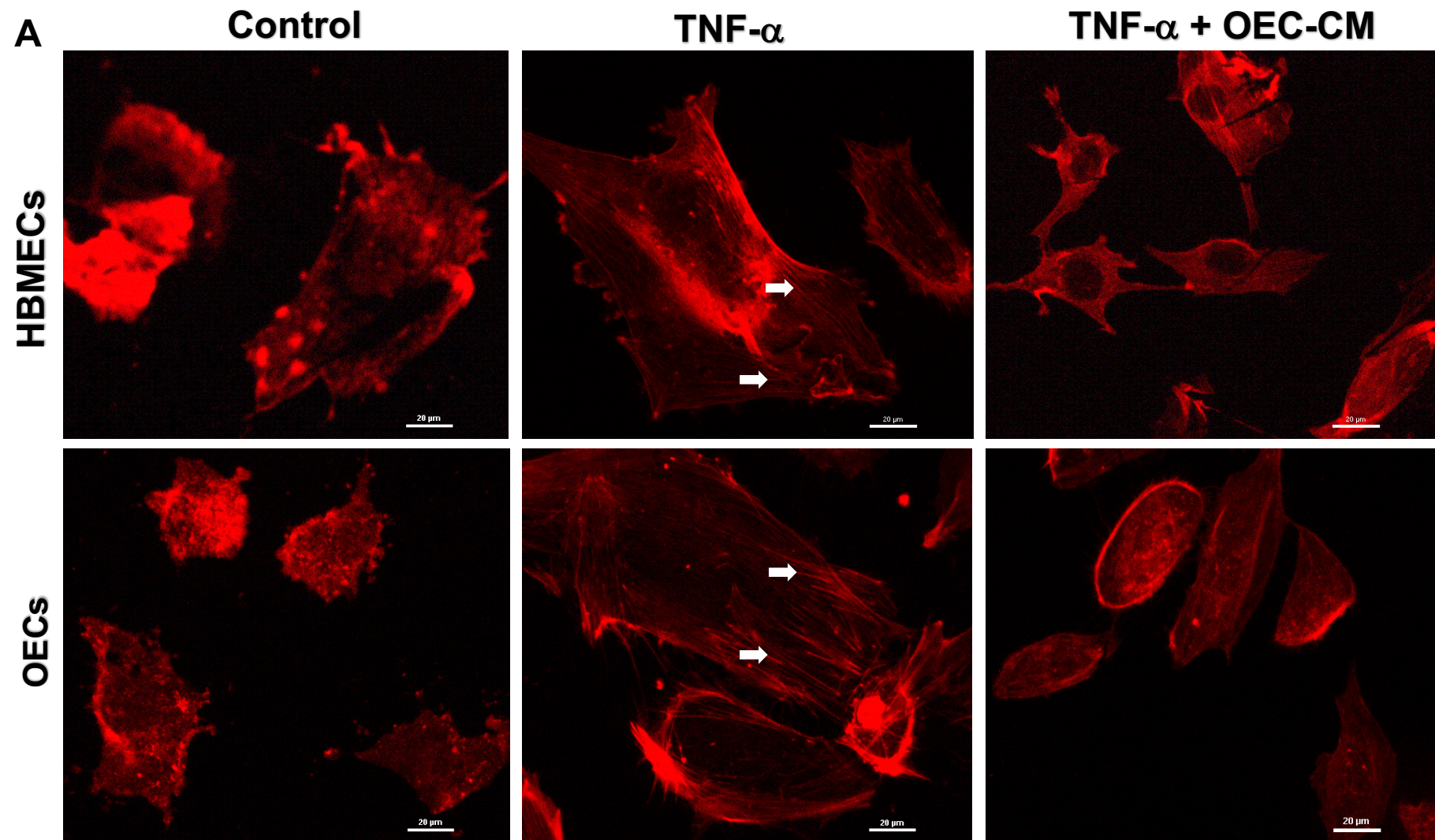


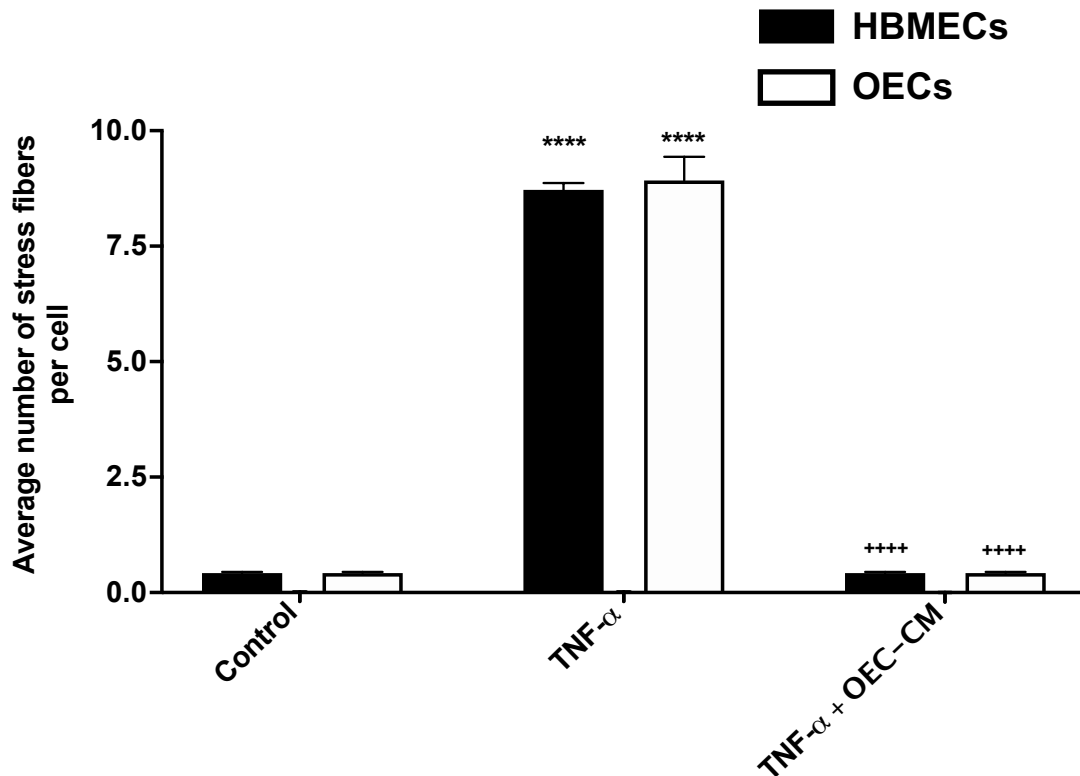
**Figure 7-1 Effect of OEC-CM on NADPH oxidase activity and superoxide anion levels in HBMECs and OECs**

HBMECs or OECs were cultured to ~90% confluence before exposure to TNF- $\alpha$  for 6 h in the absence or presence of OEC-CM. The activity of NADPH oxidase was detected by lucigenin chemiluminescence assays. Superoxide anion levels were measured by cytochrome reduction assays. Data are expressed as mean  $\pm$  SEM from three independent experiments. \*P<0.05, \*\*P<0.01 compared to control. \*P<0.05, \*\*P<0.01, \*\*\*P<0.003, \*\*\*\*P<0.0001 compared to corresponding cells exposed to TNF- $\alpha$  (one-way analysis of variance followed by Tukey's post hoc analysis).

### **7.1.3 OEC-CM inhibits TNF- $\alpha$ -induced stress fiber formation in HBMECs and OECs**

To study the effect of OEC-CM on cytoskeletal organisation, HBMECs and OECs were cultured on coverslips until they reach a confluence of  $\sim 80\%$ , then exposed to TNF- $\alpha$  (10 ng/ml) in the absence or presence of OEC-CM for 6 hours. Exposure to TNF- $\alpha$  induced the formation and number of actin stress fibres in HBMECs and OECs compared to respective controls. However, treatments with OEC-CM appeared to suppress stress fibres formation in both cell types. This was evidenced by significant decreases in the numbers of stress fibres compared to cells exposed to TNF- $\alpha$  (Figure 7.2).



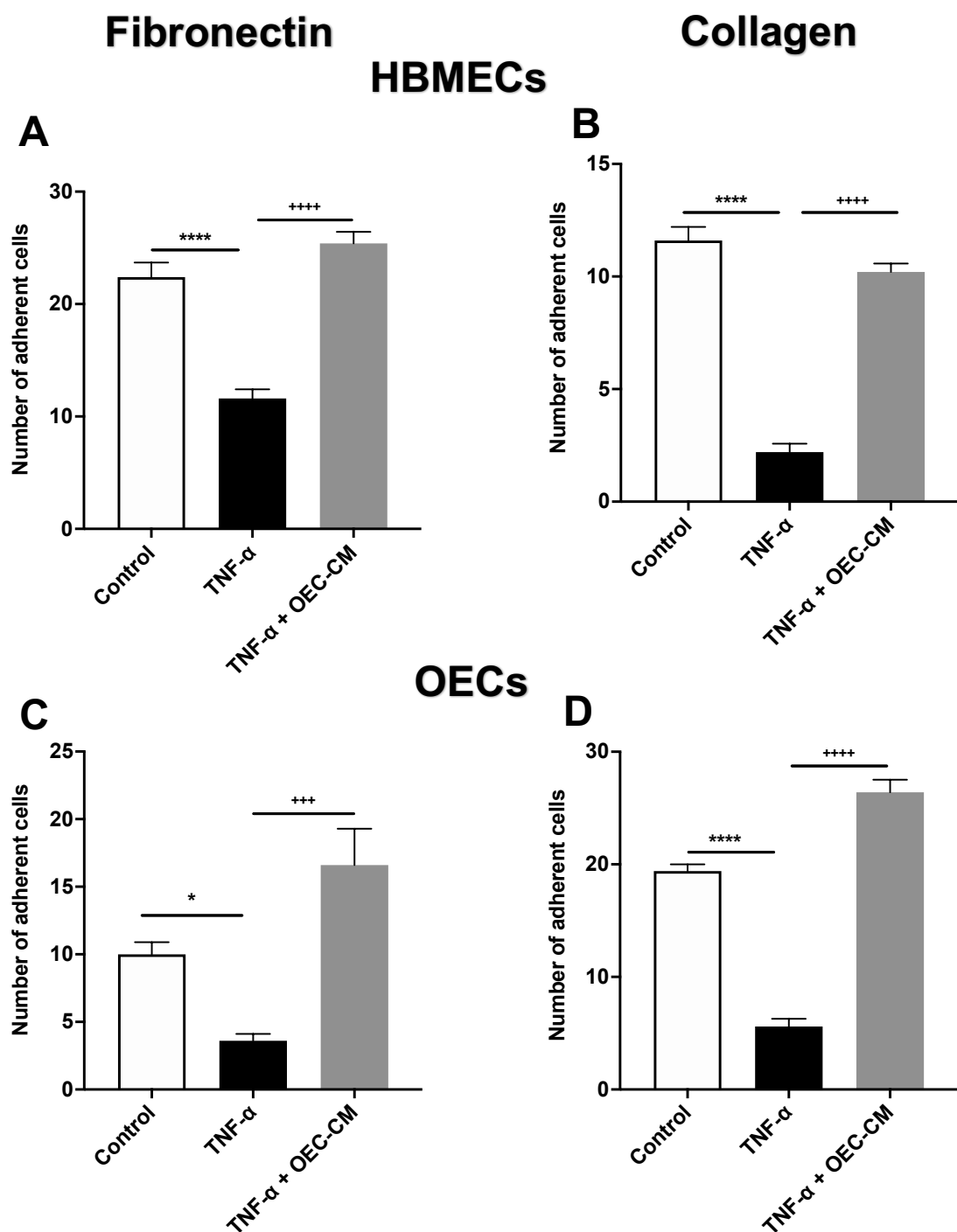
**B**

**Figure. 7-2. F-Actin microfilament staining in HBMECs and OECs subjected to TNF- $\alpha$  damage and OEC-CM treatment**

HBMECs or OECs were cultured to ~80% confluence before exposure to TNF- $\alpha$  for 6 h in the absence or presence of OEC-CM. Cells were fixed in 4% paraformaldehyde/PBS for 20 min and then permeabilised in 0.1% Triton X-100/PBS for 15 min prior to staining for 60 min in 1x Rhodamine Phalloidin. Then, cells were viewed under fluorescence microscope and the number of stress fibres were calculated manually. Data are expressed as mean  $\pm$  SEM from three independent experiments (A and B). \*\*\*\*P<0.0001 compared to control. \*\*\*\*P<0.0001 compared to corresponding cells exposed to TNF- $\alpha$  (one-way analysis of variance followed by Tukey's post hoc analysis). Scale bars: 20  $\mu$ m.

#### **7.1.4 OEC-CM induces cellular adhesion following TNF- $\alpha$ damage**

Scrutiny of the effect OEC-CM on the adhesion capacity of HBMECs or OECs subjected to TNF- $\alpha$  showed significant reductions in the number and capacity of HBMECs and OECs that adhered to plates pre-coated with fibronectin or collagen. Co-treatments with OEC-CM mitigated the effect of TNF- $\alpha$  on both cell types (Figure 7.3).

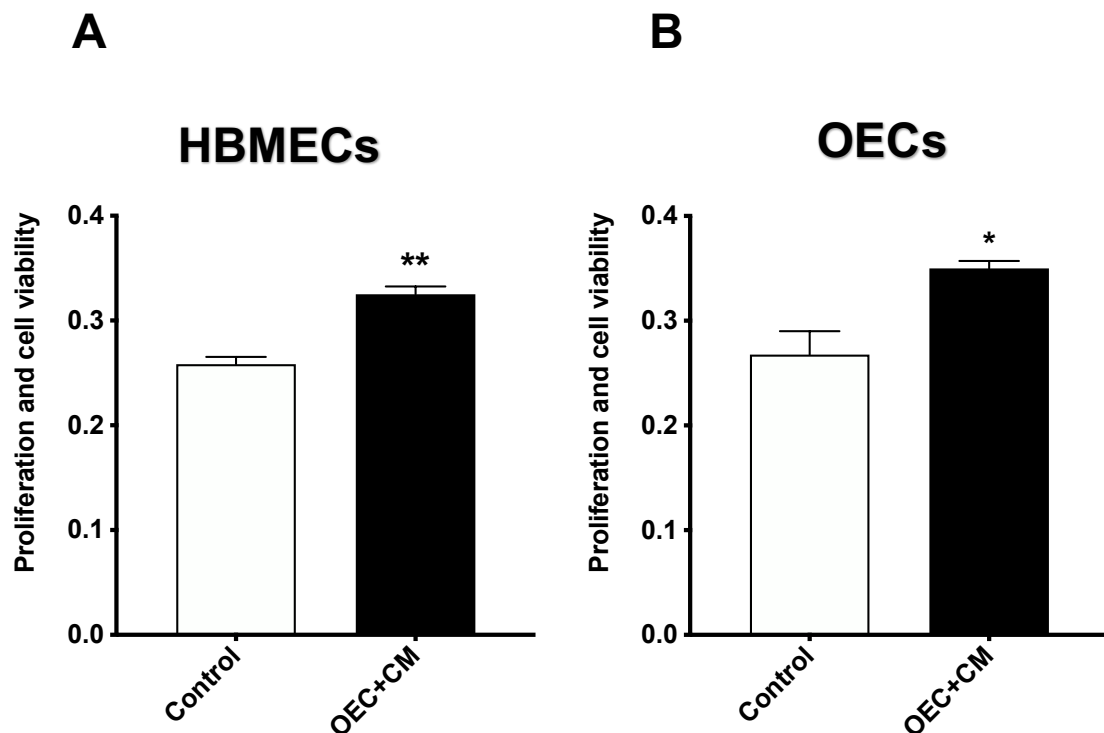


**Figure 7-3 Adhesion assay and effect of OEC-CM**

An equal number of HBMECs or OECs were seeded on 24-well plates pre-coated with fibronectin or collagen. Then cells were exposed to TNF- $\alpha$  for 30 minutes in the absence or presence of OEC-CM. Then, plates were gently washed, and adherent cells were counted (A-D). Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$  compared to control. \*\*\* $P < 0.0005$ , \*\*\*\* $P < 0.0001$  compared to cells exposed to TNF- $\alpha$  (one-way analysis of variance followed by Tukey's post hoc analysis).

### 7.1.5 OEC-CM induces proliferation of HBMECs and OECs

To determine the effect of OEC-CM on HBMECs and OECs proliferation and viability, WST-1 proliferation assays were carried out. These studies showed that exposure of HBMECs or OECs to OEC-CM significantly induced the viability and proliferative capacity in both cell types (Figure 7.4).



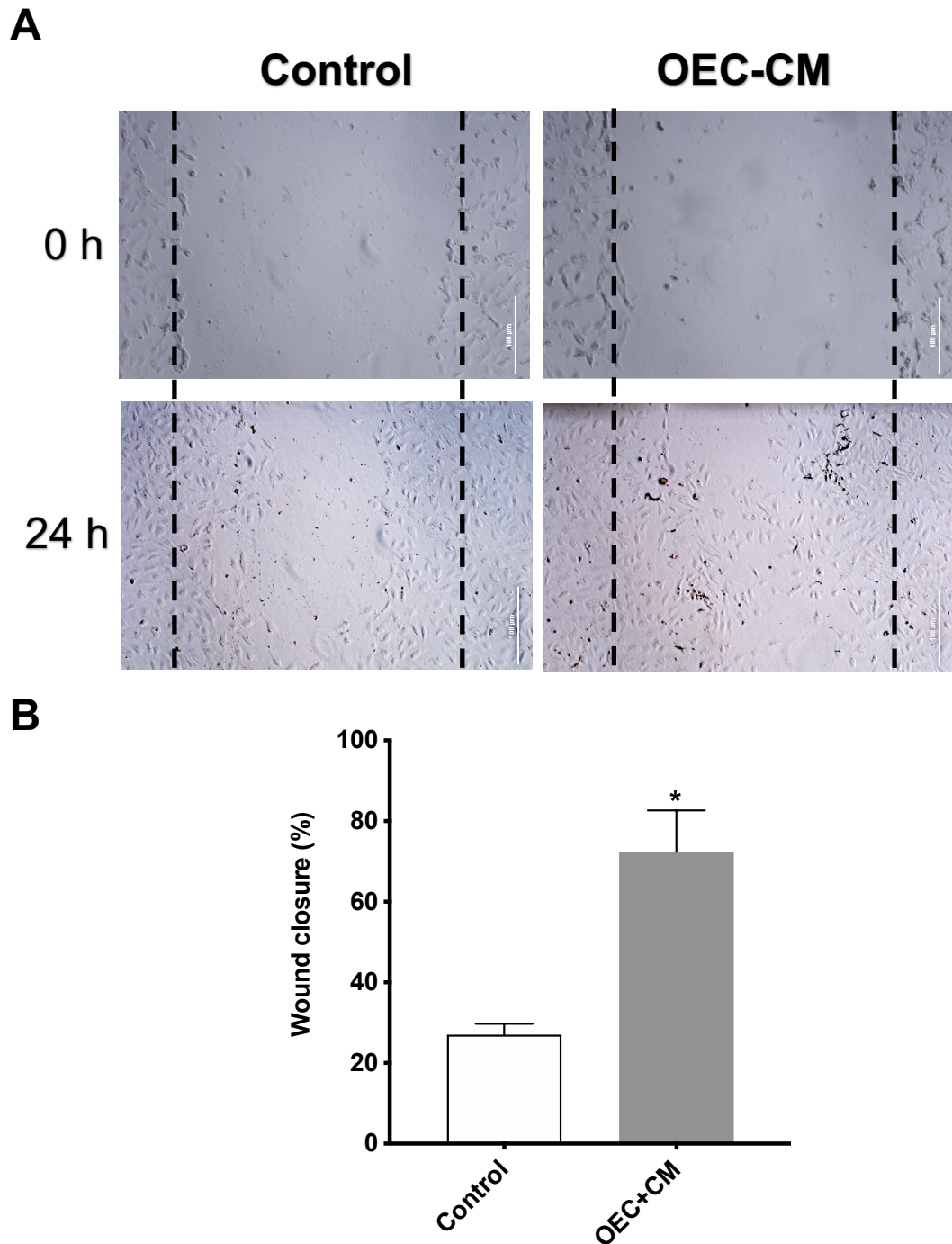
**Figure 7-4 Effect of OEC-CM on proliferation of HBMECs and OECs**

HBMECs or OECs were treated with OEC-CM before cellular proliferation and viability were assessed at 6 hr using WST-1. Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.003$  compared to control (Unpaired t test).

#### **7.1.6 OEC-CM induces proliferation and wound repair**

To determine the effect of OEC-CM on cellular proliferation, migration, and wound repair, wound scratch assays were conducted as described in chapter two. OECs were cultured until they attained full confluence and treated with OEC-CM following introduction of wound scratch. Our findings showed that OEC-CM significantly increased the percentage of OEC-mediated wound repair compared to non-treated groups. As the time of wound closure was shorter in the OEC-CM treatment group, it is safe to suggest that OEC-CM has the capacity to induce cellular proliferation as well as migration rates (Figure 7.5).



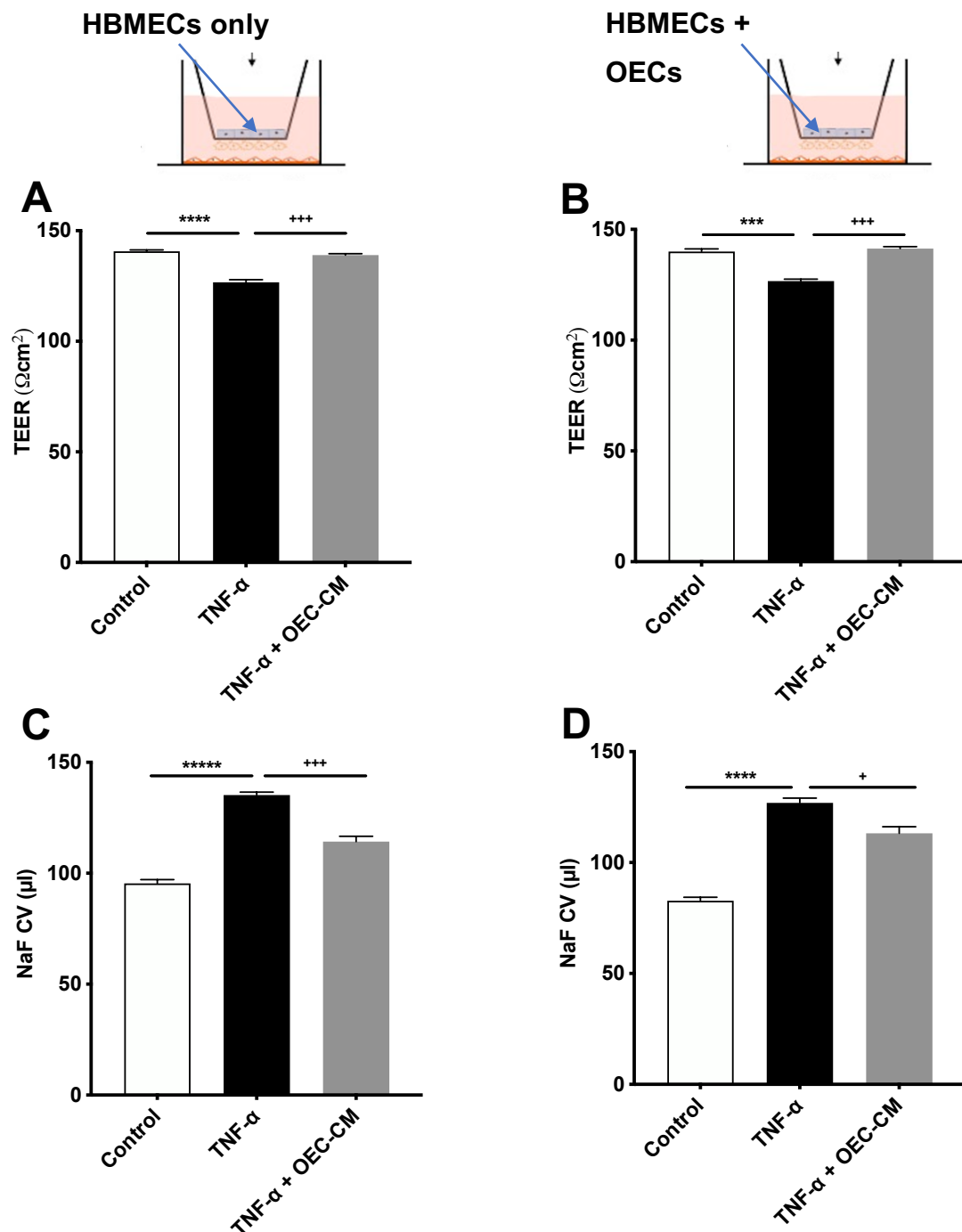


**Figure 7-5 Wound scratch assay with OECs**

OECs were cultured in 12-well plates until become confluent. Then, a scratch was made manually down the centre of the well. Then, plates were incubated in serum free medium, or OEC-CM. Images of wound closure were captured at 0- and 24-hours' time points post-scratch. Graph representing the wound closure speed in all experimental groups (B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \* $P < 0.02$  compared to control (Unpaired t test).

### **7.1.7 OEC-CM protects blood-brain barrier against TNF- $\alpha$ damage**

To determine the effect OEC-CM on TNF- $\alpha$ -evoked BBB damage, BBB models were constructed by co-culture of HA and HP with HBMECs or HBMECs + OECs as described in chapter two. Then, BBB models were exposed to TNF- $\alpha$  for 4 hours (10 ng/ml) in the absence or presence of OEC-CM. The data generated show that exposure to TNF- $\alpha$  impairs BBB integrity and function in both models as evidenced by significant decreases in TEER and increases in paracellular flux of NaF. However, co-treatment with OEC-CM protects both BBB models, constructed by HBMECs alone or with OECs, against TNF- $\alpha$ -evoked barrier damage (Figure 7.6).



**Figure 7-6 OEC-CM inhibits TNF- $\alpha$ -evoked BBB damage**

Human BBB models were established by co-culture of HAs and HPs with HBMECs or HBMECs + OECs and exposed to TNF- $\alpha$  in the absence or presence of OEC-CM. The integrity and function of models were assessed by measuring TEER and paracellular flux of NaF, respectively (A-D). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\* $P$ <0.0003, \*\*\*\* $P$ <0.0001 compared to control. + $P$ <0.02, \*\*\* $P$ <0.001 compared to TNF- $\alpha$ . (one-way analysis of variance followed by Tukey's post hoc analysis).

#### **7.1.8 OEC-CM negates the impact of TNF- $\alpha$ on HBMEC and OEC senescence**

To study whether, and to what extent, OEC-CM can inhibit TNF- $\alpha$ -induced HBMEC and/or OEC senescence,  $\beta$ -galactosidase staining was performed on same passage (P8) HBMECs and OECs. For this, cells were treated with TNF- $\alpha$  (10 ng/ml) in the absence or presence of OEC-CM. While both cell types exposed to TNF- $\alpha$  showed significantly higher  $\beta$ -galactosidase staining as determined by the appearance of blue-coloured areas within the cells, co-treatments with OEC-CM almost completely neutralised these effects in both cell types (Figure 7.7).

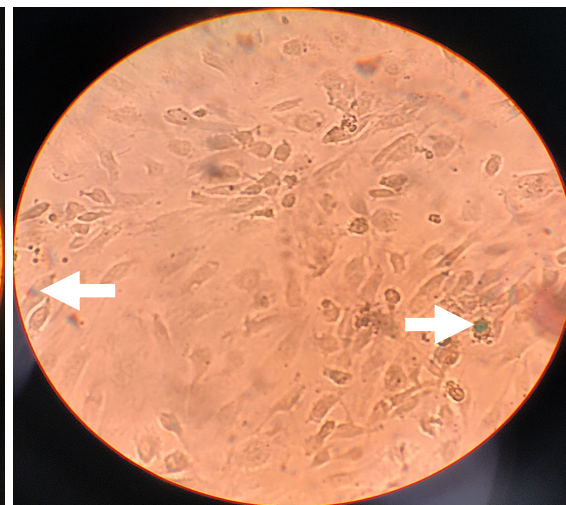
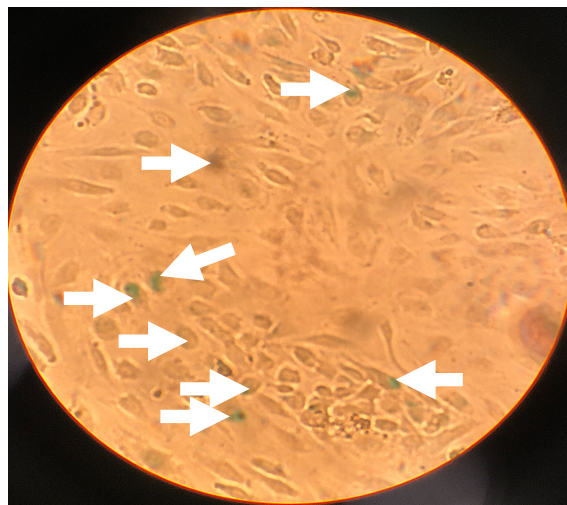
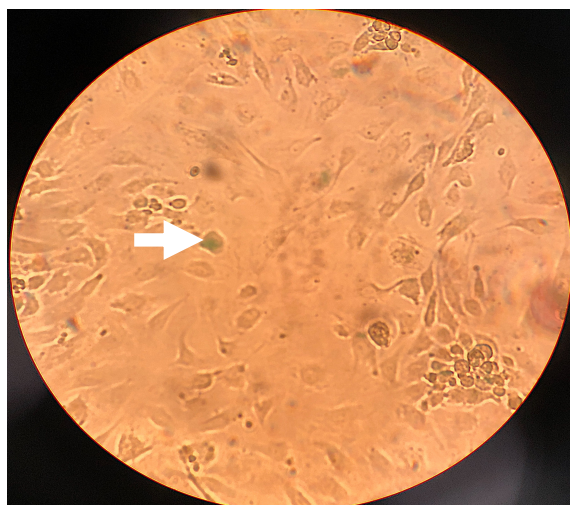
**A**

**Control**

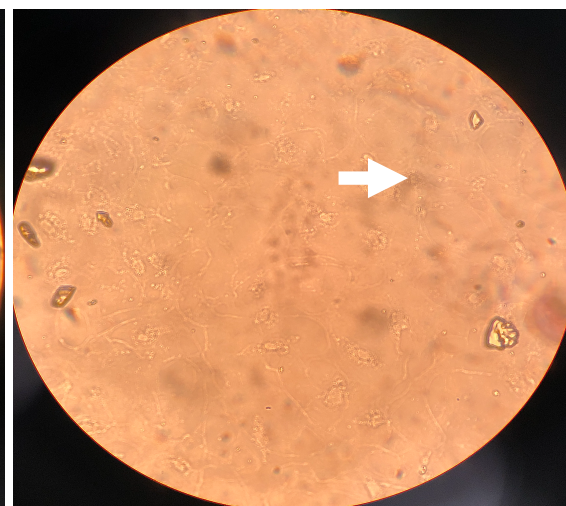
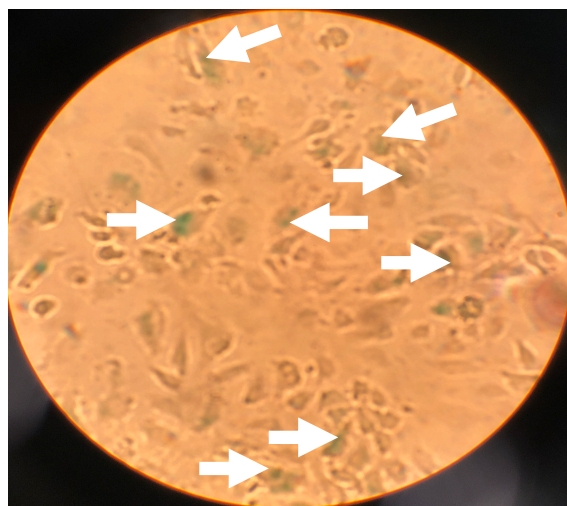
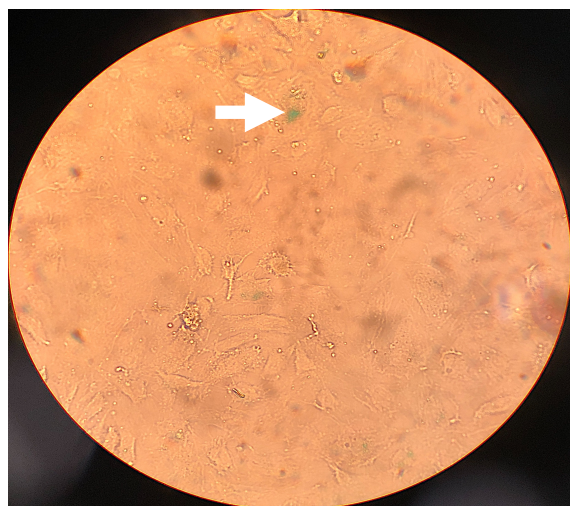
**TNF- $\alpha$**

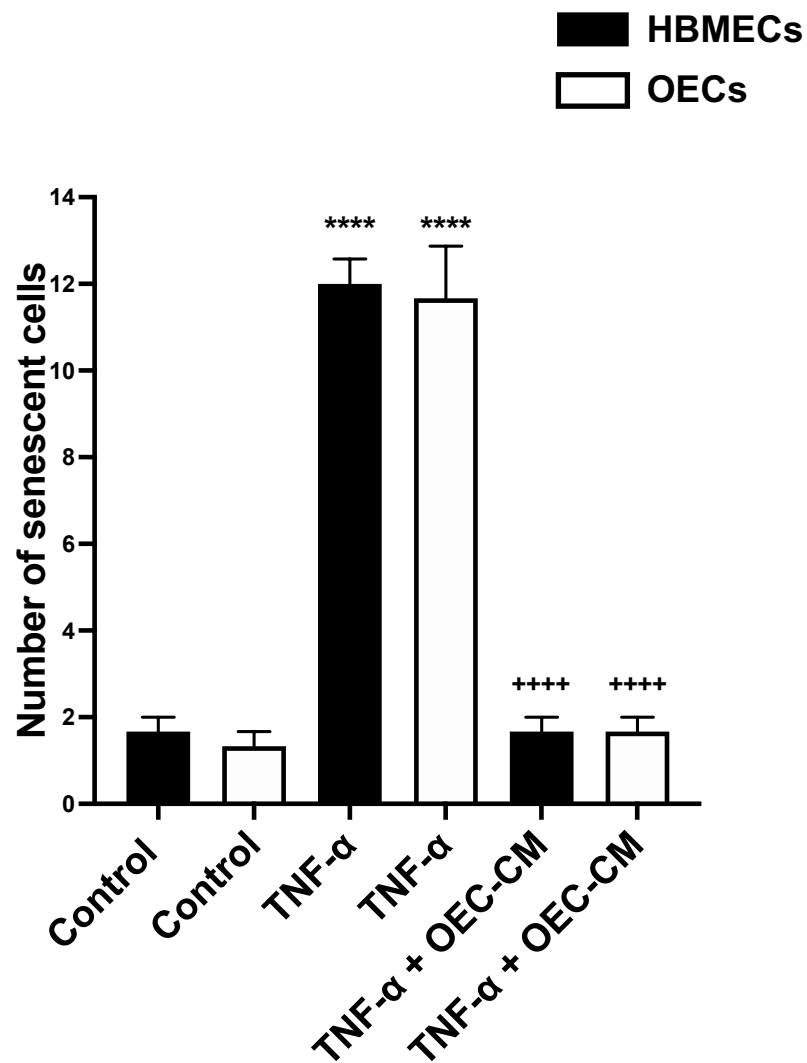
**TNF- $\alpha$  + OEC-CM**

**HBMECs**



**OECs**



**B**

**Figure 7-7 senescence-associated  $\beta$ -galactosidase staining.**

HBMECs and OECs were cultured to about 80% confluence before exposure to TNF- $\alpha$  (10 ng/ml) in the absence or presence of OEC-CM. Cells were then stained to detect  $\beta$ -galactosidase activity. Cells were observed under light microscope and the appearance of blue-coloured areas within the cells indicated  $\beta$ -galactosidase activity (A-B). Data are expressed as mean  $\pm$  SEM from three independent experiments. \*\*\*\*P<0.0001 compared to control. \*\*\*\*P<0.0001 compared to TNF- $\alpha$  (one-way analysis of variance followed by Tukey's post hoc analysis).



### 7.1.9 Discussion

OECs play vital roles in maintaining endothelial integrity and formation of new blood vessels. In addition to direct physical incorporation into the site of endothelial damage, OECs have been thought to contribute to vascular repair by inducing proliferation, migration, angiogenesis, and adhesion through secretion of what is known as paracrine factors (Di Santo et al., 2014a). In fact, the circulating OECs that can migrate to the site of ischaemic injury and repair vascular damage by replacing dead endothelial cells and restore BBB function can be potential therapeutic option for patients with ischaemic stroke. However, the very limited number of OECs in circulation may necessitate their isolation and ex-vivo expansion before being administered into an ischaemic stroke patient. Our novel findings in the previous chapters of this thesis proven that OECs can form well-established *in vitro* model of human BBB, and also restore its integrity and function following physical or ischaemic damage (Abdulkadir et al., 2020b). Neuroinflammation, manifested by an over production of pro-inflammatory cytokines, notably TNF- $\alpha$ , has been known as one of the key mechanisms that affect the integrity and function of BBB following ischaemic stroke (Abdullah and Bayraktutan, 2014). While, it is well-documented that the OECs contribute to neovascularisation through secretion of varieties of trophic factors (OEC-secretome), the effect of OEC-secretome on TNF- $\alpha$ -evoked BBB damage is not understood (Urbich et al., 2005a, Kalka et al., 2000, Urbich et al., 2003, Yamaguchi et al., 2003, Gallina et al., 2015, Di Santo et al., 2009, Felice et al., 2018). In this chapter, we investigated the role of OEC-secretome against TNF- $\alpha$  impact on cellular architecture, endothelial NADPH oxidase activity, O<sub>2</sub><sup>•-</sup> production, endothelial adhesion, and BBB integrity and function.

In addition, whether OEC-secretome can induce endothelial cells proliferation, migration, wound healing, and prevent cellular senescence.

Oxidative stress associated with overproduction of reactive oxygen species is one of the major mechanisms that affect cerebral barrier following ischaemic stroke. Acute inflammation manifested by overproduction of pro-inflammatory cytokines, notably TNF- $\alpha$ , is thought to induce BBB-associated oxidative stress damage following ischaemic stroke. The HBMECs are essential component of BBB structure and any damage to these cells will lead to disruption of barrier function and lead to brain oedema, the most common cause of death during the first week following stroke. In this chapter, first, our investigation proven that TNF- $\alpha$  induces NADPH oxidase activity and O<sub>2</sub><sup>•-</sup> release in HBMECs. Other studies, also, shown that TNF- $\alpha$  mediates NADPH and O<sub>2</sub><sup>•-</sup> release on endothelial cells (Abdullah and Bayraktutan, 2014). Our study shows that OEC-CM inhibits overproduction of NADPH oxidase and O<sub>2</sub><sup>•-</sup> in HBMECs when exposed to TNF- $\alpha$ . These novel findings are supported by previous studies which show that OECs secrete higher levels of antioxidant enzymes such as Mn-containing superoxide dismutase (Mn-SOD), glutathione peroxidase, and catalase (Dernbach et al., 2004a) which can resist oxidative stress in ischaemic or inflamed (micro)environments. In this study, we also investigated the impact of TNF- $\alpha$  on OEC-evoked NADPH oxidase and O<sub>2</sub><sup>•-</sup> release. We revealed that TNF- $\alpha$  induce the expression of NADPH oxidase and O<sub>2</sub><sup>•-</sup> but significantly lower in OECs than HBMECs. However, treatment with OEC-CM appears to reduce these increases.



Alterations in cellular architecture, associated by formation of stress fibres, during or following ischaemic injury also promote BBB dysfunction. Stress fibers attach to adherence junctions of adjacent cells. When stress fibers start to contract under stress, this will lead to formation of intracellular gaps between neighboring cells, causing an increase in BBB permeability (Millán et al., 2010, Manaenko et al., 2016). In this chapter, we show that exposure to TNF- $\alpha$  has induced the formation of stress fibres accompanying structural damage in OECs and HBMECs. Interestingly, treatment with OEC-CM protected the architecture of both cells against TNF- $\alpha$ -evoked actin microfilament formation. Both cells have shown significantly lower numbers of stress fibres when treated with OEC-CM, another prerequisite that can be added to the OEC-secretome endothelium-protective and reparative function. Other study shows that OEC-secretome protects human umbilical vein endothelial cells against oxidative stress damage by increasing the release of antioxidants such as CuZn-SOD and Mn-SOD (Yang et al., 2010).

Cellular adhesion is one of the most important physiological mechanisms for vascular repair and prevention of cardiovascular events. Endothelial repair with resident OECs or exogenous OECs therapy involves multiple complex mechanisms including cellular recruitment, proliferation, migration, and adhesion. The adhesion capacity of recruited OECs at the site of ischaemic damage is critical and requires adhesive bonds between OECs and the resident endothelial cells (Gong et al., 2011). Our study revealed that TNF- $\alpha$  significantly impairs the adherence of OECs and HBMECs. Treatment with OEC-CM induced adhesion of both cells following exposure to TNF- $\alpha$  insult. It has been,

also, found that increases in SDF-1 and VEGF, important components of OEC-secretome, can stimulate adhesion and recruitment of OECs to the sites of ischaemic damage (Anderson et al., 2015).

One of the essential processes that can help induce angiogenesis and restore vascular integrity is proliferation of resident endothelial cells and circulating OECs. By using WST-1 study, we show that OEC-CM induces viability and proliferation of HBMECs and OECs. Furthermore, we reveal that OEC-CM accelerate wound healing and cellular proliferation following physical damage induced on a single layer of OECs. Other studies shown that OEC-CM contains higher levels of mitogenic cytokines, such as angiogenin and IL-8, which supports our findings that OEC-CM can stimulate proliferative and vascular repair (He et al., 2005). Administration of OEC-secretome that contains these proliferative factors following ischaemic stroke may induce blood vessels repair by activating proliferation of endothelial cells at the site of vascular damage (Paneni et al., 2016). Other study shown that administration of OEC-secretome induced the angiogenic capacity of brain microvascular endothelial cells in *in vitro* and in a mouse model of cerebral hypoperfusion (Di Santo et al., 2014b).

As the disruption of BBB integrity and function results in cerebral oedema formation represent the major cause of mortality within the first few days following ischaemic stroke (Bayraktutan, 2019b), targeting certain mechanisms involved in the pathogenesis or worsening of oxidative stress, notably TNF- $\alpha$ , may be of paramount importance to decrease mortality and severity following ischaemic stroke events. Having considered that OECs releases trophic factors

that contain antioxidants such as CuZn-SOD and Mn-SOD (Yang et al., 2010), we investigated the OEC-secretome ability to protect BBB against TNF- $\alpha$  damage. In this study we constructed two types of *in vitro* BBB models. The first model was established with HAs, HPs, and HBMECs. The second model was formed with HAs, HPs, and HBMECs + OECs. OEC-HBMECs mixture in the second model was used to form endothelial monolayer of the barrier to investigate the therapeutic effect of OEC-CM against TNF- $\alpha$ -evoked BBB damage in presences of the OECs. Our investigations revealed for the first time that OEC-CM protected the integrity and function of both BBB models against TNF- $\alpha$  damage. These novel findings show that a therapeutic approach targeting TNF- $\alpha$  using cell-free therapy or in combination with OECs as reparative or regenerative therapeutics, can protect human cerebral barrier against damage. Previous study shown that inhibition of TNF- $\alpha$ , by a neutralising antibody, significantly blocked the deleterious effects of oxidative stress injury on barrier integrity and function like other studies showing a substantial neurological recovery and concurrent reductions in infarct size apoptosis, and haemorrhage following ischaemic insult (Abdullah et al., 2015, Barone et al., 1997, Yang et al., 1998, Yenari et al., 2006).

To use OECs as vasoreparative therapeutics for ischaemic stroke, *ex vivo* expansion of these cells is required. In the previous chapter of this thesis, we proven that further expansion of OECs revealed that they enter senescence cycle which affected their replicative potential and reparative properties. In this chapter, we also investigated the impact of TNF- $\alpha$  pathway on the OECs. We found that exposing OECs to TNF- $\alpha$  appears to increase  $\beta$ -galactosidase

activity, while treatment with OEC-CM appears to inhibit senescence-associated  $\beta$ -galactosidase on these cells. Other study shown that OECs enter stage of growth arrest at certain stage when expanded ex vivo and that late passage cells have marked increases in senescence-associated  $\beta$ -galactosidase activity (Medina et al., 2013). This, also, suggests that OEC-CM might be used to prevent OECs senescence and induce ex vivo expansion to harness OECs as therapeutics for ischaemic stroke.

#### **7.1.10 Summary**

In conclusion, our findings in this chapter demonstrate that OEC-CM reduces TNF- $\alpha$ -evoked NADPH oxidase activity and  $O_2^{\bullet}$  release in HBMECs and OECs. Co-treatment with OEC-CM protects cellular architecture in both cells against TNF- $\alpha$ -evoked damage. OEC-CM also induces adhesion, viability, and proliferation of both cells following exposure to TNF- $\alpha$ . Furthermore, OEC-CM accelerates wound healing and protects BBB integrity and function against TNF- $\alpha$  damage. Taken together, these data imply that a therapeutic approach targeting TNF- $\alpha$  by this cell-based but cell-free approach may protect human cerebral barrier against inflammatory cytokine-evoked damage. In addition, OEC-CM effectively prevents cells from going into early senescence in the presence of TNF- $\alpha$  which suggests that OEC-CM may be used to induce the ex vivo expansion of OECs, a seminal requirement for clinical application.

## **8 GENERAL DISCUSSION, LIMITATIONS, FUTURE DIRECTIONS, AND CONCLUSION**

## 8.1 General discussion

The main aim of this thesis to investigate whether OECs, a subtype of bone marrow-derived EPCs, can form functional blood-brain barrier and restore its integrity and function following ischaemic damage. In addition, to explore whether targeting oxidative stress, a major mechanism associated with BBB disruption following ischaemic stroke, can protect cerebral barrier against ischaemic damage. Also, whether inhibiting this mechanism augment the therapeutic effect of hypothermia against hyperglycaemic damage, a factor associated with higher morbidity and mortality rates among ischaemic stroke patients effected by diabetes. In addition, explore the impact of cellular senescence on the reparative capacities of OECs. Finally, whether OEC-secretome, cell-free therapy, can protect cerebral barrier against damage. The major findings of this thesis are that OECs show true endothelial characteristics, can form intracellular tight junctions, can establish human BBB models, and effectively repair and restore its integrity and function following damage. We also revealed that, compared to mature endothelial cells, OECs have significantly lower basal levels of pro-oxidants ( $O_2^{\cdot-}$  and NADPH oxidase) and higher total antioxidant capacity under normal or ischaemic conditions. This indicates that OECs may handle oxidative stress better in ischaemic (micro)environments than mature endothelial cells, a prerequisite for their endothelium-reparative and cerebral barrier-restorative function. We found that OECs perform better during the reperfusion and repair *in vitro* BBB model after 4 hours of ischaemic damage, supporting the idea that progenitor cells can work effectively in ischaemic settings and therefore be reliable therapeutics for neurovascular damage even during subacute phase. Our findings demonstrate

that inhibition of NOX2, the main source of cerebrovascular oxidative stress, in human brain microvascular endothelial cells, protects the integrity and function of *in vitro* model of human BBB against ischaemic damage. We reveal that inhibition of NOX2 aggravates the detrimental impact of ischaemic injury on BBB established with OECs by inducing cytoskeletal reorganisation and augmenting oxidative stress. Which suggests that targeting oxidative stress through inhibition of NOX2 requires close attention while OECs are being used as reparative cellular therapeutics.

We showed that NOX2 appears to play a vital role in hyperglycaemic BBB disruption. Compared to single therapy approach, the combinatory therapy of NOX2 inhibitor and therapeutic hypothermia appears to have higher protection against hyperglycaemic cerebral barrier injury. This was evidenced by reduction in oxidative stress, preservation of cellular architecture, and increases in the proliferation and migration rates of HBMECs and HAs, two main cellular elements of human cerebral barrier. In this thesis, we also found that *ex vivo* expanded OECs can enter senescence phase, as evidenced by significant reduction on proliferation, migration, adhesion capacities of these cells. Also, compared to early OECs, senescent OECs has substantially higher expression of pro-oxidants and lower NO and antioxidant capacity, which underscore their ability to resist oxidative stress in ischaemic niches. Also, we find that, unlike early OECs, senescent OECs fail to form tight junctions and establish BBB models. These ultimately can cause a significant impact on their vasculoreparative functions which highlight the importance of investigating how senescence can be prevented when expanding OECs for therapeutic usage. Furthermore, our findings show that OEC-CM, cell-free approach, has restored

human BBB integrity and function following exposure to TNF- $\alpha$ . It appears to inhibit the deleterious effect of TNF- $\alpha$ -evoked oxidative stress damage as evidenced by decreases in superoxide anion levels and NADPH oxidase activity and increases in proliferation rates and cellular adhesion in endothelial cells and OECs. Also, by protecting cellular architecture in both cell types. Interestingly, treatment with OEC-CM appears to attenuate TNF- $\alpha$ -evoked senescence in OECs and endothelial cells. Further understanding of the mechanisms of whether OEC-secretome can inhibit senescence in OECs may leads to development of new treatments that could be used to prevent cellular senescence *in vitro* or *in vivo*.

## **8.2 Limitations and future directions**

This thesis has revealed that OECs has a potential to be used as cell or cell-free therapeutics for ischaemic stroke. Studying impact of OGD, hyperglycaemia, senescence, and TNF- $\alpha$  on the cellular architecture and using different therapeutic interventions including NOX2 inhibitor, hypothermia, and OEC-CM to prevent stress fibers formation in these cells, have provided valuable but somewhat limited evidence in relation to BBB functions. We have proven that OECs can establish equally tight and functional BBB as HBMECs, as evidenced by similar TEER and NaF flux readings. However, our studies focusing on tight junctional characterisation of OECs would have benefited from the inclusion of claudin-5 and occludin in addition to ZO-1. Also, cellular migration studies have shown that OECs can incorporate at the site of damage and migrate laterally to repair wound induced in the endothelial layer. However, in relation to BBB function, migration studies are limited and not specific. Indeed, following a certain number of replication *in vitro*, OECs lose their



functional capacities and start to express some signs of cellular senescence, particularly changes in morphological characters, substantial increases in superoxide and NADPH oxidase levels, inability to establish tight junctions and human BBB models, and DNA damage. While senescence is the most useful test for tendency to generate tumour cells in *in vitro* studies, it is safe to suggest that therapeutic administration of OECs clinically would not cause such complication. However, identification of molecular pathways associated with senescence of OECs is essential to generate adequate numbers of functional OECs *in vitro* and to regulate the degree and extent of their activity *in vivo*. In addition, we show that NOX2 inhibitor protects *in vitro* models of human BBB against ischaemic insult. But the effect of targeting this pathway using gp91ds-tat in *in vivo* studies is unknown. Thus, assessing whether a therapeutic approach targeting NOX2 may be useful in an animal model of human transient ischaemic stroke. Furthermore, considering our findings that show that OECs or NOX2 inhibitor are both effective in restoring *in vitro* BBB evoked-ischaemic damage, it would be beneficial to assess whether attenuation of NOX2 during OGD and subsequent addition of OECs as therapeutics during reperfusion to same group of BBB model established with HBMECs has any subsequent protective effect, i.e., OGD+gp91ds-tat followed by 20 hrs of reperfusion with OECs therapy. More studies are required to investigate this point.

However, we show that NOX2 inhibition affects the OECs ability to maintain BBB functions under ischaemic conditions. Because OECs has lower basal level of NADPH oxidase activities than endothelial cells. Thus, further *in vitro* studies exploring oxidative stress mechanisms of these cells would provide new evidence of how these cells would get further protection when given as cellular

therapeutics in ischaemic settings. While our studies show the benefits of combinatory approach of NOX2 inhibition and therapeutic hypothermia in protecting human BBB models against hyperglycaemic insult, this study is limited. Studying the impact of this combinatory strategy against hyperglycaemic insult under ischaemic settings, in *in vivo* models, would provide further evidence to support our findings. Although, we find that OEC-CM appears to protect BBB against oxidative stress damage induced by TNF- $\alpha$ , this study also is limited. Studying the effect of OEC-CM in BBB model exposed to ischaemic insult (OGD model) would provide further evidence about whether cell-free approach can protect BBB against ischaemic damage. Also, *in vivo* studies exploring the effectiveness of this strategy during (sub)acute or chronic phase of ischaemic stroke would be with paramount importance.

### **8.3 Conclusion**

Ischaemic stroke is a common cause of morbidity and mortality worldwide. Disruption of human blood-brain barrier, formed mainly by human brain microvascular endothelial cells, constitutes the main cause of death following ischaemic stroke. In addition, NOX2, hyperglycaemia, and tumour necrosis factor alpha represent pathways that induce BBB-evoked damage following ischaemic stroke. The findings represented in this thesis reveal that OECs, subtype of endothelial progenitor cells, can repair and restore BBB integrity and function following ischaemic damage. Therefore, OECs may serve as a novel cell-based therapeutic option for ischaemic stroke. In addition, gp91ds-tat appears to protect cerebral barrier against ischaemic insult and augments the effect of therapeutic hypothermia by protecting barrier against oxidative stress

damage under hyperglycaemic conditions. Accordingly, gp91ds-tat may serve as an efficacious therapeutic strategy for ischaemic stroke patients with diabetes. Finally, while we show that senescence appears to affect the functional capacities of OECs when expanded to a certain passage *in vitro*, we reveal that OEC-CM protects cerebral barrier against tumour necrosis factor alpha damage. This cell-free therapeutic strategy may open new avenues in the field of ischaemic stroke medicine.

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