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Outgrowth Endothelial Cells (OECs): Cell-Based Therapy, Cell-Free Strategy, and Role of Senescence

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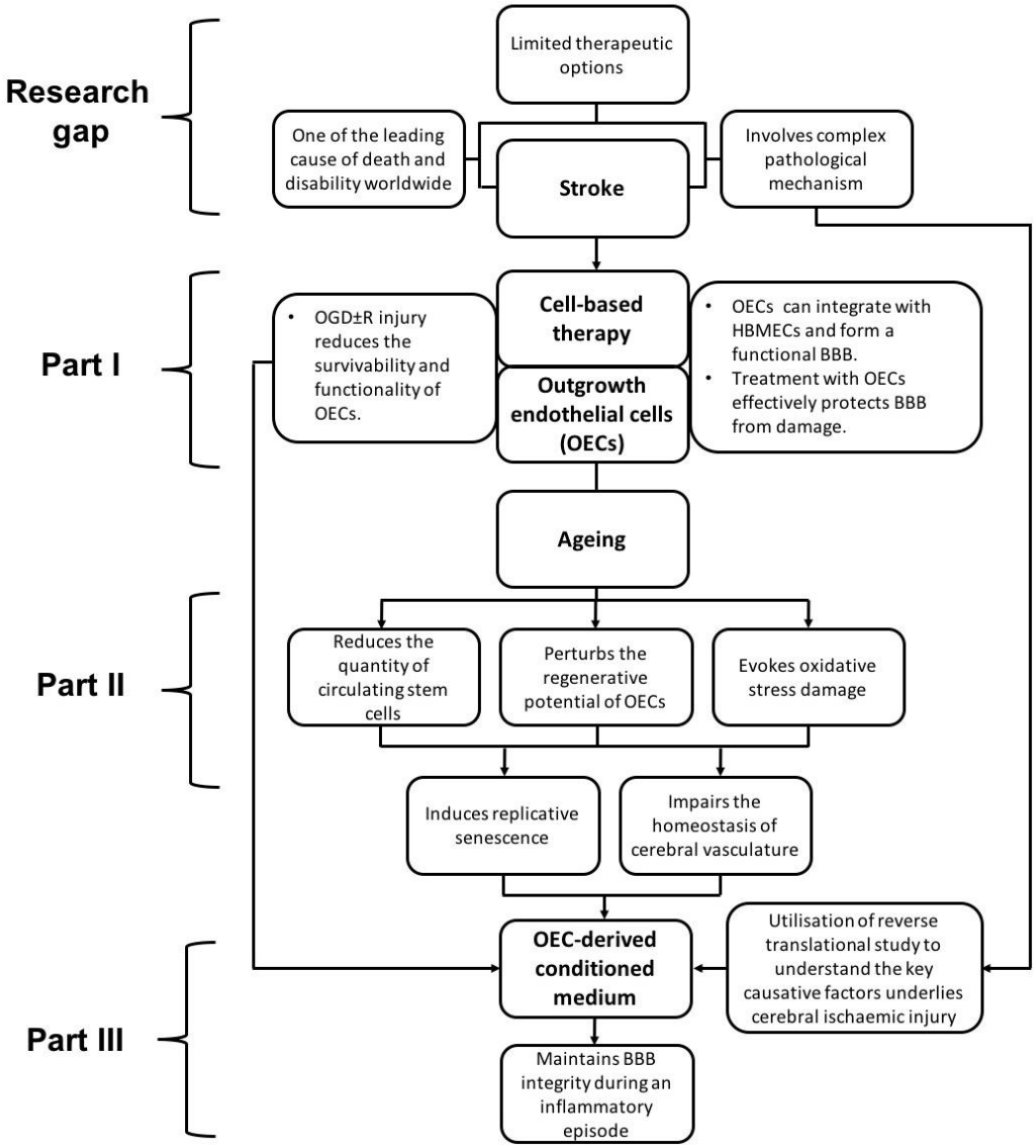
Flow of the thesis



BBB, blood-brain barrier; *OECs*, outgrowth endothelial cells; *OGD±R*, 4 hours oxygen glucose-deprivation alone or followed by 20 hours reperfusion.

Abstract

Background: Ischaemic stroke, emerging from an interference of blood supply leading to or within the brain, continues to be one of the main causes of mortality and disability worldwide. Since the disruption of the blood–brain barrier (BBB), primarily formed by brain microvascular endothelial cells (BMECs), and ensuing brain oedema have been associated with the enhanced risk of death in the early stages of ischaemic stroke, the discovery of novel agent(s) that can effectively repair endothelial integrity and overall BBB function is of paramount importance to mitigate stroke-related damages. In this regard, endothelial progenitor cells (EPCs), which are capable of replacing the dead or dying endothelial cells through directly differentiating into mature endothelial cells or indirectly via releasing various active compounds, may represent such agent.

Methods: To assess the cerebral barrier-reparative effect of outgrowth endothelial cells (OECs), the functional subtype of EPCs, an *in vitro* model of human BBB was established by co-culture of human BMECs (HBMECs), astrocytes, and pericytes before exposure to oxygen-glucose deprivation alone or followed by reperfusion (OGD±R). Using a rodent model of middle cerebral artery occlusion (MCAO), this thesis subsequently assessed the therapeutic potential of OECs *in vivo*. Since the availability and functionality of EPCs may be adversely affected by age, the thesis then investigated the level of circulating EPCs and several elements known to regulate their mobilisation and survivability in elderly (n=40, 73.3±7.2 years) and young (n=50, 40.2±14.3 years) healthy individuals. An experimental model of chronological ageing, mimicked by repetitive culture of the cells up to passage 14, was employed to assess the effects of ageing on the morphology and functional properties of OECs. To elucidate the key mechanism underlies the limited functional capacity of aged OECs,

so-called replicative senescence, the cells were treated either with specific inhibitor of NADPH oxidase, VAS2870 (5 μM), or broad-spectrum anti-oxidant, vitamin C (0.5 μM), starting at passage 12. To understand the pathological mechanisms involved in ischemic stroke and to provide a strong foundation for therapeutic target, a reverse translational research, which analysed the biochemical alteration of 90 ischaemic stroke patients compared to 81 healthy controls, was employed. The thesis eventually assessed the capacity of OEC-derived conditioned medium (OEC-CM), produced by exposure of OECs to hypoxic injury, to neutralise these changes using the aforementioned *in vitro* BBB model.

Results

Part I: Similar to HBMECs, OECs possessed classical endothelial characteristics, as observed by their typical cobblestone morphology and capacity to bind with FITC-UEA-1 and Dil-Ac-LDL. These cells also equipped with remarkable proliferative, migratory, and anti-oxidant capacity. They can integrate with resident brain endothelial cells and form a tight and functional BBB. Even so, exposing OECs to OGD±R impaired their function to a similar degree as HBMECs. Exogenous addition of OECs during OGD±R effectively repaired the integrity and function of an *in vitro* BBB model, as ascertained by the increases of transendothelial electrical resistance (TEER) and decreases of sodium fluorescein flux, respectively. Similar to these results, treatment with OECs also restored the scratch damage induced on the endothelial layer of the BBB model in serum-free conditions, and protected overall cerebral barrier integrity and function. These barrier-reparative effects have also been replicated in *in vivo* settings, with the intravenous administration of OECs 24 hours after induction to MCAO markedly decreasing the brain infarct volume in the ipsilateral hemisphere brain assessed on day 3 after treatment. The mechanistic studies

showed that the suppression of oxidative stress and apoptosis of resident cerebral endothelial cells may mediate this barrier-reparative effect of OECs.

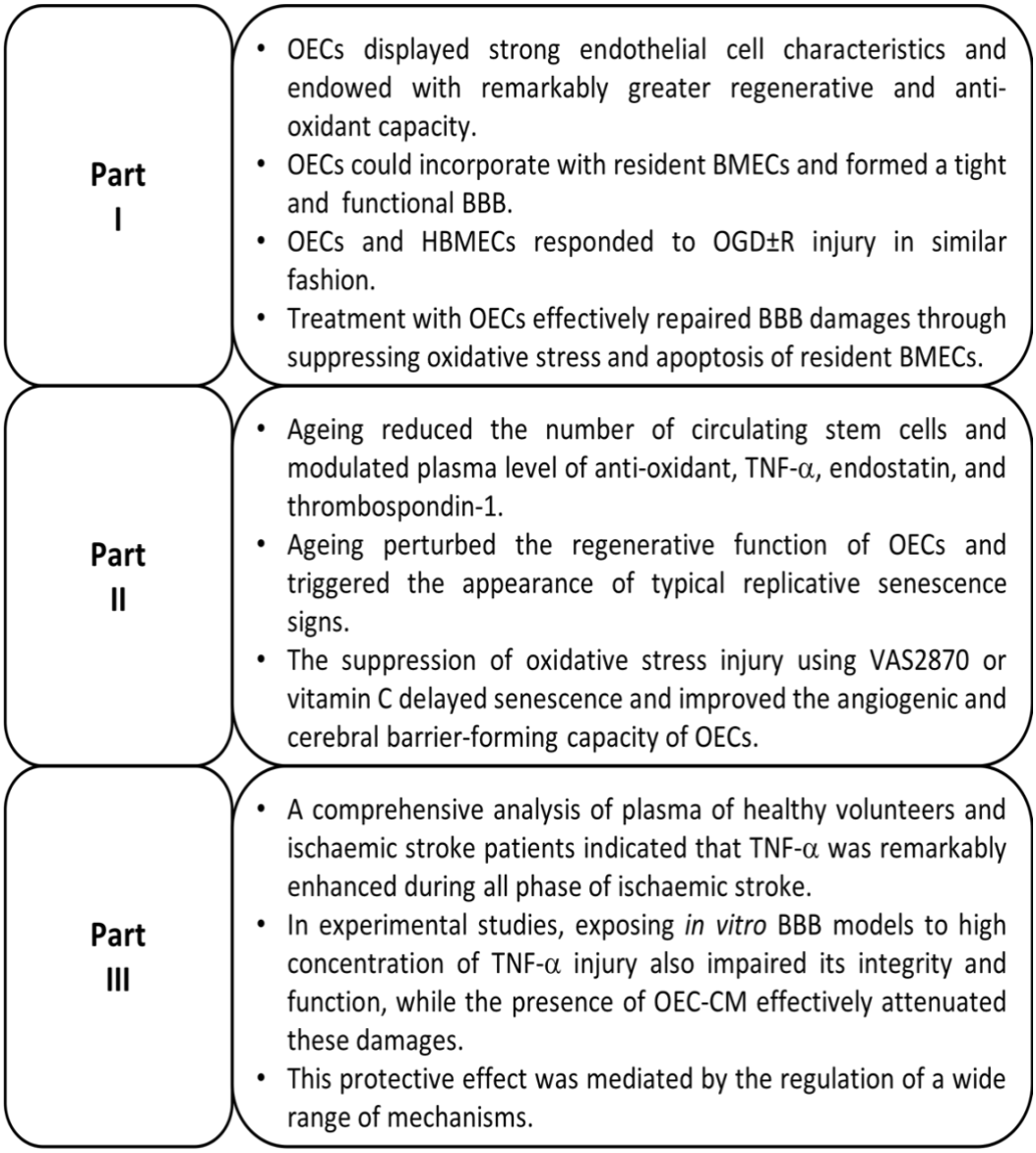
Part II: An investigation to quantify the level of circulating EPCs in older versus younger healthy participants showed insignificant differences in the level of EPCs (CD34+CD133+KDR+) between these two groups. However, the number of cells exclusively expressing stemness markers and known to possess unique capacity to differentiate into mature endothelial cells (CD34+ and/or CD133+) sharply declines in the elderly. This phenomenon was followed by a decrease in total anti-oxidant capacity and simultaneous increases in plasma levels of inflammatory mediators, TNF-α, and anti-angiogenic factors, endostatin and thrombospondin-1. The subsequent experimental studies to scrutinise the effect of ageing on molecular and functional phenotypes of OECs showed that chronological ageing profoundly perturbed the critical functions of these cells, and induced the appearance of multiple typical signs of replicative senescence. In line with the findings from clinical observational studies, senescent OECs also manifested decreased total anti-oxidant capacity along with the increased pro-oxidant NADPH oxidase activity and endostatin level. Suppressing oxidative stress level using anti-oxidants compounds, namely vitamin C or VAS2870, somewhat delayed OEC senescence and repaired their tubulogenic and BBB-forming capacities.

Part III: A comprehensive analysis of plasma samples acquired from a large number of healthy volunteers and ischaemic stroke patients showed significant increases in the levels of TNF-α during acute, subacute, and chronic phases of stroke. Further analysis from this study also showed that the level of this inflammatory mediator was significantly higher in healthy volunteers with endothelial dysfunction associated risk factors, such as hypertension, diabetes mellitus, and hyperlipidaemia than those

without. The subsequent experimental studies also showed the dramatic impairment on the integrity and function of an *in vitro* BBB model exposed to high concentration of TNF-α (10 ng/mL, 6 hours), as observed by the decreases in TEER value and concomitant increases in sodium fluorescein flux, respectively. Co-treatment with OEC-CM effectively negated the detrimental effects of TNF-α on the BBB. The remarkable suppression on endostatin level, oxidative stress, apoptosis, stress fibre formation as well as the improvements in HBMEC and OEC viability, tubulogenic, and adhesion properties appeared to contribute to this protective effect.

Conclusion: Treatment with OECs effectively repaired BBB damages in both *in vitro* and *in vivo* model of ischaemic stroke through suppressing oxidative stress and apoptosis of resident brain endothelial cells. While OECs provide the endogenous repair mechanism to counteract on-going brain endothelial injury, advanced age ultimately evokes senescence and cellular dysfunction. Nonetheless, regulating oxidative stress level appears to delay the appearance of senescence phenotype and protect overall stem cell function. An alternative cell-free strategy using OEC-CM effectively negated the detrimental effects of TNF-α, an important inflammatory cytokine that was remarkably elevated in all phases of ischaemic stroke, on BBB integrity and function by simultaneously modulating a variety of mechanisms.

Major findings



BBB, blood-brain barrier; *BMECs*, brain microvascular endothelial cells; *HVs*, healthy volunteers; *OECs*, outgrowth endothelial cells; *OEC-CM*, outgrowth endothelial cell-derived-conditioned medium. *OGD±R*, 4 hours oxygen glucose-deprivation alone or followed by 20 hours reperfusion; *TNF-α*, tumour necrosis factor-α.

Acknowledgement

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List of publications and conferences

Publications

a. Original article:

1. **Kadir R.R.A**., Alwjwaj, M., Bayraktutan, U. (2022). Treatment with outgrowth endothelial cells protects cerebral barrier against ischemic injury. Cytotherapy, 24(5), 489-499. https://doi.org/10.1016/j.jcyt.2021.11.005
2. **Kadir R.R.A**., Alwjwaj M, Othman OA, Rakkar K, Bayraktutan U (2020) Outgrowth endothelial cells form a functional cerebral barrier and restore its integrity after damage. Neural Regen Res 15(6):1071-1078. doi:10.4103/1673-5374.269029
3. **Kadir R.R.A.**, Alwjwaj M, McCarthy Z, Bayraktutan U. Therapeutic hypothermia augments the restorative effects of PKC-β and Nox2 inhibition on an *in vitro* model of human blood-brain barrier. Metab Brain Dis. 2021 Oct;36(7). doi:10.1007/s11011-021-00810-8
4. **Kadir, R.R.A**., Alwjwaj, M. & Bayraktutan, U. Protein kinase C-β distinctly regulates blood-brain barrier-forming capacity of Brain Microvascular endothelial cells and outgrowth endothelial cells. Metab Brain Dis (2022). https://doi.org/10.1007/s11011-022-01041-1
5. **Kadir, R.R.A.**, Alwjwaj, M., Othman, O. A., Rakkar, K., Sprigg, N., Bath, P. M., & Bayraktutan, U. (2022). Inhibition of oxidative stress delays senescence and augments functional capacity of endothelial progenitor cells. Brain Research, 1787, 147925.
6. Alwjwaj, M., **Kadir, R.R.A.**, Bayraktutan, U. (2022). Outgrowth endothelial progenitor cells restore cerebral barrier function following

ischaemic damage: The impact of NOX2 inhibition. European Journal of Neuroscience, 55(6), 1658-1670. https://doi.org/10.1111/ejn.15627

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8. **Kadir R.R.A**., Alwjwaj M, Othman OA, Rakkar K, Sprigg N, Bath P, Bayraktutan U. Outgrowth Endothelial Cell Conditioned Medium Negates TNF-α-Evoked Cerebral Barrier Damage: A Reverse Translational Research to Explore Mechanisms. In press. Stem Cell Reviews and Report (2022).

b. Review paper:

1. **Kadir, R.R.A.**, Bayraktutan, U. Urokinase Plasminogen Activator: A Potential Thrombolytic Agent for Ischaemic Stroke. Cell Mol Neurobiol 40, 347-355 (2020). https://doi.org/10.1007/s10571-019-00737-w
2. **Kadir, R.R.A.**, Alwjwaj, M. & Bayraktutan, U. MicroRNA: An Emerging Predictive, Diagnostic, Prognostic and Therapeutic Strategy in Ischaemic Stroke. Cell Mol Neurobiol (2020). https://doi.org/10.1007/s10571-020-01028-5
3. Alwjwaj M, **Kadir R.R.A**, Bayraktutan U. The secretome of endothelial progenitor cells: a potential therapeutic strategy for ischemic stroke. Neural Regen Res 2021;16:1483-9

c. Book chapter:

1. **Kadir, R.R.A**., Alwjwaj, M., & Bayraktutan, U. (2022). Establishment of an In Vitro Model of Human Blood–Brain Barrier to Study the Impact of

Ischemic Injury. The Blood-Brain Barrier: Methods and Protocols, 2492, 143.

Conferences

a. Oral presentation:

1. **Kadir R.R.A.**, Alwjwaj M, Bayraktutan U. Long-Term Expansion of Outgrowth Endothelial Cells Evoke Replicative Senescence and Attenuate Their Cerebral Barrier-Forming Capacity. European Stroke Journal; 2021, Vol. 6(1S) 3–513. European Stroke Conference 2021.
2. **Kadir R.R.A.**, Alwjwaj M, Bayraktutan U. Administration of Outgrowth Endothelial Cells Repair Cerebral Barrier Function Following Ischaemic Stroke Injury. European Stroke Journal; 2021, Vol. 6(1S) 3–513. European Stroke Conference, 2021.
3. **Kadir R.R.A.**, Alwjwaj M, Bayraktutan U. Outgrowth Endothelial Cells-Derived Conditioned Medium: A Potential Cell-Free Strategy to Repair Neuroinflammatory Cerebral Barrier Damage. European Stroke Journal; 2021, Vol. 6(1S) 3–513. European Stroke Conference, 2021.
4. **Kadir R.R.A.**, Alwjwaj M, Bayraktutan U. Comparative Assessment of Outgrowth Endothelial Cells and Brain Microvascular Endothelial Cells in Forming an *in vitro* Model of Human Blood-Brain Barrier: Role of Protein Kinase C-β. 4th UK Stroke pre-clinical research conference.
5. Alwjwaj M, **Kadir R.R.A.**, Bayraktutan U. Comparative Analyses of Outgrowth Endothelial Cells and Microvascular Endothelial Cells in Forming an *in vitro* Model of Human Blood-Brain Barrier. 4th UK Stroke pre-clinical research conference.

b. Poster presentation:

1. Alwjwaj M, **Kadir R.R.A.**, Bayraktutan U. NADPH Oxidase is Essential for Endothelial Progenitor Cell Migration, Proliferation and Vascular Repair. European Stroke Journal; 2021, Vol. 6(1S) 3–513. European Stroke Conference, 2021.
2. Alwjwaj M, **Kadir R.R.A.**, Bayraktutan U. Senescence attenuates the ability of endothelial progenitor cells to form blood-brain barrier and repair the damage. International Journal of Stroke. 2020; 15(1S) 3–752. World - European Stroke (WSO-ESO) Conference, 2020.
3. **Kadir R.R.A.**, Alwjwaj M, McCarthy Z, Bayraktutan U. Can Inhibition of Protein Kinase C-Beta or NADPH Oxidase Augment the Restorative Impact of Hypothermia on Blood-Brain Barrier? International Journal of Stroke. 2020; 15(1S) 3–752. World - European Stroke (WSO-ESO) Conference, 2020.
4. **Kadir R.R.A.**, Alwjwaj M, Othman OA, Rakkar K, Bayraktutan U. Outgrowth Endothelial Cells: A Cell-Based Approach to Repair Ischaemia-Evoked Cerebral Barrier Damage. International Journal of Stroke. 2020; 15(1S) 3–752. World - European Stroke (WSO-ESO) Conference, 2020.
5. Alwjwaj M, **Kadir R.R.A.**, Bayraktutan U. Can Inhibition of Protein Kinase C-beta or Nox2 Augment the *in vitro* Blood-Brain Barrier Forming Activity of Endothelial Progenitor Cells? International Journal of Stroke. 2020; 15(1S) 3–752. World - European Stroke (WSO-ESO) Conference, 2020.

6. **Kadir R.R.A.**, Bayraktutan U. Inhibition of Protein Kinase C-beta Protects *in vitro* Brain Barrier from Ischaemia-Evoked Damage. European Stroke Journal; 2019, Vol. 4(1S) 150–778. European Stroke Conference, 2019.

Table of contents

List of figures

List of tables

List of equations

List of abbreviations

AF	Atrial fibrillation
ARRIVE	Animal research: reporting of *in vivo* experiments
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad-related protein
BBB	Blood–brain barrier
CDK	Cyclin-dependent kinase
CNS	Central nervous system
CO_2	Carbon dioxide
CTCF	Corrected total cell fluorescence
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-associated protein 9 nucleases
DAPI	4′,6-diamidino-2-phenylindole
DDR	DNA damage response
Dil-Ac-LDL	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine-labelled-Ac-LDL
DM	Diabetes mellitus
DMT	The Dunhill Medical Trust
ECM	Endothelial cells media
EDTA	Ethylenediaminetetraacetic acid
EGM-2	Endothelial cell growth medium-2
EPCR	Endothelial protein C receptor
EPCs	Endothelial progenitor cells
FBS	Foetal bovine serum
FDA	Food and drug administration

FITC-UEA-1	Fluorescein isothiocyanate labelled-ulex europaeus agglutinin
G-CSF	Granulocyte-colony stimulating factor
HBMECs	Human brain microvascular endothelial cells
HVs	Healthy volunteers
ICH	Intracerebral haemorrhage
IL	Interleukin
JAMs	Junctional adhesion molecules
KDR	Kinase insert domain receptor
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MSCs	Mesenchymal stem cells
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluorescein
NOx	Total nitrate/nitrite
O_2	Oxygen
OEC-CM	Outgrowth endothelial cells-derived conditioned medium
OECs	Outgrowth endothelial cells
OGD	Oxygen-glucose deprivation
OGD±R	Oxygen-glucose deprivation alone or followed by reperfusion
OPCs	Oligodendrocyte precursor cells
PBS	Phosphate buffered saline
PDGF-BB	Platelet-derived growth factor-BB

PDT	Population doubling time
Rb	Retinoblastoma
ROS	Reactive oxygen species
RPMI 1640	Roswell park memorial institute 1640
rtPA	Recombinant tissue plasminogen activator
SA-β-gal	Senescence-associated-β-galactosidase
SASP	Senescence-associated secretory phenotype
SDF-1	Stromal cell-derived factor 1
SGLT-2	Sodium-glucose cotransporter 2
STAIR	Stroke therapy academic industry roundtable
STEPS	Stem cell therapy as an emerging paradigm for stroke
T2DM	Type II diabetes mellitus
TEER	Transendothelial electrical resistance
TIA	Transient ischaemic attack
TJs	Tight junction proteins
TNF-α	Tumour necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
WST-1	Water-soluble tetrazolium salt
ZO	Zonula occluden

Contributions

Author performed all the *in vitro* experiments throughout the thesis. The *in vivo* experiment was performed by Dr Ulvi Bayraktutan, and the clinical studies was carried out by Dr Kamini Rakkar and Dr Othman Ahmad. The author collated, analysed, and interpreted both experimental and clinical data.

The author confirms that all work in this thesis is original and their own work, unless otherwise stated.

Introduction

1. Stroke

1.1. Definition and epidemiology

Stroke is defined as an acute neurological disturbance lasting for more than 24 hours or leading to death with no apparent cause other than vascular origin (Sacco et al. 2013). Based on its aetiology, stroke is classified into two major types: ischaemic and haemorrhagic. Ischaemic stroke derives from the interference of the blood supply leading to or within the brain due to the formation of an embolus (embolic strokes) or a thrombus (thrombotic strokes). Haemorrhagic stroke, on the other hand, stems from the rupture of an artery within cerebral tissue (intracerebral haemorrhage) or on its surface (subarachnoid haemorrhage) (Campbell and Khatri 2020).

Stroke is the third leading cause of mortality and disability worldwide (Feigin et al. 2021). In the UK, over 100,000 individuals suffer from this debilitating disease each year, and only one-third of stroke survivors leave the hospital without a disability. In addition, the social and economic impacts of stroke are also severe, incurring almost £9 billion every year for direct (inpatient/outpatient care) and indirect (income loss, social benefit payments to patients, and carer costs for home nursing) expenses in the UK, accounting for about 5% of net National Health Service costs (Patel et al. 2020). The number of stroke patients as well as the costs of stroke care are also projected to increase by 60% and 300%, respectively over the next two decades, unless measures to prevent and reduce the disabling effects of this disease can be successfully developed and implemented (King et al. 2020).

1.2. Current therapeutic approaches

Despite representing massive devastating impacts (section 1.1), haemorrhagic stroke has no medical therapy, while thrombolysis with recombinant tissue plasminogen activator (rtPA) remains the solely approved pharmacotherapy for ischaemic stroke

(Kleindorfer et al. 2021). However, due to the narrow therapeutic time window (<4.5 hours of stroke onset) and markedly enhanced risk of haemorrhage beyond this point, less than 1% of patients receive this therapy each year worldwide (Anand et al. 2021; Berge et al. 2021). Recently, endovascular treatment has made its way into clinical practice and somewhat extend the therapeutic window for reperfusion therapy, but it is only effective in ischaemic stroke stemming from large vessel occlusion, and can only be performed in clinical units equipped with advanced facilities and human resources (Leischner et al. 2019; Mokin et al. 2019). Hence, it is no surprise that only very limited number of patients receive this treatment each year (Kamel et al. 2021). Such limited therapeutic options coupled with higher mortality and disability have prompted the stroke research community to develop novel treatment regimens for stroke patients that are both safer and more efficacious, particularly for those who are unsuitable for thrombolytic or endovascular treatment (Boltze et al. 2021). Intriguingly, despite showing enormous success in experimental research, more than 250 novel therapeutic agents were unable to replicate the favourable outcomes in clinical trials (Dhir et al. 2020). This fundamental failure might be due to numerous reasons, including targeting the specific pathways related to recanalisation or excitotoxicity, or the utilisation of young and healthy male animals subjected to middle cerebral artery occlusion (MCAO) in pre-clinical studies (Schmidt-Pogoda et al. 2020). In this context, considering that cells can simultaneously target several mechanisms involved in the pathophysiology of stroke, cell-based therapy has emerged as a powerful and promising concept for stroke therapy (Singh et al. 2020).

1.3. Pathophysiology of ischaemic stroke

The occlusion of the cerebral artery and decreased blood flow below a critical level following ischaemic stroke attack causes insufficient supply of oxygen and glucose in

the affected area in the brain (Figure 1). The restriction of basic nutrients and oxygen to the cells perturbs cellular mitochondrial oxidative phosphorylation, which in turn depletes the generation of ATP and evokes irreversible failure of energy metabolism (Liang et al. 2021). The unavailability of ATP impairs the physiological function of ionic pumps, leading to aberrant influx of sodium (Na^+) and calcium ions (Ca^{2+}) into the cell, and the efflux of potassium ions (K^+). The excessive concentration of extracellular K^+ leads to the opening of L-type voltage-gated calcium channels, and subsequently triggers the release of glutamate (Xing et al. 2012). Glutamate is the main excitatory neurotransmitter in central nervous system (CNS), and excessive amounts of this compound are the major cause of cell death in the early stages of ischaemic stroke (Radak et al. 2014; Rama and García 2016). In addition, the accumulation of glutamate also activates post-synaptic glutamate receptors, and further increases the influx of Ca^{2+} into the neuron, thus exacerbate neuronal damage (Stout et al. 1998).

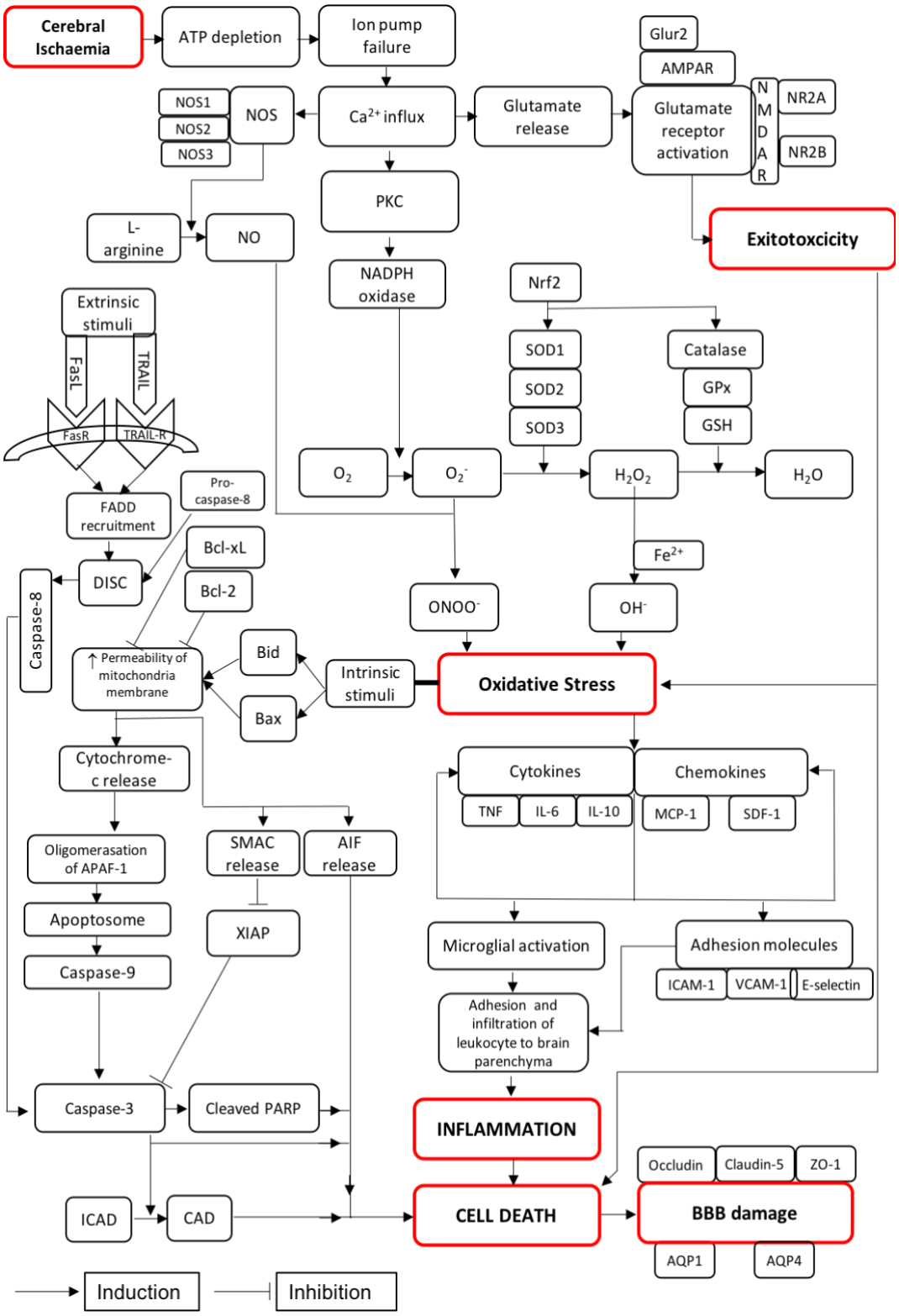


Figure 1. Pathomechanism of ischaemic stroke.

Cerebral ischaemia lessening blood supply in the brain, causing the reduction of ATP availability, which in turn triggers complex pathological events, leading to excitotoxicity and oxidative stress damage. These cascades subsequently activate a series of molecular signalling pathway that successively trigger inflammation, cell death, and BBB disruption. Figure adopted from Kadir et al. (2020).

Besides increasing glutamate concentration, the overload of calcium also increases protein kinase C level, which further activates the series of molecular mechanisms that induced excessive generation of reactive oxygen species (ROS) (Abdullah and Bayraktutan 2016; Rakkar and Bayraktutan 2016). Under physiological conditions, ROS is produced at minimal levels through the mitochondrial respiratory chain to maintain various cellular functions, such as gene expression, proliferation, migration, immune defence system, and synaptic plasticity (Samanta and Semenza 2017; Forman and Zhang 2021). However, following cerebral ischaemia, the ROS concentration remarkably increases while anti-oxidant enzymes are not able to counterbalance their level, thus causing lipid peroxidation, protein denaturation, and structural damage to DNA (Shaafi et al. 2021).

The increased level of glutamate and ROS following cerebral ischaemic injury causes primary cerebrovascular damage and activates microglia cells, the specialised population of macrophage in the CNS. The activated microglial cells subsequently release various cytokines, such as TNF-α, IL-1β, IL-1, and IL10, and chemokines, including SDF-1 and MCP-1, which further induce the expression of cell adhesion molecules on the endothelial surface, and facilitate the infiltration of leukocytes in the site of injury (Shigemoto-Mogami et al. 2018). The infiltration of immune cells into ischaemic areas of the brain further aggravates brain damages, as they induce the production of large amounts of cytotoxin compounds such as NO, ROS, and more inflammatory mediators (Pawluk et al. 2020).

TNF-α is one of the most extensively studied inflammatory mediators in the context of post-ischaemic neuroinflammation (Abdullah et al. 2015). The level of both mRNA and protein of TNF-α are significantly increased from 3 hours up to several days after stroke onset, and are strongly correlated with brain infarct size and neurological

deficit, while the inhibition of this cytokine with a monoclonal antibody significantly reduces brain damage after ischaemic stroke (Hosomi et al. 2005; Abdullah and Bayraktutan 2014; Clausen et al. 2020). Intriguingly, a recent study has also found a significant improvement of TNF-α level in old mice and humans associated with blood-brain barrier (BBB) leakage and delay post-stroke recovery. Weekly treatment with adalimumab, a TNF-α inhibitor, over a month effectively attenuates infarct volume and functional deficits of ageing mice exposed to transient MCAO, suggesting the crucial role of this cytokine in both the pathological mechanism of ischaemic stroke and ageing (Liberale et al. 2021).

The abovementioned series of detrimental events associated with the pathomechanism and progression of ischaemic stroke (i.e. calcium overload, glutamate accumulation, oxidative stress, and inflammation) lead to ultimately programmed cell death (also known as apoptosis) in the penumbra, an area surrounding the infarct, within hours to days following an ischaemia/reperfusion injury (Sekerdag et al. 2018). In general, apoptosis is activated either through the intrinsic pathway, originating from the release of cytochrome-c from mitochondria intermembrane space; or the extrinsic pathway, mediated by the activation of a death receptor that receives extracellular signals and transduces them to intracellular signals (Li et al. 2020a). Both intrinsic and extrinsic pathways interface at the point of caspase-3 activation, an executioner caspase in apoptosis, and further cause the destruction of cellular structures, including DNA fragmentation and cell death (Boland et al. 2013).

The impairment of BBB, characterised by structural disruption of tight junction proteins (TJs), is also a hallmark of the pathogenesis and progression of ischaemic stroke. The mechanisms underlying stroke-evoked BBB damages are dynamically interconnected, but it has been well-established that the excessive amount of ROS,

MMP, and pro-inflammatory mediators increases the permeability of BBB, leading to a large inflow of hematogenous fluid, notably immune cells, into the brain, hence progressively forming cerebral oedema (Abdullah et al. 2015; Abdullah and Bayraktutan 2016).

Since BBB dysfunction is associated with brain oedema, haemorrhagic transformation, and increased rates of mortality and disability in patients with ischaemic stroke, a great deal of research interest has been channelled into the investigation of cellular and molecular mechanisms involved in BBB breakdown during and/or following an ischaemic stroke-mediated injury (Bernardo-Castro et al. 2020; Arba et al. 2021). It is anticipated that better understanding of these mechanisms may open up new avenues for future therapeutic strategies for ischaemic stroke.

2. Blood-brain barrier (BBB) structure and function

2.1.1. Overview

BBB is an active interface between the peripheral circulation and CNS, preserving the neural microenvironment with a dual purpose: the barrier function to restrict transport of potentially harmful or toxic compounds from the blood to the brain, and the carrier function responsible for the transport of essential nutrients and oxygen to the brain and the removal of metabolites (Jiang et al. 2018). This distinctive barrier consists of brain microvascular endothelial cells (BMECs), adjoined by their cell-to-cell tight junction proteins (TJs). The endothelium is embedded by pericytes and surrounded by astrocytes, thereby forming an additional continuous striatum that separates cerebral tissue from peripheral blood (Figure 2). These individual components work in concert to maintain the integrity and function of BBB (Jiang et al. 2018).

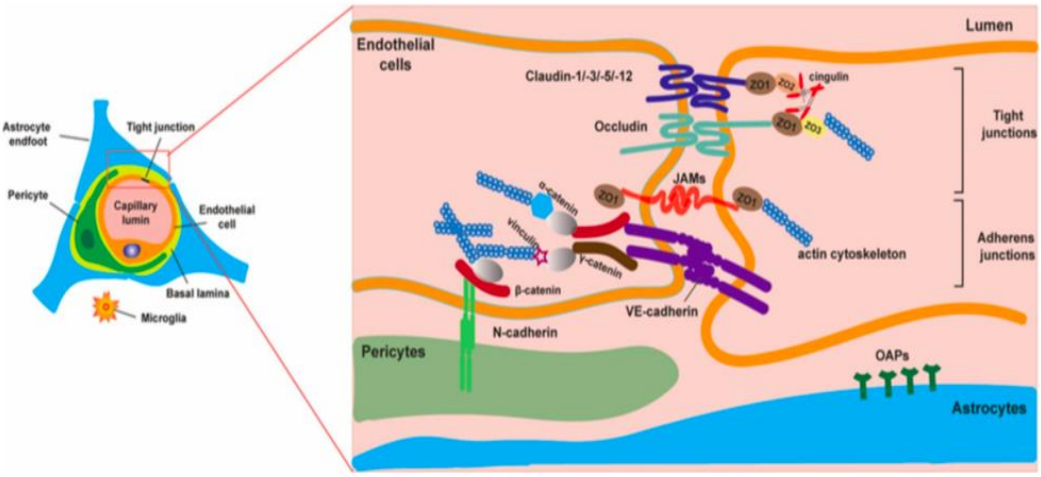


Figure 2. A schematic diagram of BBB and TJs.

Anatomically, BBB is made up of endothelial cells, astrocytes, pericytes, and basement membrane. Endothelial cells and pericytes are enclosed in the basement membrane, surrounded by astrocytes. TJs consist of integral membrane proteins (occludin, claudin, and junctional adhesion molecules) as well as cytoplasmic proteins (ZO-1, ZO-2, ZO-3, and cingulin) that link the membrane proteins to actin cytoskeleton in order to maintain the architecture and function of brain endothelium. Figure adopted from Kadry et al. (2020).

2.1.2. Brain microvascular endothelial cells

Endothelial cells, the major cellular component of the vasculature, cover the entire inner surface of all capillaries and synthesise a large number of vasoactive substances to control and maintain cerebrovascular tone and homeostasis at all times (Martinez-Majander et al. 2021). In some organs, endothelial cells differentiate and develop into highly specialised barriers to regulate vascular integrity and permeability (Segarra et al. 2021). One example is the BMECs, the specialised type of endothelial cells in the brain equipped with TJs and also have lack fenestration and pinocytotic vesicles compared to peripheral endothelial cells (Lu et al. 2021). Structurally, the TJs complex provides an essential mechanical link between individual BMECs, which involve specific transmembrane adhesion proteins and cytoplasmic accessory proteins (Stamatovic et al. 2016).

Transmembrane protein is composed of integral membrane proteins such as claudin, occludin, and junctional adhesion molecules (JAMs). Claudin is the major component of TJ complex and a member of a transmembrane protein family with more than 26 isoforms, of which claudin-5 is the primary constitutive and most abundant claudin in humans (Greene et al. 2019). The discovery of remarkably smaller infarct size and lower permeability of BBB following ischaemic injury in animal models of ischaemic stroke through targeting the level of claudin-5 further verified the critical role of this protein for BBB function (Lv et al. 2018).

Occludin is also an essential transmembrane regulator of BBB permeability. The changes in localisation of this protein following ischaemic injury led to the massive impairment of BBB and cerebral oedema in *in vivo* ischaemic stroke models (Lochhead et al. 2010; Winkler et al. 2020). Furthermore, as the fragments of occludin can be detected in the circulation after ischaemic stroke, and its levels increase proportionately to the magnitude of BBB damage, occludin may serve as a reliable early parameter for the disruption of BBB in patients with ischaemic stroke (Pan et al. 2017). JAMs are a member of the immunoglobulin protein superfamily that play important role in regulating BBB paracellular permeability, particularly with respect to immune cells (Weber et al. 2007).

The cytoplasmic accessory proteins provide a link between transmembrane proteins and actin cytoskeleton through their PDZ domains. Zonula occluden (ZO) is the most important cytoplasmic plaque member, as it contributes the most to the stability of the structure of TJs (Fanning et al. 1998). The association of dislocalisation, decreased expression, and enhanced phosphorylation of ZO with the increases of paracellular flux across the BMEC monolayer may suggested the functional importance of this protein to maintain BBB structure (Fischer et al. 2002).

The organisation of actin cytoskeleton also plays a critical role in maintaining the functional and structural integrity of BMECs. Under physiological conditions, the cortical actin is distributed throughout the endothelial cells as short filaments and diffuse monomers, while the specific pathological exposures such as hypoxic/reperfusion, free radical, or inflammation polymerised these filaments into linear stress fibres, which in turn increase cytoskeletal tension force and contracted cell morphology, leading to opening of intercellular gaps between endothelial cell and enhancing hyperpermeability (Xu et al. 2021). The disruption of actin cytoskeleton formation in the early stages of ischaemic stroke leads to the massive breakdown of BBB integrity and worsened long-term neurological outcome, confirming the crucial role of this structure in maintaining BBB permeability (Shi et al. 2016).

2.1.3. Astrocytes

Astrocytes, the most abundant glial cells in the CNS, perform multiple cerebral homeostatic functions, such as promoting angiogenesis and neurogenesis, preventing oxidative stress through generating anti-oxidant enzymes, and modulating synaptic function (Becerra-Calixto and Cardona-Gómez 2017). Morphologically, astrocytes resemble star shapes, with endfeet almost completely covers the abluminal surface of the cerebral microvasculature. Intercommunication between astrocytes and BMECs through direct contact, as shown in *in vitro* model of human BBB, exhibited remarkably better BBB integrity and function, indicating the important role of astrocyte in preserving BBB permeability (Shao and Bayraktutan 2013). It is also been reported in the recent study that cerebral ischaemic injury activates transcriptional programs in reactive astrocytes which relevant to vascular remodelling, BBB permeability, and overall neurological outcome, confirming the critical role of these

glial cells in the regulation of BBB integrity and progression of ischaemic stroke (Williamson et al. 2021).

The response of astrocytes to all forms of CNS insults including ischaemic stroke injury is through a process referred to as reactive astrogliosis. Although there is a core set of genes that is upregulated in reactive astrocytes, at least 50% of the altered gene expression depend on the type of inducing injury. For instances, while neuroinflammation insult display a phenotype that suggests they may possess disruptive impacts, ischaemic injury exhibit a molecular phenotype which indicates they possible protective (Zamanian et al. 2012). Liddelow et al. (2017) subsequently classified these different types of reactive astrocytes into A1 and A2, respectively, where the activation of the former subtypes is induced by several inflammatory factors, such as IL-1α, TNF-α, and C1q, which in turn induced neurons and oligodendrocytes death (Liddelow et al. 2017; Li et al. 2020b). In contrast, the latter is activated by IL-6/JAK/STAT3 signalling pathway, which contribute to contain the influx of systemic and local inflammatory cells (Rakers et al. 2019) and provide a supportive scaffold for axonal regeneration (Anderson et al. 2016). Since reactive astrogliosis display such double-edged sword effect, exploration of the timing and mechanism of astrocyte participation in ischaemic stroke progression is of paramount importance to discover novel therapeutic strategy in ischaemic stroke (Shen et al. 2021).

2.1.4. Pericytes

Pericytes are vascular mural cells embedded within the basement membrane of cerebral endothelial cells (Yang et al. 2017). In areas lacking basement membranes, they are directly in contact with BMECs through gap junctions and adherent junctions (Liu et al. 2012). The reduction of pericyte numbers as observed in pericyte-deficient

mouse mutants remarkably increases the permeability of BBB through regulating the expression of endothelial TJs and by promoting polarisation of astrocyte endfeet (Armulik et al. 2010).

Growing evidence shows that pericytes play different roles in different stages of ischaemic stroke. During the hyperacute stage, the constriction and death of pericytes may be the main cause of the no-reflow phenomenon in brain capillaries, and the damages of these cells in the acute phase participate in inflammatory-immunological response leads to BBB damage and brain oedema. In contrast, in the later recovery stages, pericytes express various substances, such as VEGF and nerve growth factor, to induce angiogenesis and neurogenesis, thereby promoting neurological recovery after ischaemic injury (Xiong et al. 2010).

3. Endothelial Progenitor Cells (EPCs)

3.1. Definition and identification

Endothelial progenitor cells (EPCs) were first described as CD34 and Flk1-positive cells isolated from peripheral blood by using magnetic microbeads, which displayed typical endothelial cell characteristics after plating on fibronectin-coated surface. This seminal study subsequently demonstrated an effective mobilisation, differentiation, and incorporation of exogenous EPCs with pre-existing vasculature in the ischaemic area and eventually improved neovascularisation and functional outcome of animal model of hind limb ischaemia (Asahara et al. 1997). Since *de novo* blood vessel formation was formerly thought to be limited merely on embryogenic vasculogenesis, this discovery has indeed radically changed our understanding of postnatal neovascularisation and opened up new avenue to therapeutic vasculogenesis by using these particular stem cells (Faris et al. 2020).

EPC populations can be isolated and quantified by two methods, namely immunological surface markers using flow cytometry, and *in vitro* cell culture isolation procedure. Using flow cytometry, circulating EPCs are frequently measured as the proportion of mononuclear cells expressing CD34+ (stemness marker) and KDR+ (endothelial marker). While CD34+/KDR+ cells can fully differentiate into mature endothelial cells (Pelosi et al. 2002), it is also possible that these cell populations may accidently arise from sloughed mature endothelial cells (Peichev et al. 2000), hence they are not truly representative of EPCs. In addition, CD34+ cells have been criticised as markers of EPCs since their therapeutic value in vascular damage may not originate from their capacity to differentiate into mature endothelial cells, but from paracrine factors (Popa et al. 2006).

Given that mature endothelial cells do not express CD133+ (progenitor markers) and co-expression of CD133+ and KDR+ on CD34+ cells constitute a phenotypically and functionally distinct population of circulating/sloughed endothelial cells, these cells may more correspond to the true EPCs (Peichev et al. 2000). Of note, the observation of intracellular expression of CD133 in OECs and the abolishment of post-ischaemic revascularisation of hind-limb ischemia nude mouse model administrated by OECs transfected with specific siRNA (siCD133-OECs) have addressed the discrepancies of CD133 positivity and ontogeny in endothelial progenitors (Rossi et al. 2019). In addition, increasing of evidence have also shown that CD34+ cells and CD133+ cells play an important role for endothelial repair (Jiang et al. 2021) and the expression of these markers are crucial to maintain the regenerative properties of OECs (Tasev et al. 2016; Rossi et al. 2019). Nevertheless, instead of forming EPC colonies, direct plating of CD34+/CD133+/KDR+ cells has been shown to differentiate only into hematopoietic cells, suggesting that these cells were non-angioblastic hematopoietic

progenitors, and their main therapeutic action is as paracrine factors, rather than direct differentiation into mature endothelial cells (Case et al. 2007). Considering those facts, a recent consensus therefore recommends operationally defining true EPCs as an immunophenotype depicting positive for stemness/immaturity markers (CD34, CD133) and endothelial cell markers (CD31, VE-Cadherin, von Willebrand factor, CD146, VEGFR2), and negative for hematopoietic markers (CD45, CD14) (Medina et al. 2017; Bayraktutan 2022).

In addition, recent studies have discovered multiple novel biomarkers to identify stem cells that possess unique capacity to differentiate into endothelial cells (Chambers et al. 2021). For instance, single CD157 cells, a marker of tissue-resident vascular endothelial stem cells in large arteries and veins of numerous mouse organs, can form colonies *in vitro* and produce donor-derived portal vein, sinusoid, and central vein endothelial cells following transplantation in the liver (Wakabayashi et al. 2018). Although CD157 cells have been used to identify EPCs that possess high proliferative potential, and better bleeding phenotypes have been observed following the administration of CD157+CD200+ cells to haemophilia A mice, to date, there is not yet evidence for their existence in humans (Farkas et al. 2020).

Another example of a novel marker is endothelial protein C receptor (EPCR), a glycoprotein with anti-thrombotic and cytoprotective roles. EPCR+ cells display a robust clonogenicity in culture and high vessel reconstitution efficiency upon transplantation. Importantly, they give rise to *de novo* formation of endothelial cells (Yu et al. 2016). However, careful consideration is necessary when defining EPCR+ cells as the true EPCs, since these cells can also differentiate into pericytes and this marker has been widely used as reliable markers for hematopoietic stem cells (Fares et al. 2017).

In vitro cell culture isolation procedure is based on the functionality of cells to display endothelial cell markers and promote neovascularisation. This approach consistently produces two morphologically and functionally distinct EPC subtypes, namely early EPCs (eEPCs) and late EPCs, the latter of which is also known as outgrowth endothelial cells (OECs) (Medina et al. 2010) or recently called endothelial colony forming cells (ECFCs) (Medina et al. 2017; Chambers et al. 2021). eEPCs appear within <7 days in culture, display spindle-shape morphology, and do not have the ability to differentiate into mature endothelial cells. Even so, accumulating evidence shows that eEPCs may contribute to vasculogenesis through secreting various growth factors, such as VEGF, SDF-1, HGF, and IGF-1 (Hur et al. 2004; Medina et al. 2010). In contrast to eEPCs, OECs appear later in the culture (14-28 days), and they exhibit typical endothelial cell characteristics and strong proliferative and migratory capacity. They also continuously express CD34 and CD133, but not CD45 marker (Medina et al. 2017; Reid et al. 2018). The discovery of the OECs' ability to differentiate into mature endothelial cells and then become part of the new vasculature (Medina et al. 2010; Reid et al. 2018), as well as to release various angiogenic factors to support dying endothelial cells (Maki et al. 2018), has attracted researchers to the potential use of these particular cells to regenerate vascular damages in various ischaemic diseases, including ischaemic stroke (Bayraktutan 2019; Faris et al. 2020; Bayraktutan 2022).

3.2. EPC levels as a clinical biomarker of ischaemic stroke

Considering that endogenous factors such as EPCs are involved in the cerebrovascular restoration and functional recovery of patients with ischaemic stroke, variations in their quantity and functionality may serve as clinical biomarkers for ischaemic stroke injury (Bayraktutan 2022). However, evidence supporting the utilisation of EPC numbers as diagnostic and prognostic markers remains inconsistent mainly due to the

wide variety of surface antigen markers used to measure the level of these cells. In terms of diagnostic markers, a marked increase in EPC levels (CD34+/CD133+/KDR+, CD34+/KDR+, and CD34+ cells) has been observed within the first 24 hours of ischaemic stroke onset (Dunac et al. 2007; Paczkowska et al. 2013), while other groups have reported initially lower (CD34+/CD133+ and CD34+/KDR+ cells) (Tsai et al. 2014) but steadily improving numbers (Fadini Gian et al. 2006) of EPCs (CD34+/CD133+/KDR+) in similar ischaemic stroke patient arms compared to the healthy controls. Similarly, several groups have reported a close association between EPCs level (CD34+, CD34+/CD133+, and CD34+/KDR+ cells) and the severity and neurological recovery of ischaemic stroke patients (Sobrino et al. 2007; Taguchi et al. 2008; Tsai et al. 2014), while others reported to the contrary (Zhou et al. 2009).

The scrutiny of subtypes and aetiologies in patients with ischaemic stroke revealed that EPC levels (CD34+/CD133+/KDR+) are only associated with functional outcomes in patients with large-artery atherosclerosis and small-vessel disease subtypes; however, caution should be taken when interpreting this finding, as Martí-Fàbregas et al. (2013) used a limited sample and did not recruited healthy controls. Due to controversies surrounding EPC level, functional studies focusing on the tubulogenic, proliferative, and migratory capacity of EPCs may serve as a better biomarker to predict the prognosis of patients with ischaemic stroke (Navarro-Sobrino et al. 2010; Kukumberg et al. 2020). Although data supporting this notion remains limited, accumulating evidence suggests that the impairment of EPC functions prevents the execution of their crucial role in repairing endothelial damages (Ingram et al. 2007; Chen et al. 2016). A recent study that longitudinally measured the quantity and functionality of circulating EPCs in patients with ischaemic stroke during acute,

subacute, and chronic phases of the disease may shed some lights on the actual diagnostic and prognostic role of this particular stem cells (Rakkar et al. 2020).

It is also important to note that several comorbidities of stroke, such as hypertension (Lee et al. 2011), diabetes mellitus (DM) (Rigato et al. 2015), dyslipidaemia (Li et al. 2018a), and ageing (Xia et al. 2012) may reduce the number and functional properties of EPCs, and have been associated with enhanced risk of future cerebrovascular events (Schmidt-Lucke et al. 2005; Martí-Fàbregas et al. 2015). However, since culturing EPCs is laborious and time consuming, the use of this particular stem cell as a diagnostic marker in the acute setting of ischaemic stroke may not be feasible.

3.3. EPCs serve as therapeutics for ischaemic stroke

Since brain endothelial dysfunction plays a crucial role in the initiation and progression of ischaemic stroke (Andjelkovic et al. 2019), therapeutic strategies aiming to reconstruct the damaged cerebrovascular network either by stimulating local angiogenesis or promoting *de novo* blood vessel formation have emerged as attractive and promising therapeutic approaches in stroke medicine (Li et al. 2021). In this context, cell-based therapy using OECs may represent such strategy, as they can perform many functions, including differentiating into mature endothelial cells and subsequently become part of the new vasculature (Medina et al. 2010; Reid et al. 2018), as well as secreting various soluble factors to induce angiogenesis and recruit more stem cells into injury sites (Maki et al. 2018).

In addition, the capacity of OECs to regulate almost all signalling pathways, ranging from oxidative stress and inflammation to apoptosis (Qiu et al. 2013; Geng et al. 2017), has made them exceptionally distinctive and unique compared to the many previous agents that targeted only a single molecular pathway involved in cerebral ischaemic injury (Neuhaus et al. 2017; Bayraktutan 2019). Intriguingly, since

neovascularisation appear to play critical role to prepare a fertile ground for neuronal regeneration (Taguchi et al. 2004; Fujioka et al. 2019), it has been reported that the administration of OECs also stimulate neurogenesis following cerebral ischaemic injury (Shen et al. 2004; Ding et al. 2016; Li et al. 2018b).

Although studies investigating the safety and efficacy of OECs in patients with ischaemic stroke remain limited, the administration of autologous bone marrow mononuclear stem cells (i.e. cells administrated from the same individual) in subacute stroke patients was safe and feasible (Savitz et al. 2011; Prasad et al. 2014). Recently, a randomised, two-centre, placebo-controlled phase I/IIa study involving 18 patients with acute cerebral infarction showed that intravenous administration of autologous EPCs developed fewer serious adverse events and displayed no allergic reactions or toxicity effects compared to placebo-controlled group during a four-year follow-up period (Fang et al. 2019).

Despite being relatively safe from the risks of immunological reactions, biological incompatibility, and disease transmission, the application of autologous strategy may be very challenging and even impossible in acute ischaemic stroke settings, as the generation of sufficient OEC numbers requires a prolonged period of time (approximately six weeks), and it is possible that cells acquired from patients may be dysfunctional (Kot et al. 2019; Law et al. 2021). As a result, allogeneic cell therapy, which involves administering *ex vivo* expanded cells to an unrelated recipient, may overcome these pitfalls and also offer various additional benefits.

To begin with, allogeneic therapeutic options allow for the isolation of highly functional cells from young and healthy donors, which can then be subjected to a variety of quality control measures, as well as pharmacological, epigenetic, and genetic manipulation to enhance their vasoreparative function before being

administered to patients (Faris et al. 2020). Second, the cells can be expanded in large quantities and cryopreserved (Reinisch et al. 2009). Third, expression of inherently higher levels of immunoregulator molecules, such as HLA-G and anti-inflammatory factors (e.g. TGF-β and IL-10), may render OECs very safe therapeutics, due to the expected absence of severe inflammatory reactions (Proust et al. 2020).

In support of this notion, no serious adverse events have been reported in ischaemic models of dorsal chamber immunocompetent mouse model administrated with these particular stem cells (Proust et al. 2020). The attainment of no clinical abnormalities, tumorigenesis, and cell toxicity in healthy dog or mouse models of ischaemic retinopathies transplanted with human umbilical cord-derived OECs further corroborates this notion, and confirms the safety of allogeneic therapeutic strategy for OECs (Reid et al. 2018; Lee et al. 2019b).

Finally, gene-editing technologies including clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) can be used to ablate class II transactivator (CIITA) expression, the master regulator of major histocompatibility complex (MHC) II, in effort to diminish immunogenicity of OECs (Abrahimi et al. 2015). Since immune rejection is the major hurdles in allogenic therapy, this discovery is a promising platform to regulate immunomodulatory effect of stem cell-based therapy.

4. Senescence of EPCs

4.1. Overview

Advances in public health and biomedical research over the last century have spurred a substantial growth in life expectancy around the world (Wang et al. 2016). According to the current trajectory, it is estimated that there will be more than 2 billion

individuals aged 60 years and over by 2050, thus fundamental investigations into the mechanisms of ageing and strategies to attenuate the detrimental effects associated with this condition are of pivotal importance (Chakravarti et al. 2021). Indeed, accumulating evidence shows that advanced age is strongly associated with increased risk of stroke and more severe post-stroke neurological deterioration (Androvic et al. 2020; Akyea et al. 2021; Zaidat et al. 2021). Nevertheless, recent findings indicate that ageing may be a modifiable risk factor, and it is feasible to delay age-related diseases as a group by regulating fundamental ageing mechanisms (Baker et al. 2016; Jeon et al. 2017; Bussian et al. 2018).

Since increasing of evidences have shown that senescent cells, a permanent state of cell cycle arrest that is triggered by a variety of stresses, accumulate in various tissues acquired from age and age-related diseases individuals, and these cells may actively contribute to the pathobiology of diseases, targeting senescent cell may be a logical option to delay or perhaps reverse the development of ageing or age-associated diseases (Baker et al. 2011; Schafer et al. 2017; Childs et al. 2018).

4.2. Basic mechanism of cellular senescence

Continuous rounds of cell division during the lifetime gradually induce shortening and instability of the telomere, a protective structure present at the ends of chromosomes, leading to the activation of a persistent DNA damage response (DDR), as illustrated in Figure 3 (Azarm et al. 2020).

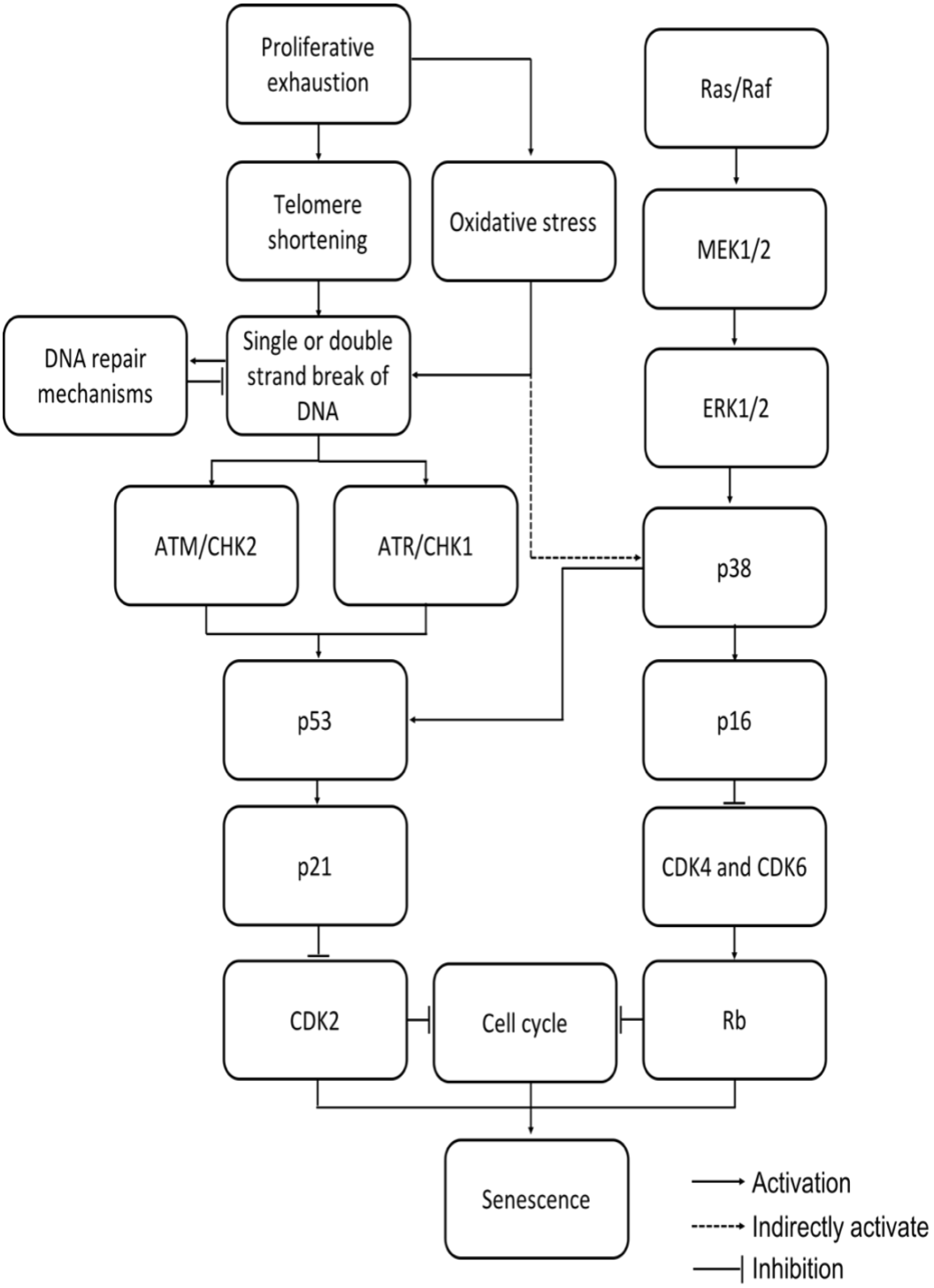


Figure 3. Overview of the molecular alterations driving cellular senescence.

Successive rounds of cell replication gradually shorten telomere length, leading to the induction of single- or double-stranded DNA break and enhanced oxidative stress level. DNA damages subsequently trigger a series of molecular alterations that eventually block the cell cycle and promote senescence. Oxidative stress or Ras/Raf signalling pathway can also induce cell cycle arrest and senescence through activating the p38 and p16 pathways.

Telomere attrition also can be induced by the excessive accumulation of ROS, as this structure is particularly rich in guanine, the most susceptible of the four DNA bases to oxidisation, due to having a low redox potential (Coluzzi et al. 2019). During DDR, either single or double-strand break of DNA is developed and can be visualised via detection of the phosphorylation of histone variant H2AX, γ-H2AX (Sławińska and Krupa 2021). This damage subsequently activates transducer protein kinases, including ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad-related protein (ATR), leading to the phosphorylation of their respective target effector kinases, Chk2 and Chk1 (Kumari and Jat 2021). In parallel, DDR also activates DNA repair signalling mechanism through several pathways, such as base excision, nucleotide excision, and mismatch repair (Simon et al. 2019).

While effective DNA-repair mechanisms can deactivate DDR signalling pathway and allow the resumption of normal cell function, the failure of this counteract system leads to the activation of transcription factor and tumour suppressor, p53, and subsequently activates p21 (Gorbunova and Seluanov 2016). Persistent stress or additional signals, such as activation of Raf/Ras/p38 pathway, can activate p16, which in turn inhibits CDK4 and CDK6, leading to the activation of Rb (Anerillas et al. 2020). The activation of either p21 or Rb concomitantly ceases the cell cycle and induces the development of senescence phenotypes, characterised by the profound morphological, biochemical, and functional alterations described in Figure 4 (Kumari and Jat 2021).

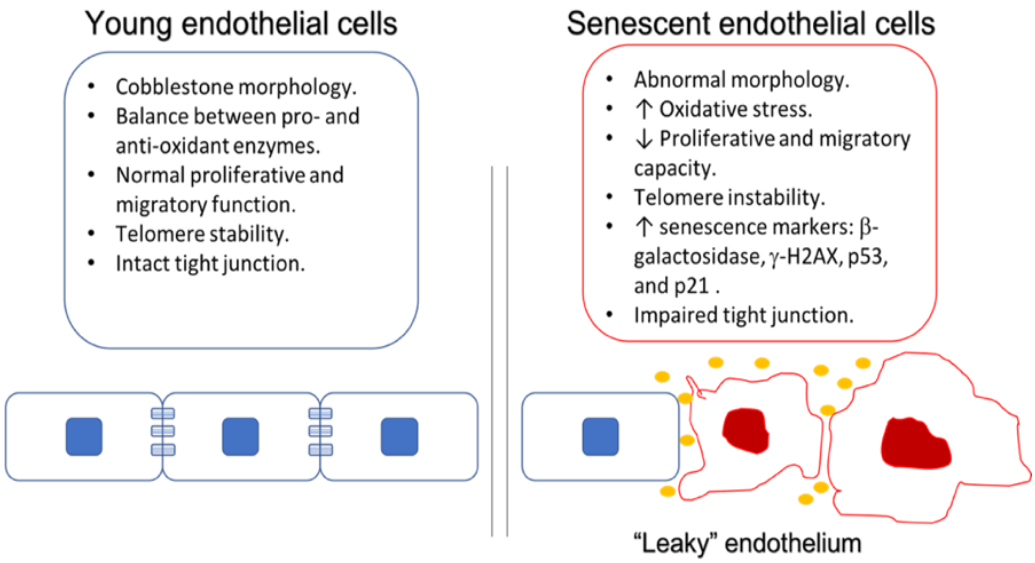


Figure 4. Changes in senescent endothelial cells.

The development of replicative senescence has profound implications for the morphological, molecular, and functional properties of endothelial cells.

4.3. The impact of senescence on EPCs

Although equipped with remarkably high anti-oxidant, proliferative, and migratory capacity, several typical senescence phenotypes (as shown in Figure 4) have been consistently observed in various stem cell types during normal and pathological ageing, implying that stem cells are not immune to the process of senescence (Lewis-McDougall et al. 2019). Considering that homeostatic maintenance and regenerative responsiveness to injury in many tissues depend on the self-renewal and restorative potential of specific stem cells, the accumulation of senescent stem cells may contribute to the development of ageing-associated tissue/organ dysfunction and disease (Ermolaeva et al. 2018; Lewis-McDougall et al. 2019).

Advanced age has been closely associated with the deterioration of EPC function, which may culminate in decreased capacity for neovascularisation in damaged vasculature, and facilitate the development and progression of cardiovascular (Rigato et al. 2016) and cerebrovascular diseases (Xia et al. 2012; Kukumberg et al. 2020).

This is supported by the fact that decreased circulating EPC availability and functionality following cerebral ischaemic injury is associated with increased extent of BBB damages and neurological deterioration, as well as the enhanced risk of future vascular events (Martí-Fàbregas et al. 2015; Sargento-Freitas et al. 2018; Kukumberg et al. 2020).

An effective regulation of intracellular redox state with Sirtuin 1 activator, glutathione, or oestrogen may attenuate the detrimental effect of chronological ageing in EPCs, as observed by the augmentation of telomerase activity and reduced of various senescence markers such as senescence-associated-β-galactosidase (SA-β-gal), γ-H2AX, p53, and p21 (Haendeler et al. 2004; Imanishi et al. 2009; Lamichane et al. 2019). Silencing one of the important pro-inflammatory chemokines, IL-8, also delays EPCs' ageing and increases their proliferative and regenerative potential (Medina et al. 2013). These findings collectively suggest that oxidative enzymes and inflammatory factors play a substantial role in regulating the ageing mechanism of EPCs, and manipulating these key mechanisms may be used in the context of stem cell rejuvenation.

5. Cell-free strategy

5.1. Overview

Accumulating evidence has shown that the restorative effects of cell-based therapy, including EPCs, are not solely realised by their differentiation into other cell lines, but also through the action of a set of molecules, including chemokines, cytokines, growth factors, lipids, free nucleic acids, microRNA, and the extracellular vesicles, so-called secretome, that they release (Maki et al. 2018; Cunningham et al. 2020). The critical role of these factors in the beneficial effects of cell therapy have been corroborated by

various studies demonstrating low EPC survival and engraftment rates in ischaemic sites in comparison to their remarkable reparative effects (Moubarik et al. 2011; Ahangar et al. 2020).

It is noteworthy here that harnessing cell transplantation-free strategies such as the one proposed by EPC-secretome may address several limitations of cell therapy. First, EPC-secretome can address the reduction of EPC vasoreparative function following introduction to hostile microenvironments (i.e. hypoxic condition) (Tasev et al. 2018). Second, using secretome as a ready-to-use product remarkably reduces the significant high number of cells required for transplantation, and overcomes the issue of replicative senescence that ultimately occurs after large-scale expansion (Medina et al. 2013; Pinho et al. 2020). Third, cell-free strategy displays lower risk of infection, immune incompatibility, and teratoma formation in comparison to living and proliferative cells (Pinho et al. 2020). Finally, secretome is relatively cheap and easy to produce, handle, and store compared to cell-based therapies, due to not requiring massive cell collection procedures (Alwjwaj et al. 2021).

5.2. Possible mechanism of action

Recent advances in cell and molecular biology have identified the crucial soluble factors in EPC-secretome as well as their specific functions in repairing vascular damages (Alwjwaj et al. 2021). To begin with, through stimulating the production of various anti-oxidant enzymes, including MnSOD and CuZnSOD, treatment with EPC-secretome neutralised hydrogen peroxide-evoked damages in endothelial cells, as shown by decreased ROS formation and more coherent tubule networks (Yang et al. 2010). In another study, intratracheal administration of EPC-exosomes to animal model of acute lung injury substantially reduced lung injury score and pulmonary oedema, partly via inhibiting the release of myeloperoxidase (MPO), a leukocyte-

derived enzyme that catalyses the formation of a number of ROS (Zhou et al. 2019). These findings collectively indicate that EPC-secretome helps to repair and maintain vascular damages through regulating oxidative stress signalling pathway.

Increasing evidence has shown that EPC-secretome may also modulate the release of various inflammatory cytokines and chemokines to execute its reparative function. For instance, treatment with EPC-secretome has been shown to decrease macrophage activation markers, such as CD86 and CD206, as well as mRNA expression of IL-1β and IL-6, in both *in vitro* and *in vivo* models of spinal cord injury (Wang et al. 2018). Likewise, the administration of an essential paracrine factor, namely thymosin B4, secreted by embryonic EPCs, has attenuated infarct size of pigs exposed to myocardial ischaemia and enhanced post-hypoxic cardiomyocyte survival by moderating post-ischaemic inflammatory responses (Hinkel et al. 2008).

In addition to oxidative stress and inflammation, EPC-secretome also effectively downregulates the release of caspase 3/7 and enhances brain endothelial cells' survivability against ischaemic/reperfusion injury, suggesting the capacity of this regimen to regulate the apoptotic process (Di Santo et al. 2016). The presence of this regimen also decreases the percentage of TUNEL-positive oligodendrocyte precursor cells (OPCs) exposed to ischaemic injury via regulating CXCL12-CXCR4 axis (Zhou et al. 2021). The observation of significant reduction in neuronal cells positively stained by propidium iodide and Hoechst 33258 during oxidative stress damages along with fewer TUNEL-positive cells in the grey matter of rats subjected to spinal cord injury following EPC-secretome treatment further verified the pivotal role of this regimen to modulate apoptotic process (Wang et al. 2018).

5.3. Therapeutic potential

The new concept of cell-free-based therapies focused on the paracrine factor of stem cells was first reported by Gnecchi et al. (2005), who showed the therapeutic role of MSC-secretome in an animal model of heart infarct. This seminal discovery then spurred an exponential increase in research utilising this regimen in various diseases in recent years, including stroke (Cunningham et al. 2020). In the context of EPC-derived factors, this regimen has been shown to enhance vascular density, myelin, and mature oligodendrocytes in white matter, and rescue cognitive function in the mouse cerebral hypoperfusion model (Maki et al. 2018). In line with these findings, treatment with EPC-secretome also improved vascular density and myelination in the corpus callosum, as well as attenuated learning and memory deficits in cerebral ischaemia neonatal rats (Zhou et al. 2021).

The regenerative potentials of EPC-secretome are not merely limited to the vascular system, but also display such neuroprotective effects. For instances, treatment with this regimen significantly enhanced the differentiation and proliferation of both neuronal cells and NPCs against oxygen glucose-deprivation injury (Park et al. 2014; Di Santo et al. 2019; Santo et al. 2020). In the animal model of ischaemic stroke, the presence of EPC-secretome promoted axonal reorganisation towards cortical peri-infarct areas two weeks after the ischaemic event (Rosell et al. 2013).

The comparative study assessing the safety and efficacy of EPC- and EPC-secretome-based therapy subsequently discovered that both agents are in fact equally safe and effective to reduce infarct volume and neurological deficits in animal model of ischaemic stroke (Rosell et al. 2013). In accordance with these findings, treatment with either bone marrow-derived autologous OECs or OEC-secretome were also

equally effective to inhibit migration and proliferation of vascular smooth muscle cells *in vitro* and regulated their arrangement (Liu et al. 2011).

While the reparative effect of the secretome of EPCs is relatively well-documented in pre-clinical settings, to our knowledge there is currently no clinical study that has assessed the putative therapeutic role of this regimen in clinical settings. Nonetheless, the clinical study assessing the safety and efficacy of MSC-secretome has shown that this regimen triggers less inflammatory signs and appears to have great osteogenic potential (Katagiri et al. 2016).

6. Reverse translational study

The conventional bench-to-bedside biomedical research paradigm, whereby mechanisms with potential relevance to human disease are elucidated using experimental model systems prior to designing and assessing novel therapeutics in clinical trial, has had mixed results. While the number of novel agents approved by the US Food and Drug Administration (FDA) has gradually increased over the last decade, only about 10% of new drugs entering phase 1 clinical trials have been successful in obtaining FDA approval (Stitziel et al. 2020).

In contrast to translational research, reverse translational study (Figure 5), also called bedside-to-bench research, begins with actual, real-life patient experiences in clinic practice or during clinical trials, and works backward to identify the key signalling molecules associated with diseases (Bix et al. 2018). Such human data are subsequently used to fuel research in experimental models for mechanistic studies, enabling the development of novel therapeutic approaches (Lourbopoulos et al. 2021).

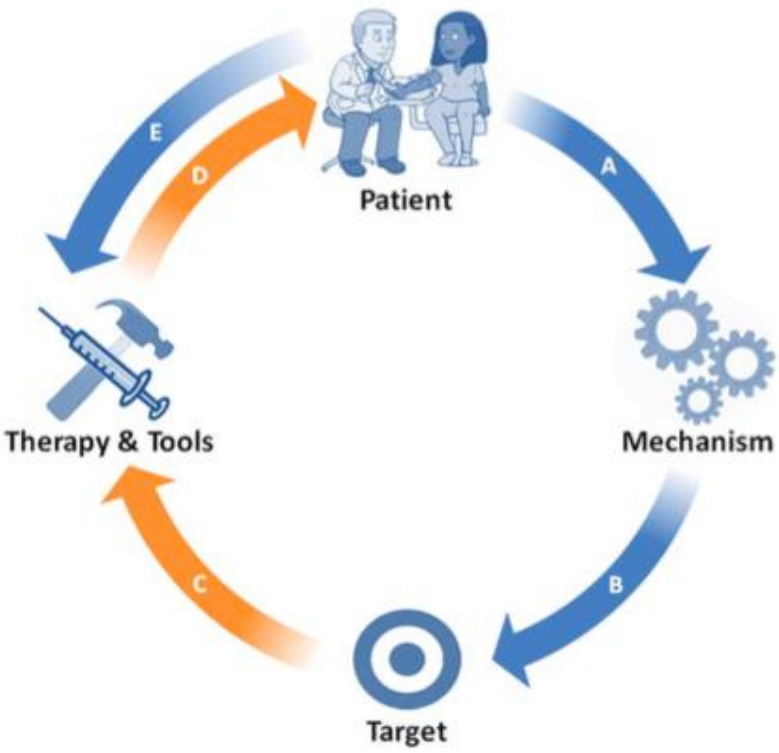


Figure 5. Iterative cycle of translational and reverse translational study.

Reverse translational research (blue) begins with extensive characterisation of the patients informing the disease mechanism (A), which in turn drives deeper understanding and better selection of targets (B). Conventional translation study (orange) advances identification of a therapeutic target to discovery and optimisation of novel potential therapies and corresponding tools, including biomarkers (C), to testing in patients (D). Reverse translational study also includes learnings from patients that are directly reflected back to drug discovery and development tools or to refinements of a therapeutic (E). Figure adopted from Wagner (2018).

The concept of reverse translational research design is not new, but it has generally been overlooked and not clearly delineated in the past. For instance, while the importance of translational research in bidirectional manner or the utilisation of human data in driving pre-clinical studies was highlighted since a decade ago by Rubio et al. (2010), they did not use the term "reverse translational research". Nevertheless, the potential applications of this strategy are crucial for the translational science community, and an increasing number of researchers in recent years have employed this approach (Soranno et al. 2019; Han et al. 2021; Maas et al. 2021; Tian et al. 2022).

In addition, Japan's Research for Allergy and Immunology strategy recently proposed reverse translational research as one of the major platforms to achieve the vision of national freedom (in so far as this is possible) from allergic and immunological diseases (Adachi et al. 2020).

The observation of a close association between better performance on memory tasks and aerobic fitness in older humans has propelled *in vitro* and *in vivo* studies to ascertain the mechanisms underlying this correlation, which further demonstrated that controlled exercise promotes profound and lasting effects on the proliferative behaviour of neural progenitor cells in the hippocampus (Erickson et al. 2009; Farioli-Vecchioli et al. 2014). This bidirectional relationship is one of the examples of the utilisation of reverse translational study that offers scope to identify novel therapeutic approaches and to identify key mechanisms involved in reparative effects.

Another example is the discovery of the cardioprotective effect of sodium-glucose cotransporter 2 (SGLT2) inhibitor, the FDA-approved drug to lower blood sugar in adults with type II diabetes mellitus (T2DM). Following the FDA recommendation in 2008 to include cardiovascular endpoints in phase II and III clinical trials of new anti-diabetic medications, the Empagliozin Cardiovascular Outcome Event Trial in T2DM Patients (EMPA-REG OUTCOME trial) unexpectedly revealed a profound 35% relative risk reduction in hospitalisation for heart failure in patients with T2DM treated with empagliozin (Zinman et al. 2015). This trial subsequently spurred a large surge *in vivo* and *in vitro* studies to ascertain the key mechanisms of the cardioprotective effects of this drug (Shao et al. 2019; Griffin et al. 2020).

In the context of ischaemic stroke, despite being one of the leading causes of death and disability worldwide (Feigin et al. 2021), rtPA remains the single approved pharmacotherapy for this disease, with the large majority of patients being excluded

from its potential benefits due to its restrictive therapeutic criteria (Anand et al. 2021; Berge et al. 2021). Given the establishment of several protocols such as ARRIVE, STEPS, and STAIR (Saver et al. 2009; Boltze et al. 2019; Percie du Sert et al. 2020), and extensive rigorous pre-clinical studies, methodological pitfalls alone may not be the single factor blocking the translation of favourable outcomes from experimental setting into clinical study (Lourbopoulos et al. 2021).

In addition, considering the fact that that many processes, ranging from excitotoxicity to oxidative stress and apoptosis, are spatially and temporally involved in the pathophysiology of ischaemic stroke, the identification of basic biological alterations following cerebral ischaemic injury is of paramount importance (Neuhaus et al. 2017; Tian et al. 2022). In this regard, a reverse translational study may represent a promising tool to establish a strong foundation for future advances and effective therapeutic strategies (Bix et al. 2018; Lourbopoulos et al. 2021).

Thesis aims

Part I:

1. To characterise the morphology and functional properties of OECs.
2. To assess the effect of OGD±R injury on OEC function.
3. To ascertain the capacity of OECs to integrate with resident brain endothelial cells and to form functional BBB.
4. To determine whether treatment with OECs can attenuate BBB damage and elucidate the molecular mechanism involved in this process.

Part II:

1. To investigate the effect of ageing on the level of circulating EPCs and various elements that regulate EPC mobilisation and survivability.
2. To assess the effect of chronological ageing on the morphology, molecular, and functional phenotypes of OECs.
3. To determine whether the inhibition of oxidative stress signalling pathway can delay senescence and preserve barrier-forming activity of OECs.

Part III:

1. To identify the biochemical alteration following cerebral ischaemic injury, and to discover whether OEC-CM can address this pathological event.
2. To scrutinise the molecular mechanism underlying OEC-CM-reparative action.

Materials and methods

1. Cell preparation

1.1. Chemicals and equipment

The details of chemical, biological, antibody, equipment, and software utilised in the thesis are listed in Appendix I.

1.2. Cell culture

Human brain microvascular endothelial cells (HBMECs), human astrocytes, and human pericytes (Figure 6) were obtained at passage 2 and were subsequently seeded in T25 flasks in a humidified atmosphere (95% relative humidity) under normal conditions (70% N_2, 20% O_2, 5% CO_2, and 5.5 mM D-glucose at 37°C). The HBMEC media (ECM) contained 10% foetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% endothelial growth supplement. Astrocyte and pericyte media contained 5% of FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% astrocyte or pericyte growth supplement. The media was changed every other day. To detach cells from the flask, the cells were trypsinised and washed two times with phosphate buffered saline (PBS), and were then incubated for 5 minutes in trypsin (2.5 gr/L) in PBS (1:4). The flasks were gently tapped to detach the cells and then viewed under light microscope to ensure detachment. The respective cell media was added to neutralise the trypsin. The cell suspension was collected in the falcon tube and the flask was washed by media to ensure the remaining cells were added to the falcon. The cells were centrifuged at 250 g for 5 minutes. The supernatant was then discharged, and the cell pellet was suspended by cell media. The cells were subsequently cultured in a fresh T75 flask. Aseptic techniques and the best tissue culture practices were used.

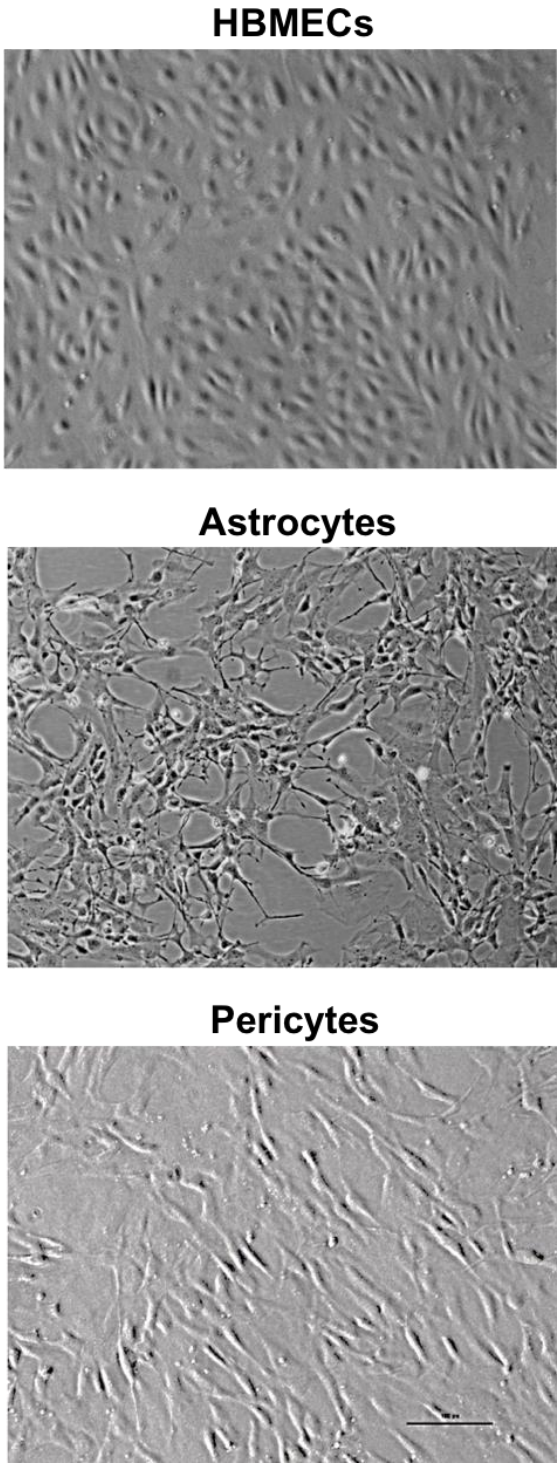


Figure 6. The morphology of HBMECs, astrocytes, and pericytes.

The HBMECs, astrocytes, and pericytes exerted cobblestone, stellate shape, and spindle shape morphology, respectively. Scale bar: 100 μM.

1.3. OEC culture and OEC-CM preparation

The blood samples of young healthy volunteers who had been recruited for The Dunhill Medical Trust (DMT) EPC study (Section 3. Clinical study) were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Blood samples were then diluted 1:1 with PBS in falcon tube and were added slowly over histopaque. The tubes were centrifuged at 400 *g* brake off for 30 minutes at room temperature, causing the buffy coat layer to appear. This layer was transferred and diluted with 1:3 PBS in a fresh falcon tube. The tubes were centrifuged at 300 *g* brake on for 15 minutes at room temperature. The supernatant was discharged and the pellets were diluted with culture medium, and were centrifuged again at 250 *g* brake on for 10 minutes at room temperature. The supernatant was removed and the cell pellets were resuspended with medium. The cells were then plated into fibronectin-coated wells and were incubated under normal conditions, as described in section 1.2. The media was changed every other day. Endothelial Cell Growth Medium-2 (EGM-2) containing 20% FBS and full supplementation (provided with the media) was used to culture the outgrowth endothelial cells (OECs). The isolation efficiency from healthy volunteers is ~25%.

OEC-derived conditioned medium (OEC-CM) was isolated according to a previously published protocol (Di Santo et al. 2014). The OEC-CM was isolated from OECs that acquired from 3 young healthy volunteers with similar OEC characteristics across the volunteers. Nearly confluent OECs were washed three times and were incubated with EGM-2 containing 1% FBS and without supplement for 48 hours under hypoxic conditions (1.5% O_2, 5% CO_2, 93.5% N_2). The conditioned medium was then collected and centrifuged at 10,000 rpm in 4°C, and the supernatant was filtered (0.22 μm) and was snap frozen at -80°C until use. Supplement-free EGM-2 containing 1% FBS was used as a control medium.

1.4. Cell counting

To address the inter-experimental errors, cells were cultured in accurate numbers and were monitored to determine cellular viability. Small aliquots of cell suspension (10 µL) were mixed with equal volume of 0.1% trypan blue for 5 minutes, then the mixture was carefully pipetted onto haemocytometer for cell counting. Cells situated on five large grids were counted with the aid of a light microscope. The number of cells per mL and cell viability were calculated using Equation 1 and Equation ***2***, respectively.

Equation 1. The total number of cells per millilitre

$$\text{Cells per mL} = \text{average number of cells per grid} \times \text{dilution factor} \times 10^4$$

Equation 2. Cell viability

$$\text{Cell viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \text{x } 100\%$$

2. Animal study

Focal ischaemia was induced in anaesthetised male Wistar rats by transient middle cerebral artery occlusion (MCAO) as described previously (Gibson et al. 2014). A 4-0 round tip and silicon coated suture was inserted from the left common carotid artery into the internal carotid artery until reaching the circle of Willis to occlude the origin of the MCA. This technique avoids transection of the external carotid artery, improves welfare, and prevents confounding effects on behavioural outcomes. Reperfusion was allowed after 60 minutes of occlusion to mimic the commonly encountered clinical sequence of thrombotic occlusion of a major vessel followed by reestablishment of blood flow. Cerebral blood flow (CBF) was measured by laser Doppler technique. Animals exhibiting >65% reduction in CBF during MCAO followed by >80% recovery after 10 minutes of reperfusion were included in the study. Rectal

temperature was monitored and kept at ~37°C during the surgery and recovery periods.

24 hours after the induction of MCAO, the animals were randomly divided into two subgroups and given either $4x10^6$ OECs or vehicle (500 μl in EBM-2 media) via tail vein injection. On day 4 of ischaemic injury (or on day 3 of OEC treatment), animals were sacrificed and their brains were removed and cut into three pieces: right and left cerebral hemispheres and cerebellum. The presence of cerebral oedema was verified in ipsilateral and contralateral brain hemispheres via the wet-dry method. For this, brain samples were immediately weighed on an electronic balance to obtain their wet weight before drying them in an oven at 100°C for 24 h to obtain their dry weight. The % of brain water content (BWC) was calculated using the following formula: BWC = [(wet weight−dry weight)/wet weight] × 100%. The experimenters were blinded to the treatment the rats had received prior to all subsequent analyse.

All animal experiments have been carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The animal study in this thesis was performed by Dr Ulvi Bayraktutan.

3. Clinical study

The clinical data was obtained from DMT EPC study (NCT02980354). This single-centre, prospective, observational case-controlled study assessed the quantity of circulating EPCs and the biochemical properties of healthy volunteers (HVs) and patients with ischaemic stroke at four different time points following ischaemic injury: days 0 (within 48 hours of ischaemic stroke symptom onset), 7, 30, and 90, covering acute, subacute, and chronic phases of ischaemic stroke. The HVs were subsequently

split into two subgroups by age: young (< 65 years old) and ageing (≥ 65 years old). The demographic and clinical data are presented in Table 1.

Table 1. Baseline characteristics of healthy volunteers and ischaemic stroke patients.

	Healthy volunteers ***n* = 90**	**Patients** ***n* = 81**	**Young healthy volunteers** ***n* = 50**	**Elderly healthy volunteers** ***n* = 40**
Age (mean±SD)	54.9±20.2	76.8±7.7	40.2±14.3	73.3±7.2
Sex				
Male (*n*)	35	56	19	19
Type of stroke				
Lacunar (*n*)	-	43	-	-
Cortical (*n*)	-	38	-	-
NIHSS on admission (mean)	-	4.85	-	-
Outcome days 90				
mRS ≤ 2	-	82.4%	-	-
BI ≥ 90	-	92.1%	-	-
History				
Hypertension (*n*)	21	49	6	15
Diabetes (*n*)	9	17	3	6
AF (*n*)	3	23	1	2
Hyperlipidaemia (*n*)	15	24	4	11
TIA (*n*)	0	15	0	0
IS (*n*)	0	12	0	0
ICH (*n*)	0	0	0	0

n, number; *NIHSS*, national institutes of health stroke scale; *mRS*, modified ranking scale; *BI*, Barthel index; *AF*, atrial fibrillation; *TIA*, transient ischaemic attack; *IS*, ischaemic stroke; *ICH*, intracerebral haemorrhage.

At each time point, 30 mL blood samples were taken from each participant and were split for the abovementioned objectives (Figure 7). The first 6 mL were used to count the number of circulating EPCs using flow cytometry, and the plasma was simultaneously extracted to analyse the biochemical profiles of participants, while the

remaining blood was used to isolate and then culture the mononuclear cells to acquire the OECs.

To quantify the CD34, CD133, and KDR positive cells in the peripheral blood, phycoeryhtrin-cyanine7, allophycocyanin, and phycoeryhtrin antibodies were used (respectively). The biochemical profile of participant plasma was assessed according to the manufacturers' protocols using the ELISA kit for the following proteins: TNF-α, G-CSF, SDF-1, total anti-oxidant, VEGF, PDGF-BB, endostatin, angiostatin, thrombospondin-1, and thrombospondin-2.

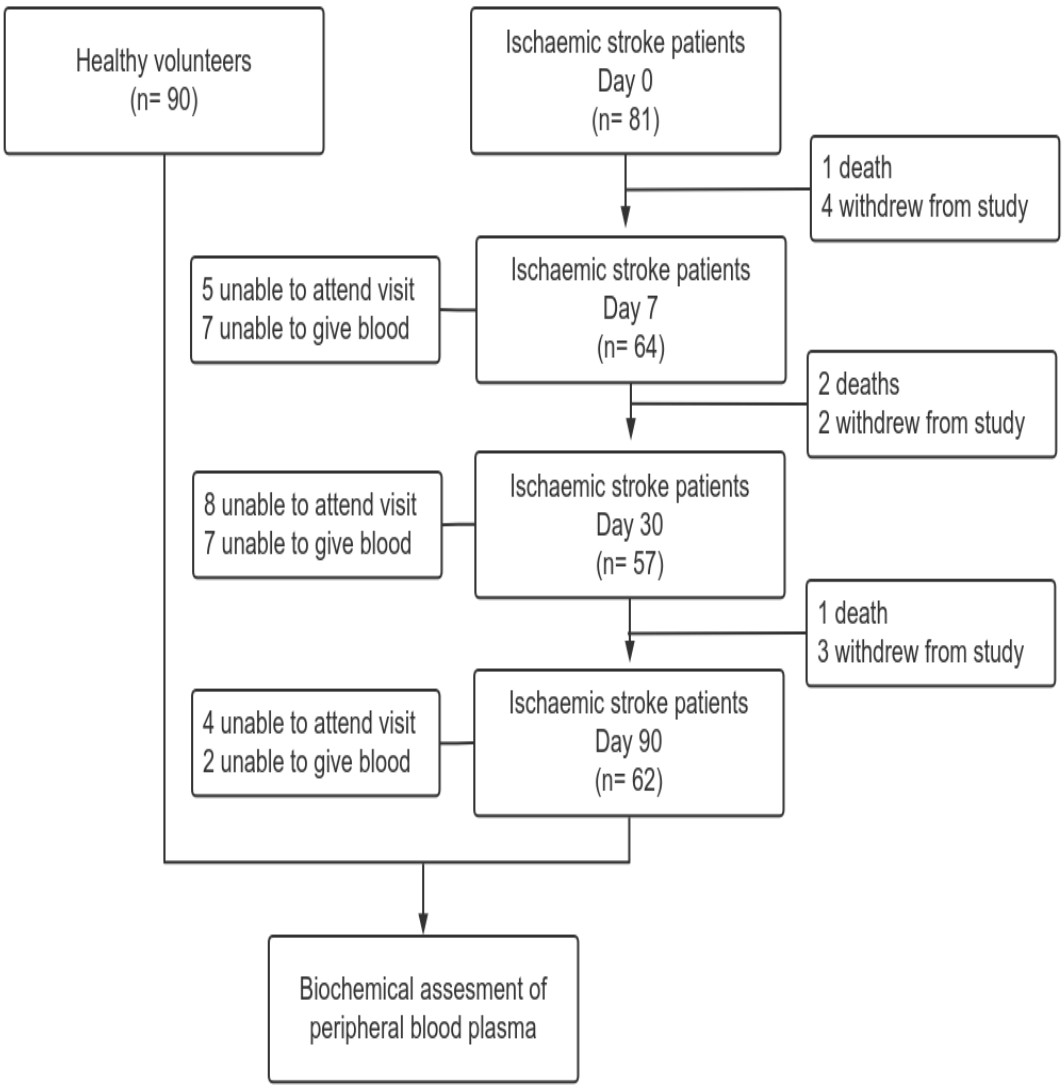


Figure 7. Study recruitment and patient pathway.

4. Functional assay of OECs

4.1. Dil-Ac-LDL and FITC-UEA-1 staining

Since mature endothelial cells can bind to 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine-labelled-ac-LDL (Dil-Ac-LDL) and fluorescein isothiocyanate labelled-ulex europaeus agglutinin (FITC-UEA-1) (Holthofer et al. 1982; Voyta et al. 1984), the endothelial phenotypes of OECs in this thesis were characterised by assessing their capacity to interact with these markers (Medina et al. 2010).

Cells (5 x 10^4) were grown on fibronectin-coated (1 mg/mL) coverslips in 24-well plates with EGM-2 medium containing VEGF (5 μl/mL). After the cells reached 95% confluence, the media was removed and the coverslip cell was washed by PBS 3x. OECs were incubated with Dil-Ac-LDL (1 mg/mL) for 4 hours, then the cells were stained with FITC-UEA-1 (1 mg/mL) for 2 hours. They were then washed and fixed by PBS for three times and 4% formaldehyde for 20 minutes, respectively. The coverslips were then laid over the mounting medium for 20 minutes at room temperature. Finally, the cells were visualised under fluorescence microscope.

The fluorescence intensity was assessed using ImageJ software and the data were presented as corrected total cell fluorescence (CTCF) calculated using Equation 3 (Migneault et al. 2020). The value was subsequently normalised to the CTCF from HBMECs.

Equation 3. Quantification of fluorescence intensity

$$\mathrm{CTCF} = \mathrm{A} - (\mathrm{B} \times \mathrm{C})$$

Where A is integrated density, B is area of selected cell, and C is mean fluorescence of background readings.

4.2. Matrigel tube formation assay

Matrigel (150 μL) was added to 48-well plates and was incubated at 37°C for 1.5 hours. The cells (9 x 10^4) were then added onto Matrigel layer and incubated at 37°C for 8 hours. Subsequently, the media was gently removed and the cells were washed with PBS. The cells were fixed with 4% formaldehyde for 20 minutes at room temperature.

In some experiments, after exposure to their respective experimental conditions, the cells (10^4) were seeded in 96 well plates-coated with 50 μL of Matrigel. The tube-like structure was subsequently observed under light microscope. The specific characteristics of the tubule networks, defined as the sum of number or length of segments, isolated elements, and branches detected in the analysed area, were then assessed using ImageJ software (Chevalier et al. 2014).

To assess the capacity of OECs to integrate and form tubule networks together with HBMECs, the former and the latter were pre-labelled through incubation with Dil-Ac-LDL (1 mg/mL) and Calcein (10 μg/mL), respectively, for 4 hours at 37°C. Matrigel (500 μL) was added in Milicell EZ Slide and was incubated at 37°C for 1.5 hours. Thereafter, the mix of pre-labelled HBMECs (2.5 x 10^5) and OECs (1.25 x 10^5) were added into the Milicell EZ Slide, followed by incubation at 37°C for 16 hours. The tube formation was finally observed under fluorescence microscope.

4.3. Proliferation assay

Cell proliferation was analysed using water-soluble tetrazolium salt (WST-1) colorimetric assay. This assessment is based on the capacity of mitochondrial dehydrogenases to cleave tetrazolium salt to formazan in viable cells (Mariko et al. 2010). These results were interpreted as a relative living cell number or viability,

hence as end-point of analysis, this value may also reflect changes in proliferation (Huttala et al. 2020).

The cells ($4x10^3$) were cultured in 96-well plates in triplicate. After exposing to their respective experimental conditions, the cells were incubated for a further 2 hours at 37°C with 100 μL medium containing 10μL WST-1 reagent. Three wells per each time point containing 100 μL of medium without cells were used as blanks. The formazan dye produced by viable cells was quantified by measuring the absorbance of the dye solution at 440 nm by spectrophotometer.

The cell growth was also counted in a Neubauer chamber. The cells ($5x10^3$) were seeded in 6-well plate, harvested by trypsinisation, and were counted every 48 hours. The population doubling time (PDT) was computed using

Equation ***4***. The mean PDT of each time point was determined as the population doubling time for each cell-type.

Equation 4. Population doubling times

$$\mathrm{PDT} = \frac{\mathrm{t\ x\ log2}}{\mathrm{logn1} - \mathrm{log\ n2}}$$

Where t is time (hours), n1 is number of harvested cells, and n2 is initial cell number

4.4. Migration assay

Transwell inserts (4.0 μm pore size) of 24-well plates were used to assess the migratory capacity of the cells. The cells were cultured in T25 flasks until reaching about 90% confluence. The medium subsequently was replaced with fresh migration medium (EGM-2 without FBS and supplements) containing 10 μg/mL Calcein AM. After 2 hours, the cells (5×10^4) were trypsinised and seeded in the upper chamber, and cultured with migration medium. Fully supplemented EGM-2 medium containing 5 μl/mL VEGF was added in the lower chamber. The plate was analysed after 18 hours

incubation at 37°C using microplate reader at excitation of 485 nm and emission of 520 nm.

4.5. Cell matrix adhesion assay

A cell adhesion assay was performed in accordance with previous protocols (Zhang et al. 2020b). Briefly, the cells were trypsinised and seeded (10^4) into fibronectin- or collagen-precoated 96-well plates and were incubated for 60 minutes, then the cells were gently washed with PBS and unattached cells were removed. The attached cells were subsequently fixed and counted in 5 random microscopic fields.

5. Assessment of BBB characteristics

5.1. Establishment of an *in vitro* model of human BBB

To establish an *in vitro* model of human blood-brain barrier (BBB), different cerebrovascular cells, namely HBMECs, astrocytes, and pericytes, were simultaneously cultured on Transwell inserts. Given that astrocytes cover 99% of brain capillaries, and there is an abundance of pericytes in the central nervous system (CNS), triple culture models consisting of all the aforementioned cells (i.e. EAP or EPA models) may anatomically and physiologically better represent human BBB than the co-culture models comprising HBMECs and astrocytes or pericytes i.e. EA or EP models (Figure 8). Indeed, discovery of a crosstalk between cerebral endothelial cells and astrocytes in EAP model has proven this particular model as the best *in vitro* model with significantly higher barrier integrity and function compared to HBMEC monoculture, EA, EP, and EPA models (Shao and Bayraktutan 2013).

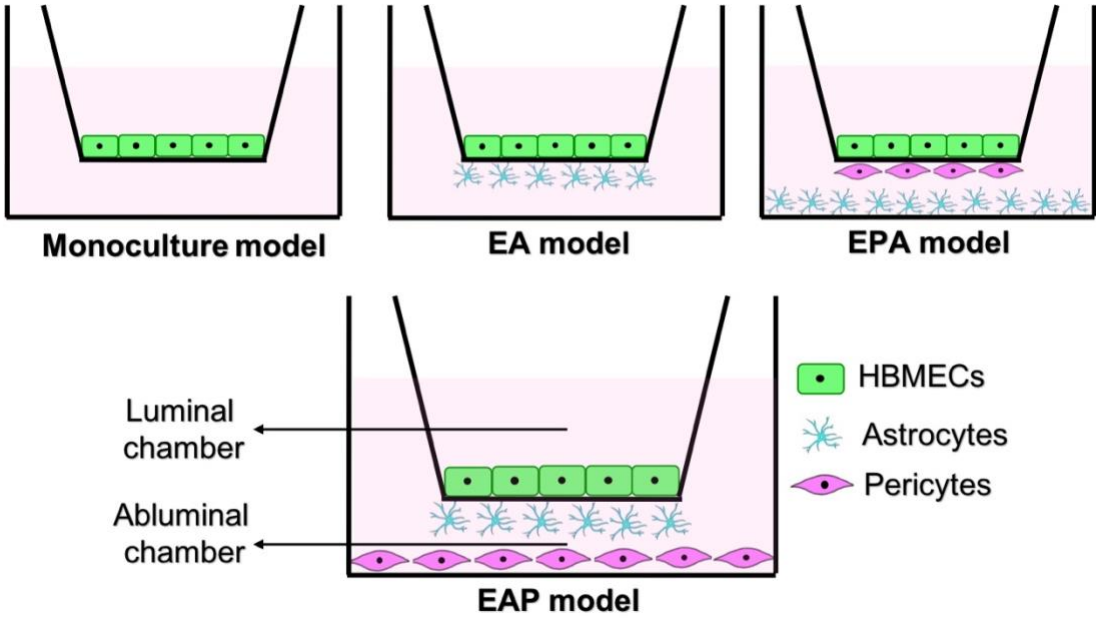


Figure 8. Schematic representation of various *in vitro* models of human BBB.

With astrocyte end-feet covering all endothelial cells and the presence of pericytes at the bottom of the 12-well plate, the EAP model is anatomically and physiologically more representative of the human BBB and produces more electrical resistance than other models.

To set up this triple culture model, astrocytes (1.5 x10^5) were seeded onto the outer surface of 12-well Transwell inserts (0.4 μm pore), seated upside down in 6-well plates, and were incubated overnight. The next day, the inserts were re-inverted into 12-well plates containing astrocyte media. HBMECs (4 x 10^4 cells) were seeded in the inner surface of the same insert, and pericytes (1.5 x 10^5) were seeded in the basement of the well. All cells lines were grown to confluence with their specific media before the experiments were conducted. The media was changed every day.

5.2. BBB integrity

Measurement of transendothelial electrical resistance (TEER), based on electrical resistance across a cellular barrier, has been extensively used to assess the integrity of BBB in live cells during various stages of their growth and development (Srinivasan et al. 2015; Stone et al. 2019). This method offers advantages over functional assay (Section 5.3), including being more rapid and non-invasive (Srinivasan and Kolli

2019). In addition, it is also an excellent strategy to assess barrier integrity, as the electrical impedance across an endothelium barrier depends on the formation of robust tight junctions between neighbouring cells. Hence, higher value of TEER readings indicate better tight junctional formation between endothelial cells (Helms et al. 2016; Srinivasan and Kolli 2019).

After the inserts were exposed to various experimental conditions, the total resistance was measured using STX2 electrode and EVOMX resistance meter (Figure 9). In this system, two electrodes with unequal length were designed to gain easy access to luminal and abluminal chambers. The longer electrode was touched to the abluminal chamber and the shorter electrode was in the luminal chamber. The EVOMX meter gave the reading of resistance when electrodes were placed into each chamber.

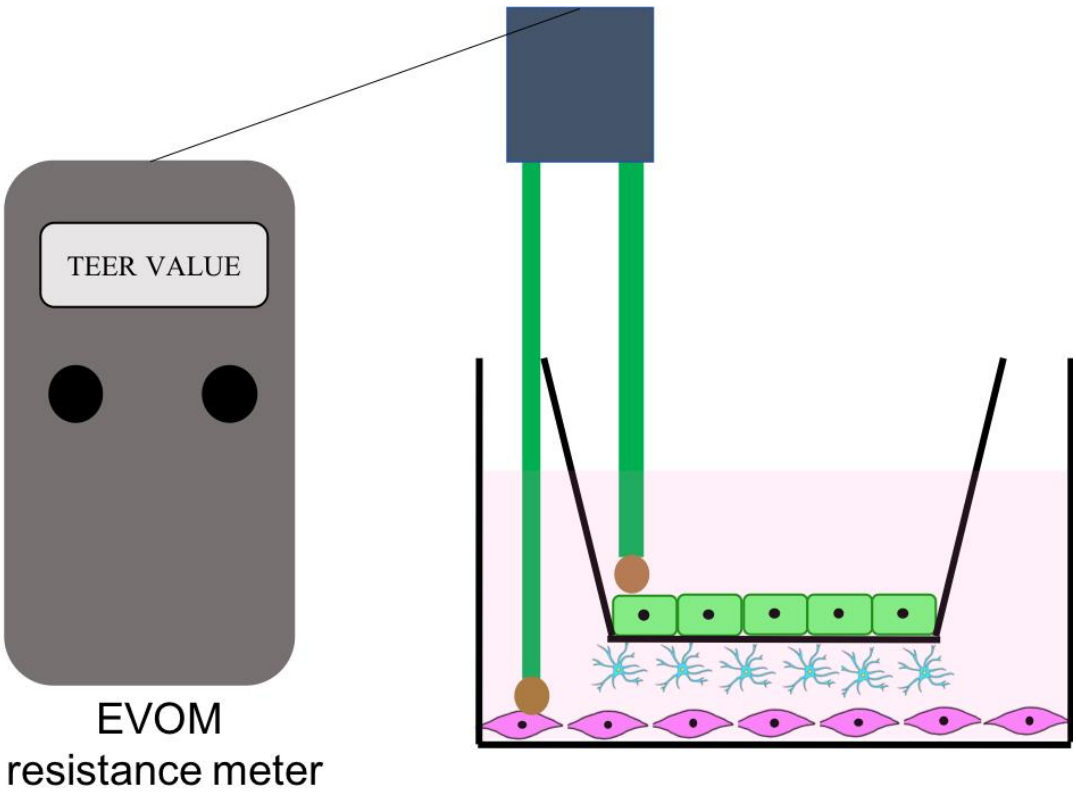


Figure 9. Measurement of TEER value in an *in vitro* model of BBB.

After exposure to experimental conditions, the integrity of *in vitro* BBB model was measured using TEER. The electrode probe should be maintained in an upright position at all times, to ensure consistent readings.

5.3. BBB permeability assay

BBB integrity is based on impedance measurements reflecting the ionic conductance of the monolayer, which cannot be extrapolated to reflect the permeability of barrier. Validation assessment through a functional assay is thus needed in addition to electronic measurement of the ion flux (Helms et al. 2016). This assay basically measures the number of solutes that can cross the cellular barrier (from luminal to abluminal compartment), thereby reflecting the functionality of the barrier to limit paracellular flux. Hence, both TEER measurement and paracellular flux assessment are equally needed for optimal characterisation of an *in vitro* model of BBB (Helms et al. 2016).

BBB function was assessed by flux of low molecular weight permeability markers, sodium fluorescein (NaF, 376 Da). After exposure to different experimental conditions, inserts were transferred into fresh 12-well plates containing 2 mL Hank's balanced salt solution (HBSS) in the abluminal compartments Figure 10).

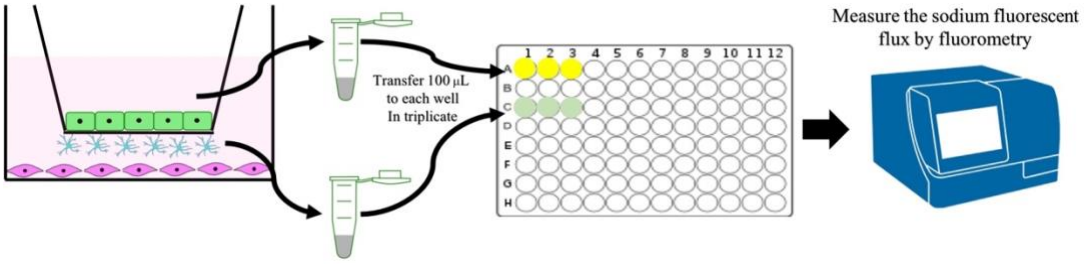


Figure 10. Measurement of sodium fluorescein flux across the *in vitro* model of human BBB.

After incubation for 1 h at 37°C, 400 µL from luminal and 400 µL from abluminal compartment is transferred into two different Eppendorf tubes. 100 µL from each compartment is subsequently transferred to a 96-well plate in triplicate. Finally, the 96-well plate is transferred to a fluorometer to measure the paracellular flux of sodium fluorescein.

In the luminal compartments, HBMEC medium was replaced by NaF (500 µL, 10 µg/mL). After 60 minutes, 400 µL of sample was taken from the luminal and abluminal compartments, and 300 µL of each sample were pipetted into 96-well plates

to study passage of NaF in triplicate (100 µL/well). The average concentration of dye in each compartment was calculated by measuring fluorescence (excitation 440 nm and emission 525 nm). Equation 5 was used to calculate the leakage of NaF.

Equation 5. Leakage of paracellular flux volume (NaF)

$$\text{Leakage of NaF} = \frac{\text{Abluminal reading concentration}}{\text{Luminal reading concentration}} \times 500$$

6. Experimental conditions

6.1. Exposure to OGD and reperfusion (OGD±R)

To somewhat replicate ischaemic injury associated with ischaemic stroke in *in vitro* settings, oxygen-glucose deprivation (OGD) alone or followed by reperfusion (OGD±R) were employed. BBB co-cultures (section 5.1) or cell monolayers were subjected to 4 hours of OGD (94.95% N_2, 0.05% O_2, and 5% CO_2) in Roswell Park Memorial Institute (RPMI) 1640 media (ischaemic culture medium lacking glucose, FBS, and pyruvate). For reperfusion, cells or BBB models were re-exposed to normoxic condition (70% N_2, 20% O_2, 5% CO_2, and 5.5 mM D-glucose at 37°C) for 20 hours, and RPMI media was replaced with normal cell media. Cells cultured under normoxic conditions served as the control. To assess the BBB-protective effect of OECs, these cells (10^4) were added during OGD±R.

6.2. Wound scratch assay

Equal number of cells (1.5 x 10^4) were seeded in 6-well plate and were cultured in their respective media to full confluence before scratching the monolayers with a p1000 micropipette tip to induce a wound. The cells were subsequently washed twice with PBS to remove the debris. The speed of wound closure was quantified as the

percentage of difference in scratch area at the beginning (immediately after scratch), and at the indicated time points using ImageJ software.

In some experiments, following the establishment of the triple culture of *in vitro* BBB model (section 5.1), a scratch was made manually down the centre of the endothelial layer using a p10 micropipette tip in one swift motion. Endothelial cell media was then replaced with serum-free media containing OECs (3.5×10^4). It is noteworthy here that the omission of serum in the culture conditions used in these experiments was to confirm that the observed healing process was solely due to OEC homing and differentiation, rather than HBMEC proliferation. The integrity (section 5.2) and function (section 5.3) of the BBB were assessed as described earlier 8 hours after the induction of scratch.

Due to difficulties regarding clear photography of endothelial monolayer in the BBB model, in additional experiments, HBMECs were seeded in 48-well plates (which provided closer growth area to inserts used: 0.95 cm^2 versus 1.12 cm^2, respectively), and were cultured to confluence in endothelial cell media. Wounds were made by scratching the monolayer with a p10 micropipette tip prior to replacing endothelial cell media with serum-free media containing OECs (3.5×10^4). Images were taken immediately after scratch and 8 hours after the addition of OECs.

6.3. Induction to replicative senescence

To simulate chronological ageing *in vitro*, the cell culture model of ageing i.e. repetitive culture was employed. For this, OECs were continuously cultured up to passage 14. OECs in passages 4-7 were defined as young OECs. In some experiments, HBMECs were co-cultured with young or senescent OECs in the ratio 2:1.

6.4. Exposure to TNF-α injury

Considering that time- and dose-escalation study showed that exposure to 10 ng/mL TNF-α for 6 hours led to an increase in oxidative stress in HBMECs and dramatically disrupted overall cerebral barrier integrity (Abdullah and Bayraktutan 2014), this particular time point and concentration were used in the current study. Since the level of TNF-α in the tissue is remarkably higher compared to peripheral blood (Li et al. 2017), the utilisation of TNF-α concentration (10 ng/mL compared to those observed in the peripheral blood, ~1 pg/mL) is somewhat replicated the real condition in human. TNF-α stock solutions were made in PBS (1.16 mg/mL) and were frozen at -20°C. The working solution (10 ng/mL) was made by dilution either in ECM or EGM-2 medium.

6.5. Treatments with VAS2870 or vitamin C

VAS2870 stock solution made in DMSO (69.3 mM) was frozen at -20°C, while vitamin C stock solution diluted with PBS (56.77 mM) was kept at 4°C. The working solution of VAS2870 and vitamin C (5 μM and 0.5 μM) was dissolved with EGM-2 medium. Such concentrations were selected according to the relevant studies showing cellular protective effects against various pathological conditions (Allen and Bayraktutan 2009; Silva et al. 2017; Lu et al. 2019).

7. Downstream signalling

7.1. Protein sample preparation and quantification

After exposure to the relevant treatment, the cells were scraped in ice cold PBS and were subsequently centrifuged to obtain a cell pellet, which was then sonicated (at 20 kHz and 50% amplitude) for 3 x 15-second bursts with a 30-second rest on ice-between in the lysis buffer containing 300 mM monobasic potassium phosphate, 50

mM EGTA, 5 μL/mL protein cocktail inhibitor (PCI), 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 0.5 mM PMSF. Total cellular protein was isolated from cell debris and membrane by centrifuging the cell lysate at 10,000 *g* for 10 minutes at 4°C. The supernatant was collected and stored at -80°C before use.

The bicinchoninic acid (BCA) assay kit was used to measure the protein concentration as manufacture protocol. Samples were run in duplicate on a 96-well pate with BSA standards (0 - 2000 μg/mL) and BCA working reagents A:B (1:50). 10 μL of the protein samples was transferred into a 96-well plate, before 200 μL of working reagent was pipetted into the well. Thereafter, the plate was incubated at 37°C for 20 minutes and the absorbance was measured at 610 nm on a FLUOstar Omega Plate Reader. The buffer blanks were subtracted and a standard curve was plotted with a second polynomial fit.

7.2. Immunocytochemistry

The presence of stress fibres formation, distribution of tight junction protein, and identification of γ-H2AX (senescence marker) were assessed through immunocytochemistry. The cells were cultured to ~90% confluence on sterile coverslips and exposed to their relevant exposure. The medium was removed and the cells were fixed, permeabilised, and blocked with formaldehyde 4%/PBS for 10-30 minutes, triton X-100 0.1% for 5 minutes, and blocking solution (1% BSA, 22.52 mg/mL glycine, and 0.1% Tween 20 in PBS) for 30 minutes, respectively. To visualise actin cytoskeleton formation, 100 μL phalloidin was added and incubated in room temperature for 20 minutes.

To detect tight junction localisation or DNA damages, ZO-1 (1:250) or γ-H2AX antibody (1:250) were added to the cells and were incubated for overnight at 4°C. The relevant secondary antibody (1:1000) was subsequently added and incubated for 1

hour at room temperature, then 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) staining was added to detect cell nuclei. The coverslips were then mounted using vector shield, and were visualised under fluorescence microscope.

In some experiments, HBMECs and OECs were cultured together at a ratio of 2:1. Prior to seeding in the co-cultured model, young OECs was pre-labelled through incubation with Dil-Ac-LDL (1 mg/mL) for 4 hours at 37°C.

7.3. Senescence-associated-β-galactosidase (SA-β-gal)

Senescence-associated-β-galactosidase (SA-β-gal) activity detection is the most commonly used biomarker to identify senescence process in cell culture system (Cai et al. 2020). Cells were fixed for 10-15 minutes in SA-β-gal fixative solution and were subsequently stained with staining solution mix containing X-gal solution and staining supplement at 37°C overnight. Cells were washed with PBS 1x, and the numbers of SA-β-gal-positive cells identified as blue-stained cells by phase contrast microscopy were manually counted in 5 different random fields in each of the triplicate wells.

7.4. NADPH oxidase activity measurement

NADPH oxidase activity was measured with the lucigenin chemiluminescence assay. The cell homogenates (~50 μg) were incubated at 37°C in assay buffer containing lucigenin (200 μM), potassium phosphate buffer (300 mM, pH 7.0), sucrose (1 M), and ethylene glycol tetraacetic acid (50 mM). The specific inhibitors for nitric oxide synthase (NG-nitro-L-arginine methyl ester, 10 mM), xanthine oxidase (allopurinol, 10 mM), mitochondrial respiratory chain complex 1 (rotenone, 10 mM) and cyclooxygenase (indomethacin, 10 mM) were also added to assay buffer, to diminish the contributions of other ROS-generating enzymes to overall superoxide anion generation. NADPH (100 μmol/L) was injected to the well plate to initiate the reaction

after 15 minutes. The reaction was subsequently monitored for 2 hours and the rate of reaction was measured using a luminometer. Equation 6 was used to calculate the NADPH oxidase activity reaction rate.

Equation 6. NADPH oxidase activity reaction rate

$$\text{Reaction rate} = (\text{maximum luminescence}/\text{time to reach maximum}) \,/\, \text{mg protein}$$

7.5. Superoxide anion level assessment

Superoxide anion level was assessed by cytochrome-*C* reduction assay. Equal amounts of homogenates (100 μg) were incubated with assay buffer containing HEPES (1 M), ethylene glycol tetraacetic acid (50 mM), sucrose (1 M), mannitol (1 M), and cytochrome *C* (800 μM), for 1 hour, at 37°C. Superoxide anion production was measured as the reduction of cytochrome *C* and monitored with the change in absorbance at 550 nm using a plate reader.

7.6. Anti-oxidant capacity

Total anti-oxidant capacity was measured in samples using the total anti-oxidant capacity assay kit as per the manufacturer's instructions. The total anti-oxidant capacity in this study was determined by the sum of food-derived and endogenous anti-oxidants, including the enzymes (glutathione, catalase, peroxidase, etc.), small molecules (GSH, ascorbate, etc.), and proteins (transferrin, albumin, etc.). The cells were homogenised with 100 μL of ddH_2O and were centrifuged at 4°C for 5 minutes at top speed. The supernatant was concomitantly collected and transferred to the clear 96-well plate. A Cu^{2+} solution was added to the samples, and they were incubated in the plate shaker at room temperature for 90 minutes. The plate then was measured in the microplate reader with 570 nm absorbance.

7.7. Total NOx detection

Total nitrate/nitrite (Nox) measurement was performed using Nitric Oxide Assay Kit. The cells were homogenised with 500 μL assay buffer and were centrifuged for 5 minutes at 10,000 rpm at 4°C. The supernatant was collected and transferred to a new tube for the following measurement. The supernatant was then pipetted into the provided microtiter plate. The nitrate reductase and enhancer were added to the well plate, and was incubated for 4 hours and 30 minutes respectively. DAN probe was also added into the well plate, followed by the addition of NaOH. The fluorescein intensity the measured in the plate reader at excitation/emission of 360/450 nm.

7.8. Cell viability and caspase 3/7 assay

Calcein AM and Apo-ONE homogeneous caspase-3/7 assay kit was used to assess cell viability and caspase 3/7 activity, respectively. The cells (10^4) were cultured in 96-well black opaque plates for 48 hours, and were then exposed to their experimental groups. To initiate the reaction, the medium was replaced with 100 μL of Calcein AM/PBS (10 μg/mL) or with 100 μL of caspase 3/7 assay buffer, containing the non-fluorescent caspase substrate, rhodamine 110, bis-(N-CBZL-aspartyl- L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110). Plates were immediately frozen at -80°C overnight, and were subsequently thawed in a plate shaker for 2 hours prior to reading the fluorescence (excitation/emission: 485/520 nm) using the luminometer.

7.9. Endostatin level measurement

Endostatin level was assessed by using endostatin human ELISA Kit according to manufacturer instruction. The cells were cultured to about 95% confluence and exposed to experimental conditions with their respective medium without growth factor and serum. The medium was transferred to Eppendorf for centrifugation at 10,000 rpm at 4°C for 10 minutes, and supernatant was collected to freeze at -80°C

until use. 100 μL of supernatant then transferred to provided 96-well plate and incubated at 4°C. The following day, biotinylated endostatin detection antibody and HRP-streptavidin solution were added to the wells, followed by incubated at room temperature for 1 hour each. The TMB One-Step Substrate Reagent was added and incubated at room temperature for 30 minutes in the dark, before adding the stop solution. All the incubation process was undertaken with gentle shaking. The absorbance (450 nm) was subsequently read using microplate reader, and was plotted with standard curve before normalisation with the amount of protein.

8. Proteome profiling

A proteome profiler human angiogenesis array was used to assess the angiogenic factors release from the cells according to manufacturer's instruction. After blocking the array membrane with blocking buffer for 1 hour in room temperature, the membrane was washed with washing buffer for three times. The mixture of supernatant (1 mg) and array detection antibody cocktail was loaded into the blocked membrane and was incubated with gentle shaking at 4°C. The following day, the membrane was washed and incubated with streptavidin-HRP buffer for 30 minutes at room temperature. The membrane was then washed, and Chemi Reagent Mixture was added to the membrane for reaction at room temperature for 1 minute. Image Studio Lite software was employed to measure the densitometry. The reading was normalised to the reference spot.

9. Statistical analysis

The statistical analyses were performed either with GraphPad Prism 8.4.3 or SPSS package 15.0. Data were tested for normality using the Shapiro-Wilk normality test. Statistical tests were performed by *t*-test, Mann-Whitney, one-way analysis of variance (ANOVA) followed by Tukey's post hoc-analysis, or two-way ANOVA followed by Sidak's post-hoc analysis. Information on specific statistical test for each experimental analysis is listed in Appendix II. The experimental data were obtained from a minimum of three independent experiments. $P < 0.05$ was considered as significant.

Results

Part I

Exogenous addition of OECs protects BBB integrity and function from damage

Abstract

Considering that the breakdown of blood-brain barrier (BBB) and ensuing cerebral oedema represents the major cause of mortality during early stages of ischaemic stroke, the discovery of agent(s) that can effectively replace dead or dying brain microvascular endothelial cell (BMECs), the main cellular component of BBB, is of paramount importance in stroke medicine. Through differentiating into mature endothelial cells, outgrowth endothelial cells (OECs), the functional subtype of endothelial progenitor cells (EPCs), may represent one such agent. In this regard, this thesis assesses whether treatment with OECs can repair BBB damages in both *in vitro* and *in vivo* models of ischaemic stroke. The initial functional analyses of these cells indicated that OECs possessed strong endothelial characteristics along with remarkable greater total anti-oxidant, proliferative, and migratory capacity. By integrating with the vascular network of resident brain endothelial cells, OECs can form a tight and functional *in vitro* BBB model. The exposure of BBB model established by either human BMECs or OECs to 4 hours oxygen-glucose deprivation (OGD) alone or followed by 20 hours reperfusion (OGD±R) perturbed BBB integrity and function in similar ways. Treatment with OECs during/after OGD±R or scratch damage on endothelial monolayer protected the structural and functional features of BBB from damages. Similarly, intravenous administration of OECs also led to better barrier protection in middle cerebral artery occlusion model of rats. Subsequent mechanistic studies showed that the reduction in oxidative stress and apoptosis of HBMECs appeared to contribute to this mending process. In conclusion, exogenous addition of OECs attenuates BBB damages by suppressing oxidative stress and resident cell apoptosis.

1. Introduction

Stroke continues to be one of the leading causes of mortality and morbidity worldwide (Kyu et al. 2018; Roth et al. 2018). Despite being the most common subtype of stroke, thrombolysis with recombinant tissue plasminogen activator (rtPA) remains the only approved pharmacotherapy for ischaemic stroke (Hurford et al. 2020). However, due to a narrow therapeutic window (4.5 h following stroke onset), stringent eligibility criteria, and the increased risk of haemorrhage beyond this time point, <1% of ischaemic stroke patients globally receive this treatment each year (Anand et al. 2021; Berge et al. 2021).

Considering that many novel substances failed to replicate the favourable outcomes from pre-clinical studies into clinical trials, many recent studies have proposed cell-based therapies as an alternative therapeutic strategy for stroke (Kawabori et al. 2020; Li et al. 2020c). Accumulating evidence has shown that cells can better respond to temporal and spatial changes affecting their environment after an ischaemic attack by interacting with other cells and synthesising various biologically active compounds to promote neurovascular regeneration (Park et al. 2020).

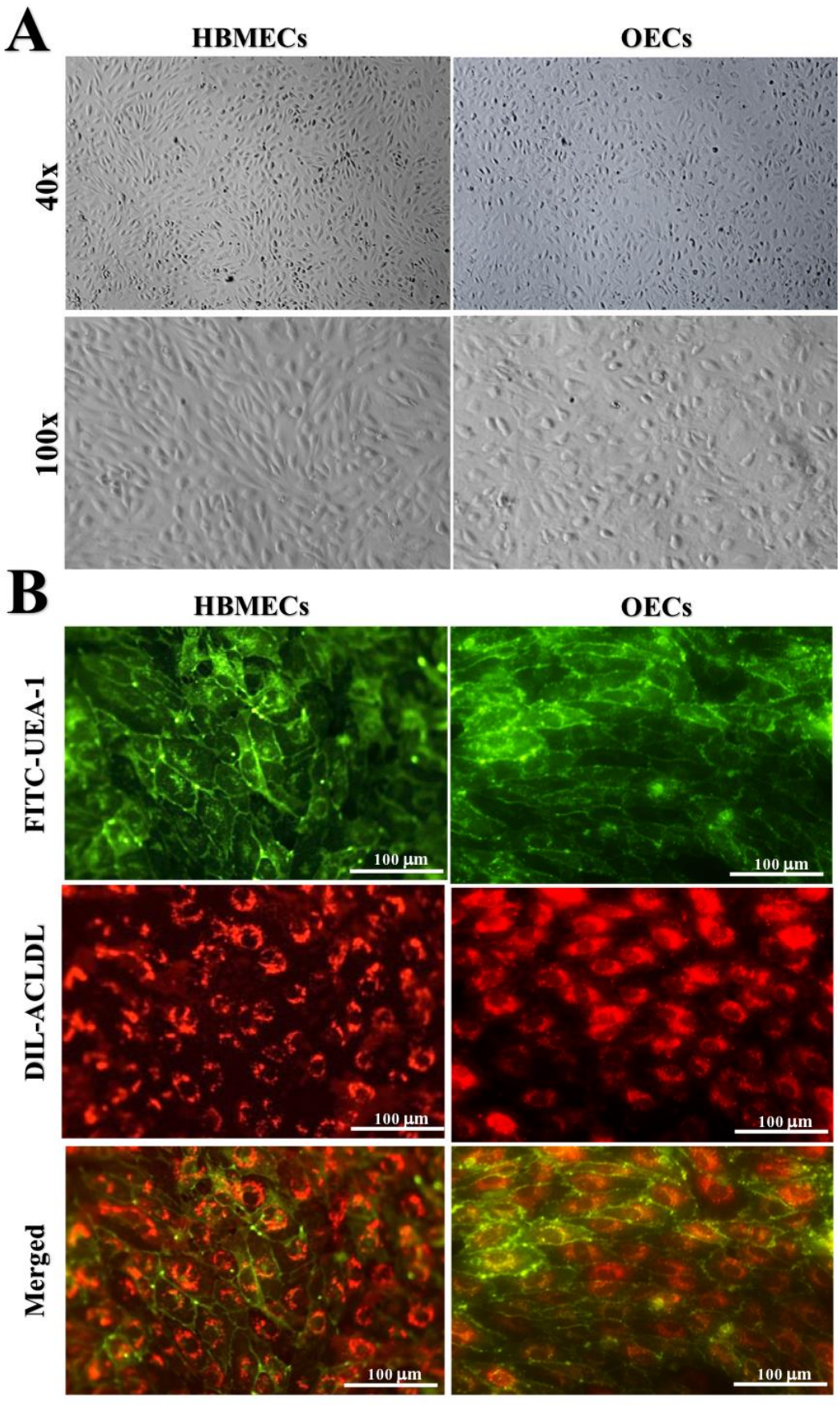
Amongst all the cell-based strategies, the utilisation of outgrowth endothelial cells (OECs), the functional subtype of endothelial progenitor cells (EPCs), may be the most promising therapeutic option for post-ischaemic restoration of vascular integrity, due to their unique capacity to detect and repair endothelial damage (Bayraktutan 2019). Indeed, the potential therapeutic role of OECs has been explored in various experimental models of ischaemic diseases such as critical limb ischaemia, ischaemic retinopathy, and myocardial infarction, where the administration of these particular stem cells effectively increased revascularisation, angiogenesis, and blood flow in the injured vasculature (Reid et al. 2018; Lee et al. 2019a; Faris et al. 2020).

In light of the above, we initially performed an extensive study of the phenotype and functional features of OECs. Considering the fact that BBB dysfunction and ensuing brain oedema represent the main cause of death and neurological deterioration in the early stage of ischaemic stroke (Dénes et al. 2011; Arba et al. 2021; Yao et al. 2021), this study subsequently investigated the cerebral barrier-forming and restorative capacity of OECs in both *in vitro* and *in vivo* ischaemic stroke models, and additionally assessed the molecular mechanism involved in the barrier-protective effects of OECs.

2. Results

2.1. OECs display endothelial cell characteristics

Similar to human brain microvascular endothelial cells (HBMECs), OECs possess strong endothelial characteristics, as shown by their typical cobblestone morphology and capacity to bind with fluorescein isothiocyanate labelled-ulex europaeus agglutinin (FITC-UEA-1) and Dil-labelled acetylated-low density lipoprotein (Dil-Ac-LDL) (Figure 11). We have previously shown that OECs isolated in this fashion also express specific markers for immaturity (CD133), stemness (CD34), and endothelial maturity (CD31), but not for hematopoietic cells (CD45) (Abdulkadir et al. 2020).


A
HBMECs
OECs
40x
100x
B
HBMECs
OECs
FITC-UEA-1
DIL-ACLDL
Merged
100 µm

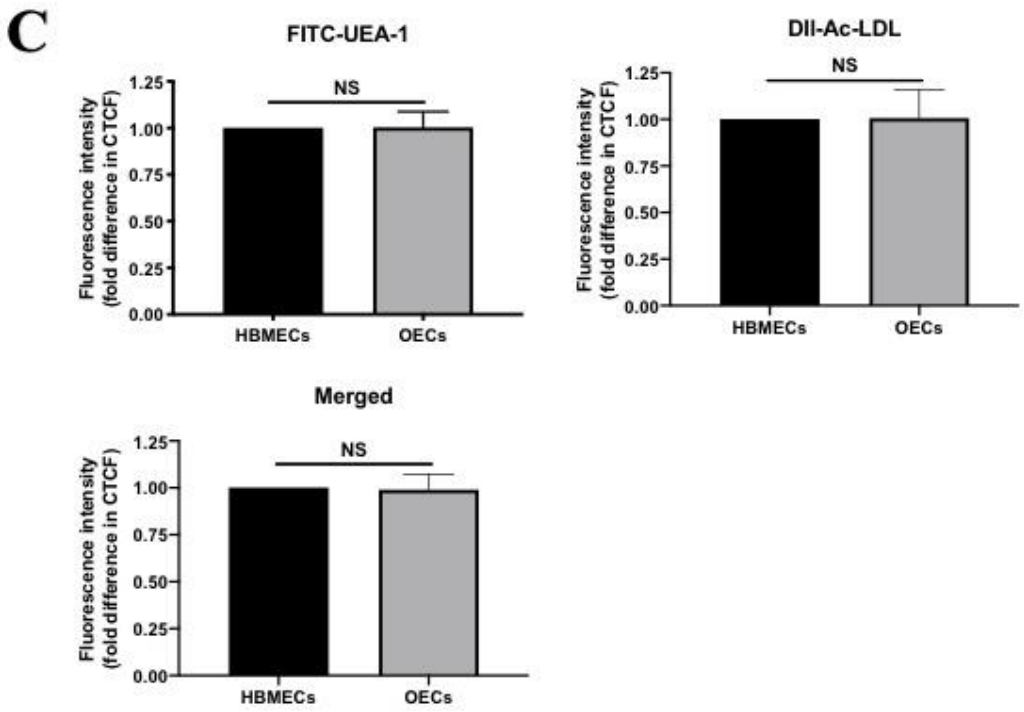
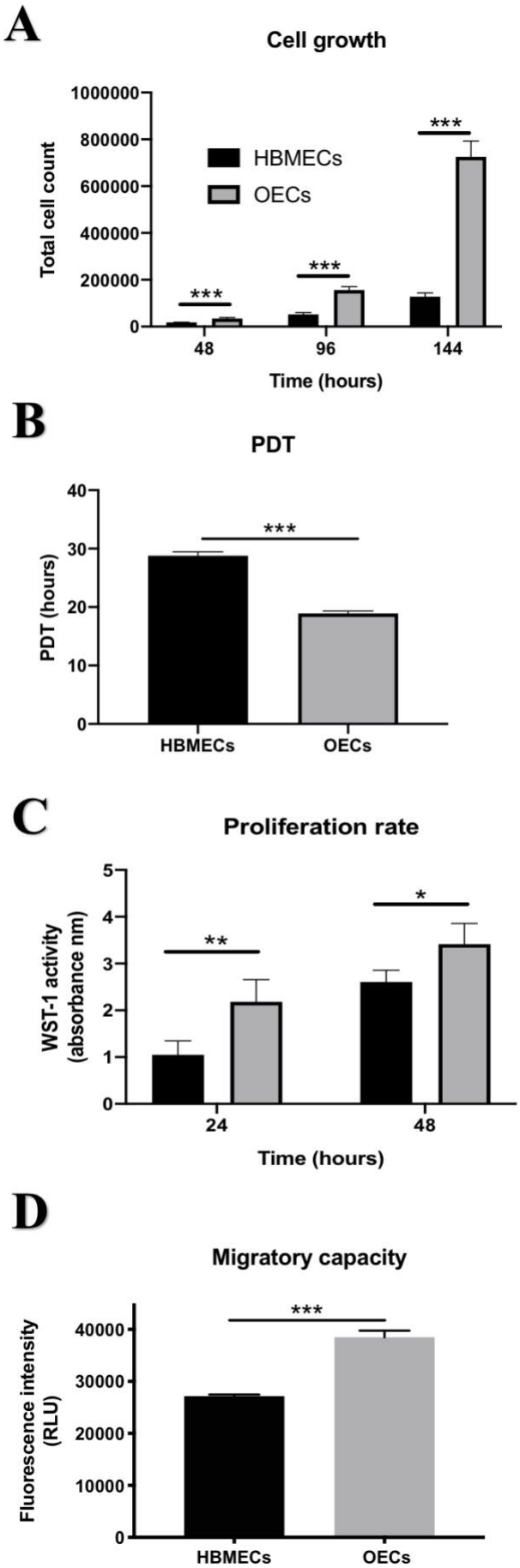


Figure 11. Characterisation of endothelial phenotypes of OECs.

(A, B) Similar to HBMECs, OECs displayed strong endothelial features, as observed by their typical cobblestone morphology and capacity to bind with FITC-UEA-1 and Dil-Ac-LDL. **(C)** Quantitative analyses of the signals, normalised by cell count, for FITC-UEA-1, Dil-Ac-LDL, and the merged images showed similar readings for HBMECs and OECs. NS: not significant. Scale bar: 100 μm. $n \geq 4$.

2.2. OECs possess greater migratory, proliferative, and anti-oxidant capacities

Since migratory and proliferative capacities are the standard criteria to distinguish true progenitor cells from mature endothelial cells (Smadja et al. 2019), these particular parameters were also assessed in the current study. Time course assessment of cell growth showed that OECs exhibited greater proliferative capacity with shorter population doubling time (PDT) compared to HBMECs (Figure 12A, B). In line with these findings, the remarkable increases in proliferative and migratory rates of OECs compared to HBMECs in additional studies specifically looking into these features (using a colorimetric assay and Transwell inserts, respectively), were also observed (Figure 12C, D), which further confirmed the high regenerative potential of these stem cells. In addition, compared to HBMECs, OECs are equipped with substantially greater basal total anti-oxidant capacity and lesser pro-oxidant NADPH oxidase activity and superoxide anion level (Figure 12E-G).


A
Cell growth
Total cell count
1000000
800000
600000
400000
200000
0
HBMECs
OECs
***
***
***
48
96
144
Time (hours)
B
PDT
PDT (hours)
40
30
20
10
0
***
HBMECs
OECs
C
Proliferation rate
WST-1 activity (absorbance nm)
5
4
3
2
1
0
**
*
24
48
Time (hours)
D
Migratory capacity
Fluorescence intensity (RLU)
40000
30000
20000
10000
0
***
HBMECs
OECs

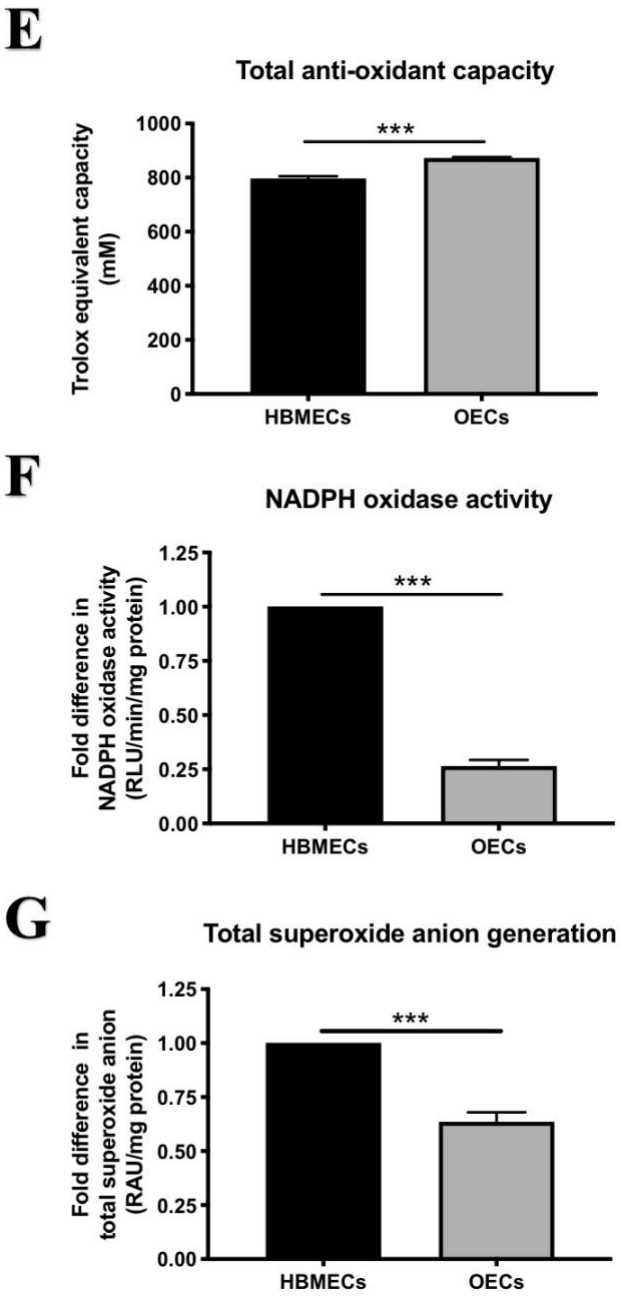


Figure 12. OECs displayed greater regenerative potential and anti-oxidant capacity.

(A, B) Comparative assessment of cell growth between HBMECs and OECs, showing that the latter displayed greater proliferation rate and shorter population doubling time (PDT). **(C, D)** In line with these findings, scrutiny of the proliferative and migratory capacity by using WST-1 and Transwell Insert assay, respectively, showed that the OECs exhibit substantial higher value in these parameters. **(E-G)** OECs possessed higher total anti-oxidant capacity and lesser pro-oxidant NADPH oxidase activity and superoxide anion level compared to HBMECs. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. $n \geq 4$.

2.3. OECs display an adequate adhesive capacity

As the homing process of OECs to ischaemic site involves the adhesion of these progenitor cells to the extracellular matrix (Zhao et al. 2016), the current study further investigated the capacity of OECs to adhere to extracellular matrix constituents, such as fibronectin and collagen matrix. As shown in Figure 13, an equal number of OECs attached in both fibronectin and collagen, suggesting the proper ability of these stem cells to adhere to extracellular matrix.

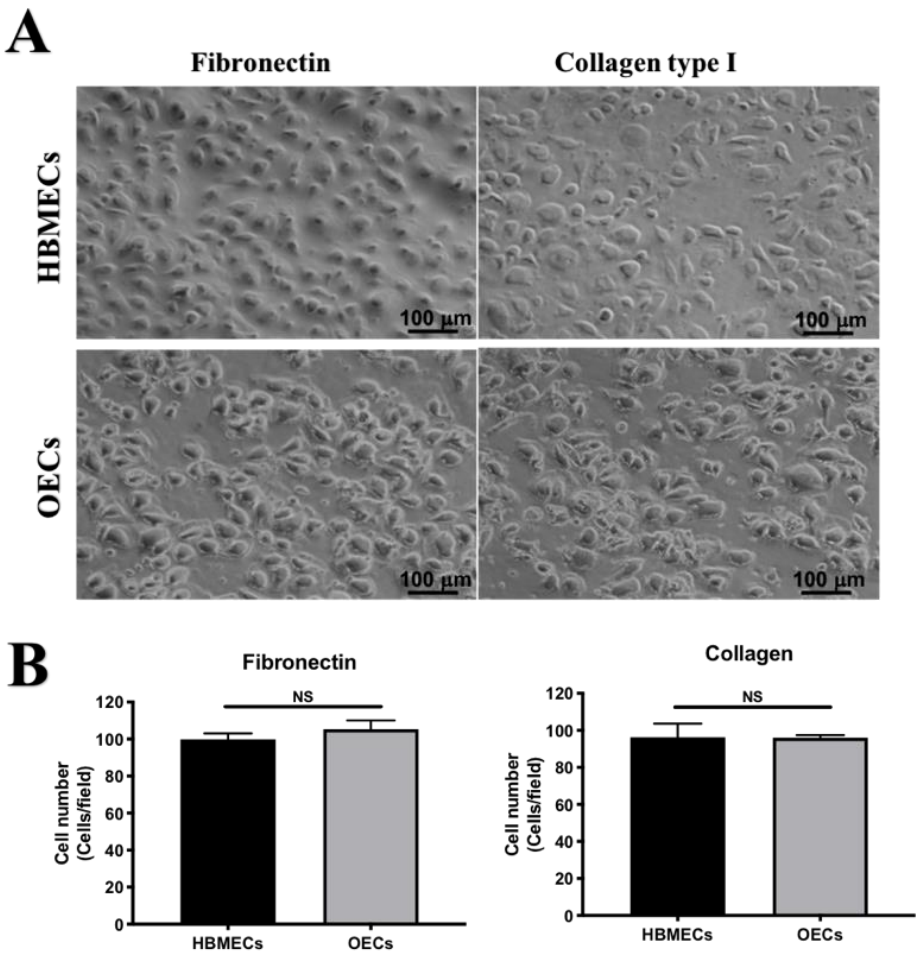


Figure 13. OECs displayed adequate adhesive capacity.

(A) Both HBMECs and OECs attached in extracellular matrix components, such as fibronectin and collagen, in equal measure. **(B)** The quantification of the number of cells adhering to extracellular matrix constituents. NS: not significant. Scale bar: 100 µm. $n \geq 3$.

2.4. OECs can fully incorporate with HBMEC vascular network and form a functional BBB

Since the vasoreparative role of OECs required the incorporation of these cells with resident cerebral microvascular network (Schwarz et al. 2012), this study concomitantly assessed whether OECs can integrate with BMECs. For this, *in vitro* BBB models consisted of astrocytes and pericytes co-cultured with HBMECs, OECs, or combination of both were established (Figure 14A). It is been shown that OECs alone or mixed with HBMECs formed equal TEER value and sodium fluorescein flux as those established with HBMECs (Figure 14B, C), indicating that OECs can form a tight and functional BBB on their own and/or through close interaction with resident brain endothelial cells. Immunocytochemical studies of HBMECs, OECs, and HBMECs mixed with Dil-Ac-LDL-labelled OECs showed an uninterrupted staining of tight junction protein ZO-1 between adjacent cells (Figure 15A), and affirmed the close interaction between progenitor and resident cells. In addition, the attainment of coherent tubule networks on Matrigel with HBMEC and Dil-Ac-LDL-labelled OEC co-cultures further proved the capacity of OECs to fully incorporate with pre-existing vascular endothelium (Figure 15B-D).

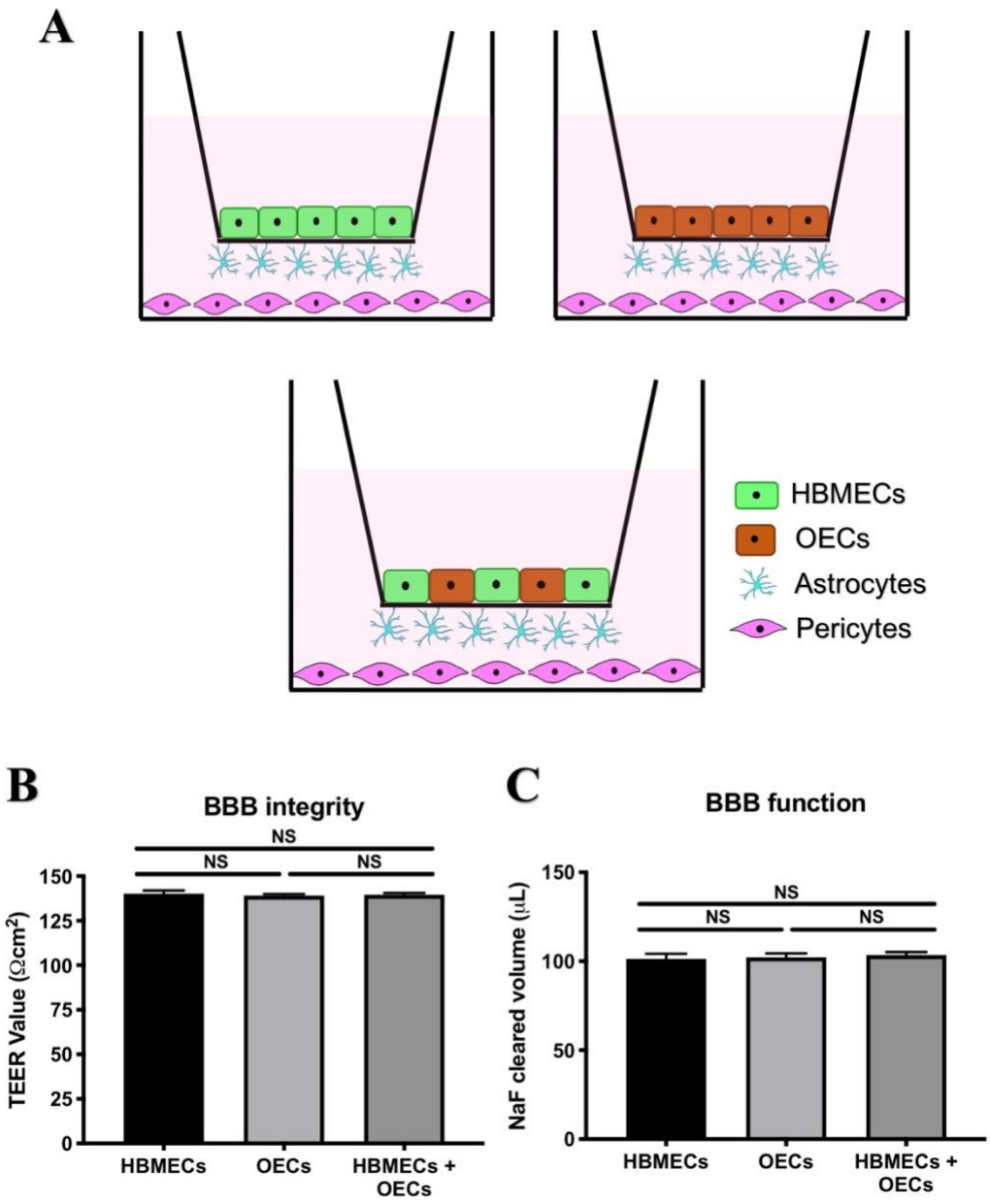
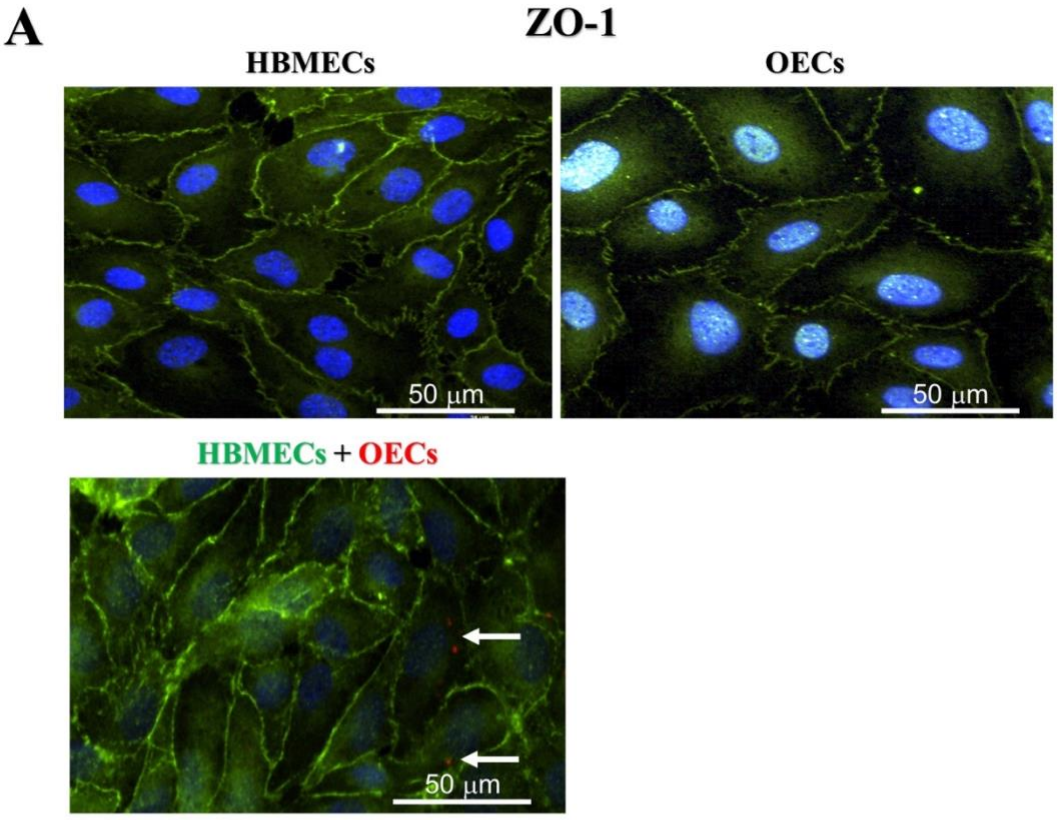
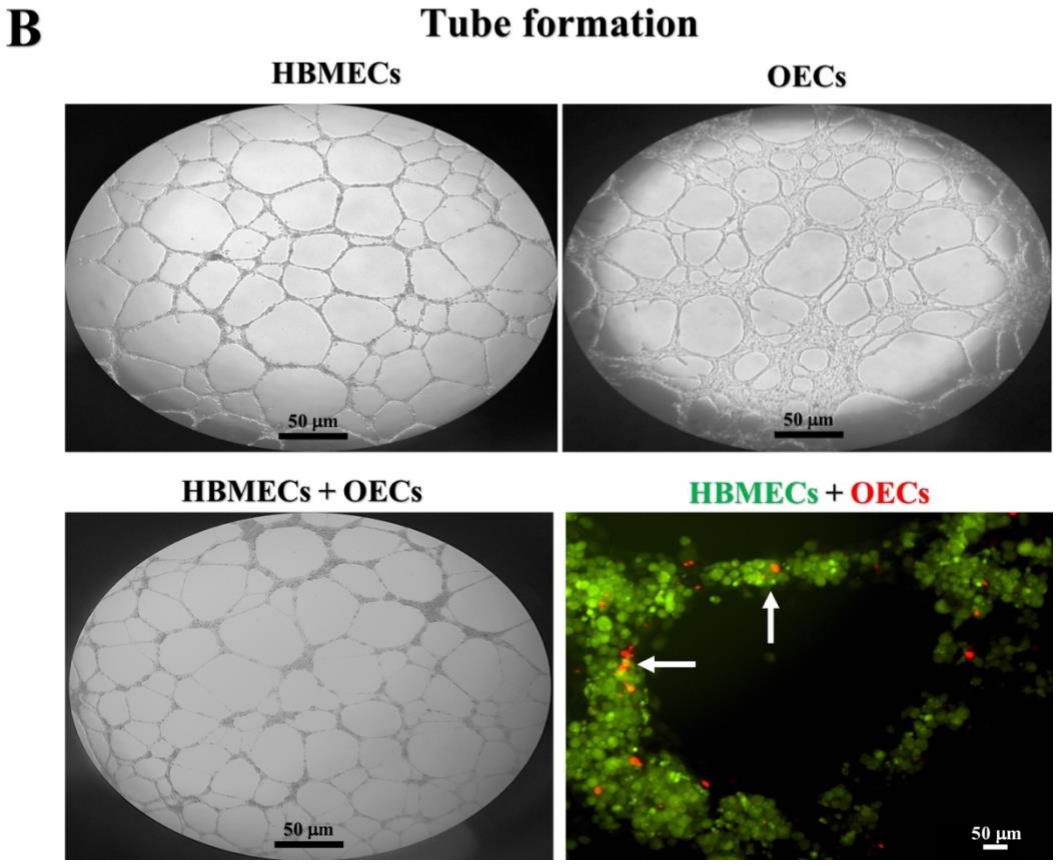


Figure 14. OECs formed tight and functional BBB.

(A) Schematic diagram of *in vitro* models of human BBB. **(B, C)** When co-cultured with astrocytes and pericytes, OECs alone or mixed with HBMECs formed equal TEER value and sodium fluorescein flux in comparison to those established by HBMECs. NS: not significant. $n \geq 4$.


A
ZO-1
HBMECs
OECs
50 μm
50 μm
HBMECs + OECs
50 μm


B
Tube formation
HBMECs
OECs
50 μm
50 μm
HBMECs + OECs
HBMECs + OECs
50 μm
50 μm

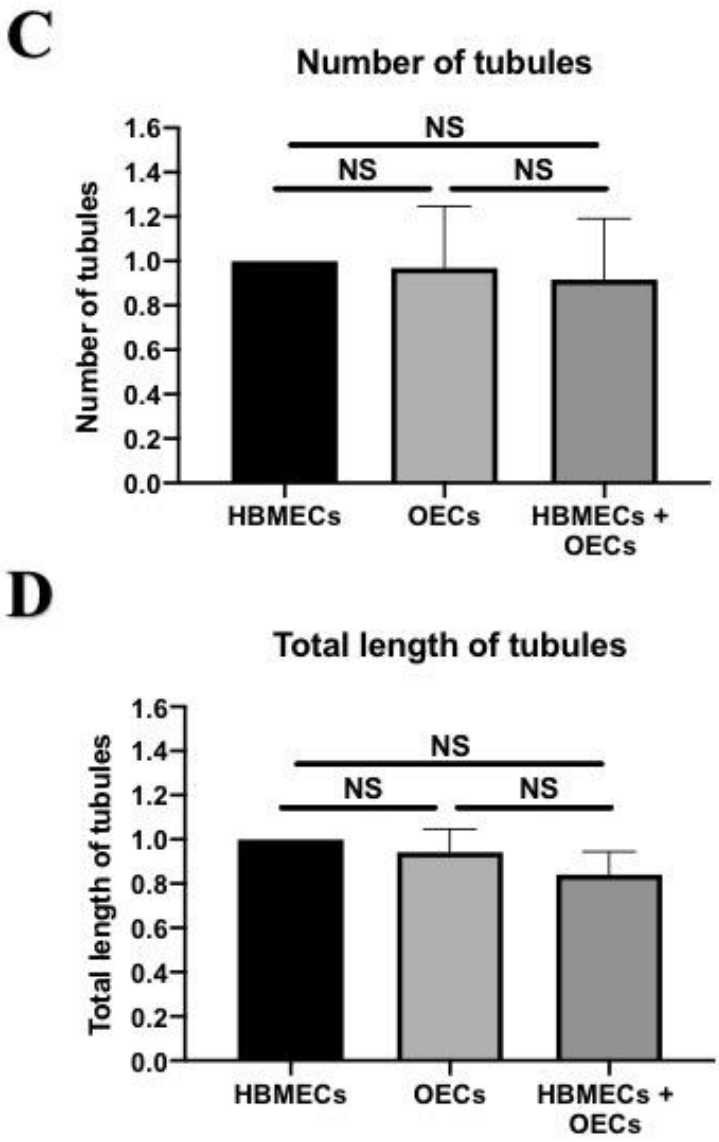
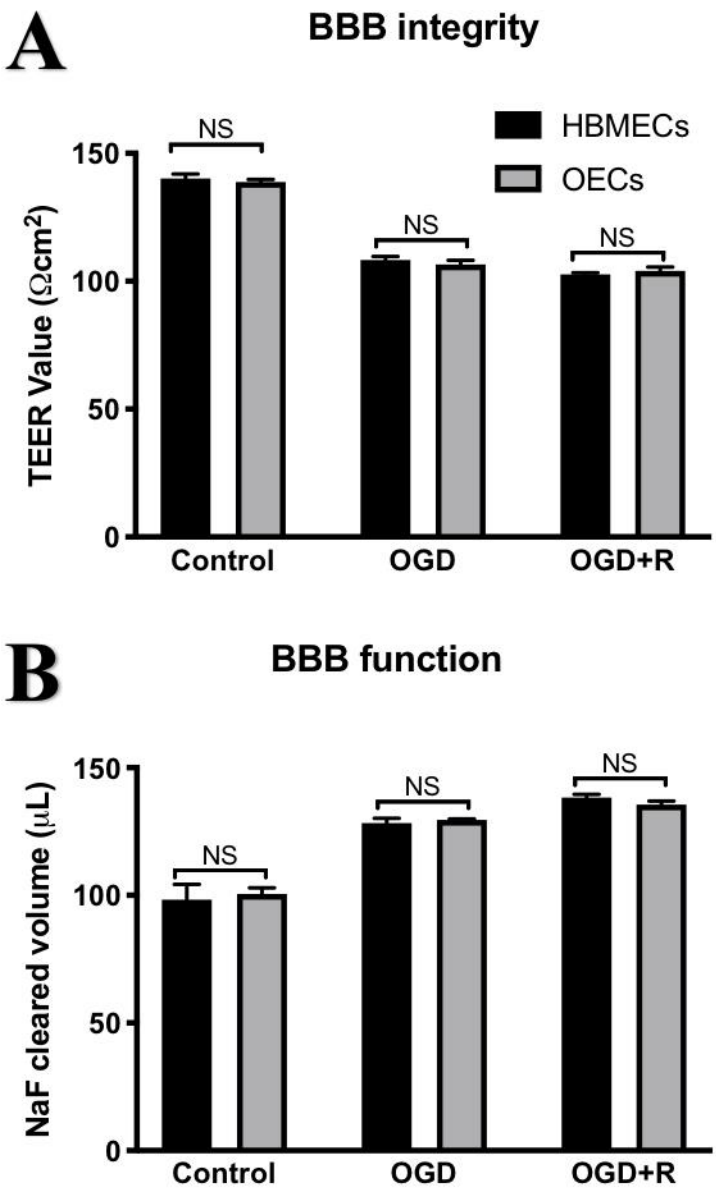


Figure 15. OECs fully integrated with mature endothelial cells network.

(A, B) Immunocytochemistry and tube formation studies showed that Dil-Ac-LDL-labelled OECs (indicated with white arrows) co-cultured with HBMECs form uniform staining of tight junction protein, ZO-1, and well-established tubules on Matrigel, similar to those observed in HBMECs and OECs. **(C, D)** Quantitative analyses of the length of tubule networks showed similar readings for HBMECs, OECs, and HBMEC+OEC co-cultures. NS: not significant. Scale bar: 50 μm. $n \geq 4$.

2.5. Ischaemia/reperfusion injury affects both HBMECs and OECs in equal measures

The integrity and function of *in vitro* model of human BBB formed either by HBMECs or OECs are similarly affected by exposure to oxygen-glucose deprivation (OGD) alone or followed by reperfusion (OGD±R) injury (Figure 16A, B). In accordance with these findings, exposure of either cell line to OGD±R also showed a similar effect on their cytoskeleton organisation, as shown by the equally increased formation of actin stress fibres traversing the cells (Figure 16C). In addition, OGD injury also reduced the viability of both HBMECs and OECs in similar manners (Figure 17). Our previous studies scrutinising the effect of OGD on the other important cellular components of BBB, namely astrocytes and pericytes, have shown that this pathological condition induced oxidative stress, inflammation, and apoptosis in these cells, suggesting the detrimental effect of OGD to the whole member of neurovascular unit (Mathur and Bayraktutan 2016; Mathur and Bayraktutan 2017).


A
BBB integrity
HBMECs
OECs
TEER Value (Ωcm²)
150
100
50
0
NS
NS
NS
Control
OGD
OGD+R
B
BBB function
NaF cleared volume (μL)
150
100
50
0
NS
NS
NS
Control
OGD
OGD+R

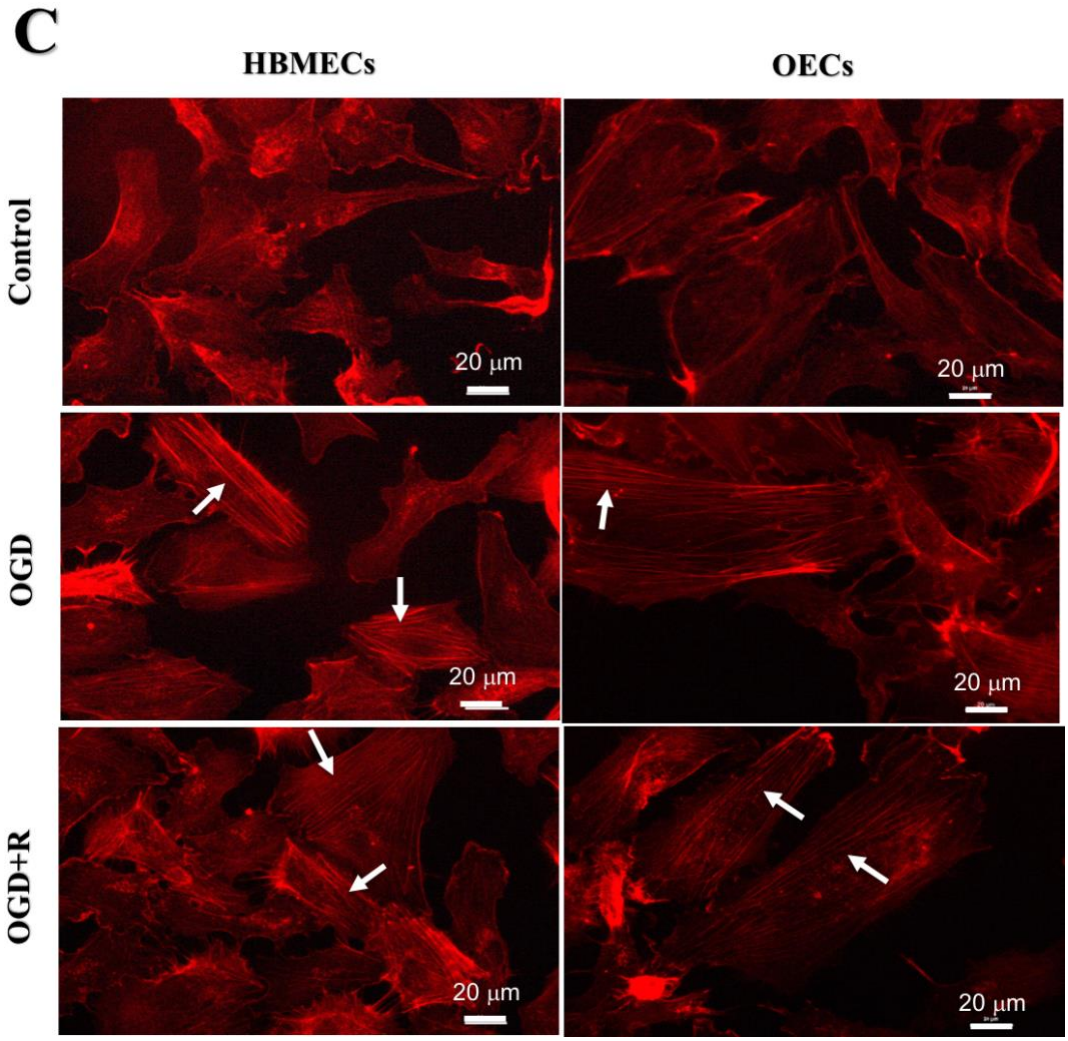


Figure 16. Ischaemic/reperfusion injury affected the barrier-forming capacity of HBMECs and OECs similarly.

(A, B) Exposure of both types of BBB models to oxygen-glucose deprivation alone (OGD) or followed by reperfusion (OGD±R) affected the integrity and function of both barriers in equal measures. **(C)** In line with these findings, the actin cytoskeleton of both cell types responded to the aforementioned injuries in a similar fashion. NS: not significant. Scale bar: 20 µm. $n \geq 3$.

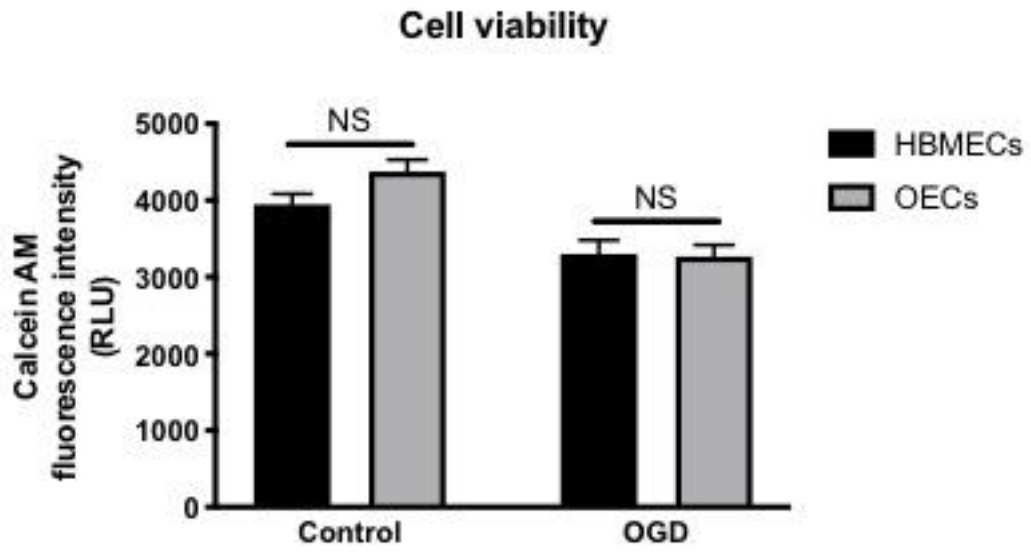


Figure 17. Ischaemic injury reduced viability of HBMECs and OECs in similar manners. Exposure of either cell line to OGD injury induced decrease cell viability in equal measures. NS: not significant. *n* = 4.

2.6. Administration of OECs restores BBB integrity and function

Ultimately the barrier-reparative effect of OECs was determined on a co-cultured model of the BBB consisted of HBMEC, astrocytes, and pericytes. OGD substantially impaired BBB integrity and function, as observed by decreased TEER and increased sodium fluorescein flux, respectively (Figure 18). Moreover, reperfusion markedly worsened deleterious effect of OGD on BBB integrity and function. Exogenous addition of OECs during OGD±R injury restored BBB integrity and function, as indicated by the improvements in readings for TEER and paracellular flux, respectively.

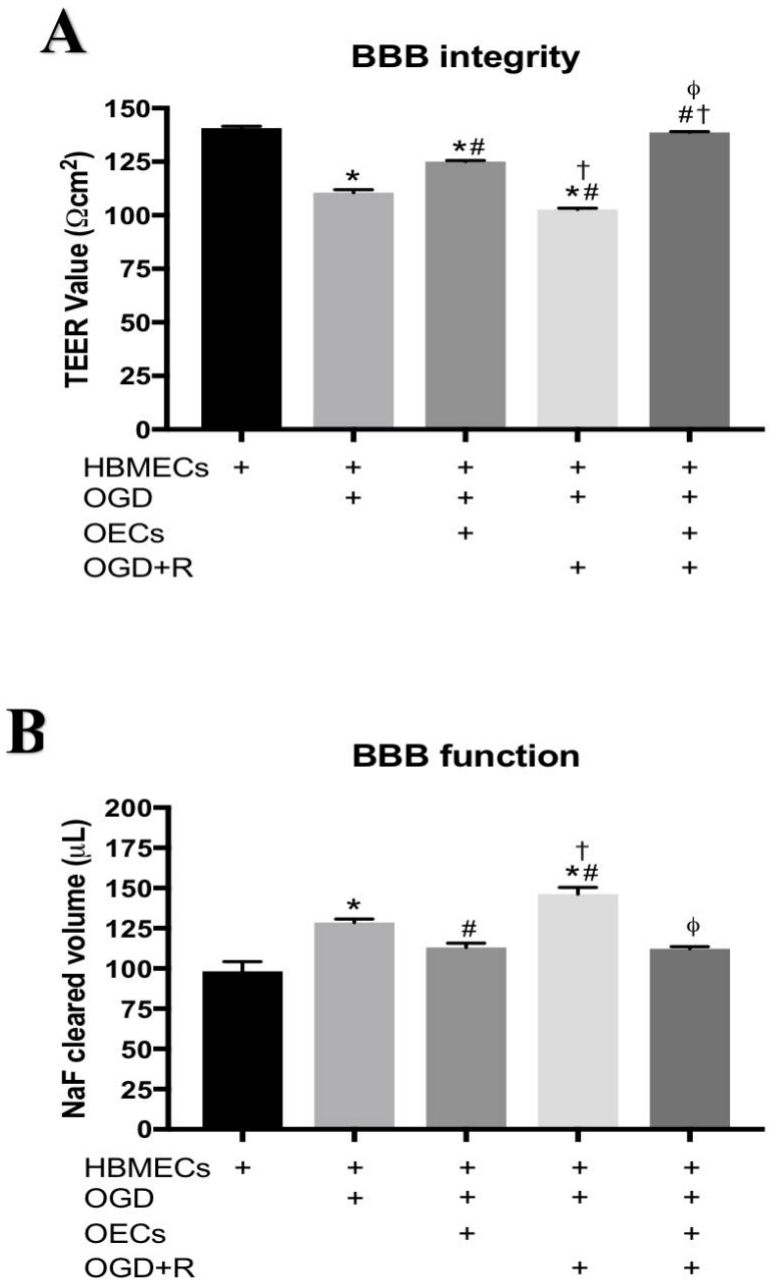


Figure 18. Treatment with OECs restored BBB integrity and function in *in vitro* setting.

(A, B) Exogenous addition of OECs during OGD±R injury repaired the integrity and function of an *in vitro* model of human BBB, as ascertained by increases in TEER and decreases in paracellular flux of NaF, respectively. *p < 0.05 compared to control, #p < 0.05 compared to OGD, †p < 0.05 compared to OGD+OECs, ϕp < 0.05 compared to OGD+R. $n \geq 4$.

To further confirm the cerebral barrier-reparative effect of OECs as well as to demonstrate their capacity to migrate into the site of injury, this study subsequently applied wound scratch in the endothelial layer of the triple culture model of BBB and treated this model with exogenous OECs. Time-course assessment of the reparative potential of OECs through wound scratch assays initially revealed that these cells restored endothelial damage considerably faster than HBMECs (Figure 19). Scrutiny of the endothelium-restorative feature of OECs in an *in vitro* model of human BBB showed that OECs effectively homed into the scratch area, repaired endothelial damage, and subsequently restored overall barrier integrity and function (Figure 20A, B). Notably, the omission of serum in the culture media used in these experiments confirmed that the observed healing process could only be attributed to effective homing and differentiation of OECs, rather than HBMEC growth. Due to the difficulties of effective visualisation of endothelial layer in the triple culture model of BBB, additional wound scratch experiments with HBMEC monolayers exposed to serum-free conditions were performed in 48-well plate to address this issue, which further substantiated the endothelial-reparative action of OECs (Figure 20C).

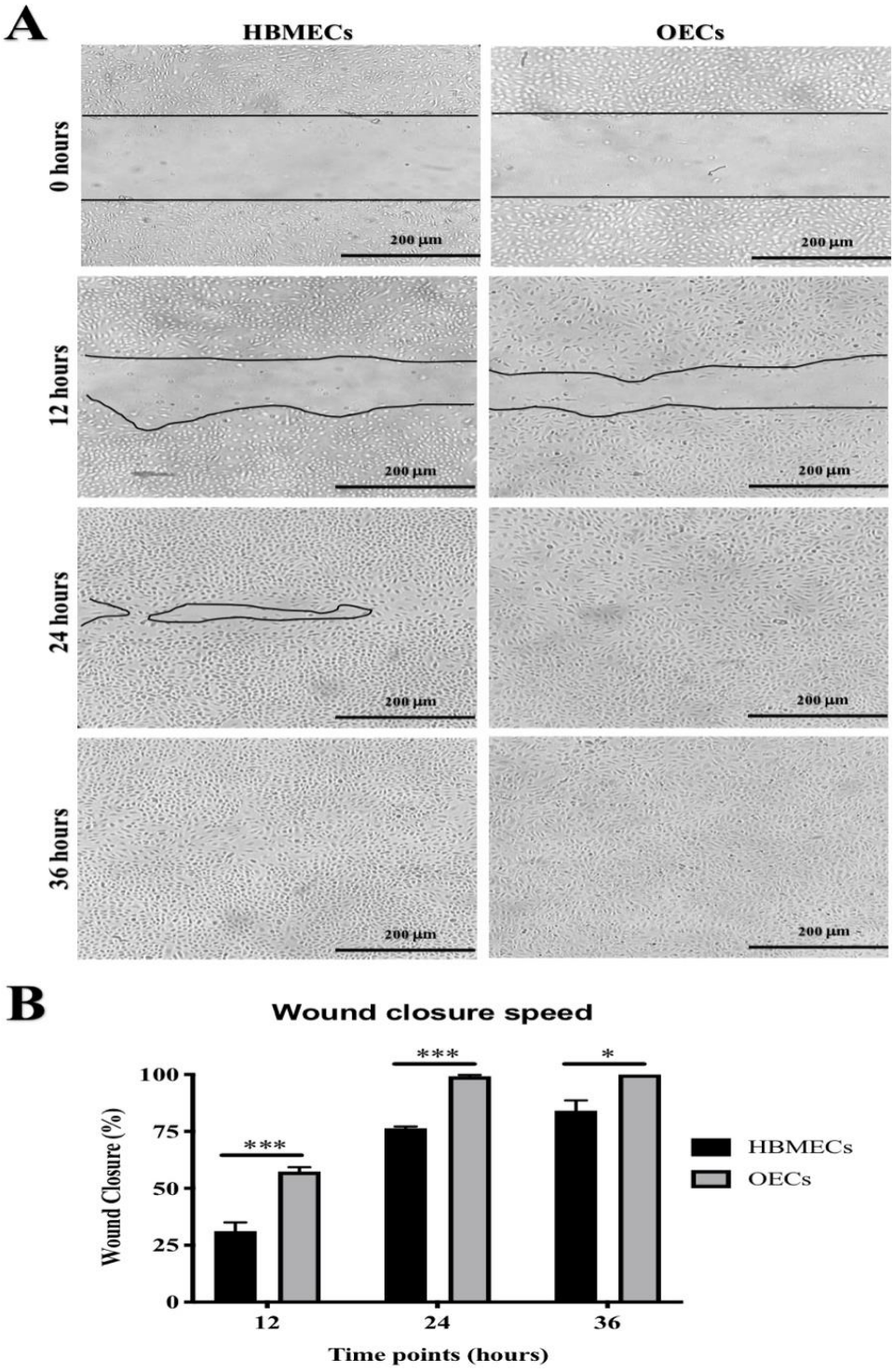


Figure 19. OECs displayed remarkably faster wound healing speed.

(A) Repair of wound damages evoked on HBMEC and OEC monolayer showed that the speed of wound closure is substantially faster with the latter at different time points following scratch than those observed in the former. **(B)** Quantification of wound healing speed of HBMECs and OECs. Scale bar: 100 µm. *p < 0.05, ***p < 0.001. Scale bar: 200 µm. *n* = 3.

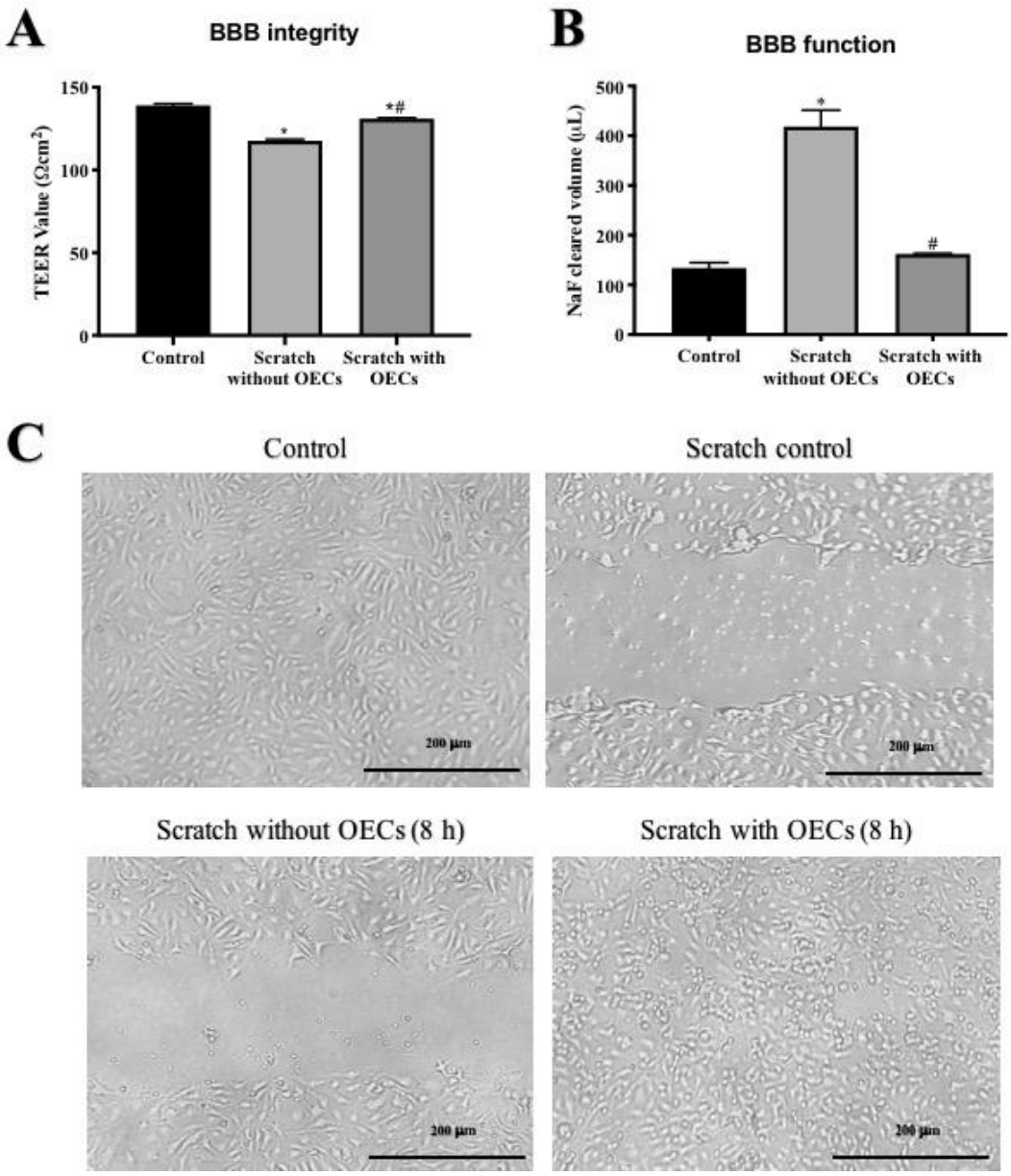


Figure 20. Analyses of endothelium-restorative function of OECs with wound scratch assay on an *in vitro* model of human BBB.

(A, B) Exogenous addition of OECs repaired the scratch induced on endothelial layer of a triple culture model of human BBB, formed by HBMECs, astrocytes, and pericytes and maintained in serum-free conditions. BBB integrity and function were determined by measurements of transendothelial electrical resistance (TEER) and sodium fluorescein flux (NaF), respectively. **(C)** Treatment with OECs restored the wound scratch evoked on HBMEC monolayer maintained in serum-free conditions. *$p<0.05$ compared to control, #$p<0.05$ compared to scratch without OECs. Scale bar: 200 µm. $n = 3$.

Having ascertained the barrier-reparative capacity of OECs *in vitro*, we subsequently assessed such therapeutic effects *in vivo*. Similar to the effects observed in the *in vitro* BBB model, intravenous administration of OECs ($4x10^6$ cells) to an *in vivo* rodent model of human transient ischaemic stroke 24 hours after induction of MCAO also significantly reduced ipsilateral, but not contralateral, brain oedema volumes on day 3 of treatment compared to vehicle-treated animals (Figure 21).

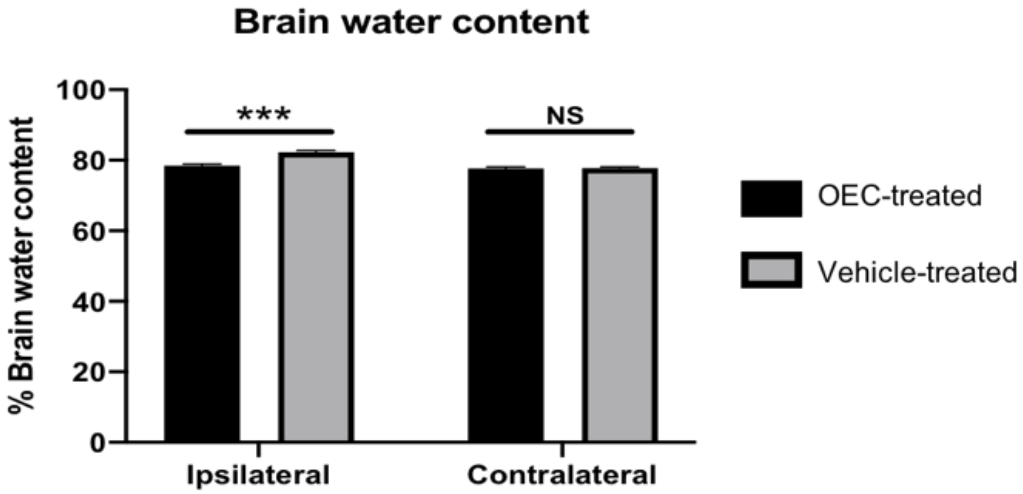


Figure 21. Treatment with OECs alleviated BBB damages of *in vivo* model of ischaemic stroke.

Intravenous administration of OECs 24 hours after MCAO suppressed brain water content in the ipsilateral, but not in the contralateral, hemisphere of the brain, observed on day 3 after cellular treatment. *** $p < 0.001$, NS: not significant. $n = 4$.

2.7. Exogenous OECs attenuate oxidative stress and cell death

To elucidate a mechanistic understanding for the putative reparative effect of OECs during ischaemic injury, the state of oxidative stress and cell death were investigated in HBMECs subjected to OGD injury in the absence or presence of OECs. While ischaemia significantly increased NADPH oxidase activity and superoxide anion levels in HBMECs, treatment with OECs reduced the level of both parameters to the levels observed in control groups (Figure 22A, B). In accordance with these findings, the presence of OECs during ischaemic injury also decreased the rate of cell death, as observed by the significant reduction in pro-apoptotic enzyme caspase 3/7 activities and improvements in HBMEC viability (Figure 22C, D).

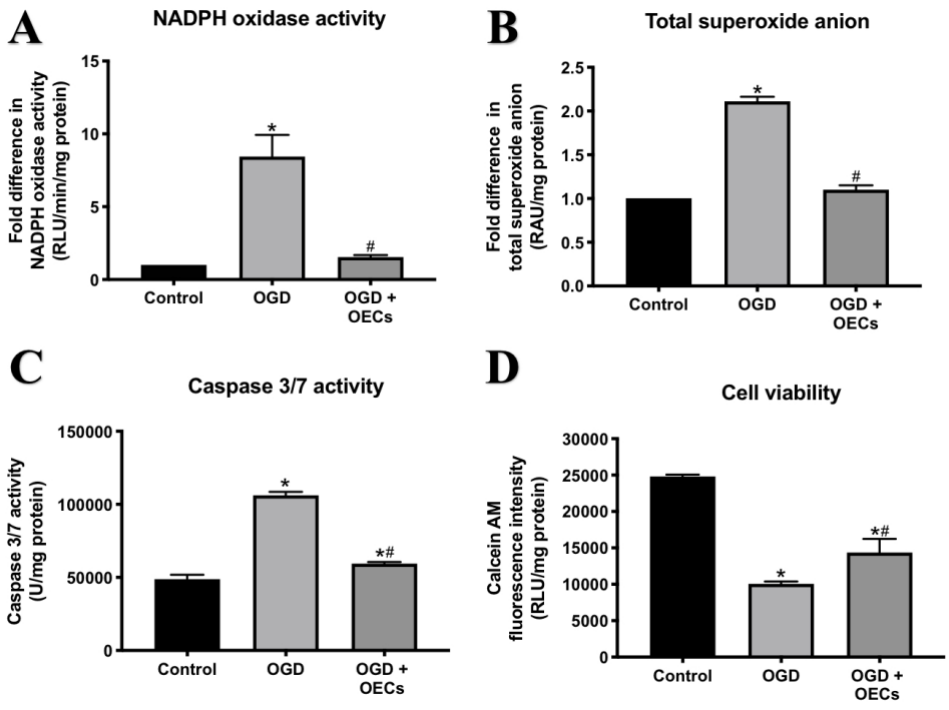


Figure 22. Treatment with OECs attenuated ischaemia-evoked oxidative stress and apoptosis of HBMECs.

(A-D) Treatment with OECs effectively inhibited NADPH oxidase activity, superoxide anion level, and caspase 3/7 activities, while significantly improving the viability of resident brain endothelial cells exposed to OGD injury. *$p < 0.05$ compared to control, #$p < 0.05$ compared to OGD. $n \geq 4$.

3. Discussion

The BBB, composed by an intimate interactions amongst cerebral endothelial cells, astrocytes, and pericytes, regulates the exchange of ions and molecules between systemic circulation and the brain parenchyma in an effort to precisely maintain the brain physiological function (Zhang et al. 2020a). Since BBB damages and ensuing brain oedema constitute the main cause of death and neurological deterioration in the early stage of ischaemic stroke (Dénes et al. 2011; Arba et al. 2021; Yao et al. 2021), restoration of this distinctive barrier may be a very effective therapeutic approach to mitigate stroke-related damage (Bernardo-Castro et al. 2020).

Re-endothelialisation of dead or dying cerebrovasculature not only requires the lateral migration and sprouting of resident BMECs, but also relies on the bioavailability of functional OECs, which can promote endothelial repair directly, through differentiation into mature endothelial cells; or indirectly, by releasing a wide range of trophic factors (Dellett et al. 2017; Reid et al. 2018). Strong associations observed between the availability and functionality of these particular stem cells and the extent of infarct volume and neurological recovery further corroborate the crucial role of OECs in the pathogenesis of ischaemic stroke, and in determining the severity of post-ischaemic damages (Tsai et al. 2014; Kukumberg et al. 2020). These encouraging findings have paved the way for a novel and promising therapeutic strategy, namely the administration of exogenous OECs after an ischaemic stroke injury, so that it can provide sufficient highly functional OECs to regenerate brain endothelial cells and maintain neurovascular integrity (Faris et al. 2020).

Data generated in the current study shows that OECs display most of the unique characteristics of BMECs, as observed by their typical cobblestone morphology, binding to specific lectin and Dil-Ac-LDL, and forming coherent tube-like structures

on Matrigel. Greater migratory and proliferation rate retained by OECs in comparison to BMECs along with an equal capacity to attach with extracellular matrix components may indicate an appropriate ability of these stem cells to migrate, detect, and adhere to injury sites.

The successful integration of OECs into the HBMEC vascular network, as observed by the well-established tubule networks on Matrigel and the uniform staining of ZO-1 when co-cultured with HBMECs, has confirmed the ability of these cells to engraft into brain capillaries. Finally, the attainment of similar TEER value and concomitant sodium fluorescein flux of simultaneous culture of HBMECs and OECs in the triple culture model of human BBB further corroborates that OECs can interact with brain endothelial cells, as well as forming a tight and functional cerebral barrier. Moreover, this equal barrier-forming capacity between both cells may also indicate that OECs are an attractive candidate to replace HBMECs to establish an *in vitro* model of human BBB, and support the results of previous studies reporting a stable and reproducible *in vitro* BBB model formed by OECs (Boyer-Di Ponio et al. 2014; Cecchelli et al. 2014).

Through using the well-established *in vitro* models of human BBB, the current study concomitantly showed that exogenous addition of OECs during OGD±R injury effectively repaired BBB integrity and function, as attested by increased TEER value and decreased sodium fluorescein flux across the barrier, respectively. Wound scratch assay showed that both HBMECs and OECs were capable of proliferation and directional migration, whereby the rate of recovery was considerably faster with OECs. Subsequent experiments investigating the restorative function of OECs on the endothelial layer of the abovementioned BBB model revealed that treatments with OECs effectively repaired the integrity and function of BBB, as ascertained by the

substantial improvements in readings for TEER and paracellular flux of sodium fluorescein, respectively. The absence of serum in the culture conditions used in these experiments confirmed that the observed healing process was solely due to OEC homing and differentiation, rather than HBMEC proliferation. In short, these *in vitro* findings collectively suggested that treatment with OECs effectively attenuates cerebral barrier damages.

These barrier-reparative effects have also been replicated in *in vivo* settings, with the intravenous administration of OECs 24 hours after induction to MCAO markedly decreasing the brain water content in the ipsilateral hemisphere brain assessed on day 3 after treatment. While the current *in vivo* study did not assess the capacity of human OECs to integrate into vasculature, the observation of reduced brain water content specifically in the ipsilateral hemisphere brain indicated the effective homing of OECs in the site of injury (Maki et al. 2018; Reid et al. 2018). The cerebral restoration effect of OECs on the outside of the rtPA therapeutic window (after 4.5 hours) in the present study has crucially important implications for the improvement of treatment options for the overwhelming number of ischaemic stroke patients who are currently not eligible for rtPA.

Although ROS in physiological concentration is a prerequisite for neurovascular stability, an excessive production of this enzyme, notably NADPH oxidase, the main source of ROS in the brain vasculature, is associated with endothelial dysfunction and neurovascular complications (Maraldi et al. 2021). The decrease of cerebral barrier damages and infarct volume in animal model of ischaemic stroke treated with various structurally distinct NADPH oxidase inhibitors (apocynin, DPI, or VAS2870) prior to induction to MCAO further substantiated the pivotal role of this oxidase enzyme in the pathogenesis of BBB damages in ischaemic stroke setting (Nagel et al. 2007; Tang

et al. 2008; Kleinschnitz et al. 2010; Zehendner et al. 2013). In this context, the present study also showed significant increases in NADPH oxidase activity and superoxide anion generation in brain endothelial cells exposed to ischaemic injury, and demonstrated an effective oxidase inhibition through using an exogenous addition of OECs. As co-treatment with OECs normalised the levels of both parameters in both settings, it is possible that modulation of oxidative stress and downstream target NF-κB may in part account for the neurovascular restorative effects of OECs (Qiu et al. 2013).

Increasing evidence show that apoptosis of brain endothelial cells exacerbates the detrimental effect of ischaemic injury by compromising tight junction formation, and thus microvascular integrity, through stimulation of a range of mechanisms, particularly the NADPH oxidase–superoxide anion-caspase 3/7 signalling pathway (Park et al. 2010; Rakkar and Bayraktutan 2016; Chignalia et al. 2020). The attainment of significant increases in the activity of these pro-apoptotic enzymes alongside a decrease in HBMEC survivability following exposing to ischaemic injury in the present study has confirmed the importance of this pathway. It was also found that treatment with OECs attenuated caspase 3/7 activity and elevated HBMEC viability, hence adds further weight to the cerebrovascular-reparative role of OECs. In addition to the regulation of caspase 3/7, the potentiation of anti-apoptotic Bcl-2 expression and accentuation of pro-apoptotic proteins Bax expression may also contribute to the neurovascular protective effect of OECs against cerebral ischaemic injury-induced apoptosis (Qiu et al. 2013; Hong et al. 2020).

The substantial increases in total anti-oxidant capacity and decreases in NADPH oxidase activity and superoxide anion level observed in the current study may suggest that these cells resist the hostile pathological microenvironment. In line with this

notion, some studies have shown that OECs are more resistant to oxidative stress damages (exposed to hydrogen peroxide) due to the high expression of intracellular anti-oxidative enzymes such catalase, glutathione peroxidase, and MnSOD in comparison to mature endothelial cells (Dernbach et al. 2004; He et al. 2004).

In contrast with these findings, we have observed that OGD±R injury affects both HBMECs and OECs in a similar fashion, as shown by the equal TEER value, paracellular flux volume, stress fibres formation, and the viability between these two cells. These findings may be a result of exposure to ischaemia/reperfusion injury involving more complex and numerous pathological pathways, rather than oxidative stress, which mainly affects single pathways (Campbell and Khatri 2020). Additionally, OECs and mature endothelial cells are known to display a similar apoptotic response to an agent that cause DNA damages (e.g. cisplatin) (Dernbach et al. 2004; He et al. 2004), consequently the exposure to ischaemic injury that causing such damage may also contribute to the similar response between these two cells (Rakkar and Bayraktutan 2016). Moreover, cell populations employed in previous studies (Dernbach et al. 2004; He et al. 2004) may include macrophages and monocytes, which are known to be resistant to oxidative stress. In support of this notion, a single-cell deposition assay showed remarkably increased apoptosis rate and profound impairment on OECs tubulogenesis in both *in vitro* and *in vivo* studies in response to oxidative stress damages (Ingram et al. 2007).

These findings may also highlight the importance of designing therapeutic strategies to enhance the vasoreparative function of OECs against pathological condition. The observation of better angiogenic capacity and restorative function of OECs subjected to hypoxic injury after transfection with various pharmacological compounds, such as miR-130 agomir (Guduric-Fuchs et al. 2021), cibinetide (a non-hematopoietic

erythropoietin mimetic) (O'Leary et al. 2019), and androgens (Lam et al. 2020), paves the way for the exploration of novel agents that can augment the regenerative potential of these progenitor cells.

Despite showing a remarkably migratory, proliferative, and wound-reparative potential, after a certain number of replications, OECs start manifesting the signs of senescence at morphological, structural, and functional levels, as ascertained by the appearance of flattened and enlarged morphology, nuclear damage, and limited regenerative capacity (Lee et al. 2020). Therefore, understanding the molecular mechanisms underlying this process is crucial to generate a large number of highly functional and homogenous OECs that can be utilised as therapeutics (Medina et al. 2013) and also to regulate the bioavailability of those are already present in circulation (Dai et al. 2017).

Given that increased oxidative stress represents one of the major driving forces in endothelial senescence, the regulation of key factors associated with this phenomenon, such as anti-oxidant and pro-oxidant enzymes may delay OEC senescence and enhance their vasoreparative function (Coluzzi et al. 2019), which further explored in the Part II. Considering that cell-based therapy may be associated with various adverse effects, including immunological reaction and tumour formation, and that paracrine factors released by OECs can initiate and potentiate neuro-angiogenesis after an ischaemic injury (Maki et al. 2018; Zhuang et al. 2021), cell-free strategy may also be considered among valid therapeutic options in regenerative stroke medicine, which will be further explored in the Part III.

4. Conclusion

This study has shown that OECs possess strong endothelial characteristics and are equipped with great anti-oxidant, proliferative, and migratory capacities. The current study also showed that OECs can integrate with brain endothelial cells and form a tight and functional BBB. Exogenous addition of OECs after cerebral barrier damages can effectively repair BBB damage by suppressing oxidative stress and resident cell apoptosis. The graphical summary of this study is shown in Figure 23.

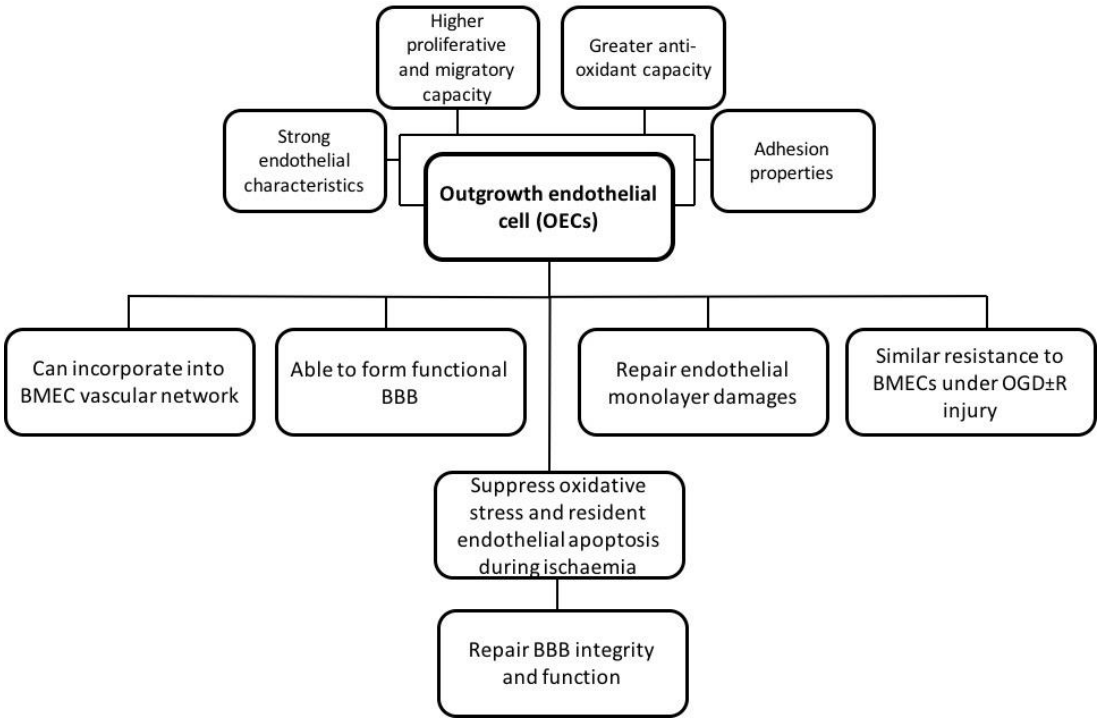


Figure 23. Graphical summary.

Part II

Inhibition of oxidative stress delays senescence and augments functional capacity of OECs

Abstract

Despite playing an integral role in repairing blood-brain barrier (BBB) damages, endothelial progenitor cells (EPCs) are prone to age-dependent changes that can accelerate their senescence and diminish their quantity and functionality. In this regard, the current study firstly measured the number of circulating EPCs in older versus younger (73.3±7.2 vs. 40.2±14.3 years) healthy volunteers and found an insignificant difference in the level of circulating EPCs (CD34+CD133+KDR+) between these two groups. However, the level of cells exclusively expressing stemness markers that were known to possess a unique capacity to differentiate into endothelial cells (CD34+ and/or CD133+) showed sharp declines in older individuals. These coincided with decreases in total anti-oxidant capacity and concomitant increases in plasma levels of pro-inflammatory cytokine, TNF-α, and anti-angiogenic factor, endostatin and thrombospondin-1. The subsequent experimental studies to scrutinise the effect of ageing on molecular and functional properties of outgrowth endothelial cells (OECs), the subtype of EPCs, showed that chronological ageing, mimicked by replicative senescence, profoundly impaired the regenerative and BBB-forming capacity of OECs. Similar to those seen in the clinical observational studies, senescent OECs also manifested decreased total anti-oxidant capacity and increased pro-oxidant NADPH oxidase activity and endostatin levels. Suppressing oxidative stress level using structurally and functionally distinct anti-oxidants, namely vitamin C or VAS2870, an NADPH oxidase inhibitor, delayed OEC senescence and repaired their tubulogenic and BBB-forming capacities. In conclusion, effective control of oxidative stress that develops during physiological ageing may somewhat delay EPC senescence and help extend healthy lifespan.

1. Introduction

Despite playing a critical role as an endogenous cerebrovascular repair system, as described in the Part I, the quantity, mobilisation, proliferation, migration, survival, and reparative capacity of EPCs may be adversely influenced by advanced age. Increasing of evidences have shown that age-related decline in the expression of various pro-angiogenic factors, including growth factors, cytokines and chemokines play an instrumental role in these processes (Zhang et al. 2009; Xia et al. 2012). Besides, with advancing age, EPCs become susceptible to internal alterations and environmental influences which gradually exhaust their replicative potential and induce cellular senescence (Kaur et al. 2018). Inevitably, any decline in EPC number and/or functional capacity is expected to contribute to age-related progressive loss of cerebral endothelium, and to the pathogenesis of several debilitating diseases, including stroke (Tsai et al. 2014; Kukumberg et al. 2020).

Hence, the determination of molecular mechanisms involved in age-mediated EPC numerical, structural, and functional aberration is of vital importance to develop novel strategies that can delay or prevent cerebrovascular dysfunction and improve lifelong health and wellbeing (Thum et al. 2007; Altabas et al. 2016). Moreover, since large-scale expansion of OECs to generate sufficient cell numbers for therapeutic purposes ultimately leads cells to replicative senescence, an improved understanding of OEC senescence mechanism may overcome the challenges of efficiently expanding cell numbers without diminishing their functionality (Medina et al. 2013).

Oxidative stress, stemming from an imbalance between the generation and metabolism of reactive oxygen species (ROS), represents the most commonly encountered pathology in aged cells and tissues (Höhn et al. 2017). At physiological concentrations, ROS regulate a diverse range of cellular functions, including

proliferation, migration, and gene expression, and modulates the response of stem cells to metabolic and environmental signals, either by keeping them in a quiescent state, or allowing them to continue cycling (Bayraktutan 2019). In contrast, prolonged exposure to excessive levels of ROS promotes senescent phenotype and stem cell dysfunction, through progressive accumulation of ROS-induced damages (Höhn et al. 2017; Wang et al. 2017). Hence, it is reasonable to suggest that the suppression of oxidative stress may delay senescent phenotype and stem cell dysfunction, and maintain cerebrovascular integrity. Indeed, treatments with broad-spectrum anti-oxidant vitamin C or an inhibitor of NADPH oxidase, the main enzymatic source of vascular ROS, have been shown to delay cellular senescence in previous studies (Sun et al. 2015; Yang et al. 2018).

In light of the above, the current study explores the quantity of circulating EPCs, and the major elements known to affect their mobilisation, number, and functionality in older versus younger healthy volunteers. By using a cell culture model of chronological ageing, it subsequently investigates the morphological, molecular, and functional changes that occur in senescent EPCs, and assesses the impact of oxidative stress management (by vitamin C or VAS2870, a NADPH oxidase inhibitor) on these changes, using a wide range of experimental methodologies, notably a well-established *in vitro* model of human BBB (Shao and Bayraktutan 2013; Kadir et al. 2021).

2. Results

2.1. Number of circulating progenitor cells are downregulated by ageing process

Peripheral blood samples obtained from 50 younger and 40 older healthy individuals were analysed by flow cytometry to investigate the correlation between chronological ageing and putative changes in circulating EPC numbers. Typically, 1.000.000 total events were acquired to determinate the percentage of the EPC subpopulation in the gate. The example of the gating strategy for the classification of a cell as EPC can be found in Figure 24.

Our study revealed substantially reduced level of undifferentiated stem cells (CD34+, CD133+, or CD34+CD133+) in older participants, but insignificant differences in the number of endothelial-committed cells (KDR+, CD34+KDR+, CD133+KDR+) and circulating EPCs (CD34+CD133+KDR+) between these two groups (Figure 25).

The level of major elements that determine the quantity, mobilisation, and functionality of EPCs also appeared to be differentially affected by the ageing process, whereby elderly people had substantially lower plasma total anti-oxidant capacity and higher levels of inflammatory cytokine (TNF-α), chemokine (SDF-1), and anti-angiogenic factors (endostatin and thrombospondin-1) compared to their younger counterparts (Figure 26A-E). In contrast, no marked differences were observed in the plasma levels of other key pro-angiogenic (VEGF and PDGF-BB), anti-angiogenic (angiostatin and thrombopondin-2), and growth factors (G-CSF) between younger and older populations (Figure 26F-J).

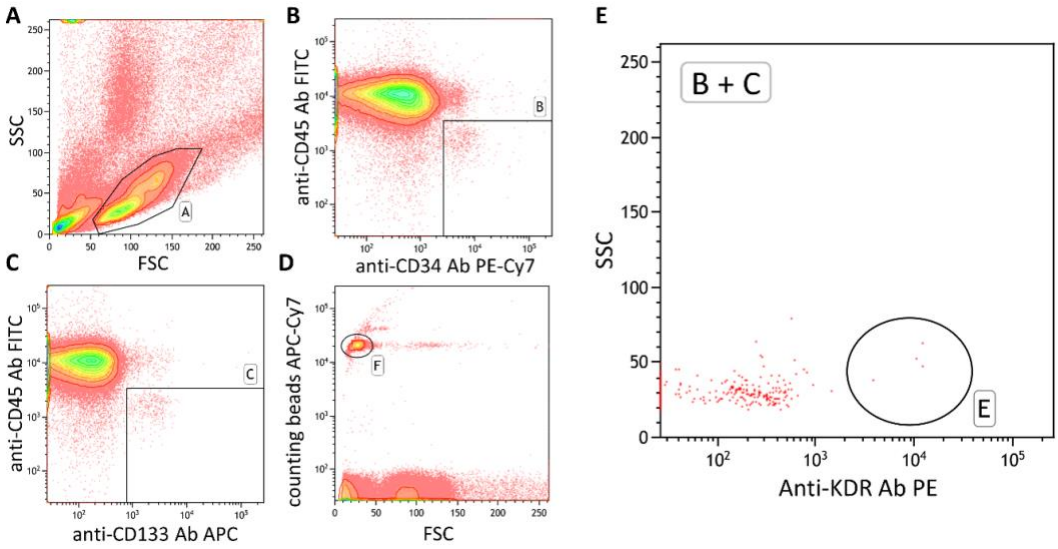


Figure 24. Example of gating strategy of EPCs by flow cytometry.

(A-E) EPCs were detected by flow cytometry and analysed using Kaluza Analysis 2.1 software. Compensation controls were used in setting up the flow protocol. Fluorescence minus one and isotype controls were used in gating for positive cells. CD45-FITC, CD34-PECy7, CD133-APC and KDR-PE. Approximately 1 million cells were counted. Cells were first gated on the monocyte and lymphocyte gate [A], then for CD45- CD34+ [B] and CD45- CD133+ [C] cells. Finally, cells were gated for [B]+[C] and KDR+ cells [E]. Counting beads were used to normalise samples to EPC counts/mL [F]. This figure was analysed and provided by Dr Kamini Rakkar.

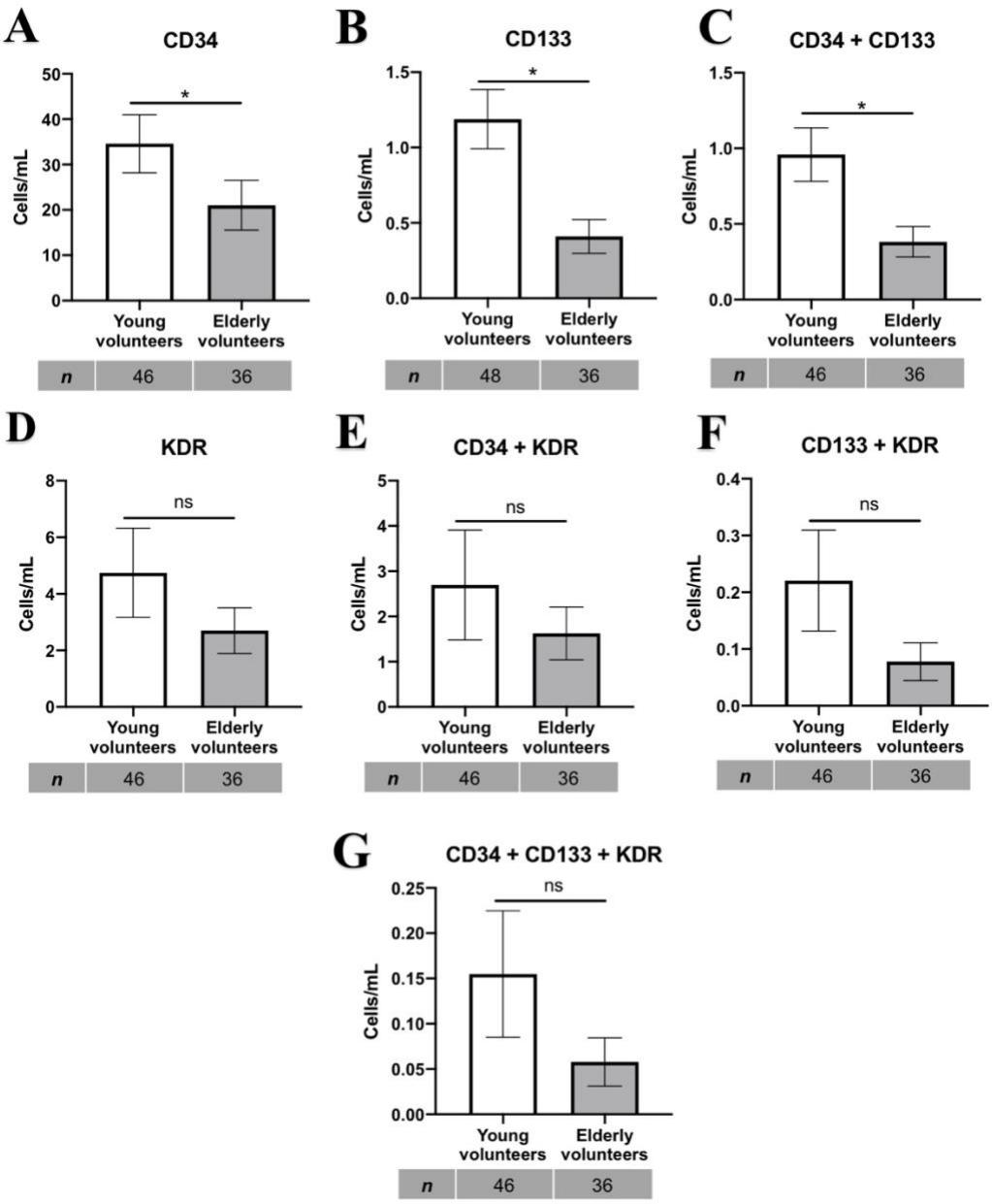


Figure 25. Plasma levels of circulating endothelial progenitor cells in young versus older healthy volunteers.

(A-C) Compared to younger individuals, older volunteers had fewer undifferentiated cells expressing CD34+, CD133+, and CD34+CD133+. **(D-G)** The number of cells expressing KDR+, CD34+KDR+, CD133+KDR+, or CD34+CD133+KDR+ did not change between older and younger healthy volunteers. *P < 0.05 compared to young volunteers. NS: not significant.

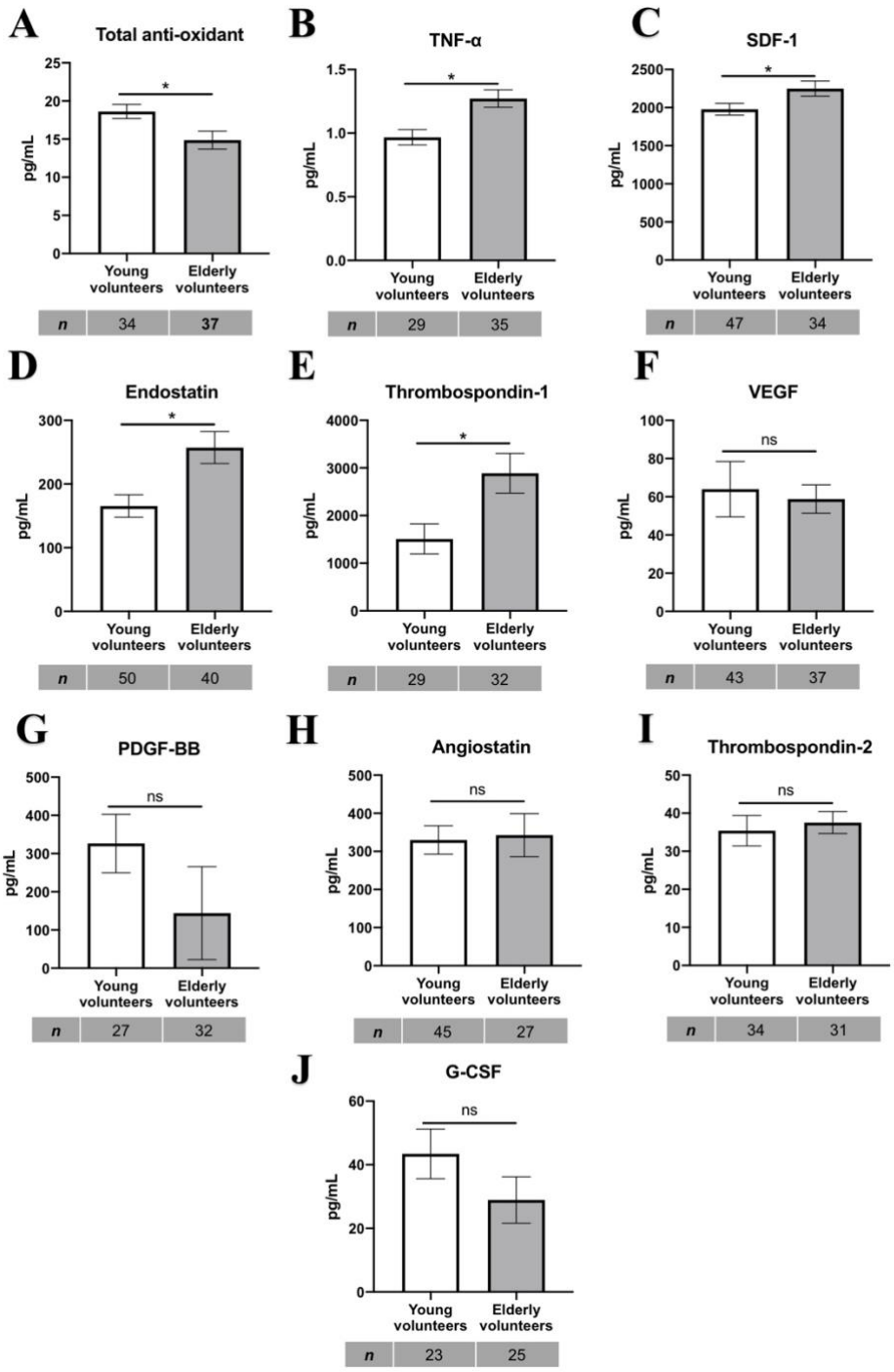


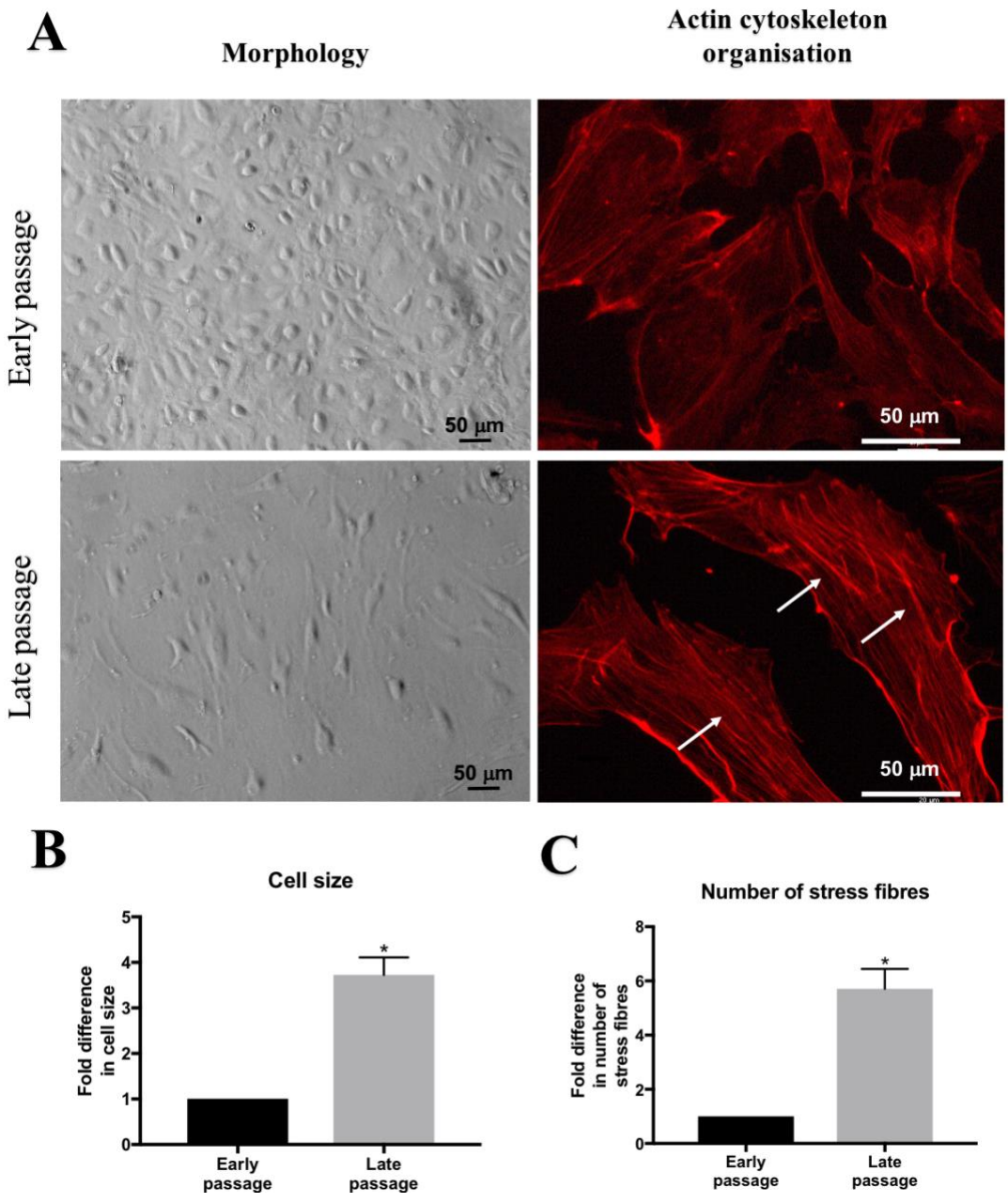
Figure 26. The level of major elements known to affect EPC number and function in plasma of young and older healthy volunteers.

(A-E) Compared to their younger counterparts, the older volunteers had significantly lower total anti-oxidant capacity but higher inflammatory and anti-angiogenic factors, namely TNF-α, SDF-1, endostatin, and thrombospondin-1. **(F-J)** In contrast, the levels of pro-angiogenic factors, VEGF and PDGF-BB, anti-angiogenic factors, angiostatin and thrombopondin-2, and growth factor, G-CSF, were not statistically different between the two groups. *P < 0.05 compared to young volunteers. NS: not significant.

2.2. Late-passage OECs exhibit typical characteristics of cellular senescence

Having ascertained the impact of chronological ageing on the numbers of circulating progenitor cells, the subsequent experimental studies were designed to examine whether or to what extent chronological ageing, induced by repetitive cell culture, might affect OEC morphological and molecular phenotype. These studies demonstrated that late-passage OECs (P14) acquired bigger and flattened morphology and developed thick actin stress fibres traversing the cells compared to the early-passage OECs (P7) (Figure 27A-C).

In addition to morphological changes, late-passage OECs also had significant increases in pro-oxidant NADPH oxidase activity and superoxide anion production, and concurrent decreases in total anti-oxidant and nitrite/nitrate (NOx) release (Figure 27D-G), thereby confirming the correlation between the advance age and oxidative stress (Höhn et al. 2017). Superoxide anion constitutes the foundation molecule of all ROS, while NOx represents the final metabolites of NO, a short-lived anti-oxidant that scavenges superoxide anion and regulates vascular tone and homeostasis (Bayraktutan 2005).


A
Morphology
Actin cytoskeleton organisation
Early passage
Late passage
50 µm
50 µm
50 µm
50 µm
B
Cell size
Fold difference in cell size
0
1
2
3
4
5
*
Early passage
Late passage
C
Number of stress fibres
Fold difference in number of stress fibres
0
2
4
6
8
*
Early passage
Late passage

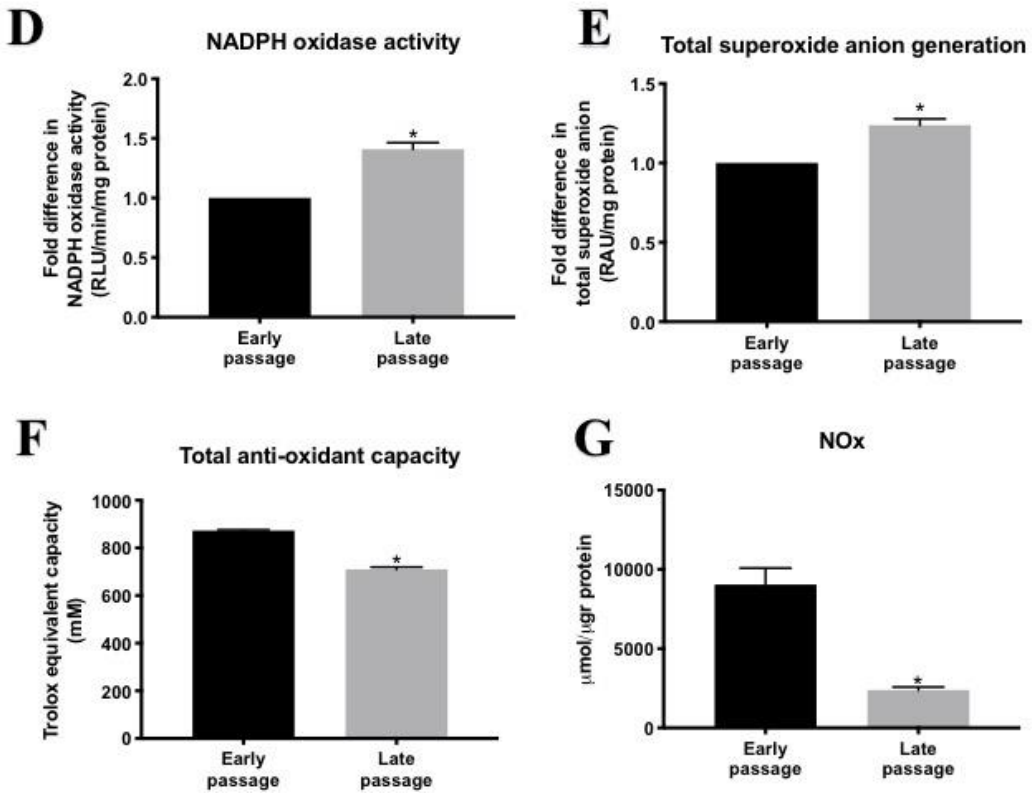
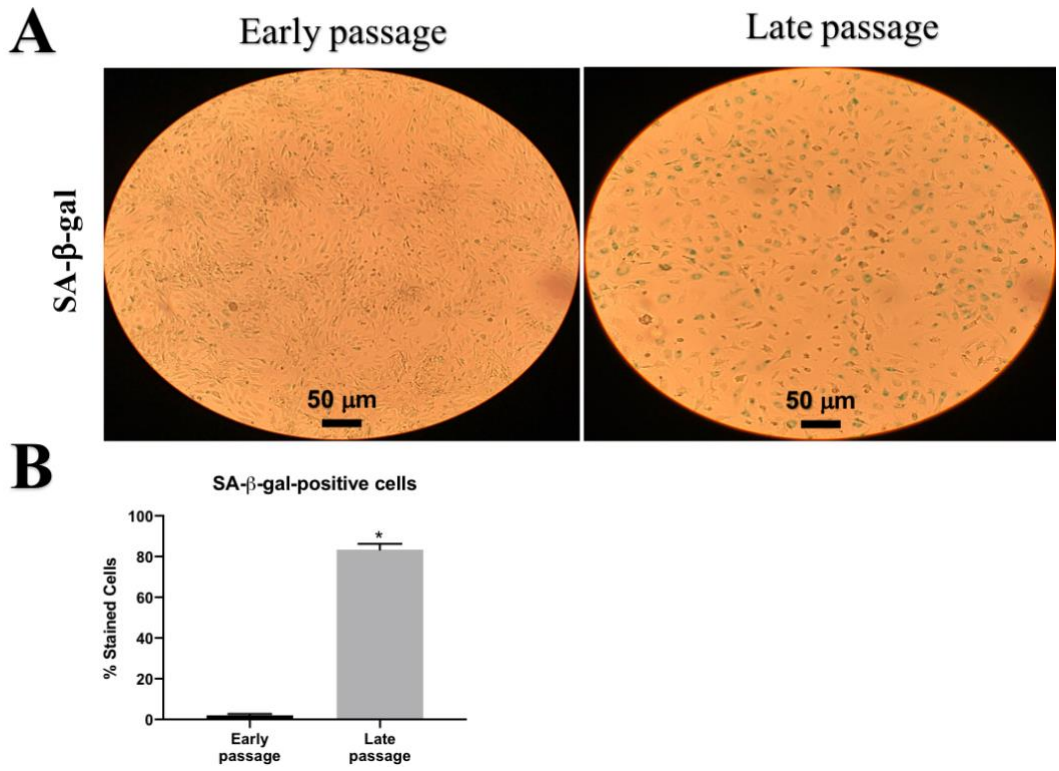


Figure 27. Exposing OECs to chronological ageing mimicked by repetitive cell culture triggered profound alterations in their morphology and evoked oxidative stress.

(A) Late-passage (passage 14) OECs took up larger and flattened morphology and displayed cytoskeletal reorganisation characterised by the appearance of actin stress fibres (white arrow). **(B, C)** The measurement of cell size and number of stress fibres in early-passage (passage 7) versus late-passage OECs. **(D-G)** Late-passage OECs possessed substantially higher NADPH oxidase activity and superoxide anion production and lower total anti-oxidant capacity and nitric oxide generation. *P < 0.05 compared to early-passage OECs. Scale bars: 50 µm. $n \geq 3$.

Studies designed to provide further direct evidence for cellular senescence demonstrated that a significantly higher percentage of late-passage OECs stained positive for senescence-associated-β-galactosidase (SA-β-gal) compared to low-passage OECs (Figure 28A, B). However, since the increased of SA-β-gal activity may not be unique to senescent cells, and cells exposed to other stimuli (such as serum starvation) can also display increased enzymatic activity (Yang and Hu 2005), the level of γ-H2AX, a sensitive marker of double-stranded DNA breaks and telomere shortening (Mah et al. 2010), was also investigated in early- and late-passage OECs. Simultaneous staining of nuclear DNA in these cells (with DAPI) showed a greater percentage of late-passage OECs were stained positive for γ-H2AX and manifested bigger nuclear size compared to the early-passage cells (Figure 28C-E). Taken together, these findings confirm that late-passage OECs possess a series of key characteristics of senescence, hereinafter described as “senescent OECs”.



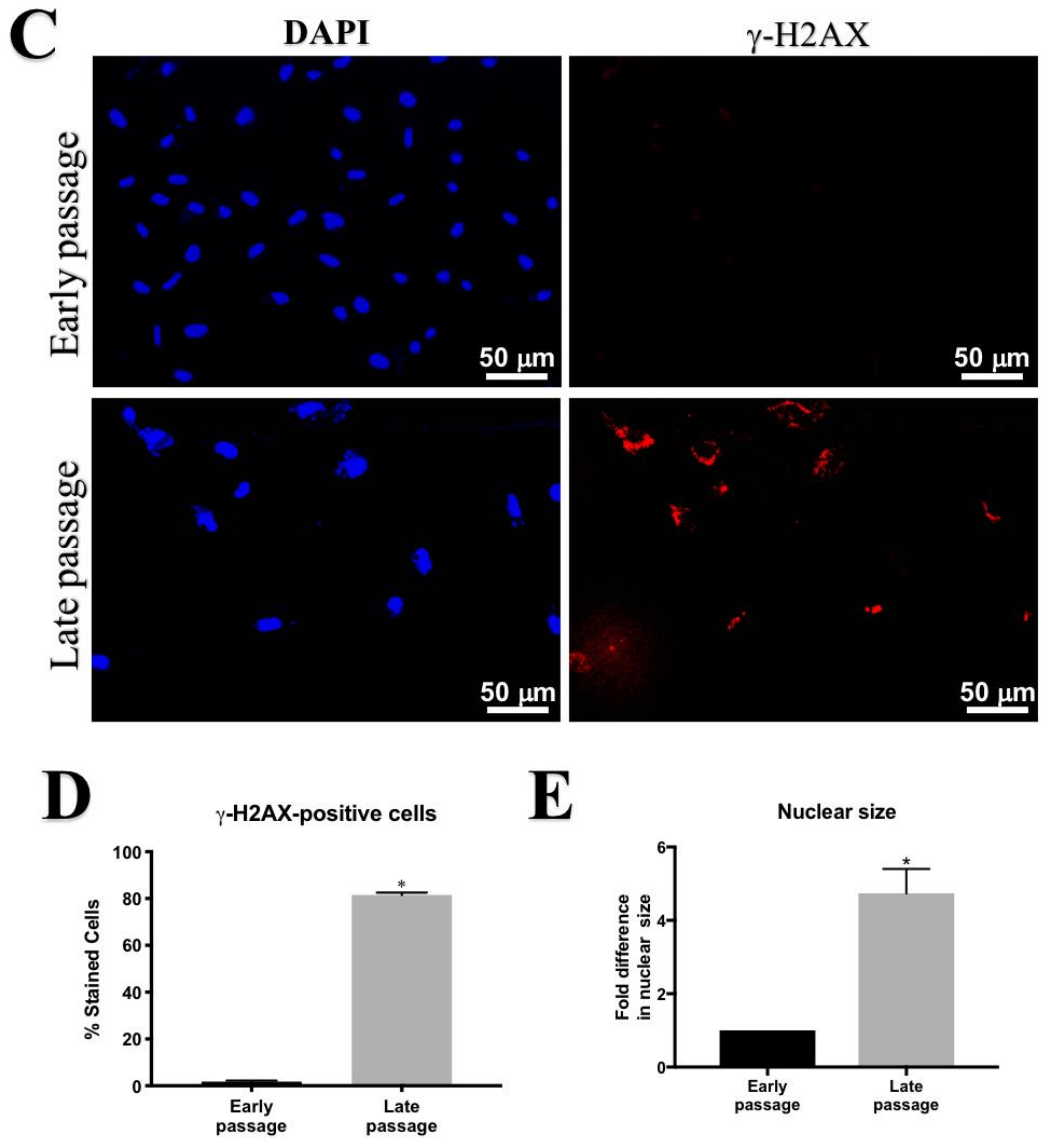
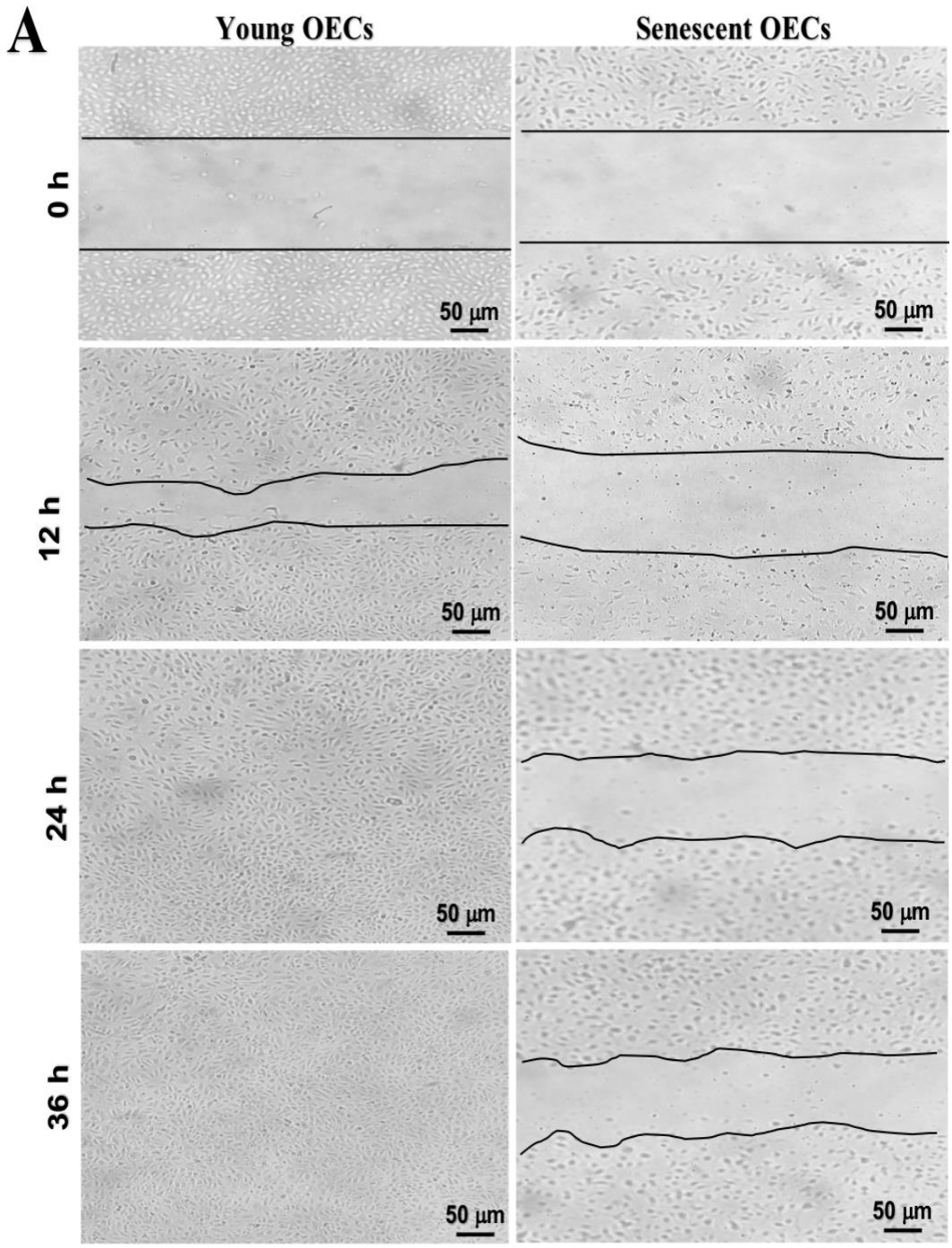


Figure 28. **Long-term expansion of OECs triggered replicative senescence.**

(A) The number of SA-β-gal-positive cells were substantially higher in late-passage OECs. **(B)** The quantification of SA-β-gal-positive cells. **(C)** Late-passage OECs showed consistent increases in γ-H2AX activity compared to early-passage ones. **(D)** The quantification of γ-H2AX-positive cells. **(E)** Late-passage OECs displayed significantly greater nuclear size compared to early-passage OECs. *P < 0.05 compared to early-passage OECs. Scale bars: 50 μm. $n \geq 3$.

2.3. Senescent OECs lose their regenerative and angiogenic potential and fail to establish functional BBB

Since enhanced migratory, proliferative, and angiogenic capacities of OECs are essential pre-requisites for their vasculo-reparative function, it was important to establish the impact of senescence on these crucial properties. Wound scratch assays, simultaneously assessing the migratory and proliferative potential of OECs, showed that while senescent OECs were unable to repair damage during the entire experimental period, i.e., 36 hours, young OECs covered much of the scratch area within 12 hours, and completely repaired it within 24 hours, proving the highly functional nature of young cells in the process (Figure 29A, B). In line with these findings, substantial reductions were also observed in migratory and proliferation rates of senescent versus young OECs in additional studies specifically looking into these features using a Transwell inserts and colorimetric assay, respectively (Figure 29C, D). It is noteworthy here that rather than showing a persistent state of cell cycle arrest, the senescent cells depicted low proliferative potentials. The presence of small number (approximately 20%) of non-senescent cells in the senescent cell monolayer (Figure 28) may contribute to this phenomenon. Scrutiny of tubulogenic properties of OECs by Matrigel assay also showed that senescent OECs formed considerably fewer number of tubes with shorter lengths and generated significantly greater levels of endostatin compared to young OECs (Figure 30).


A
Young OECs
Senescent OECs
0 h
12 h
24 h
36 h
50 µm

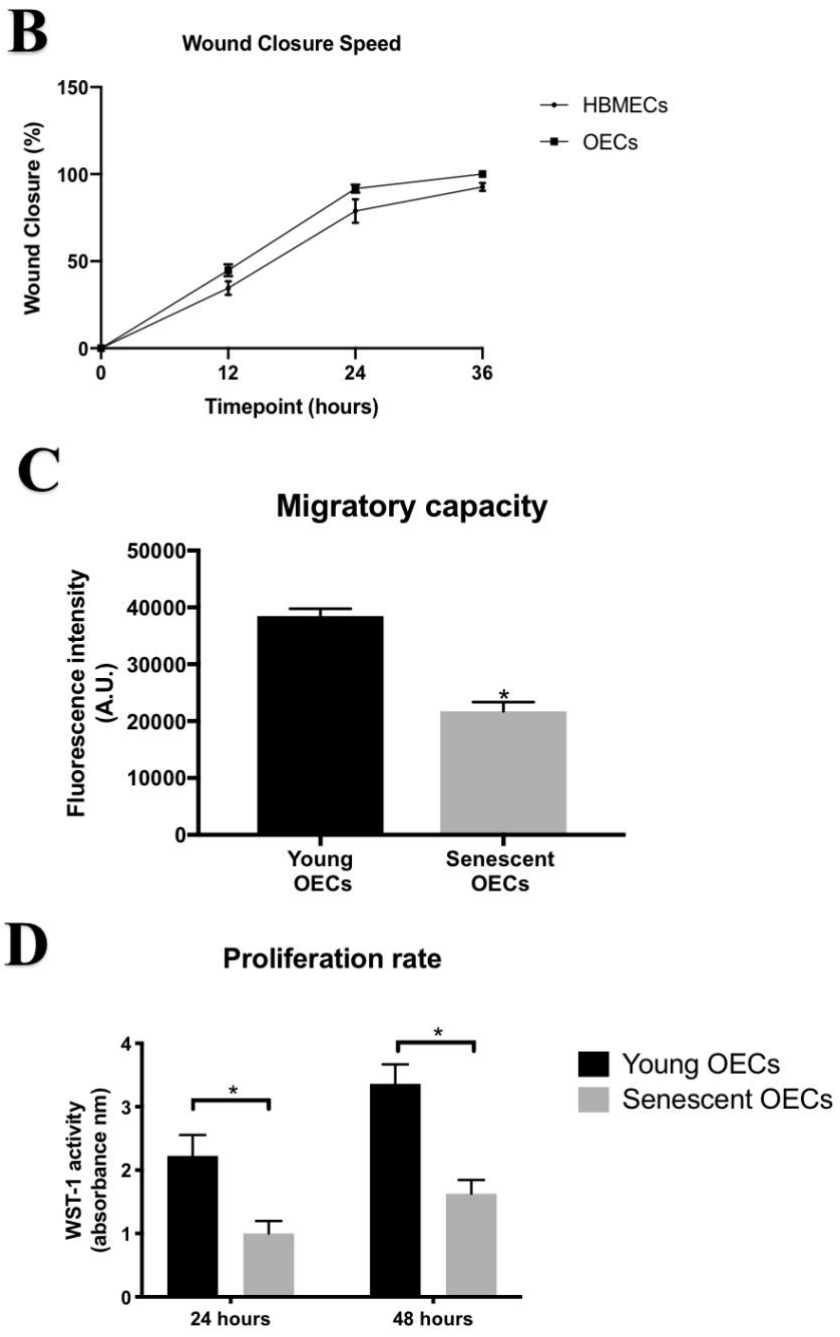


Figure 29. Senescent OECs displayed limited proliferative and migratory capacity.

(A) Time-course assessment of cell proliferation and migration through wound scratch assays showed the duration of wound closure was considerably shorter with senescent OECs compared to their young counterparts. **(B)** The quantification of wound healing speed in young versus senescent OECs. **(C, D)** Assessment of the cell migratory and proliferative capacity via Transwell Insert and WST-1 assay, respectively, also showed the substantial reduction in these parameters for senescent OECs. *P < 0.05 compared to young OECs, #P < 0.05 compared to HBMECs. Scale bars: 50 μm. $n \geq 4$.

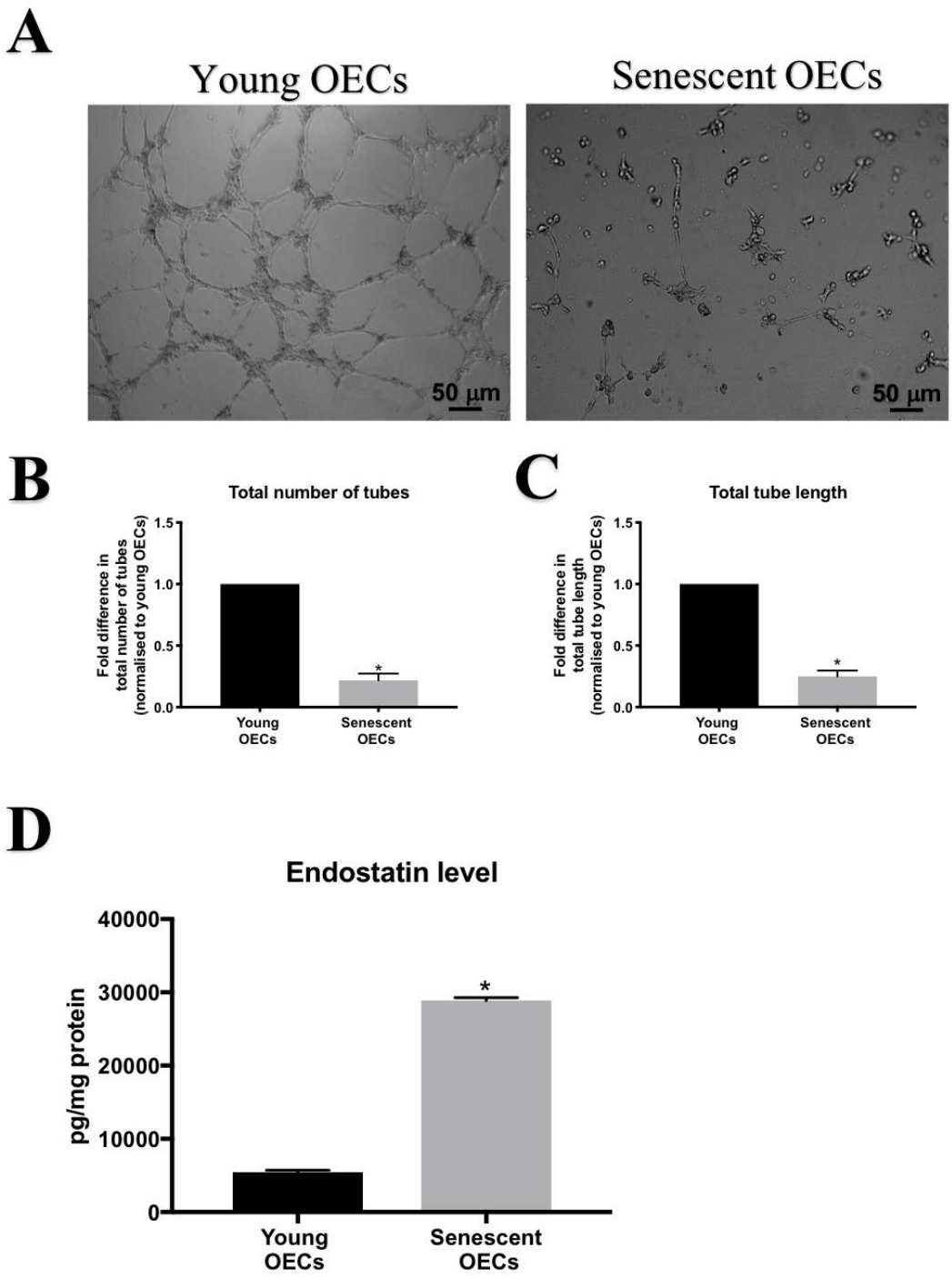


Figure 30. Senescent OECs conferred impaired tubulogenic activity.

(A) Senescent OECs formed a less organised tubule networks on Matrigel compared to the young OECs. **(B, C)** The quantification of the number and length of tubule networks in both cells. **(D)** Level of anti-angiogenic factor endostatin was substantially higher in senescence compared to those observed in young OECs. *P < 0.05 compared to young OECs, #P < 0.05 compared to HBMECs. Scale bars: 50 μm. $n \geq 4$.

Through the replacement of dead or dying endothelial cells, OECs play an important role in preventing structural and functional vascular complications (Bayraktutan 2019). The data generated up to this point suggest that senescent OECs are unlikely to be as protective or functional as younger ones. Unlike HBMECs and young OECs, senescent OECs were unable to form tight and functional human BBB models with astrocytes and pericytes (Figure 31A, B), which supports this notion. Changes in barrier integrity and function were measured by transendothelial electrical resistance (TEER) and paracellular flux of sodium fluorescein across the barrier, respectively (Kadir et al. 2021). Interruptions in the staining of tight junction protein zonula occludens-1 (ZO-1) indicated the inability of senescent OECs to form a uniform cell monolayer, and provided an explanation for the abovementioned disruptions in BBB integrity and function (Figure 31C).

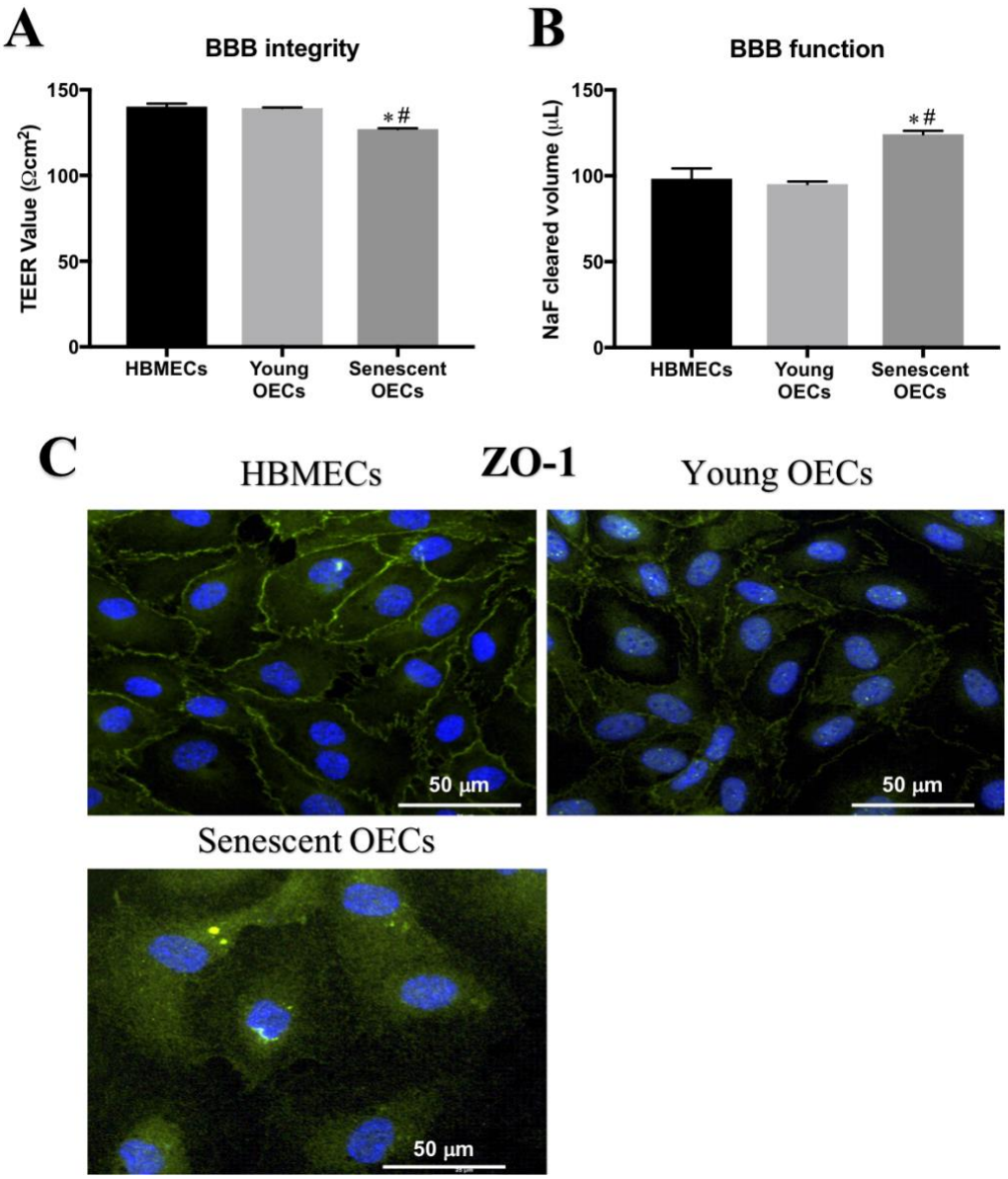


Figure 31. Senescent OECs failed to form a tight and functional cerebral barrier.

(A, B) Senescent OECs displayed remarkably lower TEER value and higher sodium fluorescein flux when utilised to establish *in vitro* model of human BBB. **(C)** Senescent OECs also showed an interrupted distribution of ZO-1. *P < 0.05 compared to young OECs, #P < 0.05 compared to HBMECs. Scale bars: 50 μm. $n \geq 4$.

2.4. Senescent OECs adversely affect the functionality of resident endothelial cells

Given that OECs are in constant close contact with resident endothelial cells *in vivo* and continuously replace them to maintain vascular homeostasis (Xie et al. 2016), it was important to probe the impact of young and senescent OECs on resident endothelial cell functionality. For this, HBMECs were mixed with either cell line in a ratio of 2:1 before setting up the aforementioned *in vitro* model of human BBB. As anticipated, the presence of senescent OECs significantly perturbed the integrity and function of the BBB (Figure 32A, B). Similarly, the presence of senescent OECs in a largely non-senescent monolayer disrupted tight junction morphology, as evidenced by incomplete ZO-1 staining (Figure 32C). In line with these findings, interaction with senescent OECs also nullified the tubulogenic activity of HBMECs, as observed by the substantially diminished total number and length of tubes formed (Figure 33).

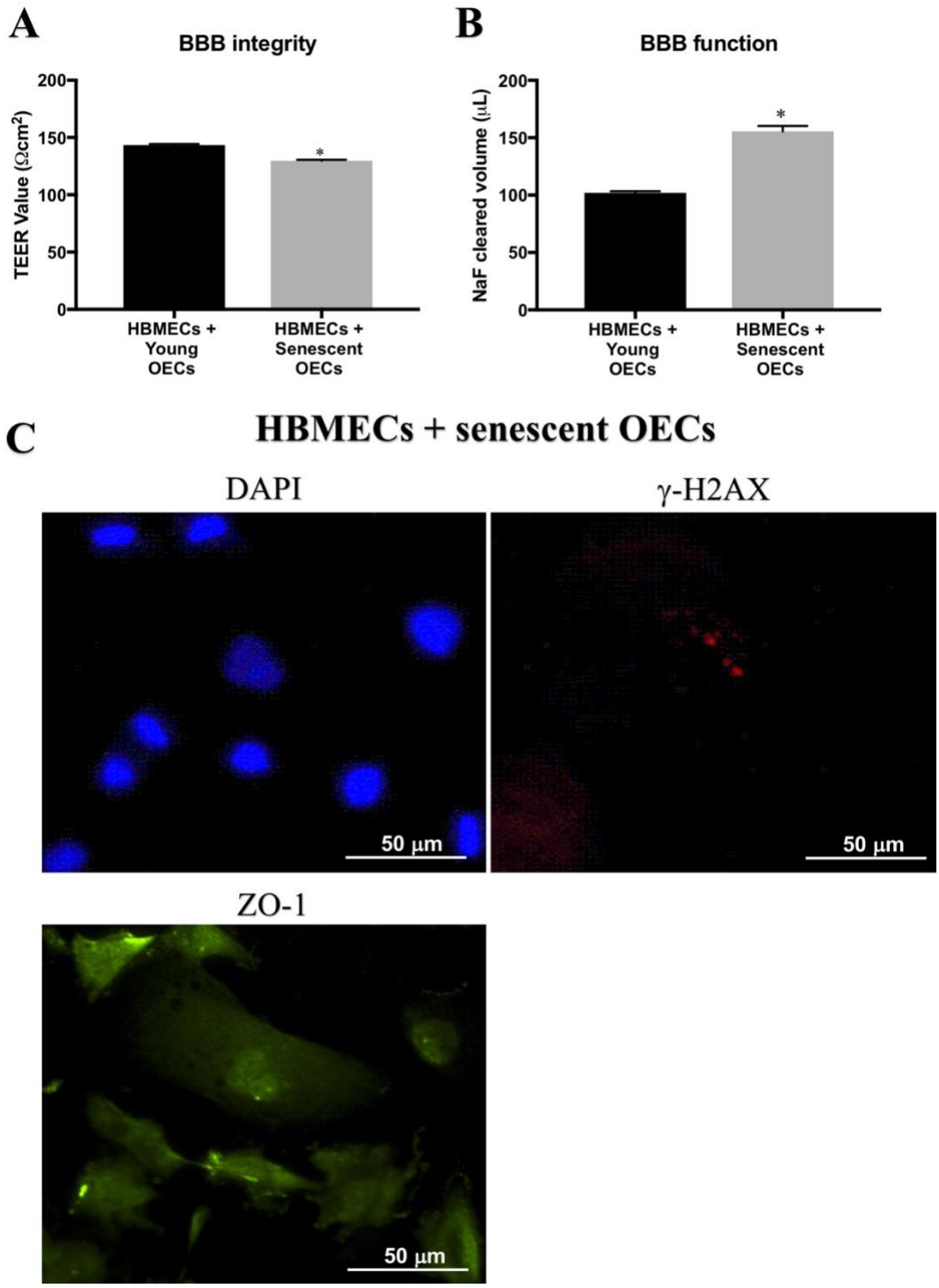


Figure 32. The presence of senescent OECs adversely affected endothelial monolayer and overall BBB integrity.

(A, B) Co-culture of young HBMECs and senescent OECs in a ratio of 2:1 in an *in vitro* model of human BBB decreased the barrier integrity and function, as evidenced by changes in TEER and paracellular flux of permeability marker, sodium fluorescein. **(C)** Senescent OECs impaired the distribution of ZO-1 in HBMECs. *P < 0.05 compared to HBMECs + young OECs. Scale bars: 50 µm. n = 3.

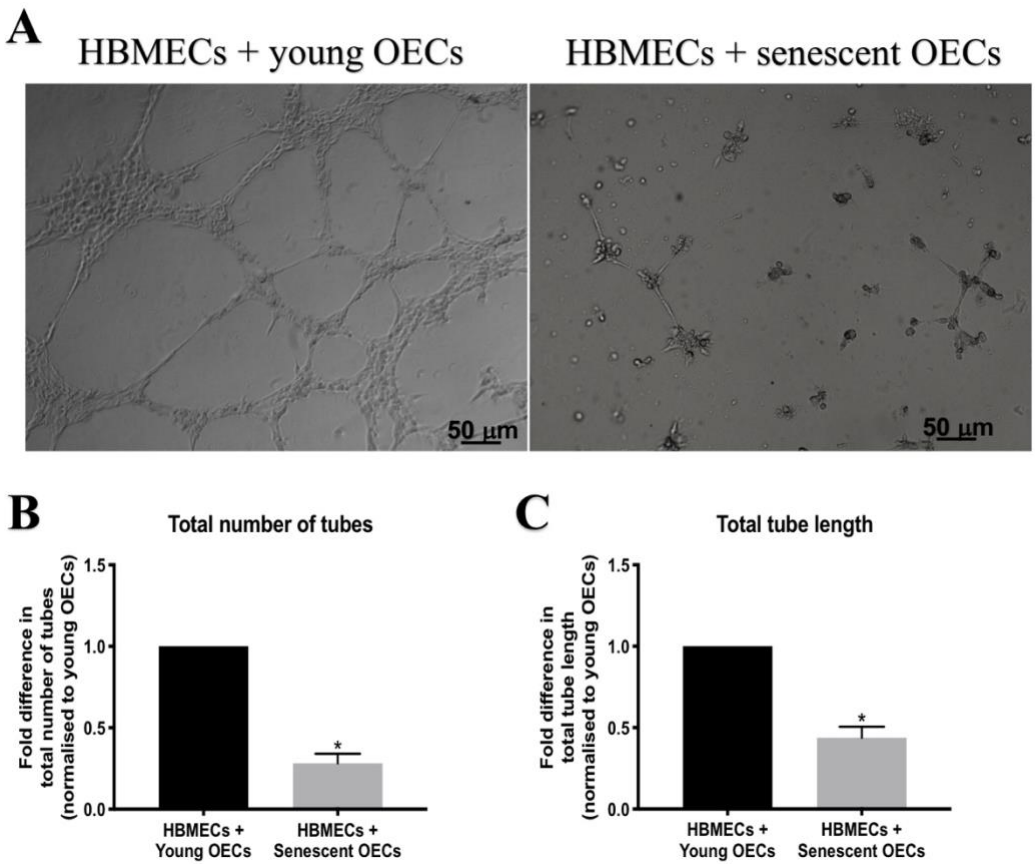


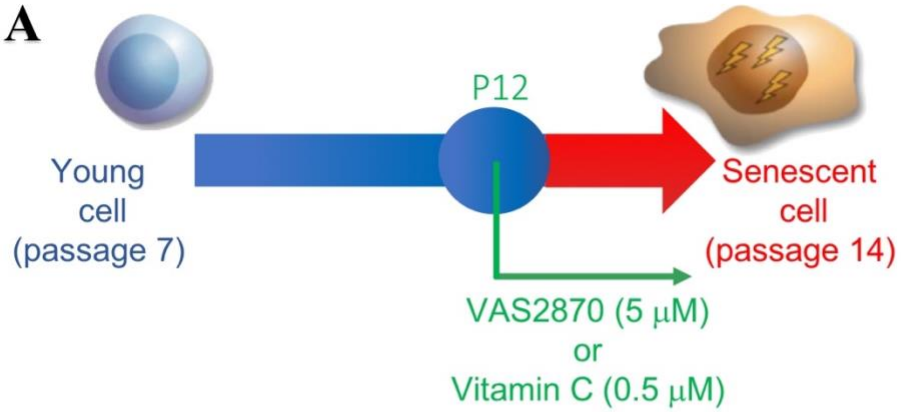
Figure 33. The presence of senescent OECs perturbed the tubulogenic activity of HBMECs.

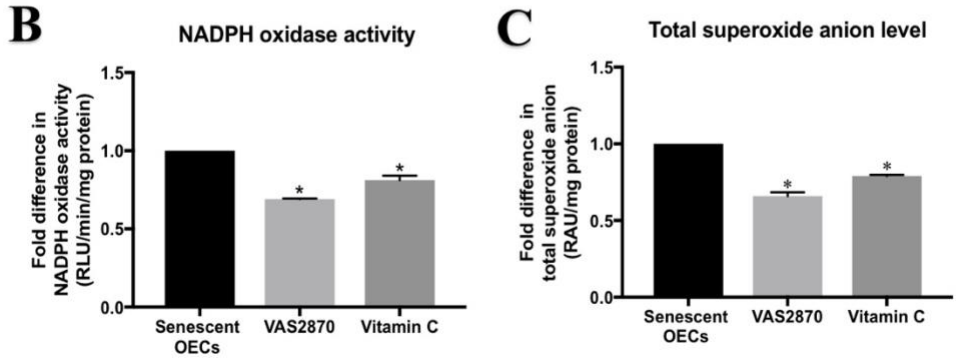
(A) Co-culture of HBMECs with senescent OECs, but not young OECs, disrupted appropriate tubule formation. **(B, C)** The quantification of total number and length of tubule networks in HBMEC and young or senescent OEC co-cultures. *P < 0.05 compared to HBMECs + young OECs. Scale bars: 50 µm. $n \geq 3$.

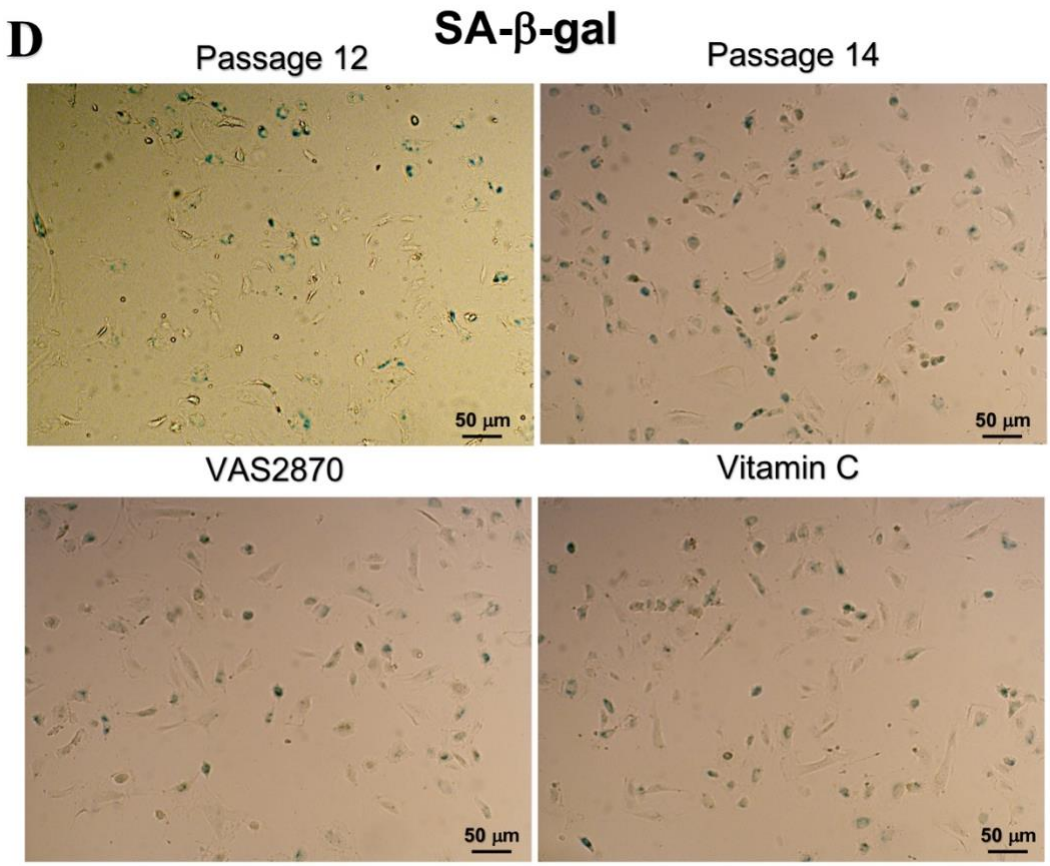
2.5. Suppression of oxidative stress modulates OEC senescence and potentiates their functional characteristics

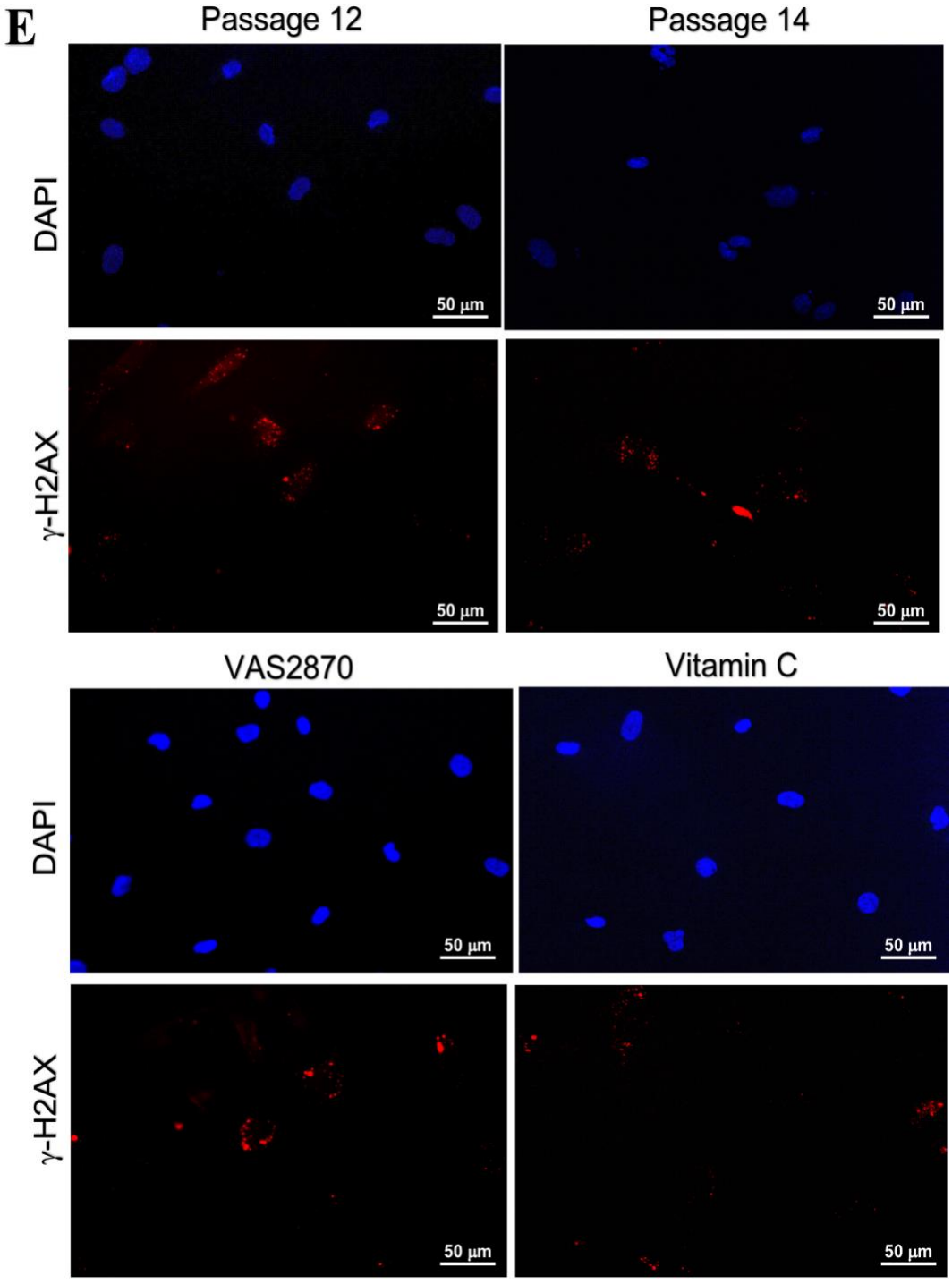
Having established that senescent OECs possess elevated NADPH oxidase activity and diminished total anti-oxidant capacity, we subsequently investigated whether effective control of oxidative stress might delay OEC senescence and consequently improve functionality. As the overwhelming majority of OECs at passage 14 displayed the aforementioned signs of replicative senescence, we treated relatively late-passage OECs (passage 12) with either vitamin C, a broad-spectrum anti-oxidant, or VAS2870, a specific inhibitor of NADPH oxidase, until passage 14 (Figure 34A). It was found that both treatment regimens significantly attenuated the level of NADPH oxidase activity and superoxide anion level, and reduced the number of OEC positively stained for SA-β-gal activity and the DNA damage marker γ-H2AX by approximately 20% (Figure 34B-G).

Furthermore, both regimens increased the cellular proliferation rate and tubulogenic activity, as ascertained by increased number and length of tubule networks (Figure 35A-D). Considering the elevations observed in endostatin levels in both senescent OECs and older individuals, and that previous studies attributed a crucial role to this anti-angiogenic factor in OEC functionality (Ai et al. 2020), we also investigated the level of endostatin in cells treated with VAS2870 or vitamin C. In line with tubulogenic study data, suppression of oxidative stress effectively prevented senescence-evoked increases in endostatin levels (Figure 35E). Finally, suppression of oxidative stress, irrespective of the mechanism targeted, led to better integrations between senescent OECs and HBMECs, and improved the tightness and functionality of the cerebral barrier, as evidenced by increases in TEER and decreases in the paracellular flux of NaF (Figure 35F, G).


A
P12
Young
cell
(passage 7)
Senescent
cell
(passage 14)
VAS2870 (5 μM)
or
Vitamin C (0.5 μM)


B
NADPH oxidase activity
Fold difference in NADPH oxidase activity (RLU/min/mg protein)
1.5
1.0
0.5
0.0
*
*
Senescent OECs
VAS2870
Vitamin C
C
Total superoxide anion level
Fold difference in total superoxide anion (RAU/mg protein)
1.5
1.0
0.5
0.0
*
*
Senescent OECs
VAS2870
Vitamin C


D
SA-β-gal
Passage 12
Passage 14
50 μm
50 μm
VAS2870
Vitamin C
50 μm
50 μm


E
Passage 12
Passage 14
DAPI
γ-H2AX
50 μm
VAS2870
Vitamin C

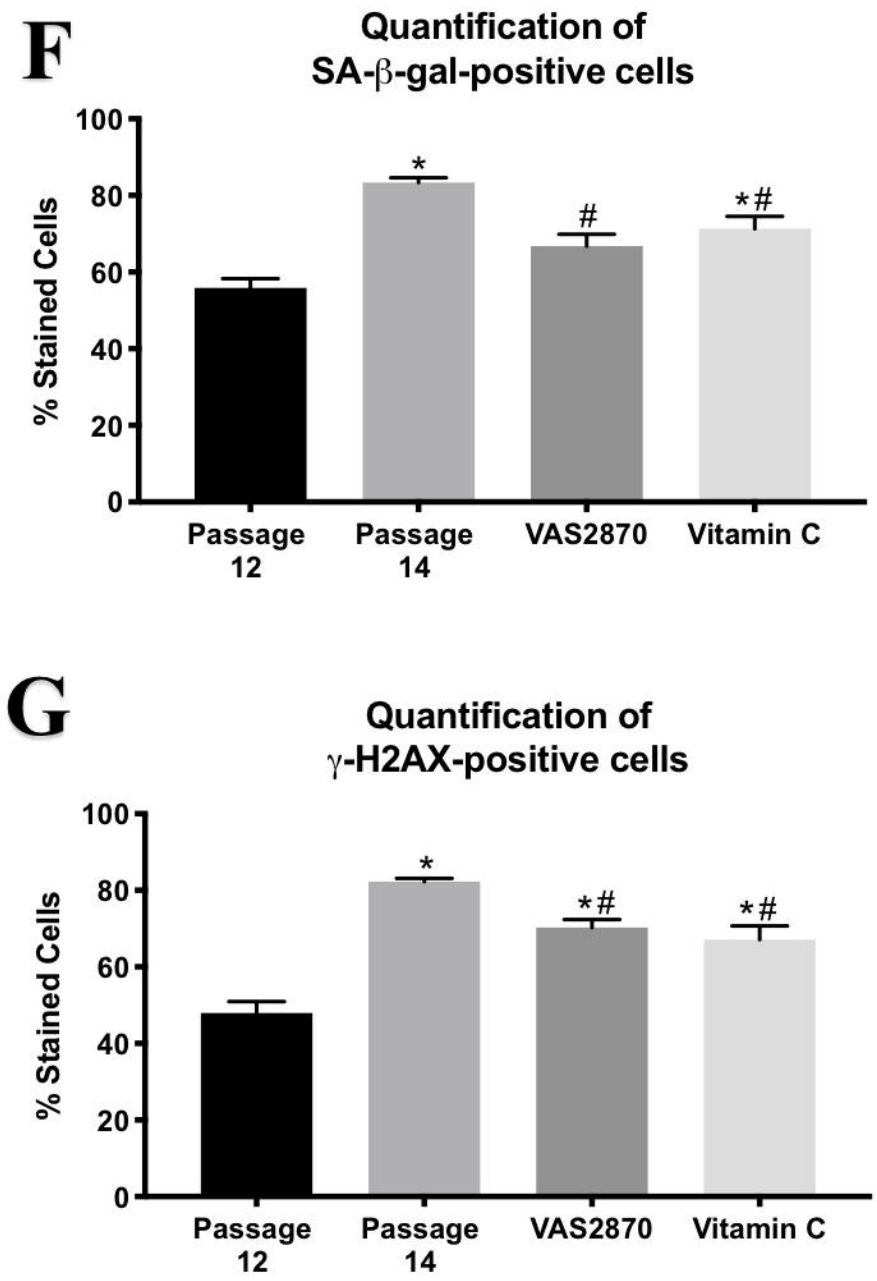
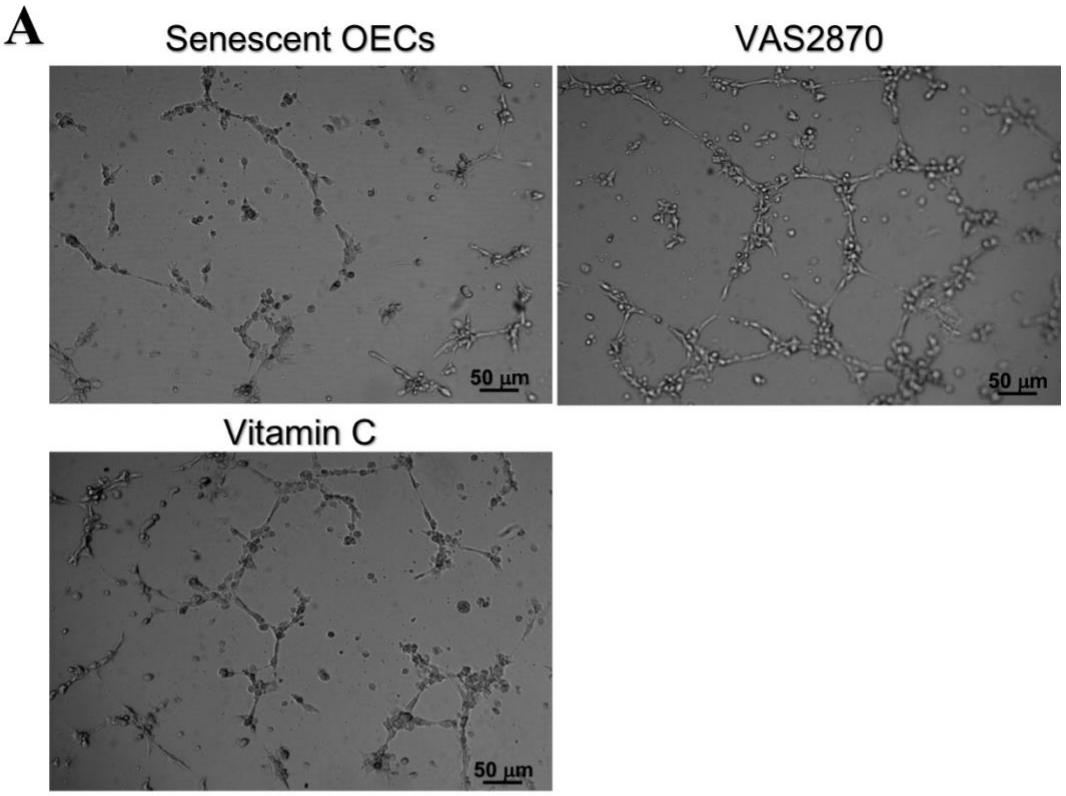
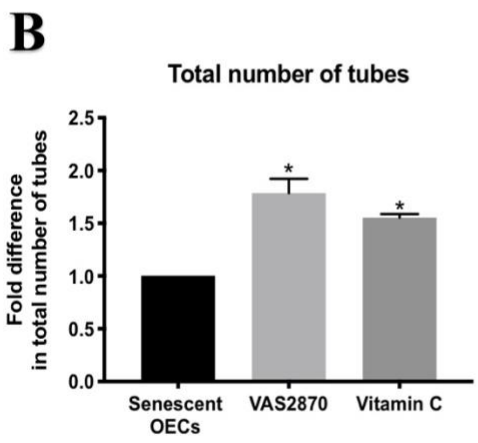
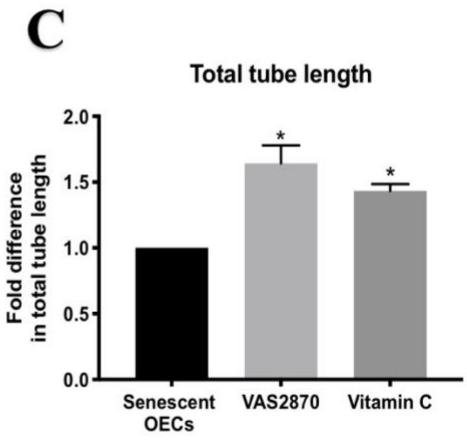


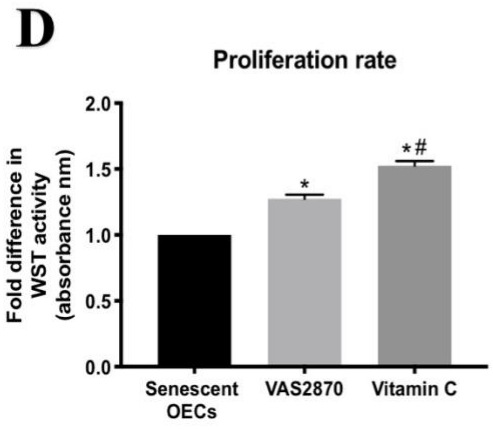
Figure 34. Suppression of oxidative stress delayed OEC senescence.

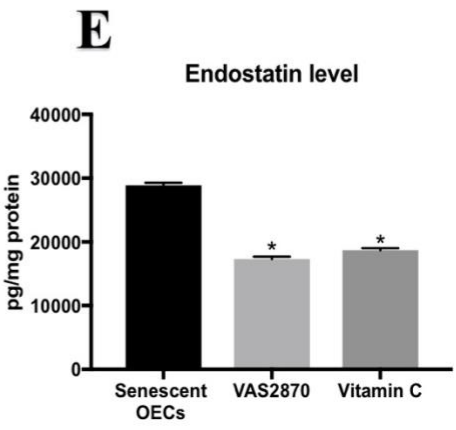
(A) Schematic diagram showing the treatment protocol with vitamin C or VAS2870, an inhibitor of NADPH oxidase. (**B, C)** The presence of VAS2870 or vitamin C inhibited NADPH oxidase activity and superoxide anion production. **(D, E)** Treatment with either agent decreased the number of SA-β-gal- and γ-H2AX-positive cells. **(F, G)** The quantification of cells stained by SA-β-gal and γ-H2AX. *P < 0.05 compared to passage 12, #P < 0.05 compared to passage 14. Scale bars: 50 μm. $n \geq 3$.


A
Senescent OECs
VAS2870
Vitamin C
50 µm
50 µm
50 µm


B
Total number of tubes
Fold difference in total number of tubes
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Senescent OECs
VAS2870
Vitamin C


C
Total tube length
Fold difference in total tube length
2.0
1.5
1.0
0.5
0.0
*
*
Senescent OECs
VAS2870
Vitamin C


D
Proliferation rate
Fold difference in WST activity (absorbance nm)
2.0
1.5
1.0
0.5
0.0
*
*#
Senescent OECs
VAS2870
Vitamin C


E
Endostatin level
pg/mg protein
40000
30000
20000
10000
0
*
*
Senescent OECs
VAS2870
Vitamin C

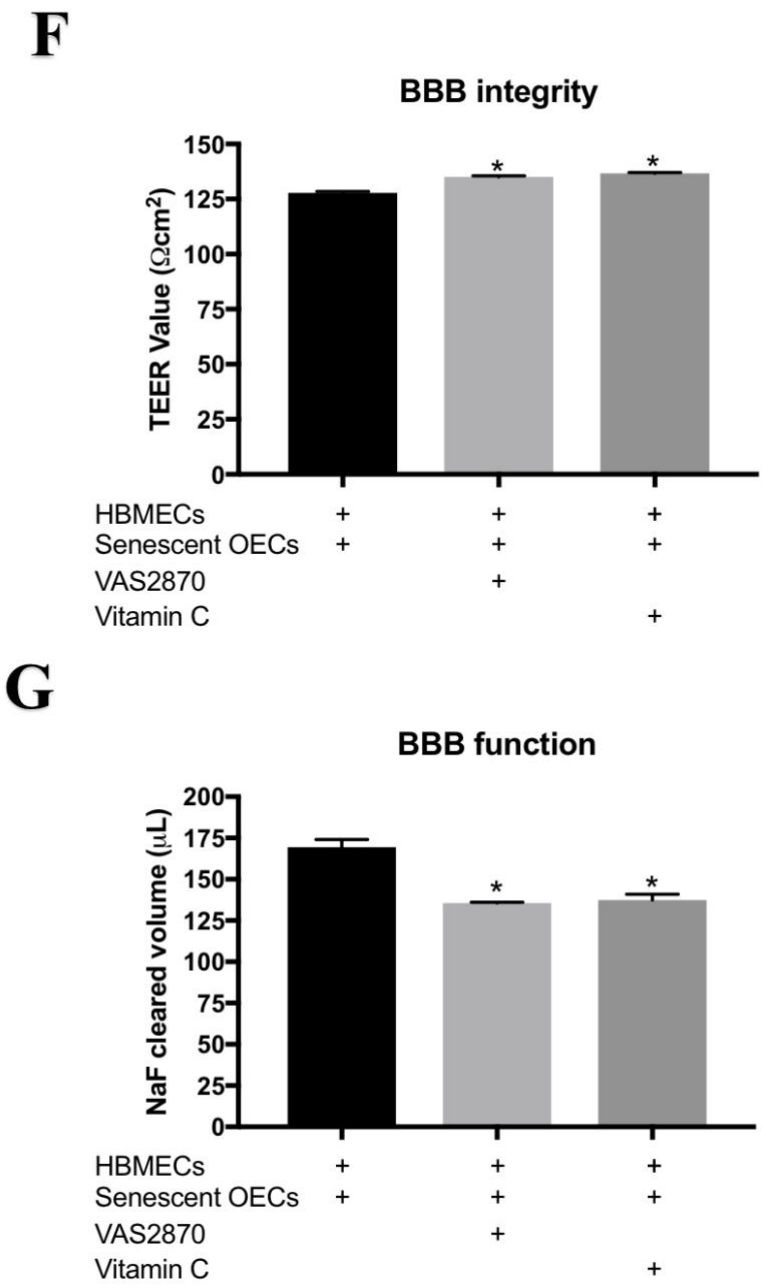


Figure 35. Inhibition of oxidative stress restored the critical functions of OECs.

(A-C) The presence of VAS2870 or vitamin C repaired the angiogenic properties of senescent OECs, as indicated by the higher number and total length of tubule networks. **(D, E)** Treatment with either compound also decreased the level of angiogenic inhibitor, endostatin, and increased the proliferation of senescent OECs. **(F, G)** Treatment with VAS2870 or vitamin C enhanced the integration between senescent OECs and HBMECs, leading to improvements in BBB tightness and functionality, as shown by increased TEER and decreased paracellular flux of sodium fluorescein. *P < 0.05 compared to senescent OECs, #P < 0.05 compared to VAS2870. Scale bars: 50 μm. $n \geq 3$.

3. Discussion

Alterations in vascular endothelial cell function play a critical role in the pathogenesis of various life-changing diseases, such as stroke, cerebral small vessel disease, and vascular dementia, and are commonly observed in otherwise healthy older individuals (Martinez-Majander et al. 2021; Toya et al. 2021). Given that the number of circulating EPCs has been positively correlated with endothelial repair capacity, vasculogenesis, and post-stroke recovery (Chang et al. 2007; Kukumberg et al. 2020), it is possible that diminished availability of EPCs may be involved in age-dependent endothelial cell dysfunction and associated vascular abnormalities.

This study found that the number of circulating endothelial-committed cells (i.e., cells expressing an endothelial cell marker, namely KDR alone or together with CD34 and/or CD133) did not significantly differ between younger and older healthy volunteers, despite observing consistent decreases in the latter group. It is noteworthy here that a similar pattern was also observed for circulating CD34+CD133+KDR+ cells, the particular cells considered as the true EPCs (Medina et al. 2017; Smadja et al. 2019).

A relevant previous study (Heiss et al. 2005) reporting similar results to ours attributed age-dependent endothelial cell dysfunction to diminished functional capacity, rather than numbers, of circulating endothelial-committed cells, implying the importance of functional assessments in age-related studies. The limited presence of EPCs in circulation (1 cell per ~20 mL of peripheral blood) does not permit the isolation of adequate EPC numbers for functional assays (Ingram et al. 2004), thus cell culture remains the only option to generate large numbers of homogenous cells to perform these assays.

In contrast to those findings, the number of circulating stem cells (CD34+, CD133+, and CD34+CD133+), which possess a unique capacity to differentiate into mature endothelial cells (Yeh et al. 2003; Suuronen et al. 2006) and contributes to overall vascular health (Shimizu et al. 2020; Jiang et al. 2021), showed substantial decreases in otherwise healthy older compared to younger individuals. These findings may suggest the reduction of endogenous reparative system performed by stem cells occurs in ageing individuals and add further weight to those of the Framingham Heart Study, a large-scale prospective cohort study involving 1786 participants (Cohen et al. 2013). A chronic pro-inflammatory status, in the absence of an overt infection, represents one of the pervasive features of ageing. Amongst the possible causes of age-related chronic low-grade inflammation, cell senescence attracts a great deal of attention, as it persists even after the initial stimuli (e.g., LDL-cholesterol and smoking) are removed (Arnson et al. 2010; Sanada et al. 2018). Increased circulating levels of TNF-α may be a key factor that shift the balance towards a pro-inflammatory state in older people (Davizon-Castillo et al. 2019), leading eventually to OEC premature senescence and reduced regenerative potential (Zhang et al. 2009).

Age-related decline in the expression of pro-angiogenic chemokines and growth factors may also be involved in EPC/OEC dysfunctionality (Zhang et al. 2019; Kapoor et al. 2021). Despite increases in SDF-1, slight but consistent decreases observed in VEGF, PDGF-BB, and G-CSF plasma levels are likely to contribute to the impaired EPC mobilisation, proliferation, and migration in older people, as they were correlated with perturbed EPC trafficking to the sites of ischaemia in aged tissue (Chang et al. 2007). Specific increases observed in plasma levels of endostatin and thrombospondin-1 in older populations are also likely to impair EPC mobilisation and clonogenic potential. Besides, through inhibition of resident and circulating

endothelial cell proliferation, these anti-angiogenic factors create fertile conditions to facilitate senescence (Capillo et al. 2003; Meijles et al. 2017).

The oxidative stress theory of ageing postulates that age-related decline in physiological functions is triggered by a slow steady accumulation of oxidative damage to macromolecules, notably DNA, which increases with age (Lin and Flint Beal 2003). At the cellular level, increased ROS leads to senescence. While OECs express substantially greater total anti-oxidant capacity compared to mature endothelial cells (Cai et al. 2006; He et al. 2009), prolonged exposure to low level oxidative stress reduces EPC numbers and seriously damages their capacity to self-renew, proliferate, and differentiate, thereby evoking premature senescence (Höhn et al. 2017; Wang et al. 2017).

As shown in the Part I, OECs are considered as the functional subtype of EPC due to their remarkable migratory, proliferative, and tubulogenic potential as well as their unique ability to differentiate into mature endothelial cells. These cells counteract endothelial cell dysfunction and help maintain vascular homeostasis in both physiological and pathological settings (Bayraktutan 2019). However, such functional capacity of OECs to restore endothelial integrity may attenuate with age which inevitably increases the risk for vascular disease in ageing individuals (Xia et al. 2012; Kaur et al. 2018).

In this regard, to further scrutinises the impact of senescence on OEC morphological, molecular, and functional characteristics, we then exposed OECs to repetitive culture, an *in vitro* model of chronological ageing (Phipps et al. 2007). It is acknowledged that such a procedure may not accurately represent ageing in organisms. However, observations that the propagative lifespan of skin fibroblasts in cell culture declines in parallel with donor age attribute a critical role for replication-mediated cellular

senescence in ageing and suggest that the limited proliferative capacity of cells may indeed be an appropriate system to scrutinise human ageing (Cristofalo et al. 2003). Of course, caution needs to be exercised when translating *in vitro* findings to the entire organism.

Morphological changes accompanying replicative senescence in cultured OECs included a flatter phenotype and marked increases in cell size, numbers of cytoplasmic microfilaments, and nuclear size. Increases in the intracellular content of RNA and proteins, resulting from diminished protein degradation and RNA turnover, may in part explain the cellular and nuclear enlargements. The blocking of senescent cells in late G1 cell cycle phase may also contribute to these enlargements, whereby the exhaustion of DNA replication cycles and telomere shortening due to repetitive cell division play important roles by activating persistent DNA damage response (DDR) and transducer protein kinases. These in turn activate transcription factor p53 and cyclin-dependent kinase inhibitor p21 to block the cell cycle at G1 – S interphase (Kim et al. 2017).

In addition to phenotypic changes, senescent OECs also presented other typical signs of senescence and possessed remarkably higher levels of SA-β-gal activity, permanent DNA damage associated with increased deposition of γ-H2AX, and oxidative stress. The latter was characterised by elevations in NADPH oxidase activity and superoxide anion production, and reductions in NOx availability and total anti-oxidant capacity. Taken together, with the decreased total anti-oxidant capacity in older versus younger individuals, these data confirm the seminal role of oxidative stress in both cellular senescence and chronological ageing.

Superoxide anion is known as the foundation molecule for all ROS. By scavenging NO, the most important endogenous vasodilator and anti-oxidant, and generating a

more harmful ROS called peroxynitrite in the process, superoxide anion severely affects telomere stability. The low redox potential of guanine nucleobase in telomeres renders them very susceptible to oxidative damage, and provokes the formation of 8-oxoguanine, which in turn exacerbates telomere shortening by reducing the protein expression of telomere repeat-binding factor 1/2 and as a result promotes DDR (Coluzzi et al. 2019). Hence, the neutralisation of superoxide anion has long been regarded as a key therapeutic target in the modulation of senescence (van der Loo et al. 2000; Haendeler et al. 2004).

Indeed, suppression of NADPH oxidase activity and superoxide anion in relatively late-passage OECs (passage 12) with VAS2870 or vitamin C, in the present study, led to marked reductions in the number of cells stained positive for γ-H2AX and SA-β-gal at passage 14, where most untreated cells appeared to be senescent. The decreases in senescence markers coincided with improvement in proliferative and tubulogenic capacity. While the current study did not scrutinise the molecular mechanism underlies this beneficial effect, recent studies have shown that specific regulation of SA-β-gal or ROS level reduced the number of senescent cells, promotes the regenerative potential of stem cells, alleviated local and systemic inflammation, and restored physical function of aged mice (Liao et al. 2019; Cai et al. 2020). Since cellular senescence is characterised by the irreversible growth arrest and treatment with either VAS2870 or vitamin C in the current study was administrated prior to senescence state, these compounds may specifically targeted and prevented non-senescent cells to become senescent cells. Moreover, findings from this thesis also support the previous data, showing that modulation of redox state with vitamin C or an NADPH oxidase inhibitor (DPI or apocynin) can attenuate the release of ROS, and

delay cell cycle arrest in various stem cells, such as mesenchymal stem cells and neural progenitor cells (Li et al. 2016; Maraldi et al. 2021).

The formation and/or restoration of the BBB to prevent vascular leakage and cerebral oedema remains one of the most critical functions of OECs *in vivo* (Bayraktutan 2019). Significant decreases observed in the integrity and function of an *in vitro* model of human BBB, established with human astrocytes, pericytes, and senescent OECs, indicate that senescence dramatically compromises the regenerative function of OECs, and may adversely affect the function of neighbouring cells. It is widely recognised that senescent cells may act as a major stress contributor to the surrounding environment. Unlike apoptotic cells, which are rapidly removed from a given tissue by tissue-resident or neighbouring phagocytes, senescent cells remain metabolically active for a prolonged period of time, and continue to accumulate (Nelson et al. 2012; da Silva et al. 2019).

As alluded to above, the present study demonstrates that the presence of senescent OECs drives their microenvironment, formed in large part by resident microvascular endothelial cells, into a senescent state, and impairs the overall barrier integrity and function. This is evidenced by decreases in TEER and increases in inter-endothelial cell flux, due to interrupted staining of tight junction protein ZO-1 and perturbed tight junctional formation. Contrary to relevant studies which combined senescent cells in 1:1 ratio with endothelial cells (Nelson et al. 2012; Nelson et al. 2018), in the current study, senescent cells were mixed with HBMECs in a ratio of 1:2. Induction of a similar degree of HBMEC dysfunctionality with comparatively lower quantities of senescent OECs highlights the highly disruptive nature of senescent cells in a cerebral barrier model.

Adoption of senescence-associated secretory phenotype (SASP) coupled with exaggerated release of IL-1β, IL-6, and TNF-α, may be one of the mechanisms whereby senescent cells affect adjacent cells and surrounding tissues (da Silva et al. 2019). Alternatively, senescent cells may influence neighbouring cells through a cell contact mechanism mediated by NOTCH/JAG1 signalling pathway (Hoare et al. 2016), or via direct intercellular protein transfer, which occurs through the formation of cytoplasmic bridge (Biran et al. 2015). Accumulating evidence show that ROS can influence both the release of SASP from senescent cells and activate cell contact mechanism (Ho et al. 2011; Nelson et al. 2018). The improvements observed in the integrity and function of BBB formed with a mixture of senescent OECs and HBMECs, and subjected to VAS2870 or vitamin C, verify the importance of regulating redox status, to negate the deleterious effects of senescent cells on vasculature.

Another mechanism by which OECs contribute to vascular homeostasis is their contribution to angiogenesis, a process in which new blood vessels develop from existing ones, to help deliver oxygen and nutrients to tissues (Medina et al. 2010). Advancing age adversely affects the function of a series of components, including OECs and the aforementioned growth factors and angiogenic factors involved in neovascular processes, thereby exacerbating the severity of vascular abnormalities (Chang et al. 2007; Xia et al. 2012). The current study demonstrates the inability of senescent OECs to form tubes in *in vitro* settings. It also shows that inhibition of oxidative stress with VAS2870 or vitamin C significantly improves the capacity of senescent OECs to proliferate and establish tubular networks. Amongst all the angiogenic factors studied in younger and older individuals, the level of endostatin appeared to fluctuate the most, suggesting a prominent role for this factor in the

initiation and progression of age-related vascular problems (Ärnlöv et al. 2013). In line with the tubulogenesis studies discussed above, this study found that the suppression of oxidative stress in senescent OECs significantly attenuates endostatin levels, and confirmed the modulatory role of oxidative stress signalling pathway in angiogenesis

4. Conclusion

The current study demonstrates that OEC senescence constitutes a key phenomenon in age-related vascular dysfunction. Targeting oxidative stress using vitamin C or VAS2870 effectively delays OEC senescence, while potentiating their angiogenic properties and vascular barrier-forming capacity to help restore and maintain vascular homeostasis. The graphical summary of this study is shown in Figure 36.

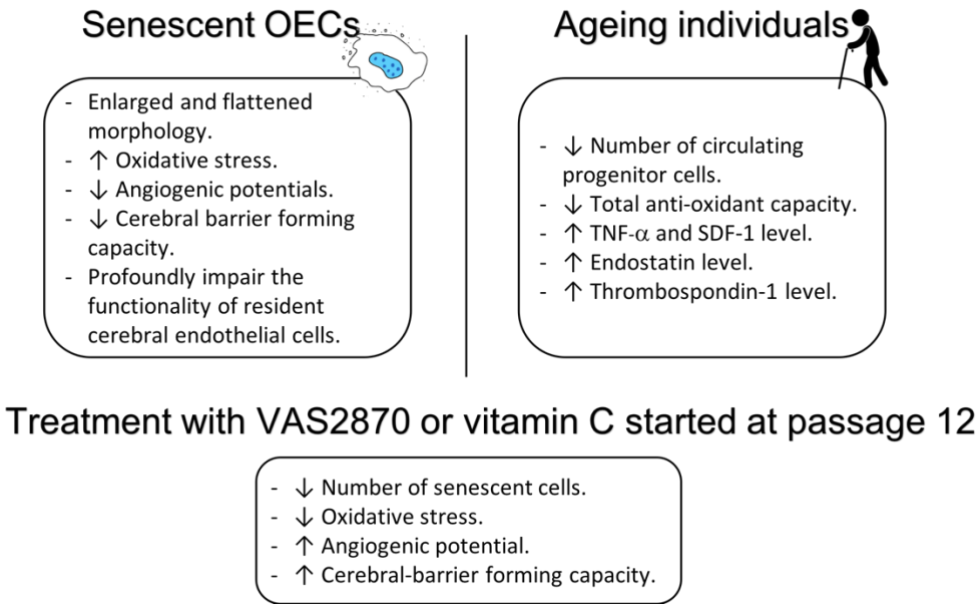


Figure 36. Graphical summary.

Part III

OEC-derived conditioned medium negates TNF-α-evoked blood-brain barrier damage: a reverse translational study to explore mechanisms

Abstract

The identification of effective and powerful new stroke therapies requires an improved understanding of the key mechanisms underlying cerebral ischaemic injury. In this regard, through in-depth analysis of plasma samples obtained from a large number of healthy volunteers and ischaemic stroke patients collected for The Dunhill Medical Trust EPC study, the present study observed significant elevations in the levels of TNF-α during acute, subacute, and chronic phases of stroke. It subsequently probed the impact of this inflammatory cytokine on an *in vitro* model of human blood-brain barrier (BBB) and found dramatic impairments in both barrier integrity and function. In similar experiments, the presence of outgrowth endothelial cell-derived conditioned media (OEC-CM), generated by exposure of OECs to hypoxic injury, negated the detrimental effects of TNF-α on BBB. Effective inhibition of the anti-angiogenic factor endostatin, oxidative stress, actin stress fibre organisation, and apoptosis of brain microvascular endothelium and OECs played a crucial role in OEC-CM-mediated benefits. OEC-CM-mediated benefits are also realised due to concomitant improvements in the extracellular matrix adhesive (a pre-requisite for angiogenesis) and tubule network properties of both cells. In conclusion, this reverse translational study identifies TNF-α as a significant mediator of post-ischaemic cerebral barrier damage, and corroborates the position of OEC-CM as an emerging vasculoprotective therapeutic strategy by demonstrating its ability to regulate a wide range of mechanisms associated with BBB integrity and function.

1. Introduction

The failure of thousands of clinical trials assessing the efficacy of hundreds of intended therapeutics in stroke medicine necessitated the adaption of dynamic and innovative strategies to better understand the mechanisms involved in the pathogenesis and outcome of stroke (Schmidt-Pogoda et al. 2020). Reverse translational study, working from bedside to bench, is considered to be of enormous help to identify the key causative factors of the disease (Tian et al. 2022) and inform the design of future therapeutic strategies (Bix et al. 2018; Lourbopoulos et al. 2021). In this context, through detailed analysis of plasma levels of inflammatory cytokines and angiogenic factors in ischaemic stroke patients and healthy volunteers (HVs) recruited for The Dunhill Medical Trust EPC study (DMT EPC study), the current study has identified inflammatory cytokine tumour necrosis factor-α (TNF-α) as an important factor for the initiation and progression of ischaemic stroke, by showing significant elevations in its level during all disease phases (acute, subacute, and chronic).

Again, since the majority of mortalities and disabilities within the early stage of an ischaemic stroke are attributed to the cerebral oedema stemming from blood-brain barrier (BBB) damages (Dénes et al. 2011; Arba et al. 2021; Yao et al. 2021), restoration of normal BBB function represents a crucial therapeutic priority (Rakkar and Bayraktutan 2016; Bayraktutan 2019). As explored in the Part I, outgrowth endothelial cells (OECs), the functional subtypes of endothelial progenitor cells (EPCs), may be such therapeutics. They are capable of releasing a wide range of trophic factors and differentiating into mature endothelial cells to preserve BBB integrity and vascular homeostasis (Moubarik et al. 2011; Abdulkadir et al. 2020). While stem cell therapies require the generation of large-scale *ex vivo* expansion to

generate sufficient cell numbers, such expansion is consistently limited by the exhaustion of proliferative potential and associated with replicative senescence (Medina et al. 2013). This phenomenon (senescence-related cellular dysfunction) coupled with the risk of emboli or tumour formation and immunological reaction indeed have profoundly hindered the utilisation of these particular stem cells in clinical practice (Boltze et al. 2015).

Increasing experimental data attribute the post-ischaemic cerebrovascular reparative function of OECs to a wide range of released active compounds (e.g., HGF, IL-8, angiogenin, thrombopoietin, PDGF-BB, and VEGF), implying that OEC-derived conditioned medium (OEC-CM) may serve as an alternative therapeutic strategy (Di Santo et al. 2014; Alwjwaj et al. 2021). In addition to eradicating most of the side effects associated with cell-based therapies, this therapeutic approach also offers additional advantages such as ease of production, handling, and storage (Harrell et al. 2019; Alwjwaj et al. 2021).

In light of the above, by using well-established *in vitro* models of human BBB, this reverse translational study aims to investigate whether OEC-CM can protect BBB from the detrimental effects of TNF-α, and attempts to ascertain the mechanisms underlying its restorative effects.

2. Results

2.1. Patients with ischaemic stroke display significant alteration in the generation of inflammatory and angiogenic modulators

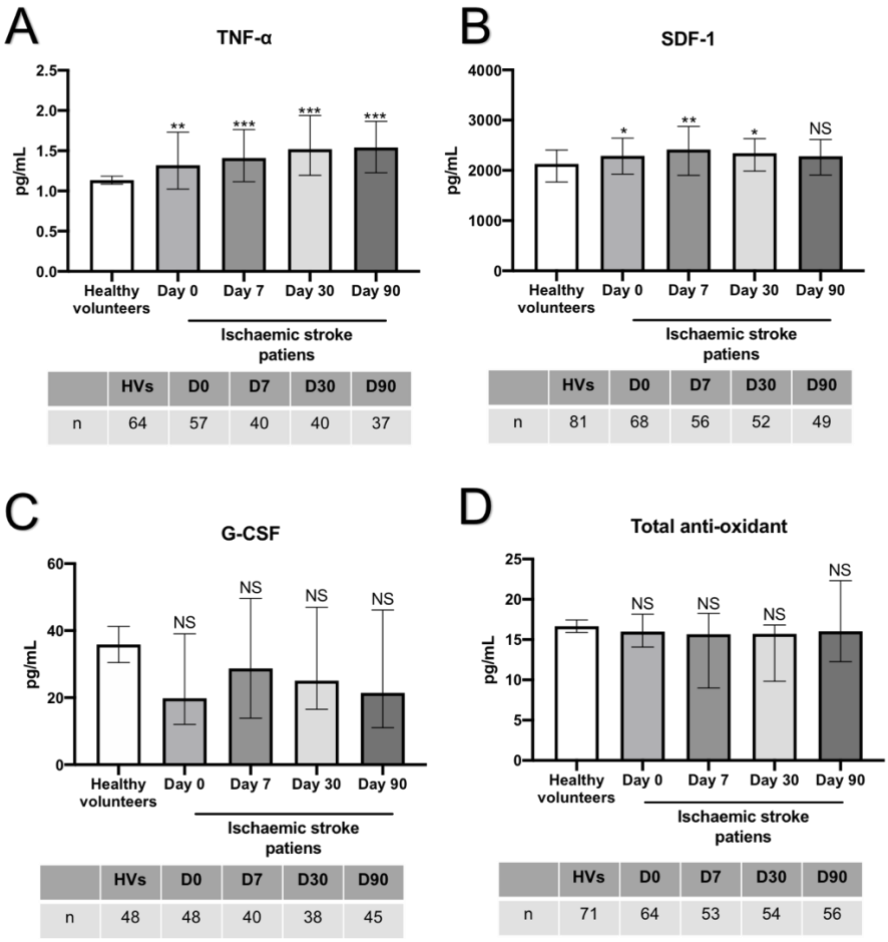
Compared to HVs, patients with ischaemic stroke have substantially higher levels of TNF-α, which remained elevated at all time points covered in this study (Figure 37A). Patients also exhibited elevations in the level of SDF-1, the chemokine that mediates

EPC trafficking and homing to the injury site, which peaked on day 7 and then steadily decreased until normalisation at day 90 (Figure 37B). In contrast, HVs and patients had no differences in plasma levels of cytokine G-CSF, which is known to mobilise circulating EPCs (Figure 37C).

Accumulating of evidence show that oxidative stress, emerging from an imbalance between endogenous pro-oxidants and anti-oxidants, profoundly affects the pathogenesis and outcome of ischaemic stroke (Chen et al. 2020; Griendling et al. 2021). However, analyses of the plasma total anti-oxidant capacity, which represents the accumulation of enzyme systems (GSH reductase, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, etc.), and proteins (albumin, transferrin, etc.), showed slight but insignificant decreases in ischaemic stroke patients compared with HVs (Figure 37D).

Effective neovascularisation in the infarction area, triggered by the release of pro-angiogenic factors in the injured tissue, is essential to improve cerebral blood flow, neuronal repair and regeneration, and functional recovery (Hatakeyama et al. 2020). In this regard, the current study comprehensively assessed the level of angiogenic modulators, which showed gradual decreases in plasma levels of major pro-angiogenic factors, VEGF and PDGF-BB, and reached significance compared to HVs on day 90 after stroke on day 90 after stroke (Figure 38A, B). Interestingly, plasma levels of anti-angiogenic factors thrombospondin-1 and thrombospondin-2 also significantly declined following ischaemic stroke, specifically at days 7, 30, and 90 for thrombospondin-1; and at days 0, 7, and 90 for thrombospondin-2 (Figure 38C, D). There were slight but insignificant decreases in plasma level of anti-angiogenic factor angiostatin in patients with ischaemic stroke at all time points compared to HVs (Figure 38E), while the level of endostatin was substantially elevated during the acute

and chronic phases of ischaemic stroke (Figure 38F). It is noteworthy here that the variety in *n* number between time points or parameters due to the collection of inappropriate blood sample (the small volume of blood sample acquired from the participants and haemolysis that occurred during measurement) or some participants discharged from the study.



	HVs	D0	D7	D30	D90
n	64	57	40	40	37

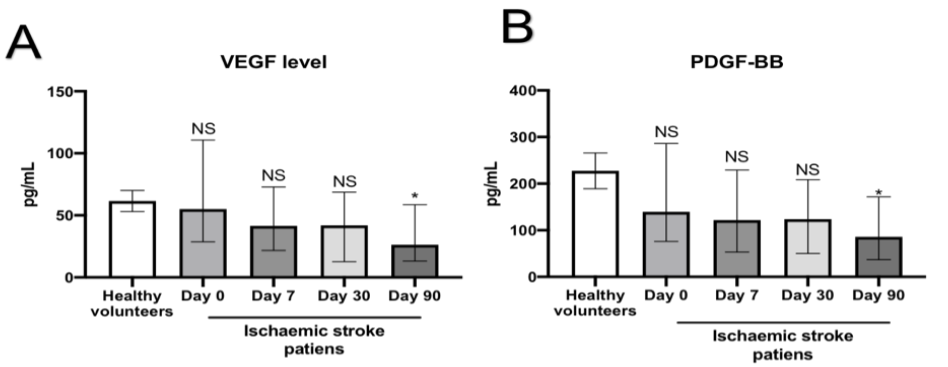
	HVs	D0	D7	D30	D90
n	81	68	56	52	49

	HVs	D0	D7	D30	D90
n	48	48	40	38	45

	HVs	D0	D7	D30	D90
n	71	64	53	54	56

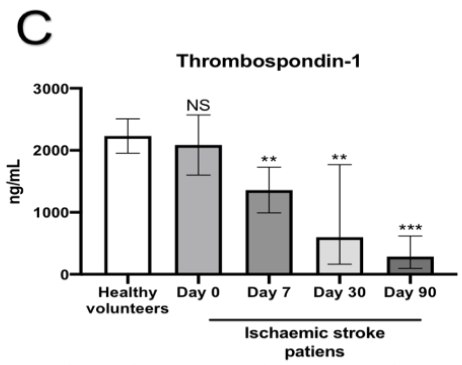
Figure 37. The serial changes of inflammatory mediators and total anti-oxidant capacity of ischaemic stroke patients compared to HVs.

(A) Pro-inflammatory cytokine TNF-α consistently elevated in all phases of ischaemic stroke injury. **(B, C)** Other than at day 90, the level of chemoattractant SDF-1 substantially increased following cerebral ischaemic injury, while G-CSF levels remained flat in all time points studied. **(D)** Total anti-oxidant capacity of patients with ischaemic stroke appeared flattened at all time points during the study. *P < 0.05, ** P < 0.01, *** P < 0.001 versus HVs. NS: not significant compared to HVs.

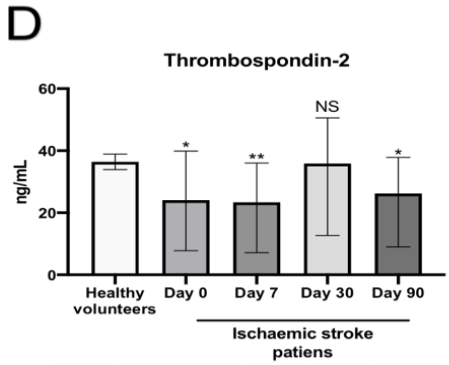


	HVs	D0	D7	D30	D90
n	80	73	52	54	52

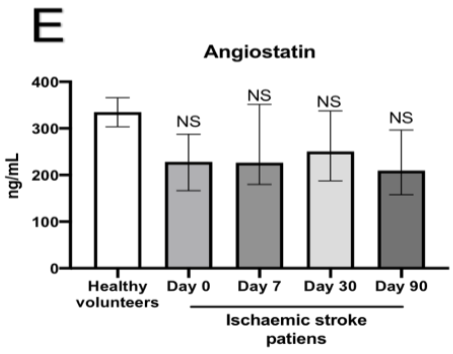
	HVs	D0	D7	D30	D90
n	59	47	40	43	49



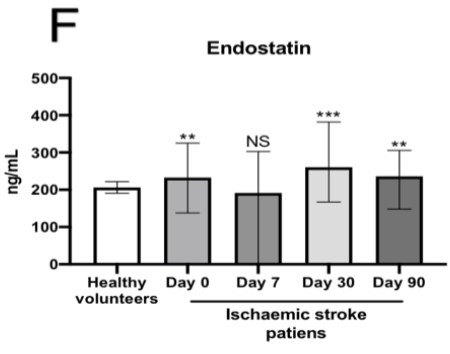
	HVs	D0	D7	D30	D90
n	61	37	31	35	31



	HVs	D0	D7	D30	D90
n	65	41	39	38	42



	HVs	D0	D7	D30	D90
n	72	64	49	47	46



	HVs	D0	D7	D30	D90
n	90	81	63	57	61

Figure 38. The serial changes of angiogenic modulators of ischaemic stroke patients compared to HVs.

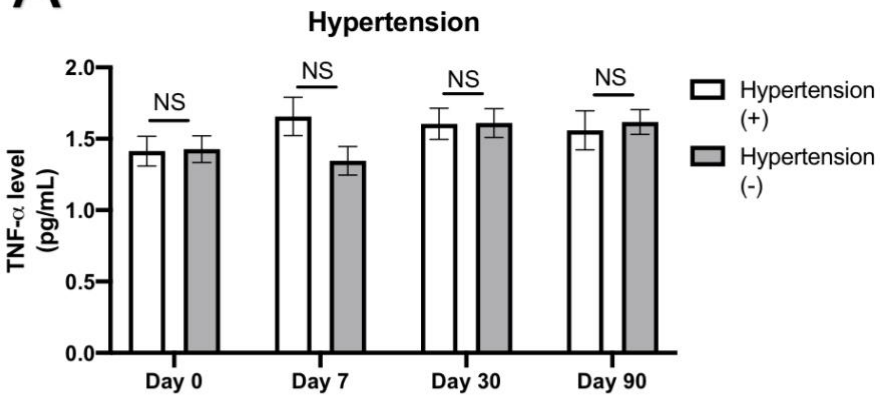
(A, B) Gradual decreases observed in pro-angiogenic factors VEGF and PDGF-BB, reaching significance at day 90 following ischaemic stroke. **(C-F)** Ischaemic stroke differentially regulated the levels of angiogenic suppressors with constantly increased levels of endostatin after ischaemic injury, and decreased levels of thrombospondin-1 and thrombospondin-2. No change was observed in angiostatin levels. *P < 0.05, ** P < 0.01, *** P < 0.001 versus HVs. NS: not significant compared to HVs.

2.2. Level of TNF-α increases in healthy volunteers, but not stroke patients, with vascular risk factors

All major vascular risk factors trigger the pathology of endothelial cell dysfunction, and consequently predispose vasculature to a heightened pro-inflammatory state and oxidative stress, both of which are critical determinants of disease severity and outcome in ischaemic stroke patients (Hou et al. 2021; Shaafi et al. 2021). Since patients with ischaemic stroke possess at least one comorbidity (Gallacher et al. 2019), it was anticipated that patients with hypertension, diabetes mellitus (DM), atrial fibrillation (AF), hyperlipidaemia, and transient ischaemic attack (TIA) might display greater levels of TNF-α. Detection of equal TNF-α levels in patients with and without risk factors, other than those with TIA at baseline and with DM on day 7, refuted this hypothesis and proposed cerebral ischaemic injury as the main generator of TNF-α in stroke patients (Figure 39A-E).

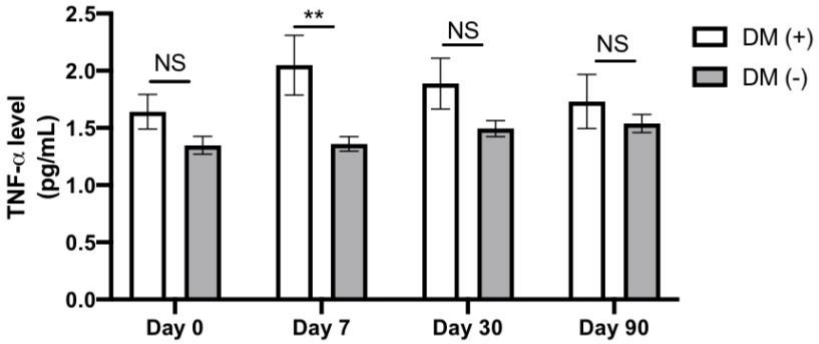
On the other hand, the observation of higher TNF-α concentrations in HVs with DM, hypertension, and hyperlipidaemia proved these risk factors as the major promoters of TNF-α in the absence of an overt ischaemic injury. It is of note that similar levels of TNF-α observed in HVs with and without AF could be an artefact of the limited number of participants with AF included in the study (Figure 39F-I); since HVs with previous history of TIA were excluded from the study, it was not possible to assess the correlation in this subgroup.

A



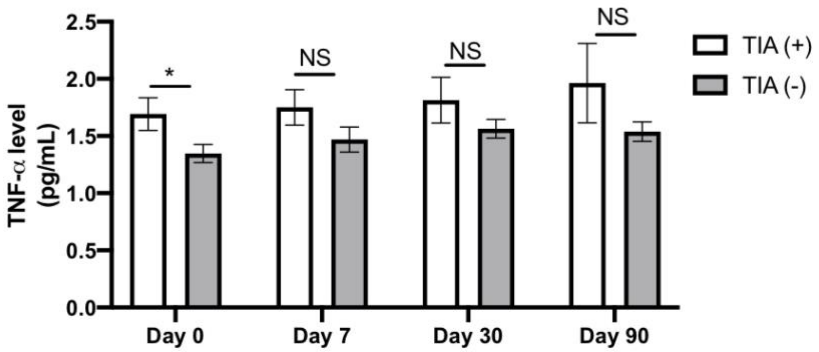
	D0	D7	D30	D90
(+)	33	24	23	21
(-)	24	16	17	16

B

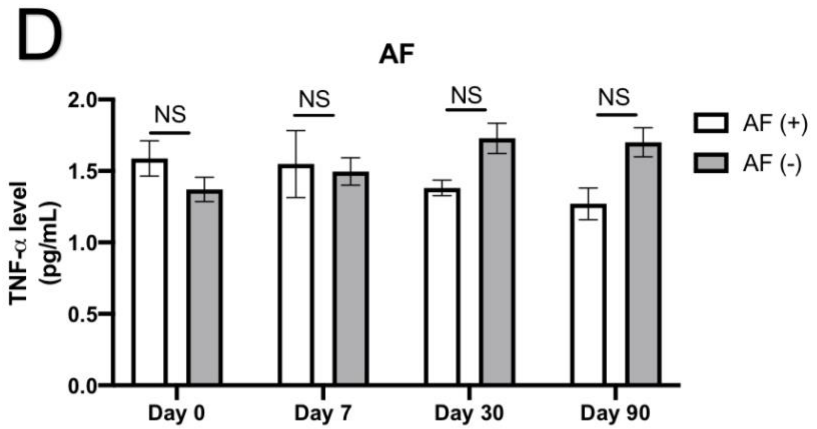


	D0	D7	D30	D90
(+)	14	10	9	9
(-)	43	30	28	33

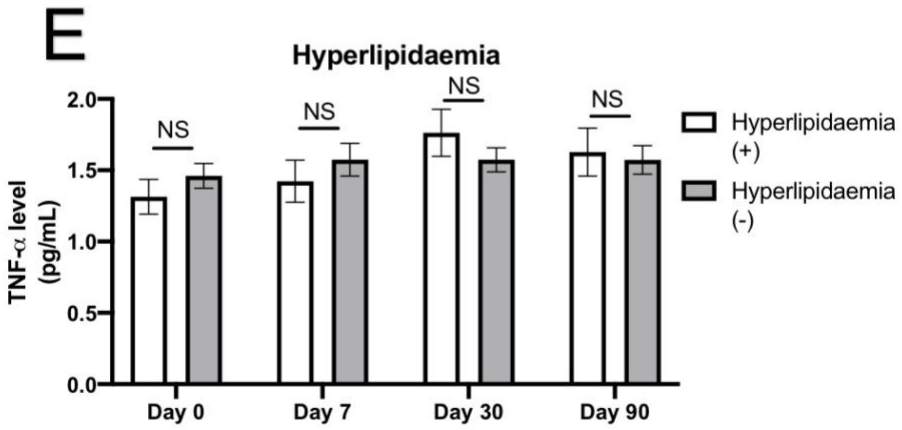
C



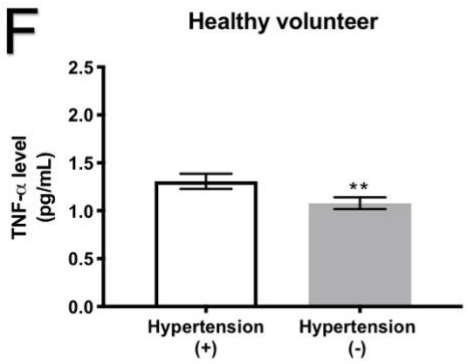
	D0	D7	D30	D90
(+)	12	9	7	4
(-)	45	31	33	33



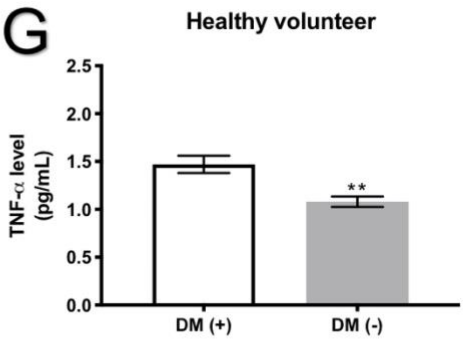
	D0	D7	D30	D90
(+)	16	11	14	10
(-)	40	30	26	27



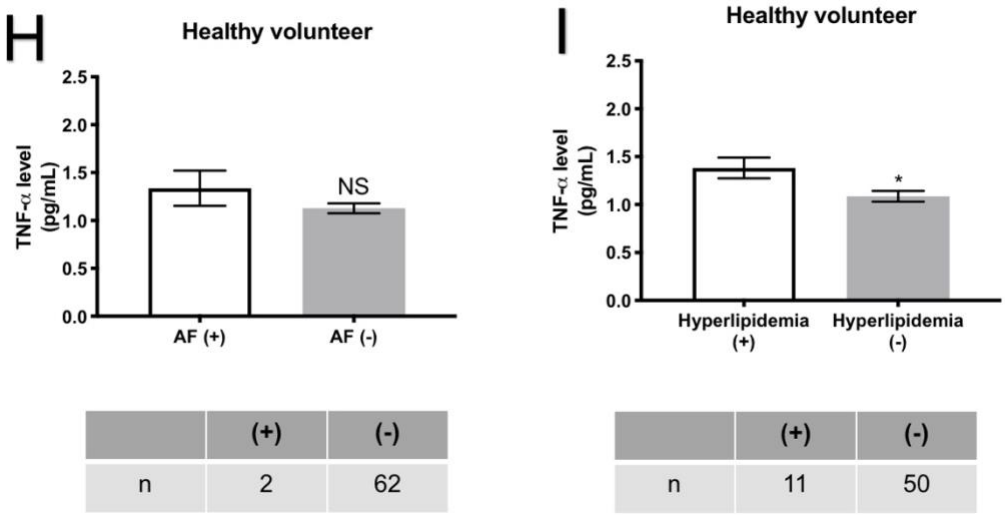
	D0	D7	D30	D90
(+)	16	11	7	8
(-)	41	29	33	29



	(+)	(-)
n	19	46



	(+)	(-)
n	9	54



	(+)	(-)
n	2	62

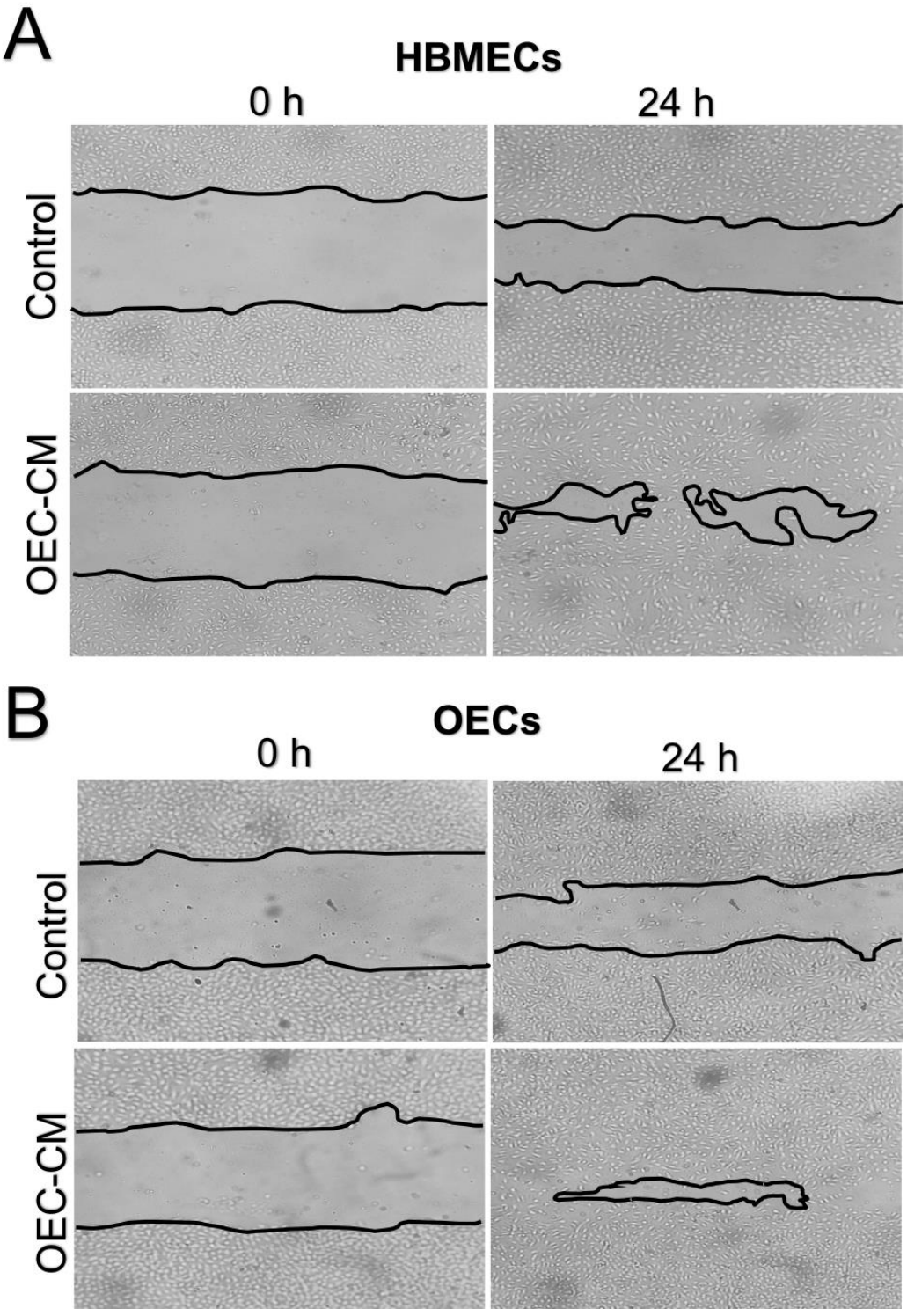
	(+)	(-)
n	11	50

Figure 39. The biochemical profile of participants with various conditions associated with endothelial dysfunction, including hypertension, DM, TIA, AF, and hyperlipidaemia.

(A-E) Analysis of the TNF-α level in ischaemic stroke patients with the abovementioned conditions revealed that this inflammatory mediator in similar level compared to those without these in all time points, except patients with TIA at day 0 and DM at day 7, who exhibited significantly declined TNF-α level. **(F-I)** To dispel the effect of cerebral ischaemic injury and to probe the factual effect of these vascular dysfunction-associated diseases, the subsequent study analysed the association between TNF-α and the presence of these conditions specifically in HVs. In this subgroup analysis, the concentration of TNF-α was significantly higher in participants with hypertension, DM, and dyslipidaemia history. *P < 0.05, **P < 0.01, *** P < 0.001 versus history (-). NS: not significant compared to without history.

2.3. OEC-CM potentiates the regenerative potential of HBMECs and OECs

Prior to assessing the protective impact of OEC-CM against TNF-α, we initially probed its impact on the regenerative potential of resident endothelial cells (i.e., HBMECs) and OECs using wound scratch assay, which found significantly accelerated wound closure speed in both cell types within 24 hours after scratch (Figure 40A-C). In line with these findings, treatment with OEC-CM also elevated the proliferation rates of both cells at 24 of exposure, as observed by marked increases in optical density, reflecting the number of metabolically active cells, compared to the cells treated by control medium (Figure 40D).


A
HBMECs
0 h
24 h
Control
OEC-CM
B
OECs
0 h
24 h
Control
OEC-CM

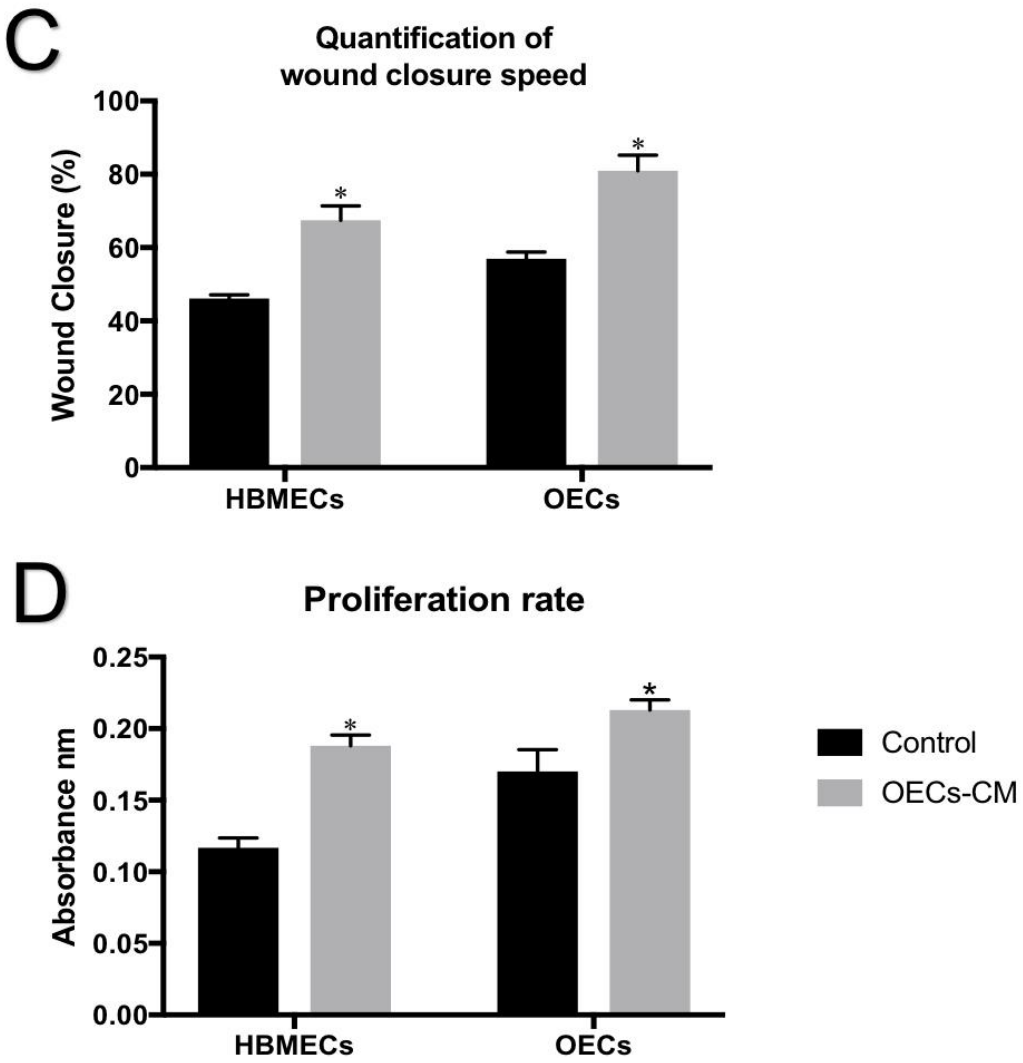


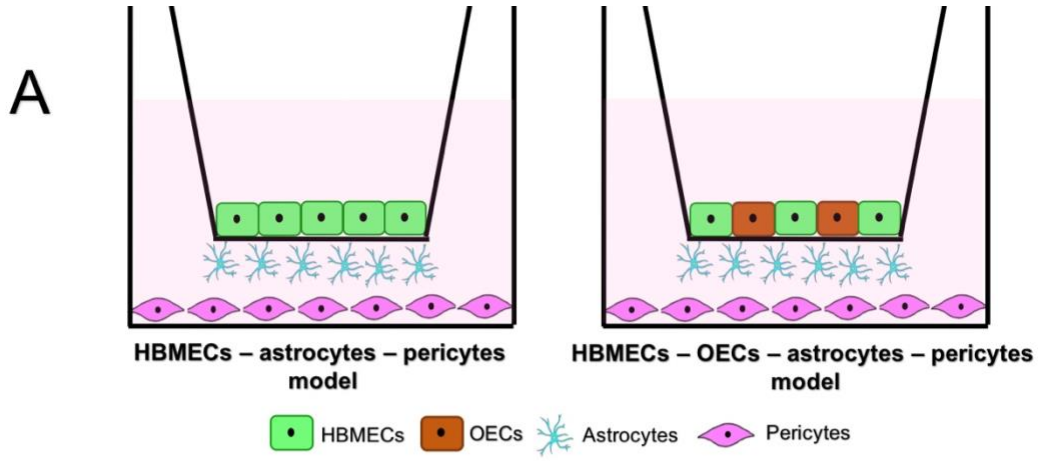
Figure 40. OEC-CM augmented the regenerative potential of HBMECs and OECs.

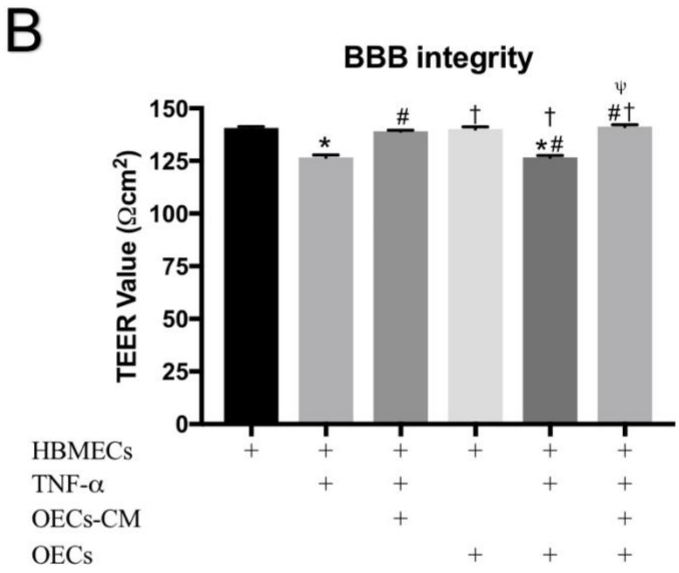
(A, B) Representative images showing the increases of wound closure speed in both cells treated with OEC-CM. **(C)** Graph showing the wound healing speed across the experimental groups. **(D)** WST-1 assay showed that the proliferative capacity of both cells was markedly enhanced in the presence of OEC-CM. * $P < 0.05$ versus control. $n \geq 4$.

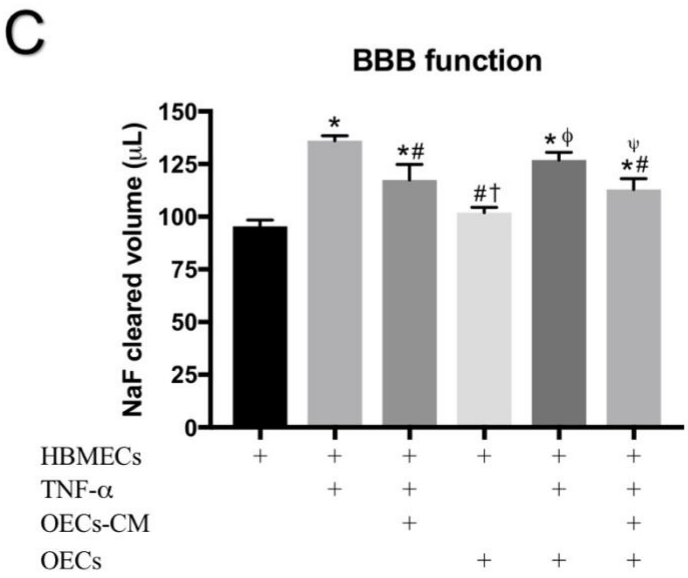
2.4. OEC-CM protects BBB integrity and function against TNF-α injury

The remarkable increases in TNF-α levels in ischaemic stroke patients and in HVs with cardiovascular risk factors strongly support its crucial involvement in the initiation and/or progression of cerebral ischaemic injury and endothelial dysfunction. As cerebral oedema following BBB damages represents the main cause of early death after an ischaemic stroke, and thrombolysis with rtPA remains the only pharmacotherapy for ischaemic stroke (Kleindorfer et al. 2021), this study assessed the therapeutic potential of an emerging strategy, employing hypoxic-primed OEC-CM, in mitigating the previously reported barrier-disruptive effects of TNF-α. For this, two triple cell co-culture models of human BBB, consisting of astrocytes, pericytes, and HBMECs alone or mixed with OECs (Figure 41A), were exposed to TNF-α (10 ng/ml) for 6 hours with and without OEC-CM. It is noteworthy here that the BBB model consisted of an HBMEC–OEC mixture developed to closely mimic the physiological structure of brain vasculature. Reversal of TNF-α-induced decreases in TEER readings and increases in paracellular flux of sodium fluorescein evinced the protective effect of OEC-CM on structural and functional properties of BBB, respectively (Figure 41B, C).

Since the cytoskeleton establishes and preserves cellular architecture, and plays a crucial role in the maintenance of BBB integrity and function (Abdullah and Bayraktutan 2014; Abdullah and Bayraktutan 2016), we further probed the effect of OEC-CM on the organisation of actin filaments of HBMECs and OECs during TNF-α injury. Treatment with TNF-α showed the appearance of thick actin stress fibres traversing both HBMECs and OECs, which were effectively prevented by co-administration of OEC-CM (Figure 41D).


A
HBMECs – astrocytes – pericytes model
HBMECs – OECs – astrocytes – pericytes model
HBMECs
OECs
Astrocytes
Pericytes


B
BBB integrity
TEER Value (Ωcm²)
150
125
100
75
50
25
0
HBMECs
TNF-α
OECs-CM
OECs


C
BBB function
NaF cleared volume (μL)
150
125
100
75
50
25
0
HBMECs
TNF-α
OECs-CM
OECs

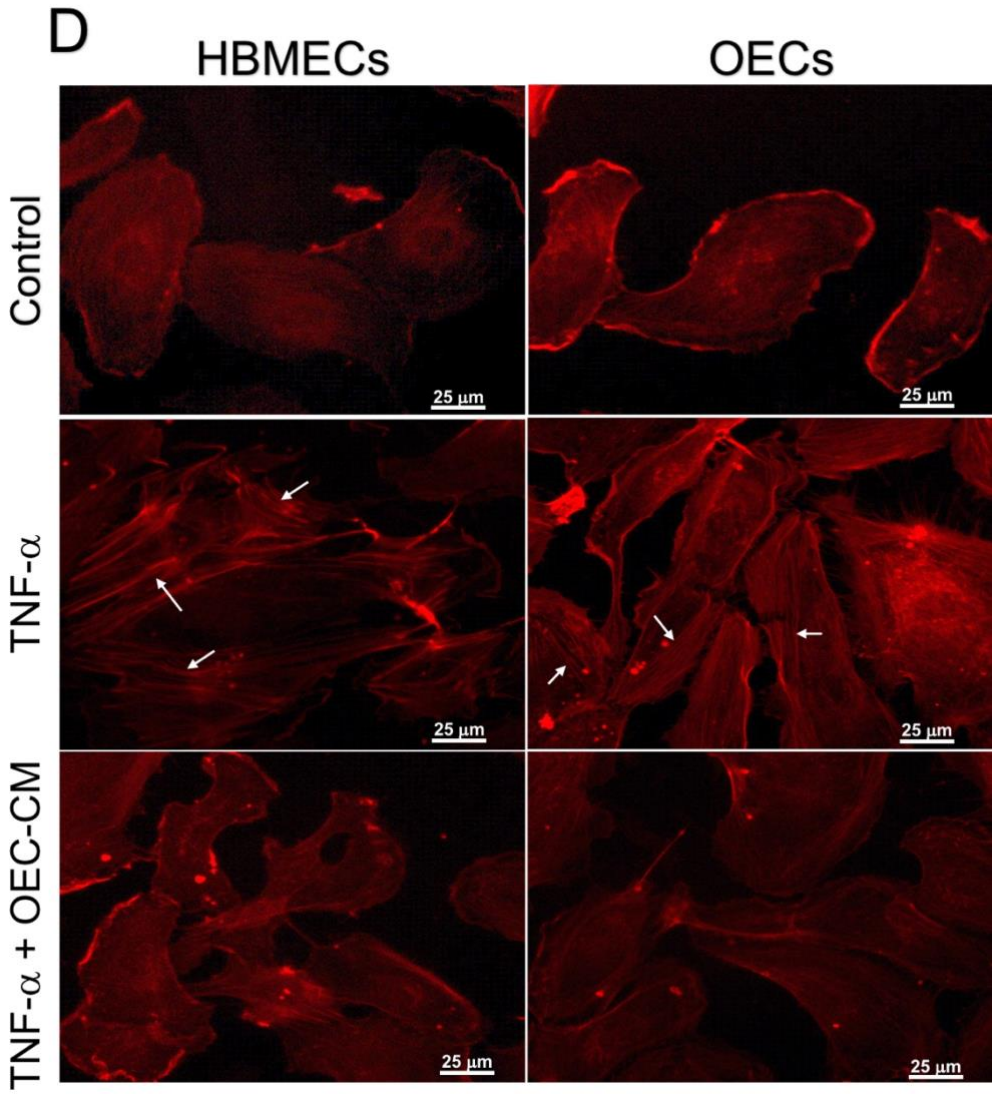


Figure 41. Schematic diagram of an *in vitro* model of human BBB and the protective effect of OEC-CM on BBB integrity and function and actin cytoskeleton organisation of HBMECs and OECs.

(A) An *in vitro* model of human BBB consisting of astrocytes, pericytes, and HBMECs, alone or mixed with OECs. **(B, C)** TNF-α substantially impaired BBB integrity and function, as observed by decreases in TEER and concomitant increases in sodium fluorescein flux volumes, respectively, which were prevented by OEC-CM treatment. **(D)** Treatment with OEC-CM repaired cytoskeleton organisation in both cells, as evidenced by the decreased of stress fibre formation (white arrows). *P < 0.05 versus BBB formed by HBMECs, #P < 0.05 versus BBB formed by HBMECs exposed to TNF-α, †P < 0.05 versus BBB formed by HBMECs exposed to TNF-α and OEC-CM, φP < 0.05 versus BBB formed by HBMECs and OECs, ψP < 0.05 versus BBB formed by HBMECs and OECs exposed to TNF-α. Scale bar: 25 µm. $n \geq 4$.

2.5. OEC-CM attenuates TNF-α-evoked oxidative stress

Redox status, defined by the perfect balance between pro- and anti-oxidant enzymes, represents one of the key regulators of the BBB function (Chen et al. 2020; Griendling et al. 2021). Since there was no difference in total anti-oxidant capacity observed between HVs and patients with ischaemic stroke, the subsequent experiments specifically assessed the effect of TNF-α with and without OEC-CM on the most prominent pro-oxidant enzyme, NADPH oxidase, and superoxide anion, the foundation molecule of all reactive oxygen species (ROS). Again, treatment with OEC-CM effectively negated the effects of TNF-α on oxidase activity and superoxide anion production in both cells (Figure 42).

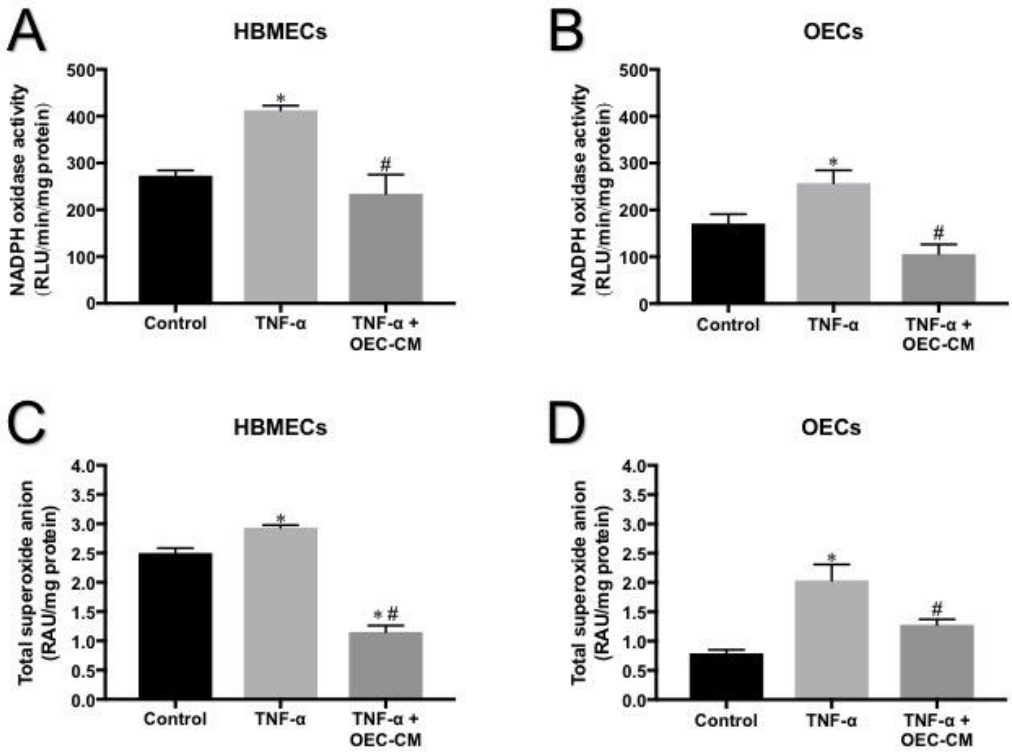


Figure 42. OEC-CM suppressed the level of oxidative stress.

(A-D) TNF-α enhanced NADPH oxidase activity and superoxide anion level in both HBMEC and OECs, which were markedly reduced by treatments with an OEC-CM. *P < 0.05 versus control, #P < 0.05 versus TNF-α. $n \geq 3$.

2.6. OEC-CM reduces apoptosis and maintains cell viability

TNF-α released from endothelial cells after a cerebral ischaemic injury may also exacerbate BBB breakdown, by inducing apoptotic process in resident endothelial cells and circulating progenitor cells tasked with the repair of endothelial damage (Du et al. 2014; Abdullah et al. 2015). In this regard, we also assessed the effect of OEC-CM on several parameters associated with apoptosis, including pro-apoptosis enzyme caspase 3/7 and cell viability during an inflammatory episode. Exposure to TNF-α substantially increased caspase-3/7 activity, while co-treatment of cells with OEC-CM markedly suppressed this parameter (Figure 43A, B). Treatments with OEC-CM also negated the effects of TNF-α on HBMEC and OEC survivability, as shown by the increased relative luminescence intensity, indicating the higher percentage of viable cells (Figure 43C, D).

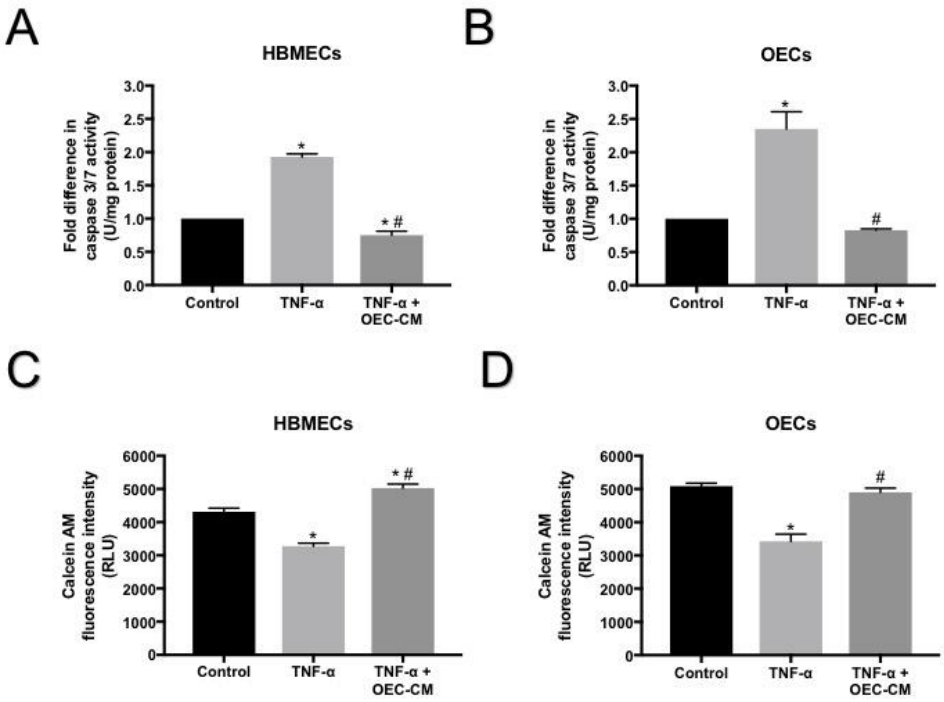


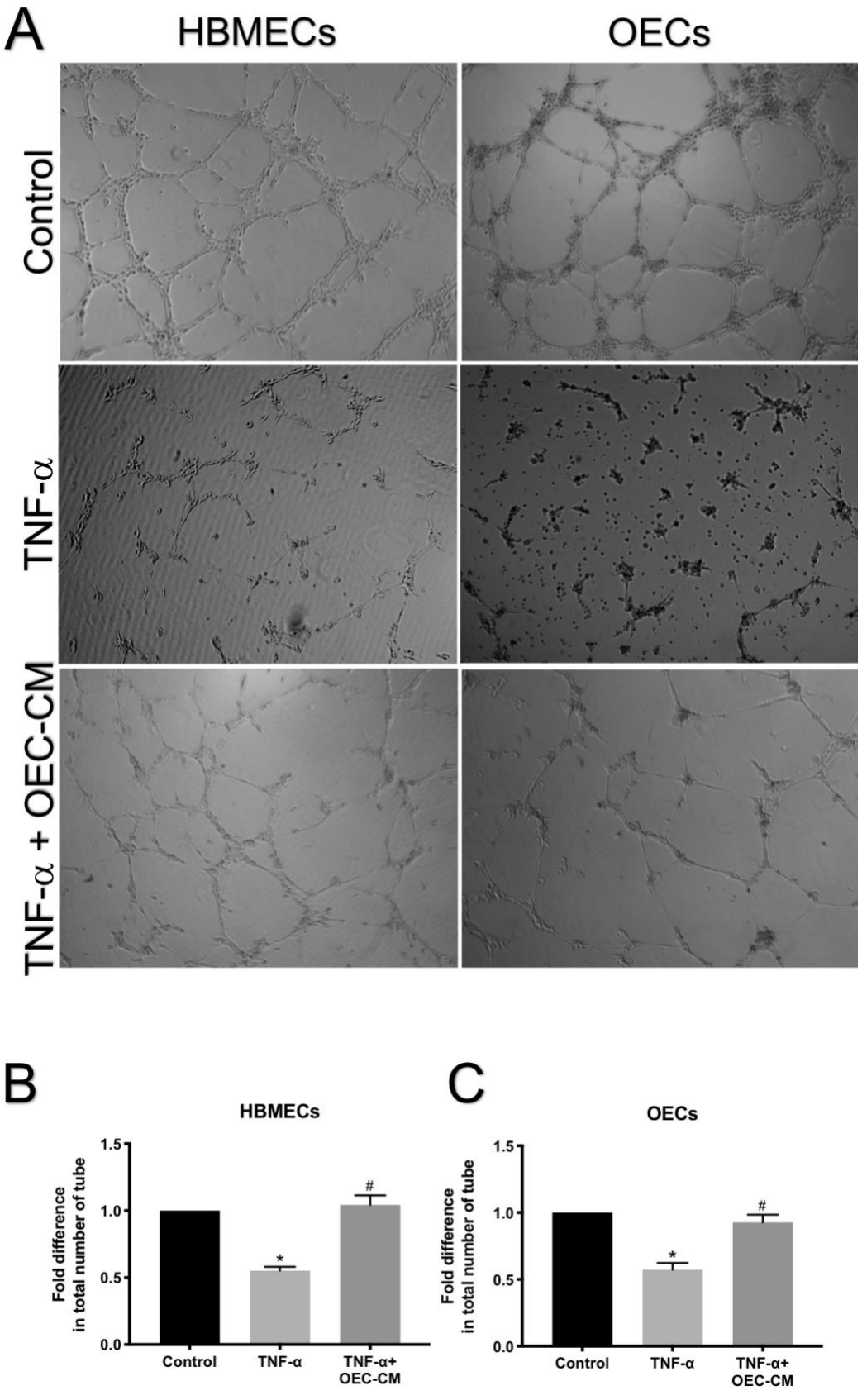
Figure 43. OEC-CM inhibited apoptosis and maintains cell viability.

(A-D) The presence of OEC-CM negated the enhancement of caspase 3/7 activity and improved the viability of HBMECs and OECs. *P < 0.05 versus control, #P < 0.05 versus TNF-α. $n \geq 3$.

2.7. OEC-CM promotes cellular mechanisms required for angiogenesis

Angiogenesis, the formation of new blood vessel from pre-existing vasculature by process of sprouting and splitting, is a physiological process in the earlier stages of vasculogenesis (Hatakeyama et al. 2020). It is controlled by a complex network of mechanisms, stimulated by signals received from other cells and/or the extracellular environment. Albeit cytokine signalling is acknowledged as one of the key drivers of angiogenesis; signalling mechanisms induced by cell adhesion interactions are also crucially important in cytokine signalling (Zhu et al. 2021). Since angiogenesis also contributes to BBB remodelling, thereby helping diminish the detrimental impact of cerebral ischaemic injury (Yang and Torbey 2020), this study assessed the effect of OEC-CM on a number of parameters closely associated with the process of angiogenesis, namely proliferation and tubulogenesis in HBMECs and OECs subjected to TNF-α, in the absence or presence of OEC-CM.

As shown in Figure 44A-G, treatment with OEC-CM attenuated the deleterious impact of TNF-α on the tubular network and proliferative capacity of both cells, as ascertained by significant increases in number and length of tubes and reading of optical density, respectively. Given the discernible differences in endostatin levels obtained in HVs and ischaemic stroke patient plasma samples, as well as the major role of this anti-angiogenic factor in regulating availability and functionality of EPCs and post-stroke angiogenesis (Schuch et al. 2003; Malik et al. 2020), the current study investigated the effect of OEC-CM on endostatin level of HBMECs and OECs. As expected, treatment with OEC-CM effectively inhibited the generation of endostatin level in both cells subjected to TNF-α (Figure 44H, I).


A
HBMECs
OECs
Control
TNF-α
TNF-α + OEC-CM
B
HBMECs
Fold difference in total number of tube
1.5
1.0
0.5
0.0
*
#
Control
TNF-α
TNF-α+ OEC-CM
C
OECs
Fold difference in total number of tube
1.5
1.0
0.5
0.0
*
#
Control
TNF-α
TNF-α+ OEC-CM

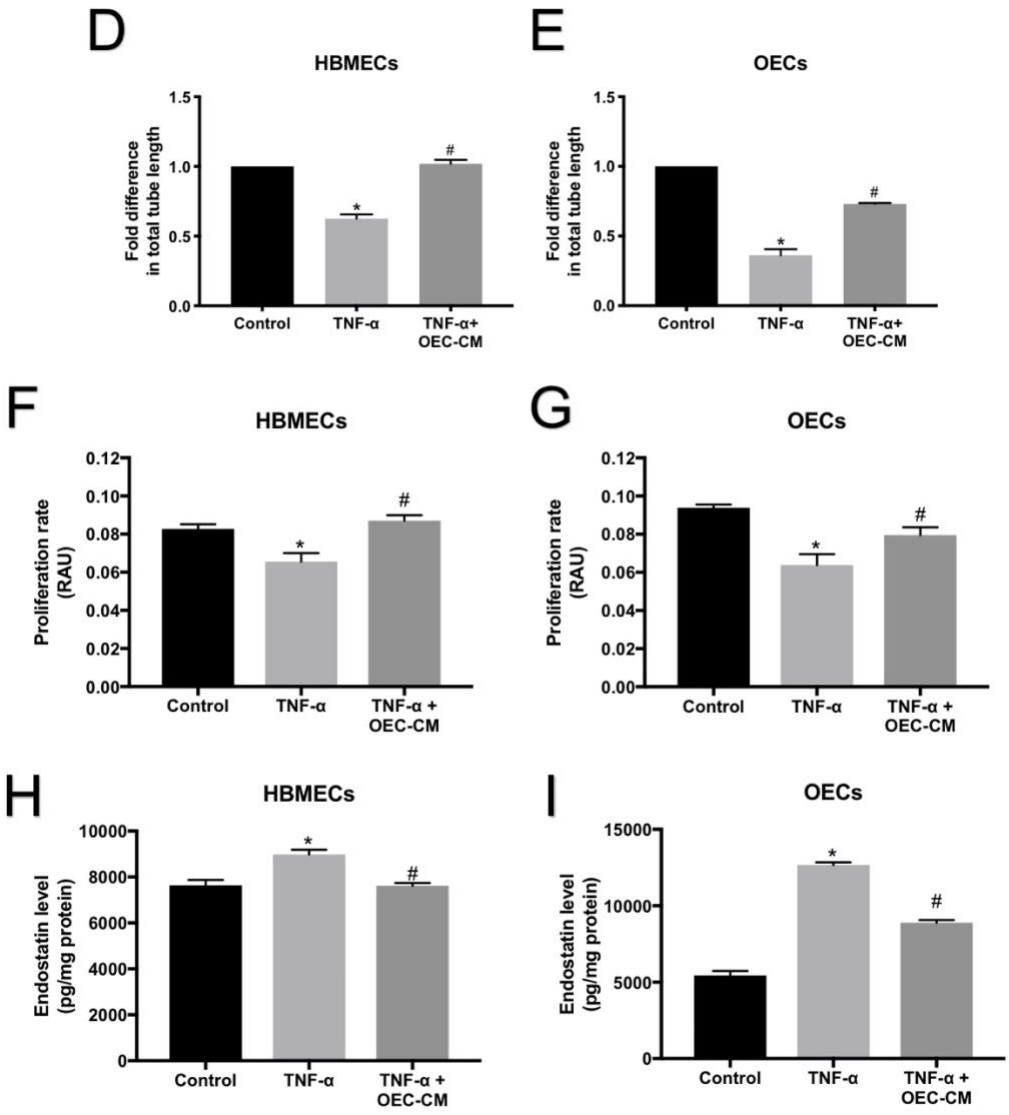
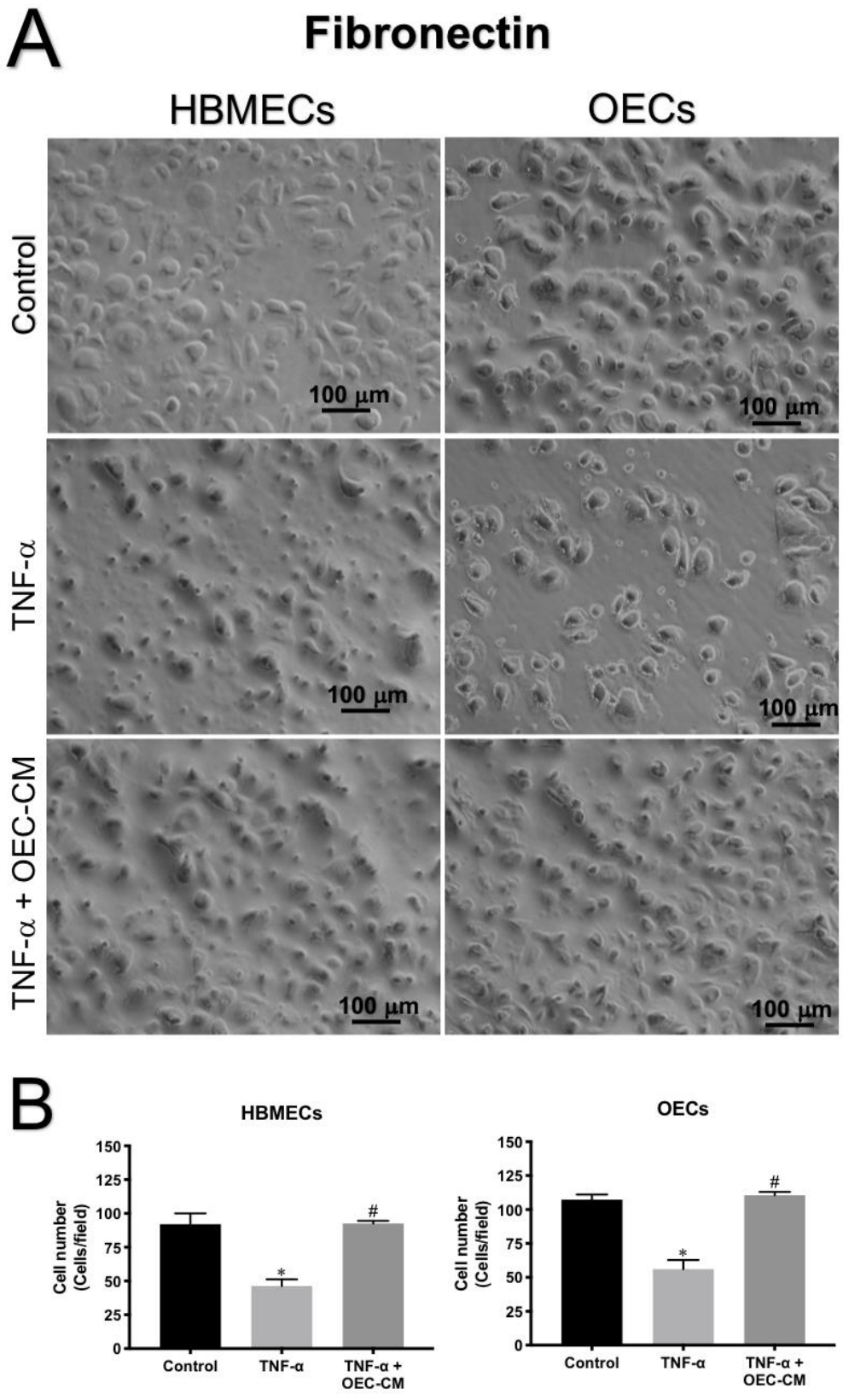


Figure 44. OEC-CM protected angiogenic properties of HBMECs and OECs.

(A-G) Exposure to TNF-α impaired the proliferation rate and tubular structure of both cells, whereas treatment with OEC-CM alleviated the detrimental effects of TNF-α in the abovementioned parameters. **(H, I)** The presence of OEC-CM also negated the improvement of angiogenic inhibitor evoked by TNF-α. *P < 0.05 versus control, #P < 0.05 versus TNF- α. $n \geq 3$.

The integrity of the vascular system after a cerebral ischaemic injury is maintained by both lateral migration of resident endothelial cells and the recruitment of circulating EPCs into the site of injury (Xie et al. 2016). This implies the importance of the adherence of both HBMECs and OECs to the underlying extracellular matrix, and represents an important early event for the successive efficacious stimulation of tubulogenesis and angiogenesis (Mongiat et al. 2016; Michalski et al. 2020). As expected, the current study found that TNF-α decreased the number of HBMECs and OECs adhering to two different seminal components of the extracellular matrix, fibronectin and collagen, in equal measure, while co-treatment with OEC-CM almost completely restored the adhesion of both cells to both extracellular matrix components (Figure 45).


A
Fibronectin
HBMECs
OECs
Control
TNF-α
TNF-α + OEC-CM
100 μm
B
HBMECs
OECs
Cell number (Cells/field)
150
125
100
75
50
25
0
*
#
Control
TNF-α
TNF-α + OEC-CM

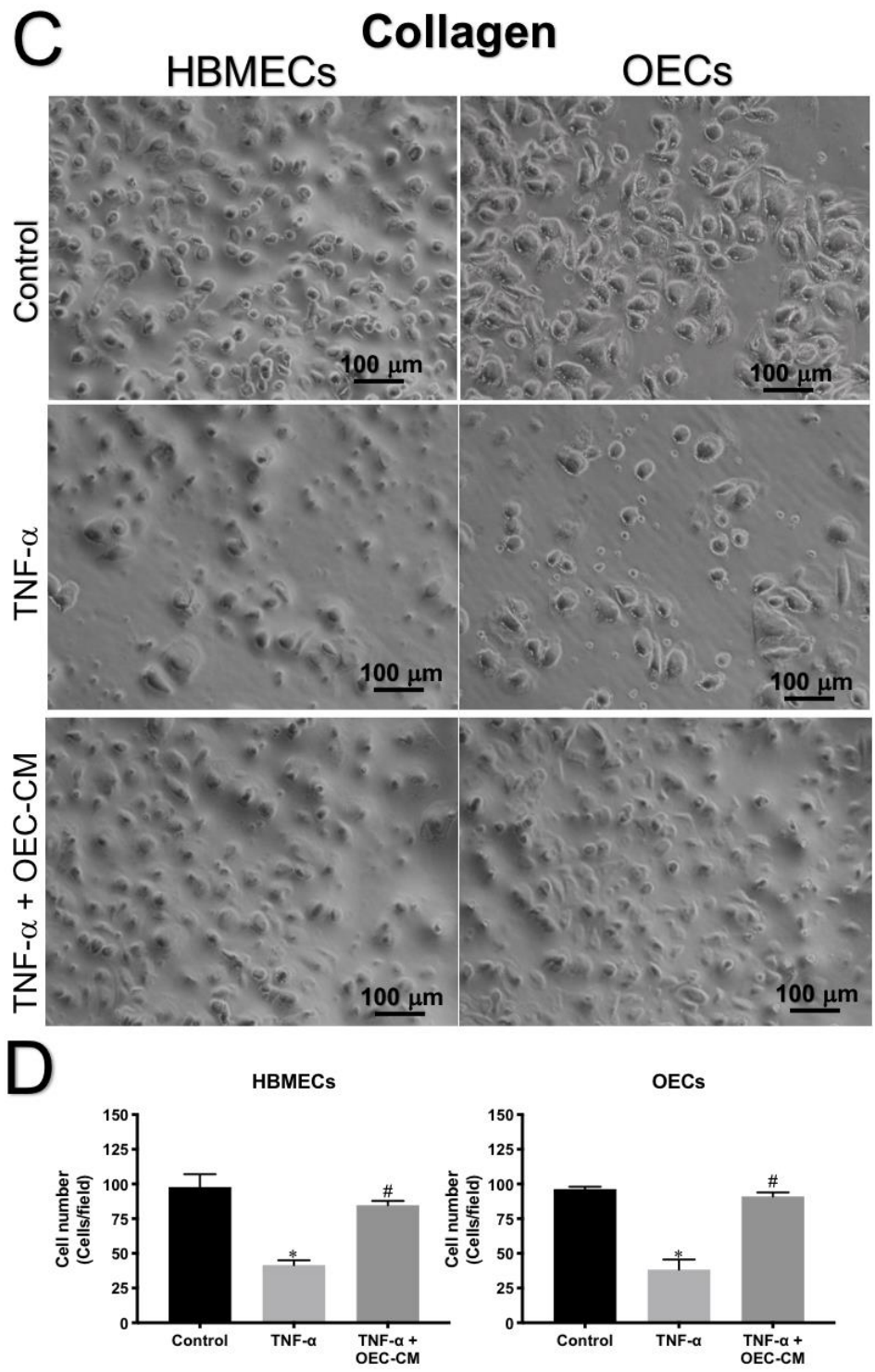


Figure 45. OEC-CM enhanced adhesion capacity of HBMECs and OECs.

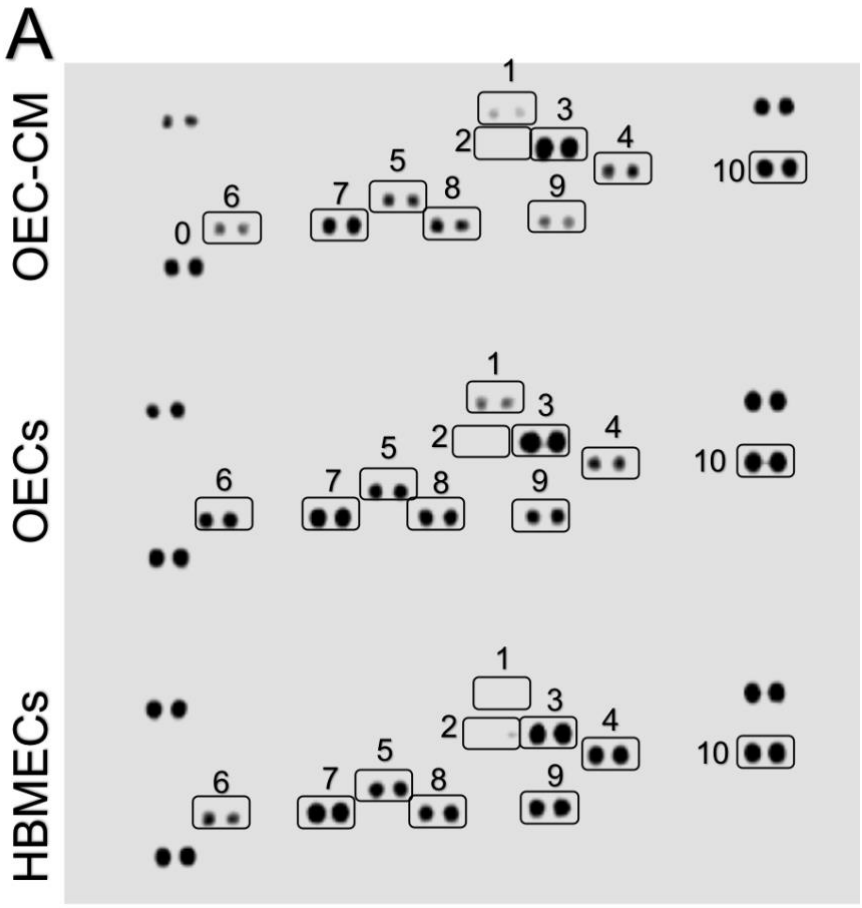
(A, C) TNF-α dramatically decreased the number of cells attached to fibronectin or collagen matrix, while treatment with OEC-CM neutralised these effects to almost normal conditions. **(B, D)** The quantification of the number of cells adhering to fibronectin and collagen matrix. *P < 0.05 versus control, #P < 0.05 versus TNF-α. Scale bars: 100 μm. *n* = 4.

2.8. OEC-CM displays greater quantities of several elements that spur cell proliferation and migration

To identify the specific elements involved in OEC-CM-mediated restorative effects as indicated above, the levels of angiogenesis-related proteins were comparatively assessed in secretome of HBMEC, OEC, and OEC-CM using a proteome profiler array. This assessment of OEC-CM showed substantial increases in the pro-angiogenic factor endothelin-1 and monocyte chemoattractant protein-1 (MCP-1), and a dramatic decrease in the anti-angiogenic factor endostatin (Figure 46). Endothelin-1 is known to stimulate the mobilisation and proliferation of endothelial cells through both ETA and ETB receptor activation (Morbidelli et al. 1995; Dong et al. 2005). Specifically, the activation of ETA receptor leads to an increase in VEGF expression, which directly or through activation of MCP-1 promotes endothelial cell proliferation and migration, and therefore participates in angiogenesis (Matsuura et al. 1998; Yamada et al. 2003; Wu et al. 2014).

Compared to OECs, a specific increase in interleukin-8 (IL-8) and decreases in serpin-E1, urokinase plasminogen activator (uPA), pentraxin-3, and angiopoietin-2 levels were found in OEC-CM. Similar to endothelin, IL-8 also directly enhances endothelial cell proliferation and survival, and thus regulates angiogenesis (Li et al. 2003). Serine protease inhibitor-1 (serpin-E1) acts as the main inhibitor of uPA, an enzyme that catalyses the conversion of plasminogen to plasmin. Attenuation of uPA in ischaemia-induced OEC-CM may help to preserve normal cellular phenotype and endothelial permeability through regulation of MMP-2 and NADPH oxidase (Rakkar et al. 2014). Likewise, angiopoietin-2 and acute phase protein pentraxin-3 also play important roles to maintain endothelial physiology and permeability, as well as to regulate vascular dysfunction and angiogenesis in conditions associated with TNF-α release (Akwii et

al. 2019). Observation of similar levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and thrombospondin-1 in OEC-CM and HBMEC and OEC supernatants rule out the possibility that these proteins are involved in OEC-CM-mediated cell proliferation and migration (Figure 46).



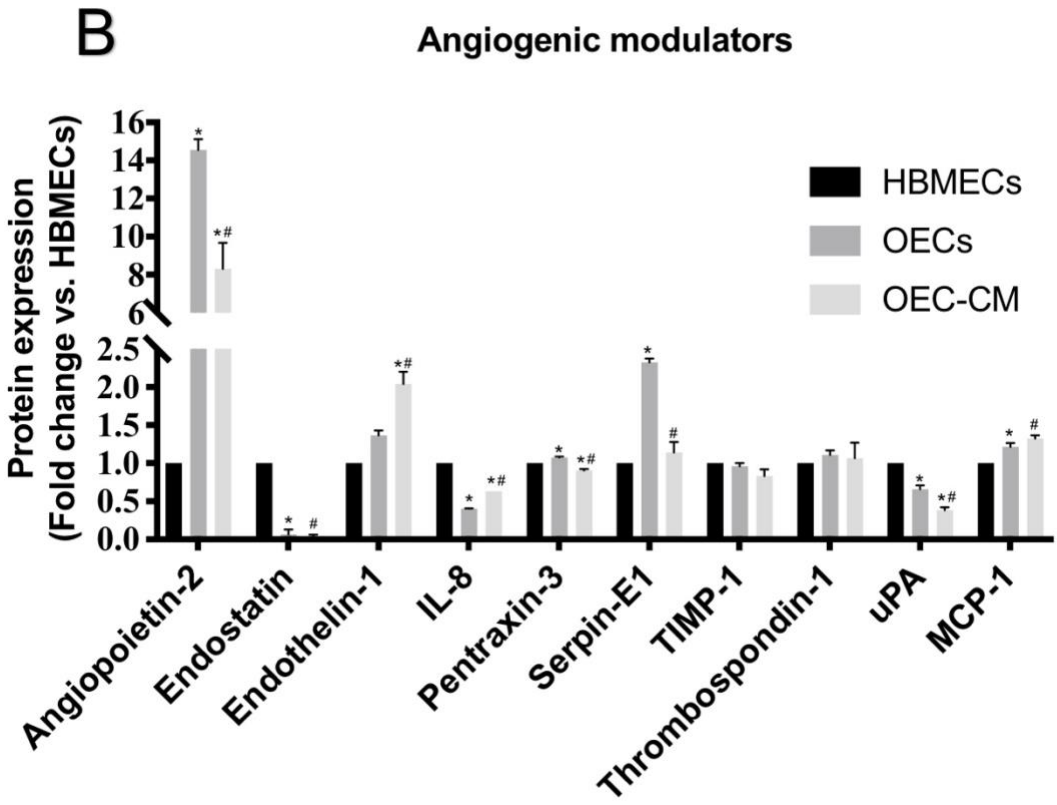


Figure 46. Identification of angiogenic modulators contains in secretome of HBMEC, OEC, and OEC-CM.

(A) Angiogenic protein arrays from HBMECs and OECs secretome as well as OEC-CM revealed that the latter release high concentration of pro-angiogenic endothelin-1 and MCP-1. **(B)** Graphs showing the quantification of pro- and anti-angiogenic factors of secretome of HBMECs, OECs, and OEC-CM. *P < 0.05 versus HBMECs, #P < 0.05 versus HBMECs. *n* = 2.

3. Discussion

Various guidelines including STEPS, ARRIVE, and STAIR have been introduced to address key issues regarding methodologies, experimental models, and statistical analyses utilised in stroke research in an effort to facilitate extrapolation of experimental data to clinical settings (Saver et al. 2009; Boltze et al. 2019; Percie du Sert et al. 2020). However, the majority of clinical trials have not successfully replicated the favourable outcomes observed in relevant pre-clinical studies (Schmidt-Pogoda et al. 2020). Since experimental pitfalls cannot be the only factors blocking translation from pre-clinical models to clinical outcomes, extensive scrutiny of the causes of past failures is of pivotal importance to develop effective and clinically relevant novel therapeutics (Lourbopoulos et al. 2021). In this regard, comprehensive analysis of clinical and biochemical alterations brought about by cerebral ischaemic injury may help to identify novel therapeutic targets (Tian et al. 2022) and inform the design of pre-clinical research (Bix et al. 2018; Lourbopoulos et al. 2021).

Through extensive assessment of a large set of clinical, demographic, and biochemical data acquired from 90 HVs and 81 patients with ischaemic stroke, the present study has shown consistent increases in the levels of TNF-α, a prominent pro-inflammatory mediator, across all phases (acute, subacute, and chronic) of ischaemic stroke compared to levels among HVs. The attainment of equal levels of TNF-α in ischaemic stroke patients with and without vascular risk factors indicates that cerebral ischaemic injury is the major stimulus for enhanced concentrations of TNF-α across all phases of stroke. In contrast to the patient group, HVs with DM, hypertension, and hyperlipidaemia manifested substantially higher levels of TNF-α than those without. It is noteworthy here that the trend of TNF-α in patients with AF tend to decrease over

time which might be caused by the anti-inflammatory effects of the medication commonly used by AF patients (Lin et al. 2020).

Endothelial dysfunction, characterised by changes in endothelial cell architecture and function, refers to the disruption in vascular permeability, coagulation, immune reaction, and platelet/leukocyte adhesion (Lee et al. 2021). Specifically, in case of ischaemic stroke, profound abnormalities in endothelial function include BBB breakdown and the concomitant appearance of vasogenic oedema or haemorrhagic transformation, an extremely dangerous condition that can increase the patient mortality rate by 80% (Yao et al. 2021).

In addition to TNF-α, cerebral ischaemia also significantly enhances levels of SDF-1 during acute, subacute, and early chronic phases of ischaemic stroke. SDF-1 promotes migration and homing of EPCs to the injury site, and increased release in the immediate aftermath of stroke may counter the detrimental effects of stroke on cerebrovasculature, and reduce infarct volume and neurological deficits (Zhao et al. 2019; Kukumberg et al. 2020). Since cell damage and inflammation are mostly settled in the chronic phase of ischaemic stroke, SDF-1 may not be required as much as during the initial onset, explaining the gradual reduction of its level during the chronic phases of stroke.

By employing the well-established *in vitro* model of human BBB formed by co-culture of HBMECs, astrocytes, and pericytes, the present study has shown that treatment with OEC-CM effectively mitigates the detrimental effects of TNF-α on BBB integrity and function, as indicated by increased TEER readings and decreased paracellular flux of sodium fluorescein. Given that OECs continuously replace dead or damaged cerebral endothelial cells, the establishment of model that closely mimics this physiological structure is of paramount importance (Stone et al. 2019). Bearing this in

mind, both HBMECs and OECs were subsequently co-cultured in the luminal chamber of the aforementioned BBB model. Again, the attainment of improved readings for TEER and paracellular flux volume of the cerebral barrier model subjected to TNF-α in the presence of OEC-CM further confirmed that this regimen can effectively neutralise the barrier-detrimental effects of this inflammatory cytokine. The previous investigations focusing on the molecular mechanisms underlying TNF-α-evoked BBB breakdown showed concurrent inductions of NADPH oxidase activity, the main enzymatic source of ROS in cerebral vasculature, and superoxide anion production, as the instrumental factors (Abdullah and Bayraktutan 2014; Abdullah et al. 2015). The observation of flattened total anti-oxidant capacity in patients with ischaemic stroke at all time points in the present study may also indicate that pro-oxidant (rather than anti-oxidant) play a prominent role following cerebral ischaemic injury. Indeed, the significant reduction in BBB damage and infarct volumes observed in NADPH oxidase-knockout compared to wild-type mice, as well as those treated with different NADPH oxidase inhibitors (apocynin, DPI, or VAS2870) before induction of middle cerebral artery occlusion (MCAO), confirms the barrier-disruptive effect of this oxidase in ischaemic stroke settings (Nagel et al. 2007; Tang et al. 2008; Kleinschnitz et al. 2010; Zehendner et al. 2013).

In line with these findings, the current study exhibited substantial increases in NADPH oxidase activity and superoxide anion production in both cerebral endothelial cells and OECs exposed to TNF-α, and demonstrated that OEC-CM can effectively suppress its activity. Suppression of ROS and inflammatory mediator production as well as apoptosis in endothelial cells subjected to hydrogen peroxide-evoked oxidative stress (500 μM for 8 hours) following treatment with OEC-CM reinforces the modulatory role of this therapeutic regimen on pro-oxidative mechanisms (Yang et al. 2010).

Considering that the physical loss of cells might also contribute to endothelial dysfunction and cerebral barrier damage (Tu et al. 2021; Wang et al. 2021), analyses of a range of markers focusing on different aspects of cell viability/death were also performed in both HBMECs and OECs subjected to TNF-α, which showed significant increases in DNA fragmentation and caspase 3/7 activities along with decreases in cell viability. Subsequent studies concomitantly showed that OEC-CM effectively protect both cells from the apoptotic effects of TNF-α, markedly increase cell viability, and hence help maintain appropriate BBB function. In support of our findings, treatment of oligodendrocyte precursor cells exposed to oxygen-glucose deprivation with EPC-CM has also been shown to preserve cell viability and inhibit apoptosis (Zhou et al. 2021).

Taken together, the data outlined thus far indicates that the biochemical cascade of apoptosis leading to BBB failure is initiated and compounded by inflammatory stimulus and activation of NADPH oxidase/superoxide anion pathway, respectively (Abdullah and Bayraktutan 2014; Abdullah et al. 2015). The existence of similar cascades has also previously been noted in various types of endothelial and neuronal cell, whereby TNF-α-mediated NADPH oxidase/superoxide anion pathway provoked apoptosis via both direct and indirect mechanisms (through the generation of other radicals, namely H_2O_2 and $ONOO^-$) (Basuroy et al. 2009; Mathur and Bayraktutan 2017; Sandoval et al. 2018).

In addition to EPC-mediated reparative processes, neurological recovery from cerebral ischaemic injury also relies on regenerative processes, such as angiogenesis, which entails several steps ranging from endothelial proliferation to tube formation, branching, and anastomosis and eventually restoration of cerebral blood flow (Hatakeyama et al. 2020). Progressive decline in plasma levels of endostatin,

thrombospondin-1, and thrombospondin-2 observed in the current study indicate that those anti-angiogenic factors (rather than pro-angiogenic factors) may be more important in the pathogenesis and outcome of ischaemic stroke (Zhu et al. 2021). Since increasing evidence have shown that endothelial proliferation and subsequent angiogenesis following ischaemic stroke injury mainly occur in the hypoperfused tissue surrounding the hypoxic core, so-called penumbra, this area is regarded as the principal target in acute phase of ischaemic stroke (Kanazawa et al. 2019). Moreover, intervention to rescue this zone potentially promotes axonal outgrowth and neurological outcome following ischaemic attack (Ito et al. 2006), which further substantiates the importance of salvaging penumbra in the context of ischaemic stroke. However, the concept of ischemic core as well as penumbra has been challenged in recent years due to the inability of computed tomography and magnetic resonance imaging, which are mostly used in the acute stroke setting, to precisely determine whether and to what extent brain tissue is infarcted (core) or still viable (Goyal et al. 2020). Therefore, extensive studies that utilised recent technologies, including machine learning and artificial intelligence, are urgently needed to devise a reliable and clinically relevant gold standard for ischaemic core imaging (Hakim et al. 2021). Comparative analyses of the plasma levels of these anti-angiogenic factors in patients with ischaemic stroke and HVs reveal substantial increases only in endostatin levels in patients. Intriguingly, while angiostatin level did not statistically change, the level of both thrombspondin-1 and thrombospondin-2 actually appeared to be lower in stroke patients. This and *in vitro* findings showing that exposure to endostatin ultimately induces the dissolution of tube formation and apoptosis in brain endothelial cells, supporting the notion that excessive generation of endostatin may attenuate angiogenesis after cerebral ischaemia (Dixelius et al. 2000; Rege et al. 2009). Impaired

tubule network and proliferative capacity concurred with endostatin overproduction in both HBMECs and OECs treated with TNF-α affirm an intimate relation between the increased availability of TNF-α and the process of angiogenesis. Normalisation of all these parameters by OEC-CM displays the modulatory role of OEC-CM in the synthesis or release of anti-angiogenic factor, and further confirms its reparative function against TNF-α.

The enhancement observed in the adhesion of resident endothelial cells and their progenitors to extracellular matrix constituents, namely fibronectin and collagen, may also be instrumental in mediating angiogenesis-stimulatory effect of OEC-CM. Indeed, a strong attachment to the underlying substratum is recognised as an important pre-requisite for the homing of lateral brain endothelial cells as well as circulating EPCs (Wang et al. 2002; Chu et al. 2018). A previous study assessing the specific mechanisms involved in ischaemic- or TNF-α-evoked vascular damage showed that the activation of matrix metalloproteinases (MMP) was one of the most influential factors in this pathology (Rakkar et al. 2014; Abdullah et al. 2015). These and others have also shown that NADPH oxidase acts upstream to this protease (Hsieh et al. 2012). Hence, by suppressing NADPH oxidase activity and downstream MMPs, OEC-CM may neutralise the basement membrane-degrading effects of MMPs and maintain the adhesive capacity of both HBMECs and OECs.

Deep analysis of the elements released by OECs upon hypoxia-priming implicates a cocktail of factors in the angiogenesis-inducing function of OEC-CM, particularly endothelin-1, MCP-1, and IL-8. Decreased expression of Ang2, uPA, serpin-1, endostatin, and pentraxin-3 also appears to responsible for hypoxic-augmented pro-angiogenic effects of OEC-CM.

Despite cell-free strategy using OEC-CM could be a great biomedical product, as it contains highly biological substances that may be easily manufactured, it has been very challenging to fully define and standardised its biochemical compounds, as well as to measure the activity and half-life time of its components (Teixeira and Salgado 2020). Donor characteristics (age, gender, and metabolic state) along with culture conditions (seeding cell density, oxygen tension, shear stress, pH, (bio)mechanical forces, electromagnetic, and chemical stimulus) may substantially contribute to the diversity of OEC function and its derivates (Alwjwaj et al. 2021). It is therefore of paramount importance to standardise OEC source and isolation/expansion as well as conditioned-medium production, collection, and bioprocessing procedure to ensure a homogenous, scalable, and effective OEC-CM quality production (Ahangar et al. 2020; Teixeira and Salgado 2020).

In addition, cell culture medium, that contain foetal bovine serum (FBS), may also present high variability in composition depending on where, when, and how it was collected and can be contaminated with animal-derived protein, which potentially cause an immunologic response when the cell products are transplanted (Phelps et al. 2018). Alternative options for cell culture medium, including human platelet lysate (HPL) supplementation or serum-free media, is also another critical point to be explored prior to translate OEC-CM into clinical trial (Fang et al. 2017; Subbiahanadar Chelladurai et al. 2021).

4. Conclusion

A comprehensive analysis of ischaemic stroke patients and HV plasma samples recruited for The Dunhill Medical Trust EPC study has shown that ischaemic cerebral injury causes a remarkable increase in the levels of the pro-inflammatory mediator

TNF-α. *In vitro* studies assessing the impact of a high but clinically-relevant, concentration of TNF-α on BBB has shown that it attenuates barrier integrity and function through a series of mechanisms, including apoptosis, oxidative stress, cytoskeletal reorganisation, angiogenesis, and cell-matrix adhesion of both brain endothelial cells and their progenitors, OECs. Scrutiny of OEC-CM as an emerging cell-free therapeutic strategy has proven very effective in negating the deleterious effects of TNF-α and restoring BBB integrity. The graphical summary of this study is shown in in Figure 47.

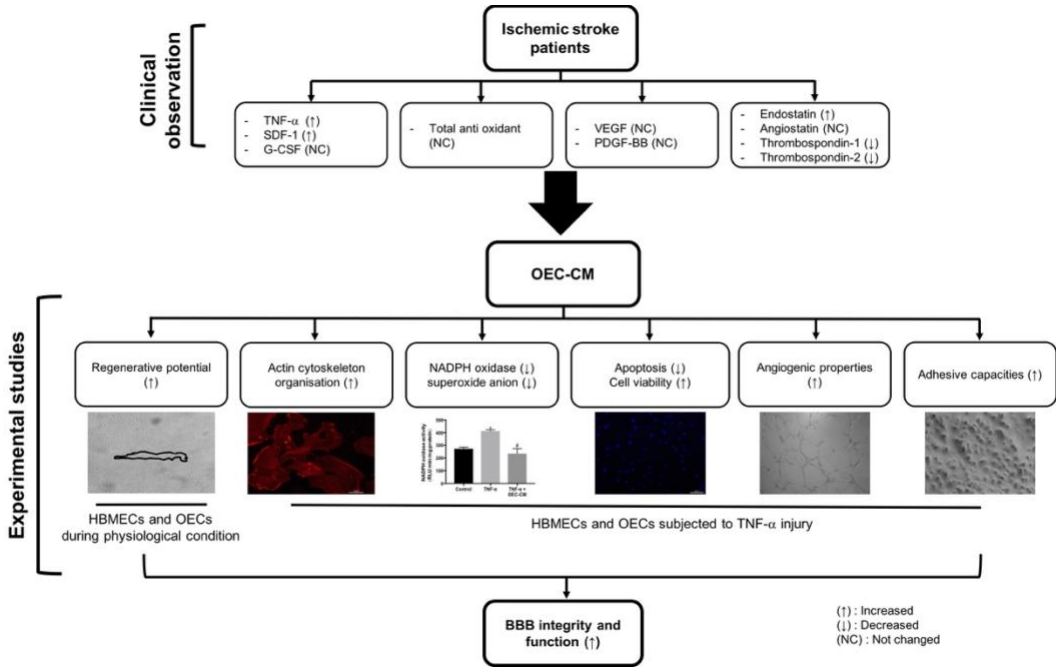


Figure 47. Graphical summary.

General discussion

Ischaemic stroke emerging from the occlusion of blood vessels leading to or within the brain continues to be one of the major causes of mortality and morbidity worldwide (Feigin et al. 2021). Thrombolysis with recombinant tissue plasminogen activator (rtPA) can effectively break up clots that restrict cerebral blood flow, but merely < 1% of patients worldwide receive this drug each year due to issues surrounding the therapeutic time window (4.5 hours), and markedly elevated risk of intracranial bleeding beyond this point (Anand et al. 2021; Berge et al. 2021).

Mechanical thrombectomy has recently emerged in clinical practice as an alternative treatment with a prolonged effective therapeutic window, but this approach is only applicable for ischaemic stroke stemming from large vessel occlusion, and it requires advanced facilities and specialised skills in neurology, neuroradiology, and anaesthesiology etc. (Mortimer et al. 2021). As a result, similar to treatments with rtPA, only limited number of patients can benefit from this new strategy (Kamel et al. 2021).

Considering the limited availability of therapeutic options along with the requirement of appropriate angiogenesis, neurogenesis, and re-endothelialisation for optimum neurological recovery in ischaemic stroke, it is of paramount importance to discover and test new agent that can safe and effective to maintain neuro(vascular) function during or after a cerebral ischaemic injury (Faris et al. 2020; Singh et al. 2020).

In this regard, the outlined studies in this thesis set to investigate a population of EPC subtypes, referred to as outgrowth endothelial cells (OECs), as an emerging alternative therapeutic approach to restore cerebrovascular integrity following ischaemic stroke injury. Since the structural and functional breakdown of BBB accounts for many ischaemic stroke-related mortalities and disabilities during (sub)acute phase of the

disease (Krueger et al. 2019; Yao et al. 2021), this thesis assessed the capacity of these stem cells to repair cerebral barrier integrity and function during ischaemic damages. To this end, a well-established *in vitro* model of human BBB consisting of human brain microvascular endothelial cells (HBMECs), astrocytes, and pericytes was established and subjected to 4 hours of oxygen-glucose deprivation alone or followed by 20 hours reperfusion in the presence or absence of OECs. While these pathological conditions dramatically impaired BBB integrity and function, as ascertained by the significant decrease in TEER value and concomitant increase in sodium fluorescein flux, respectively, treatment with OECs substantially ameliorated these damages.

In line with these results, exogenous addition with OEC also markedly improved readings for TEER and paracellular flux of the aforementioned BBB model exposed to scratch damages on its endothelial monolayer during free-serum condition, suggesting an effective homing and differentiation of OECs when repairing cerebral barrier integrity.

Building upon these *in vitro* works, the efficacy of OEC treatment was subsequently probed in an *in vivo* model of ischaemic stroke using intraluminal filament MCAO model, which showed substantial decreases in infarct volume on day 3 after treatment. Mechanistic studies then showed that exogenous addition of OECs during ischaemic damages significantly reduced the oxidative stress and apoptosis rates of resident brain endothelial cells. In short, these findings collectively demonstrated the decisive role of OECs in repairing cerebral barrier damages in the experimental setting of ischaemic stroke.

While circulating EPCs serve as a critical source of replenishment for the damaged brain endothelial cells, advanced age may adversely affect their self-renewal/reparative ability (Xia et al. 2012; Paneni et al. 2016), leading to increased

risk of various age-related vascular diseases, including stroke (Kukumberg et al. 2020). In this context, this thesis concomitantly assessed whether and to what extent ageing might impact the quantity and functionality of EPCs. The observation from 50 young and 40 elderly healthy individuals revealed insignificant differences in EPC levels (CD34+CD133+KDR+) and endothelial-committed cells (CD34+KDR and CD133+KDR+). In contrast, the quantity of circulating undifferentiated stem cells (CD34+ and/or CD133+), which possess a unique capacity to differentiate into mature endothelial cells (Yeh et al. 2003; Suuronen et al. 2006) and contributes to overall vascular health (Shimizu et al. 2020; Jiang et al. 2021), significantly declined in the latter arm.

Since a relevant study reporting similar results to ours attributed age-dependent endothelial cell dysfunction to diminished functional capacity, rather than numerical changes in circulating EPCs (Heiss et al. 2005), this thesis subsequently assessed the effect of ageing on the morphological, molecular, and functional features of EPCs. In this context, we subsequently exposed the cells to an *in vitro* model of chronological ageing, mimicked by repetitive culture, which evoked the appearance of multiple typical signs of replicative senescence, including the flattened and enlarged morphology, high levels of senescence-associated-β-galactosidase (SA-β-gal) and γ-H2AX, as well as reduction in their migratory, proliferative, and cerebral barrier-forming capacity.

In addition to these phenotypic changes, senescent OECs also presented several signs of oxidative damage, such as the elevations in NADPH oxidase activity and superoxide anion production, along with reductions in their total anti-oxidant capacity and nitric oxide availability. The observation of substantial decreased of total anti-

oxidant capacity in the plasma of elderly individuals further confirmed that an imbalance of redox status actually occurred in the process of ageing.

Having ascertained the importance and relevance of various enzymes associated with oxidative stress during ageing process, we then assessed whether regulation of this pathological pathway using specific inhibitors for NADPH oxidase activity (VAS2870, 5 μM), the major source of ROS in brain endothelial cells, or the addition of wide-spectrum anti-oxidant (vitamin C, 0.5 μM) can delay the appearance of senescence phenotypes, and repair overall stem cell function. The attainment of a decreased number of cells stained positive with senescence markers along with the marked improvement in proliferative, angiogenic, and cerebral barrier-forming activity following treatment with either compounds at relatively late-passage (passage 12) verified this notion. In addition, attenuation of oxidative stress-evoked senescence development in mesenchymal stem cells and neural progenitor cells by treatment with radical inhibitor indeed substantiated the involvement of oxidative stress signalling pathway in the progression of senescence in stem cells (Li et al. 2016; Maraldi et al. 2021).

Given that long-term culture of OECs ultimately lead to replicative senescence and cellular dysfunction, *ex vivo* expansion to generate large number of cells for therapeutic purpose may be hindered by this unavoidable phenomenon (Medina et al. 2013). Considering this, coupled with the fact that OEC-mediated reparative effects are realised by a wide range of soluble trophic factors released by these cells, the cell-free strategy using OEC-derived conditioned medium (OEC-CM) might be considered as an alternative therapeutic option in regenerative stroke medicine (Alwjwaj et al. 2021). Revelation of equally enhanced peri-infarct capillary density and functional outcome in a mouse model of permanent focal cerebral ischaemia by OECs and OEC-

CM further support the utilisation of cell-free strategy to initiate, modulate, and potentiate neuro-angiogenesis after an ischaemic injury (Di Santo et al. 2009).

Despite the establishment of several protocols, namely ARRIVE, STEPS, and STAIR (Saver et al. 2009; Boltze et al. 2019; Percie du Sert et al. 2020), to address key issues regarding methodologies, statistical analyses, and experimental models employed in stroke research, hundreds of so-called novel therapeutic agents remained fail to replicate the favourable outcomes from pre-clinical into clinical settings (Chamorro et al. 2021). Since experimental pitfalls cannot be the only factors that hampered the translation from pre-clinical models to clinical outcomes, comprehensive analysis of the causes of previous failures is critically important to develop clinically relevant novel therapeutics (Lourbopoulos et al. 2021).

Indeed, a better understanding of mechanisms involved in set of complex intertwined pathologies evoked by cerebral ischaemia is required to explore more safe and efficacious treatments (Campbell and Khatri 2020). In this context, reverse translational study, a dynamic and innovative approach that works backward from bedside to bench, may offer enormous help to identify such fundamental mechanisms underlying cerebral ischaemic injury and inform the design of future pre-clinical research (Lourbopoulos et al. 2021).

Through comprehensive assessment of a large set of clinical and biochemical data of 90 healthy volunteers and 81 ischaemic stroke patients, we have identified that TNF-α play a prominent role in the initiation and progression of endothelial dysfunction and ischaemic stroke injury. This study corroborates recent findings showing the central role of this pro-inflammatory cytokine in promoting post-ischaemic neuroinflammation and BBB breakdown (Clausen et al. 2020; Liberale et al. 2021;

Maciejczyk et al. 2021), and emphasises the importance of discovering agents that can effectively neutralise this pathological event (Bonetti et al. 2019).

In this regard, the final part of this thesis was designed to assess whether and to what extent OEC-CM can address the detrimental effect caused by this inflammatory mediator in the experimental setting of ischaemic stroke. While exposure of an *in vitro* model of human BBB to TNF-α injury (10 ng/mL, 6 hours) significantly impaired its integrity and function, the presence of OEC-CM during this inflammatory episode effectively attenuated the damages, as attested by the increases in TEER value and decreases in sodium fluorescein flux across the barrier. Treatment with OEC-CM also inhibited the level of endostatin, an anti-angiogenic factor that remarkably elevated during acute and chronic phases of ischaemic stroke, and concomitantly repaired tubule networks and adhesive properties of HBMECs and OECs. The suppression of actin stress fibre formation, oxidative stress, and apoptosis of both cells also appeared to contribute to this barrier-restorative effect of OEC-CM. Taken together, the data generated in this thesis have confirmed that OEC-CM can effectively attenuate the deleterious effect of TNF-α and therefore highlighted the potential utilisation of this regimen as a viable option to stem cerebrovascular damages in ischaemic stroke injury.

In conclusion, this thesis has shown that treatment using OEC cell-based strategy following cerebral ischaemic injury can effectively repair BBB damage and suppress brain oedema formation through suppressing oxidative stress and resident cell apoptosis. While having such crucial roles in counteracting the damages of brain endothelial cells, advanced age ultimately induced the development of senescence phenotypes and cellular dysfunction. Even so, effective control of oxidative stress level somewhat delayed the appearance of senescence signs and enhanced their

regenerative potential. An alternative strategy using OEC-CM cell-free therapy effectively protected structural and functional properties of BBB against TNF-α, an inflammatory cytokine that was significantly enhanced during all phases of ischaemic stroke, via simultaneously regulating a wide range of mechanisms

Limitations and future research

1. Limitations

While this study utilised an *in vitro* model of human BBB consisted of human brain microvascular endothelial cells (HBMECs), astrocytes, and pericytes, a cerebral barrier model which encompass the whole structure of neurovascular unit, including neuron and glia cells, with a mechanism to look at fluid flow and shear stress would be more appropriate and provided more realistic conditions (Lyu et al. 2021). The utilisation of fluorescent-labelled of various molecular weights rather than single marker of paracellular transport, namely sodium fluorescein (376 Da), may also shed some light on the size of intercellular openings.

The employment of cognitive and behavioural test after outgrowth endothelial cell (OEC) treatment also remains critical to assess the effect of these stem cells on the well-being and functional outcome in the cerebral ischaemic setting. Extensive assessment on other important tight junction proteins (occludin and claudin-5) and adherent junctions between HBMECs and OECs as well as following receiving OEC, OEC-derived conditioned medium (OEC-CM), VAS2870, or vitamin C treatment can also be explored to comprehensively examine the effect of these agents on cerebral barrier integrity.

It is also important to note that the protective effect of OEC-CM in the current study was acquired from *in vitro* study, implied the need to explore its therapeutic effect in *in vivo* model of ischaemic stroke. We also acknowledge that the OEC-CM used in the present study acquired from a limited sample. In addition, determination of protocol to isolate OEC-CM (source, duration of hypoxic exposure, and type of culture medium) is also critical in the further study in effort to provide an effective therapeutic compound.

This study has identified various senescence features, such as cell and nuclear enlargement and the presence of SA-β-gal and γ-H2AX. However, further works using other senescence markers, such as telomere length, p16, p21, and p53 would provide a better understanding of the molecular pathway of the senescence process in OECs. While this study has utilised cell culture model of ageing to assess the effect of this condition on the functionality of OECs, an extensive study comparing molecular and regenerative phenotype of OECs isolated from elderly and younger individuals may reflect the actual impact of ageing on the stem cells function in human.

2. Future directions

While the data presented in this thesis unanimously confirmed the cerebral barrier-protective role of OEC-CM in an *in vitro* model of ischaemic stroke, further exploration in *in vivo* settings remains critical to validate such therapeutic effects. It is noteworthy here that consideration of experimental model (sex, age, comorbidities, circadian rhythms, and replication in large species) and methodologies (randomisation, multicentre, and blinded studies) are of utmost importance in further animal works using this novel strategy (Esposito et al. 2020; Lyden 2021).

Since the survivability of OECs could be further reduced by the harsh microenvironment of ischaemic tissues (Hookham et al. 2016; Tasev et al. 2018), new strategies to increase the vasoreparative efficiency and survivability under pathological environment are needed. Immune rejection of transplanted allografts caused by a genetic mismatch of MHC and following recipient T cell activation is the common adverse event following stem cell therapy (Otsuka et al. 2020). Better understanding of the mechanisms of graft rejection and the development of innovative

strategy to regulate immunological response following allogenic transplantation are indeed critical to address this hurdle.

Moreover, extensive pre-clinical assessment and optimisation pertaining to the dose, effective time window, and route of administration of OECs and OEC-CM are also required to determine their efficacy (in the context of regenerating damaged tissues and improving neurological function) and safety profile (risk of immunological reactions, tumour formation, and other serious adverse events) (Boltze et al. 2019; Lyden 2021). While intralesional delivery (e.g. intracerebral, intraventricular, and subarachnoid route) associated with an effective engraftment in the site of injury, this approach is very invasive and can result in increased side effects. Systemic delivery, on the other hand, is less invasive and exogenous OECs can still reach the damaged area in the brain and restore the functional outcome of ischaemic stroke animal model (Moubarik et al. 2011). However, some studies reported a risk of vascular occlusion linked to this approach caused by clumping of transplanted cells (Rodríguez-Frutos et al. 2016). In order to address these hurdles, precise dosage and infusion velocity are therefore crucial to be established.

Rigorous and well-designed clinical studies involving ischaemic stroke patients who fall beyond the revascularisation treatment window, along with a practically acceptable standard protocol (e.g. intravenous rather than intracerebral administration, and allogenic instead of autologous approach) are desperately needed to develop off-the-shelf allogenic cell products for the vast number of patients who currently go untreated (Boltze et al. 2019; Law et al. 2021).

In addition to the clinical challenges to applying OEC-based therapy for ischaemic stroke, manufacturing processes are also critical to ensure the identity, quality, and safety of the cells (Krause et al. 2019). When manufacturing cell-based products,

materials and reagents should be selected for good manufacturing practices (GMP) compatibility (Zhou et al. 2022). Optimal aseptic processing is a requisite to avoid contamination of cell products with microbes or physical/chemical agents that can change potency and safety. Early passage OECs are preferable for clinical use, and phenotypic and genetic stability should be carefully controlled (Medina et al. 2013). Finally, quality control testing is required prior to clinically used these stem cells in order to monitor their biological or therapeutic activity and to demonstrate the absence of microorganism or microorganism's residues (e.g., endotoxins) in the final product (Fernandez-Muñoz et al. 2021).

Again, in spite of the outlined findings advancing understanding of pathways that lead to senescence and pinpointing possible interventional targets, it remains critical to confirm the role of VAS2870 or vitamin C to forestall this pathological condition in animal models. Furthermore, through administrating the senescent OECs into animal models of cerebral ischaemic injury (Kim et al. 2020) or observing the association between the number of senescent OECs and neurological recovery in patients with ischaemic stroke (Lewis-McDougall et al. 2019), questions regarding the role of senescent OECs in determining progression and severity of stroke may be addressed. Alternatively, the transgenic model mice that allow to selectively detect and eliminate senescent cells, including INK-ATTAC (Baker et al. 2016) or P16-3MR model (Jeon et al. 2017), can be used to accelerate our understanding of the contribution of senescent cells to various age-associated alterations and pathologies.

Since the senescence-associated secretory phenotype (SASP) that released by senescent cells is an important driver of and promising therapeutic target for various age-related conditions (Schafer et al. 2020), a comprehensive characterisation of SASP molecules using the most recent technology, such as mass spectrometry-based

proteomics, multiplexed proteomic assay using modified aptamers, and proximity extension assay, is also needed (Basisty et al. 2020; Moaddel et al. 2021).

While many senescent cells can attract, anchor, and modulate immune cells in order to eliminate them, the physiological mechanism to counteract the presence of senescent OECs is currently unknown, implying the need for further exploration of this system (Prata et al. 2018). Indeed, the discovery of delayed ageing progression and enhancement in OEC proliferative and vasculoreparative capacity after silencing one of the proven immunomodulators of OEC senescence, namely IL-8, may be considered as a crucial stepping stone to understand immune surveillance and the clearance system of senescent OECs (Medina et al. 2013). The comprehensive assessment of the quantity and functionality of these cells as well as overall vascular health in IL-8 knockout/overexpress mice may provide important proof-of-concept evidence for the direct impact of inflammatory mediator on vascular regeneration.

Extensive studies to identify any serious adverse events after targeting senescent OECs and determination of when to begin treatment, and whether intermittent or continuous treatment is optimal, are also urgently needed. It is also of paramount importance to build collaborative teams consisting of scientists with diverse specialisations, including the biology of ageing, geriatricians, neurologists, and clinical trialists, to understand the actual nature of senescent stem cells, investigate their specific liabilities, and eventually implement therapeutic approaches.

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Appendices

Appendix I

Equipment and chemicals

Apparatus	
Materials	**Company**
Cell scrapers	TPP
Centrifuge IEC central CL3	Thermo scientific
Cryogenic vials	Nalgene
EVOMX meter	World precision instruments
Falcon tubes – 50 mL, 15 mL	BD Biosciences
Fluorescence microscope	Carl Zeiss GmbH
FLUOstar omega plate reader	BMG labtech
Glass coverslips	Cover glass
Glass slides	Thermo scientific
Hypoxic O_2/CO_2 incubator	Sanyo
Microcentrifuge MiniSpin	Eppendorf
Milicell EZ Slide	Merck Millipore
Mr frosty freezing container	Nalgene
N_2 cylinders	Airproducts
Orbital plate shaker	Stuart scientific
Sonicator and probe sonopuls HD 2070	Bandelin
STX2 electrode	World precision instruments
Temperature controlled centrifuge 5417R	Eppendorf
Tissue culture CO_2 incubator	Sanyo
Tissue culture flasks – T25, T75	Corning life sciences

Tissue culture plates – 6, 12, 24, and 96 well	Corning life sciences
Tissue culture plates – 96 well black	Nunc
Tissue culture plates – 96 well white	Nunc
Transwell 12-well 0.4 μm pore polyester inserts	Corning life sciences
Transwell 4.0μm pore size inserts	Corning life sciences
UV light	UVP
Water bath	Fisher scientific
Tissue culture	
Astrocyte growth media	ScienCell research laboratories
Collagen, Type I solution from rat tail	Sigma-Aldrich
Endothelial cell media (ECM)	ScienCell research laboratories
Endothelial basal medium-2	Lonza
Calcein AM	Calbiochem
Fibronectin	Sigma-Aldrich
Foetal bovine serum	Sigma-Aldrich
Hank's balanced salt solution	Sigma-Aldrich
Human brain microvascular endothelial cells	ScienCell research laboratories
Human astrocytes	ScienCell research laboratories
Human pericytes	ScienCell research laboratories
Penicillin/streptomycin solution	Sigma-Aldrich
Pericyte growth media	ScienCell research laboratories
Phosphate buffered saline	Sigma-Aldrich
RPMI 1640 free-glucose media	ThermoFisher scienific
Trypsin	Sigma-Aldrich

Antibodies	
Anti γ-H2AX antibody	Abcam
Anti ZO-1 antibody	Abcam
Dil-AcLDL	Invitrogen
FITC-UEA-1	Sigma
Goat Anti-Rabbit IgG	Abcam
Activators/inhibitors	
Allopurinol	Sigma-Aldrich
Indomethacin	Sigma-Aldrich
L-NAME	Sigma-Aldrich
Rotenone	Sigma-Aldrich
TNF-α	Bio-techne
VAS2870	Calbiochem
VEGF	Fisher Scientific
Vitamin C	Sigma-Aldrich
Assay kits	
Apo-ONE homogenous caspase-3/7 assay	Promega
BCA protein assay kit	Thermo Fisher
Beta-galactosidase kit	Abcam
Endostatin Assay Kit	Abcam
Matrigel	BD Biosciences
Nitric Oxide Assay Kit	Abcam
Proteome profiler human angiogenesis array kit	R&D Systems
Total Antioxidant Capacity Assay Kit	Abcam

WST-1	Roche
Biological materials	
Aprotinin	Sigma-Aldrich
Bovine serum albumin	Fisher scientific
Cytochrome *C*	Sigma-Aldrich
Gelatine	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
Chemicals	
DAPI	Sigma-Aldrich
Dibasic potassium phosphate	Sigma-Aldrich
DMSO	Sigma-Aldrich
EDTA	Sigma-Aldrich
EGTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Glycerol	Sigma-Aldrich
HEPES buffer	Calbiochem
Lucigenin	Sigma-Aldrich
Mannitol	Sigma-Aldrich
Monobasic potassium phosphate	Sigma-Aldrich
NADPH	Sigma-Aldrich
Nitrogen	Airproducts
Paraformaldehyde	Sigma-Aldrich
Protease cocktail inhibitor	Sigma-Aldrich
Rhodamine phalloidin	Abcam

Sodium chloride	Fisher scientific
Sodium dodecyl sulphate	Sigma-Aldrich
Sodium fluorescein	Sigma-Aldrich
Sucrose	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trypan blue	Invitrogen
Tween-20	Sigma-Aldrich
Software	
GraphPad Prism 8.4.3 statistical software package	GraphPad Software Inc.
SPSS package 15.0 for Windows	SPSS Inc.
ImageJ (version 1.52k)	National institute of health (NIH)
Image Studio Lite	LI-COR

Appendix II

Details of statistical test

Figure	Panel	Statistical test
11.	**C**	Unpaired *t*-test
12.	**A**	Unpaired *t*-test
	B	Unpaired *t*-test
	C	Unpaired *t*-test
	D	Unpaired *t*-test
	E	Unpaired *t*-test
	F	Unpaired *t*-test
	G	Unpaired *t*-test
13.	**B**	Fibronectin: Unpaired *t*-test; Collagen: Mann-Whitney test
14.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
15.	**C**	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
16.	**A**	Two-way ANOVA followed by Sidak’s post-hoc
	B	Two-way ANOVA followed by Sidak’s post-hoc
17.	**B**	Two-way ANOVA followed by Sidak’s post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
18.	**A**	One-way ANOVA followed by Tukey's post-hoc
	B	One-way ANOVA followed by Tukey's post-hoc
19.	**B**	Two-way ANOVA followed by Sidak’s post-hoc

20.	**A**	One-way ANOVA followed by Tukey's post-hoc
	B	One-way ANOVA followed by Tukey's post-hoc
21.	**-**	Two-way ANOVA followed by Sidak’s post-hoc
22.	**A**	One-way ANOVA followed by Tukey's post-hoc
	B	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
23.	**-**	Not applicable
24.	**A**	Mann-Whitney test
	B	Mann-Whitney test
	C	Mann-Whitney test
	D	Mann-Whitney test
	E	Mann-Whitney test
	F	Mann-Whitney test
	G	Mann-Whitney test
25.	**A**	Mann-Whitney test
	B	Unpaired *t*-test
	C	Unpaired *t*-test
	D	Mann-Whitney test
	E	Mann-Whitney test
	F	Mann-Whitney test
	G	Mann-Whitney test
	H	Mann-Whitney test
	I	Mann-Whitney test

	J	Mann-Whitney test
26.	**B**	Unpaired *t*-test
	C	Unpaired *t*-test
	D	Unpaired *t*-test
	E	Unpaired *t*-test
	F	Unpaired *t*-test
	G	Unpaired *t*-test
27.	**B**	Unpaired *t*-test
	D	Unpaired *t*-test
	E	Unpaired *t*-test
28.	**B**	Two-way ANOVA followed by Sidak’s post-hoc
	C	Unpaired *t*-test
	D	Two-way ANOVA followed by Sidak’s post-hoc
29.	**B**	Mann-Whitney test
	C	Unpaired *t*-test
	D	Unpaired *t*-test
30.	**A**	One-way ANOVA followed by Tukey's post-hoc
	B	One-way ANOVA followed by Tukey's post-hoc
31.	**A**	Unpaired *t*-test
	B	Unpaired *t*-test
32.	**B**	Unpaired *t*-test
	C	Unpaired *t*-test
33.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc

	F	One-way ANOVA followed by Tukey's post-hoc
	G	One-way ANOVA followed by Tukey's post-hoc
34.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
	E	One-way ANOVA followed by Tukey's post-hoc
	F	One-way ANOVA followed by Tukey's post-hoc
	G	One-way ANOVA followed by Tukey's post-hoc
35.	**-**	Not applicable
36.	**A**	D0, D7, D30, D90: Mann-Whitney test
	B	D0, D90: Unpaired t-test; D7, D30: Mann-Whitney test.
	C	D0, D7, D30, D90: Mann-Whitney test
	D	D0, D7, D30, D90: Mann-Whitney test
37.	**A**	D0, D7, D30, D90: Mann-Whitney test
	B	D0, D7, D30, D90: Mann-Whitney test
	C	D0, D7, D30, D90: Mann-Whitney test
	D	D0, D7, D30, D90: Mann-Whitney test
	E	D0, D7, D30, D90: Mann-Whitney test
	F	D0, D7, D30, D90: Mann-Whitney test
38.	**A**	Two-way ANOVA followed by Sidak’s post-hoc
	B	Two-way ANOVA followed by Sidak’s post-hoc
	C	Two-way ANOVA followed by Sidak’s post-hoc
	D	Two-way ANOVA followed by Sidak’s post-hoc
	F	Unpaired *t*-test

	G	Mann-Whitney test
	H	Mann-Whitney test
	I	Mann-Whitney test
39.	**C**	Two-way ANOVA followed by Sidak’s post-hoc
	D	Two-way ANOVA followed by Sidak’s post-hoc
40.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
41.	**A**	One-way ANOVA followed by Tukey's post-hoc
	B	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
42.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
	E	One-way ANOVA followed by Tukey's post-hoc
	F	One-way ANOVA followed by Tukey's post-hoc
	G	One-way ANOVA followed by Tukey's post-hoc
43.	**B**	One-way ANOVA followed by Tukey's post-hoc
	C	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
	E	One-way ANOVA followed by Tukey's post-hoc
	F	One-way ANOVA followed by Tukey's post-hoc
	G	One-way ANOVA followed by Tukey's post-hoc
	H	One-way ANOVA followed by Tukey's post-hoc

	I	One-way ANOVA followed by Tukey's post-hoc
44.	**B**	One-way ANOVA followed by Tukey's post-hoc
	D	One-way ANOVA followed by Tukey's post-hoc
45.	**B**	One-way ANOVA followed by Tukey's post-hoc

Appendix III

Recipe for lysis buffer:

Materials	Per 1 mL (μL)
KH_2PO_4 300mM	67
EGTA 50 mM	20
PCI 5 μgr/mL	5
Aprotinin 10 μgr/mL	2
Leupeptin 0.5 μgr/mL	0.5
PMSF 0.05 mM	5
H_2O	900

Appendix IV

Recipe for buffers used in NADPH oxidase assay:

Materials	Per 1 well (μL)
Potassium buffer 300 mM	42
EGTA 50 mM	5
Sucrose 1M	37.5
L-NAME 10 mM	2.5
Rotenone 10 mM	1.25
Allopurinol 10 mM	2.5
Indomethacin 10 mM	1.25
H_2O	1.75
Lucigenin 200 μM	6.25

Appendix V

Recipe for buffers used in cytochrome *C* assay:

Materials	Per 1 well (µL)
HEPES 1M	5
EGTA 50 mM	5
Sucrose 1 M	17.5
Mannitol 1 M	52.5
Cytochrome *C* 800 µM	16
H_2O	104