

Outgrowth endothelial progenitor cells restore cerebral barrier function following ischaemic damage: the impact of NOX2 inhibition

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Short title: role of NOX2 in brain barrier repair

Funding: Part of this study has been supported by a grant to Dr Bayraktutan from The Dunhill Medical Trust (R459/0216).

Author's contributions: MA and RRA equally contributed to all experiments and data analysis. MA wrote the first draft of the manuscript. UB designed and supervised the study, interpreted the data, and edited the manuscript. All authors have approved the final version of the publication.

Acknowledgements: None.

Conflicts of interest: The authors declare that they have no conflicts of interest.

List of abbreviations

BBB	Blood-brain barrier
CV	Cleared volume
DiI-AcLDL	DiI-labelled acetylated low density lipoprotein
DMSO	Dimethyl sulphoxide
ddH₂O	Double distilled water
EBM2	Endothelial cell basal medium-2
EPCs	Endothelial progenitor cells
EVOM	Epithelial Volt/Ohm Meter
EGTA	Ethylene glycol tetraacetic acid
FITC-UEA-1	Fluorescein isothiocyanate-conjugated ulex europaeus agglutinin-1
FBS	Foetal bovine serum
HAs	Human astrocytes
HBSS	Hank's balanced salt solution
HPs	Human pericytes
H₂O₂	Hydrogen peroxide
HBMECs	Human brain microvascular endothelial cells
NOX	NADPH oxidase
NVU	Neurovascular unit
NADPH	Nicotinamide adenine dinucleotide phosphate
N₂	Nitrogen
OECs	Outgrowth endothelial cells
O₂	Oxygen
OGD	Oxygen-glucose deprivation
OGD+R	Oxygen-glucose deprivation followed by reperfusion
ROS	Reactive oxygen species
rtPA	Recombinant tissue plasminogen activator
NaF	Sodium fluorescein
O₂^{•-}	Superoxide anion
SEM	Standard error mean
SOD	Superoxide dismutase
TEER	Transendothelial electrical resistance

Abstract

Disruption of blood-brain barrier (BBB), formed mainly by human brain microvascular endothelial cells (HBMECs), constitutes the major cause of mortality following ischaemic stroke. This study investigates whether OECs (outgrowth endothelial cells) can restore BBB integrity and function following ischaemic damage, and how inhibition of NOX2, a main source of vascular oxidative stress, affects the characteristics of BBB established with OECs and HBMECs in ischaemic settings. *In vitro* models of human BBB were constructed by co-culture of pericytes and astrocytes with either OECs or HBMECs before exposure to oxygen-glucose deprivation (OGD) alone or followed by reperfusion (OGD+R) in the absence or presence of NOX2 inhibitor, gp91ds-tat. The function and integrity of BBB were assessed by measurements of paracellular flux of sodium fluorescein (NaF) and transendothelial electrical resistance (TEER), respectively. Treatment with OECs during OGD+R effectively restored BBB integrity and function. Compared to HBMECs, OECs possessed lower NADPH oxidase activity, superoxide anion levels, and had greater total antioxidant capacity during OGD and OGD+R. Inhibition of NADPH oxidase during OGD and OGD+R restored the integrity and function of BBB established by HBMECs. This was evidenced by reductions in NADPH oxidase activity and superoxide anion levels. In contrast, treatment with gp91ds-tat aggravated ischaemic injury-induced BBB damage constructed by OECs. In conclusion, OECs are more resistant to ischaemic conditions and can effectively repair cerebral barrier following ischaemic damage. Suppression of oxidative stress through specific targeting of NOX2 requires close attention while using OECs as therapeutics.

Keywords: Oxidative stress; NADPH oxidase; NOX2; Outgrowth endothelial cells; OECs; ischaemic stroke; blood-brain barrier; cell therapy; cytoskeleton; reactive oxygen species.

Introduction

Ischaemic stroke continues to be a major cause of morbidity and mortality worldwide (1, 2). Thrombolysis with recombinant tissue plasminogen activator (rt-PA) remains the only pharmacological therapeutic option for this devastating disease (3, 4). Unfortunately, due to the narrow therapeutic window to administer rt-PA (within the first 4.5 h of symptoms onset) and the increased risk of haemorrhage beyond this point, each year less than 1% of patients receive this therapy worldwide (5, 6). Since many clinical trials evaluating the therapeutic capacity of a vast range of chemicals have failed to discover a new agent that can be used with or as an alternative to rt-PA, a large number of studies are now exploring the therapeutic role of an array of cell-based approaches such as that with endothelial progenitor cells (EPCs). By differentiating into few cell lines, notably endothelial cells, EPCs may help repair stroke-induced (neuro)vascular damage and reduce the volume of cerebral oedema, the main cause of morbidity and mortality after an ischaemic stroke (7). EPCs are released into circulation from the bone marrow in response to an ischaemic injury (8). Depending on the time required for them to emerge on cell culture plates after seeding, the EPCs are classified into two subtypes, namely early EPCs which display spindle-shaped morphology and late EPCs or outgrowth endothelial cells (OECs) which display cobblestone morphology and possess angiogenic properties (9-11). Our recent study assessing the reparative effect of OECs on endothelial layer of a well-established *in vitro* model of human blood-brain barrier (BBB), composed of human brain microvascular endothelial cells (HBMECs), astrocytes and pericytes, has shown for the first time that exogenous OECs can restore integrity and function of BBB by physically incorporating into the site of endothelial injury. In addition, this study has also shown that, similar to HBMECs, when co-cultured with astrocytes and pericytes, OECs can also form a functional BBB (12, 13). Since endothelial cells constitute the major cellular element of human

BBB, any pathology capable of altering their phenotype or viability can also affect the function and integrity of this distinctive barrier (7, 14, 15).

Oxidative stress, associated with overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\bullet-}$), is considered as one of the main mechanisms that adversely affect cerebral barrier after an ischaemic stroke (3). ROS not only influence the intracellular redox environment but also play a major role in the regulation of cellular proliferation, differentiation, self-renewal, senescence, and apoptosis (16-18). NADPH oxidase (NOX) constitutes the major enzymatic source of ROS in vasculature, with many studies reporting that NOX2 (previously known as gp91^{phox}) is the main element required for ROS generation (16, 19-21). Compared to mature endothelial cells, EPCs have been shown to express higher levels of antioxidant enzymes catalase, Mn-containing superoxide dismutase (Mn-SOD), and glutathione peroxidase (22) which naturally potentiate their vasoreparative and angiogenic capacity in ischaemic (micro)environments, suggesting that EPCs may be efficacious therapeutics following ischaemic stroke. Using a triple culture model of human BBB, composed of astrocytes and pericytes with HBMECs, the current study has explored how OECs can tolerate unfavourable microenvironment of ischaemic injury-induced oxidative stress, compared to HBMECs. In addition, it has investigated whether inhibition of oxidative stress by specifically targeting NOX2 subunit of NADPH oxidase can preserve the barrier integrity and function in experimental settings of ischaemic injury. It has also assessed whether such suppression of NADPH oxidase activity in OECs might regulate barrier characteristics in a different manner. In the latter studies, to assess the precise impact of NADPH oxidase-mediated oxidative stress on barrier-forming capacity of OECs, these cells were used on their own rather than in mixture with HBMECs.

Materials and Methods

Cell culture

HBMECs, human pericytes (HPs), and human astrocytes (HAs) were purchased from TCS CellWorks Ltd (Buckingham, UK) and cultured in their specialised media (Sciencell, Caltag Systems, Buckingham, UK) in a humidified condition (20% O₂, 75% N₂, 5% CO₂) at 37°C.

Isolation and expansion of outgrowth endothelial cells

Peripheral blood mononuclear cells isolated from a 24-mL blood sample were seeded on 0.01 mg/mL fibronectin-coated 12-well plates and cultured in endothelial basal medium-2 (EBM-2) containing 20% foetal calf serum and all the supplements provided with the media. Non-adherent cells were removed after 48 hours, and culture media was changed every other day until colonies appeared. Once, sufficient numbers of cells sprouted from these colonies (~2000 cells/per colony determined by a rough visual estimate or 1 week after the identification of the first outgrowth colony), the cells were trypsinised and cultured to confluence in EBM-2. This protocol was very effective in identifying the true EPCs with actual endothelial properties as previously shown (6, 12).

Ethics approval and consent

OECs obtained from the peripheral blood samples of ten healthy volunteers (18-64 years old; 6 males and 4 females) recruited for The Dunhill Medical Trust EPC study (NCT02980354) were used in this study. The study protocol was reviewed and approved by West Midlands - Coventry & Warwickshire Research Ethics Committee (16/WM/0304). Written informed consent was obtained from all participants. Details of the study rationale, design, and inclusion/exclusion criteria have already been reported elsewhere (23).

Oxygen-glucose deprivation

Cells cultured to ~90% confluence before their exposure to 4 h of oxygen-glucose deprivation (OGD; 94.95% N₂, 0.05% O₂, 5% CO₂) in the absence or presence of gp91ds-tat (50 µM) (24-26). During OGD, cells were maintained in D-glucose free medium (RPMI 1640, Sigma, Dorset, UK). To induce reperfusion (OGD+R) which lasted for 20 h, cells were returned to normoxic conditions and OGD media was replaced with relevant specialised media for each cell type. To assess the endothelium-reparative capacity of OECs during ischaemic injury, in some experiments OECs (10⁴ cells) were added to serum-free media during OGD+R.

Immunocytochemistry

HBMECs and OECs (5x10⁴) were cultured to about 95% confluence on coverslips coated with fibronectin (1mg/ml, Sigma) using endothelial growth medium 2 (EGM-2 medium) containing vascular endothelial growth factor (VEGF) (5 µl/ml, Fisher Scientific, Loughborough, UK). Cells were then successively stained with DiI-labelled acetylated-low density lipoprotein for 4 h (DiI-AcLDL, 1mg/ml, Invitrogen, Loughborough, UK) and with FITC-conjugated Ulex europaeus agglutinin (FITC-UEA-1, 1mg/ml, Sigma) for 2 h. The cells were then fixed with 4% formaldehyde before mounting coverslips on slides using a mounting medium (Vector Laboratories, Peterborough, country).

To study cytoskeletal organisation, F-actin filaments were stained in ~80% confluent cells grown on coverslips. All cells were fixed in 4% paraformaldehyde/PBS for 20 min and then permeabilised in 0.1% Triton X-100/PBS for 15 min prior to staining for 60 min in 1x rhodamine phalloidin (Abcam, Cambridge, UK) and viewing with a fluorescence microscope (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK). The stress fibres were counted manually in five random microscopic fields using ImageJ software. The average numbers were calculated for each experimental group using data collected from three independent experiments.

Matrigel tube formation assay

To assess the *in vitro* angiogenic activity of OECs and HBMECs, same passage number cells were seeded in 48-well plates (9×10^4 cells/well) pre-coated with growth factor-reduced Matrigel (BD Biosciences). Following 8 h of culture, tube formation was studied using a light microscope. Pictures of tubes, defined as the appearance of a circle-like structure, were taken from five randomly selected fields per well.

Measurements of NADPH oxidase activity and total superoxide levels

The activity of NADPH oxidase and total $O_2^{\bullet-}$ levels were detected by lucigenin chemiluminescence and cytochrome C reduction assays, respectively (27-29). Cell pellets were resuspended in 200 μ l of ice-cold lysis buffer containing monobasic potassium phosphate (20 mM, pH 7.0), ethylene glycol tetraacetic acid (EGTA, 1mM), leupeptin (0.5 μ g/ml), aprotinin (10 μ g/ml), phenylmethylsulfonyl fluoride (PMSF, 0.5 mM) and protease inhibitor.

For NADPH oxidase activity, an equal amounts of homogenate (100 μ g) were incubated in assay buffer containing lucigenin (5 μ M), sucrose (150 mM), EGTA (1 mM) and potassium phosphate buffer (50 mM) at 37 °C. Specific inhibitors for other ROS-generating enzymes, namely L-nitro-arginine methyl ester (100 μ M, nitric oxide synthase), rotenone (50 μ M, mitochondrial respiratory chain complex 1), allopurinol (100 μ M, xanthine oxidase) and indomethacin (50 μ M, cyclooxygenase) were then added to assay buffer for 15 min. NADPH (100 μ M) was then added to initiate the reaction. The luminescence was monitored for 2 h using FLUOstar Omega plate reader (BMG labtech Ltd., UK). Buffer blank readings were subtracted from the experimental readings before the final rates were obtained per mg protein. For the $O_2^{\bullet-}$ levels, an equal amounts of protein 100 μ g were incubated for one hour with 50 μ M cytochrome C at 37°C before absorbance measurement at 570 nm on plate reader and blanks were also subtracted to obtain final readings.

Measurement of total antioxidant capacity

A total antioxidant capacity assay kit (Abcam, Cambridge, UK) was used to quantify the antioxidant capacity of both HBMECs and OECs. The assay was carried out according to the manufacturer's instructions. In brief, about 90% confluent cells subjected to different experimental conditions were washed with ice-cold PBS and resuspended in double distilled water (ddH₂O). The mixture was then homogenised by pipetting up and down for few times. The homogenate was then incubated for 10 minutes on ice before centrifugation for 4 minutes at 4°C. The supernatant was then transferred to a new tube kept on ice. Working solution was prepared by adding 1 part of the Cu²⁺ reagent to 49 parts of the assay diluent. Also, 1 mM of Trolox Standard solution was used to prepare standard curve dilution as described by the manufacturer. 100 µl of working solution was added to all standard and sample wells before being incubated at room temperature for about 90 minutes. Then plates measured in microplate reader at absorbance of OD 570 nm.

Construction of *in vitro* model of human blood-brain barrier

In vitro models of human BBB were established by co-culture of HAs and HPs with HBMECs or OECs (28, 30-32). First, about 7.5x10⁴ HA were seeded on the outer surface of Transwell inserts (polyester membrane, 0.4 µm pore size, 12 mm diameter, Corning Costar, High Wycombe, UK) placed upside down in a 6-well tissue culture plate. On the following day, the inserts were inverted and placed in a sterile 12-well plate containing fresh medium to grow to ~90% confluence. HBMECs or OECs (~5x10⁴ cells) were then cultured in the inner part of the membrane in their specialised medium until both cell layers reached full confluence. The inserts were then transferred to a fresh 12-well plate containing confluent HPs to establish the BBB model.

Assessment of BBB integrity and function

The integrity and function of the BBB were assessed by measurements of transendothelial electrical resistance (TEER) and paracellular flux of low molecular weight permeability marker, sodium fluorescein (NaF, 376 Da), respectively. An EVOM resistance meter and STX electrodes (World Precision Instruments, Hertfordshire, UK) were used to assess TEER. Then the inserts were washed with Hank's Balanced Salt Solution (HBSS, Sigma) and placed in a fresh 12-well plates containing 2 ml of HBSS. NaF (10 $\mu\text{g/mL}$) was then added to the luminal chambers of the BBB and samples were removed every 20 min from luminal and abluminal chambers to measure NaF levels by fluorometry (excitation/emission at 440/525 nm) using a FLUOstar Omega plate reader (BMG labtech Ltd., UK). The flux across the inserts was calculated as previously described (27).

Statistical analyses

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

Results

OECs display true endothelial characteristics

Similar to mature endothelial cells i.e., HBMECs, OECs also display cobblestone morphology and form well-established and clearly identifiable tubules on Matrigel (**Fig. 1A**). Furthermore,

both HBMECs and OECs stain positively with *Ulex europaeus* agglutinin and endocytose DiI-AcLDL (**Fig. 1B**).

Oxygen-glucose deprivation differentially regulates NADPH oxidase activity and $O_2^{\bullet-}$ release in HBMECs and OECs

Compared to HBMECs, the OECs possess significantly lower basal levels of NADPH oxidase activity. OGD significantly increased NADPH oxidase activity in both HBMECs and OECs. Even so, level of activity in OECs remained significantly lower than HBMECs. While reperfusion diminished OGD-mediated increases in enzyme activity in HBMECs, the activity of this enzyme remained significantly higher than similar OEC group. Treatments with gp91ds-tat substantially diminished the effects of OGD and OGD+R on NADPH oxidase activity in HBMECs. In contrast, gp91ds-tat enhanced NADPH oxidase activity in OECs subjected to OGD but not OGD+R compared to controls (**Fig. 2A**).

Similar to NADPH oxidase, OGD increased $O_2^{\bullet-}$ release in both cell lines. The impact of OGD+R on $O_2^{\bullet-}$ release was cell-specific in that it slightly but insignificantly increased $O_2^{\bullet-}$ levels in HBMECs compared to OGD alone group while normalising its release in OECs. Treatments with gp91ds-tat, on the other hand, substantially reduced $O_2^{\bullet-}$ release in HBMECs exposed to OGD or OGD+R. In contrast, gp91ds-tat produced a slight but insignificant increase in $O_2^{\bullet-}$ levels in OECs subjected to OGD or OGD+R (**Fig. 2B**).

Effect of oxygen-glucose deprivation followed by reperfusion on cytoskeletal formation

Under normal conditions, both HBMECs and OECs display cortical actin staining. Exposure of cells to OGD or OGD+R induced greater actin stress fibres formation in HBMECs compared to OECs. Treatments with gp91ds-tat (50 μ M) suppressed stress fibre formation in HBMECs

subjected to either OGD or OGD+R. The impact of OGD or OGD+R on stress fibre formation in OECs was insignificant. Treatments with gp91ds-tat exacerbated stress fibre formation in both settings (**Fig. 3A-B**).

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

Compared to HBMECs, the OECs possess significantly higher basal total antioxidant capacity. While exposure to OGD or OGD+R had no effect on OEC total antioxidant capacity, exposure to OGD produced a significant decrease in HBMECs total antioxidant capacity which was normalised by reperfusion (**Fig. 4**).

OECs restore cerebral barrier integrity and function following OGD+R

In our previous studies, we had shown that exogenously added OECs could repair wound scratch-induced damage to endothelial monolayers and endothelial layer of a triple culture model of BBB composed of HAs, HPs and HBMECs (12). The present study shows that OECs added to the abovementioned triple culture model during ischaemic injury, mimicked by OGD+R, also negates the deleterious effects on barrier integrity and function as ascertained by measurements of TEER and NaF paracellular flux (**Fig. 5A-B**).

Effect of NOX2 inhibition on BBB in experimental settings of ischaemic injury

Similar to previously established triple culture model of human BBB consisting of HBMECs, HAs and HPs, concurrent culture of OECs with HAs and HPs also led to formation of an equally tight and functional *in vitro* model of human BBB, indicating establishment of tight junction complexes between adjacent OECs. Exposure to OGD significantly impaired the BBB integrity and function in both models as evidenced by decreases in TEER and increases in paracellular flux of NaF. While restoration of normal oxygen and glucose levels (reperfusion for 20 h)

worsened barrier damage established with HBMECs, it significantly improved barrier integrity and function in OECs compared to corresponding OGD treatment groups but failed to normalise them. Inhibition of NADPH oxidase by gp91ds-tat improved the integrity and function of BBB established with HBMECs in both ischaemic and ischaemia/reperfusion settings without improving them in OEC model subjected to OGD or OGD+R (**Fig. 6A-B**).

Discussion

Oxidative stress, characterised by the excessive availability of ROS, represents one of the key mechanisms involved in the pathophysiology of BBB damage following an ischaemic stroke (33-39). As the breakdown of BBB and ensuing formation of cerebral oedema constitute the main cause of death within the first week after an ischaemic stroke (10), targeting specific mechanisms or elements implicated in the pathogenesis or exacerbation of oxidative stress may be of pivotal importance to reduce mortality as well as the risk and severity of post-ischaemic cerebral events. Given that endothelial cell dysfunction and opening of the inter-endothelial junctional complexes account for much of the early BBB damage, restoration and preservation of vascular endothelium are thought to be of significant therapeutic potential. Re-endothelialisation of cerebral barrier either by proliferation and lateral migration of resident endothelial cells or by incorporation of circulating progenitor cells (OECs) into endothelial layer play a pivotal role in this context. By releasing trophic factors and differentiating into mature endothelial cells, OECs induce angiogenesis following ischaemic damage and help restore normal endothelial function (13). In this regard, our recent study has demonstrated that OECs can readily home into the site of endothelial injury and restore *in vitro* BBB integrity and function. Indeed, in accordance with our previous studies, the present study also shows that, similar to mature endothelial cells like HBMECs, OECs also display cobblestone morphology,

bind to specific lectins, endocytose Dil-AcLDL and form tube like structures on Matrigel (11, 12).

Through employment of an *in vitro* model of human BBB in previous studies, we have successfully identified various signalling pathways involved in ischaemic injury-mediated breakdown of the BBB such as protein kinase C- β and NADPH oxidase (12, 27, 39, 40). As continuation of these studies, the current study has explored whether OECs function differently in experimental settings of ischaemia-reperfusion injury, mimicked by oxygen-glucose deprivation (OGD) alone or followed by reperfusion (OGD+R) and effectively restore BBB integrity and function. In addition, this study has explored whether targeting NOX2 activity, a key member of NADPH oxidase family which mainly responsible for vascular oxidative stress, may augment their barrier-forming capacity. In these studies, OECs, rather than OEC-HBMECs mixture, were used to establish endothelial monolayer to scrutinise the precise impact of NADPH oxidase-derived oxidative stress on barrier-forming characteristics of OECs. In this context, the current study has shown that, like HBMECs, OECs can form an equally functional *in vitro* model of human BBB when co-cultured with human astrocytes and pericytes. The study has also shown that exposure to OGD (4 h) significantly compromises the integrity and function of BBB established with either OECs or HBMECs (12). While OGD+R (20 h of reperfusion following OGD) has aggravated OGD-induced damage of BBB established with HBMECs, the BBB models established with OECs have shown a significant improvement in integrity and function following reperfusion. Intriguingly, while suppression of oxidative stress by an NOX2 inhibitor, i.e., gp91ds-tat (50 μ M), attenuated the deleterious effects of OGD and OGD+R on BBB established with HBMECs, it somewhat augmented the deleterious effects of both phenomena; OGD and OGD+R on BBB established with OECs. These results imply that a therapeutic approach targeting NOX2 while employing OECs as regenerative or restorative

compounds may be counterproductive rather than vascular protective. Studies demonstrating that NOX2 is required for the regulation of neovascularisation, VEGF and SDF-1 α -mediated angiogenesis and migratory and vasculoreparative function of EPCs add further weight to this hypothesis (16, 41-43). Furthermore, ROS released by NADPH oxidase have been shown to be a prerequisite for lineage commitment of EPCs (44). However, reports showing that inhibition of NADPH oxidase can negate hypoxia-induced EPCs dysfunction also exist (45). The present study shows that compared to mature endothelial cells, OECs possess significantly lower basal levels of O₂^{•-} and NADPH oxidase activity and higher total antioxidant capacity under normal conditions. In line with these findings, EPCs have previously been shown to possess significantly lower basal levels of ROS and higher level of antioxidant manganese superoxide dismutase (46), glutathione peroxidase and catalase activities compared to ECs (22, 47). In addition, the current study shows that, compared to mature ECs, OECs also express significantly higher total antioxidant capacity and lower NADPH oxidase activity and O₂^{•-} production during ischaemic insult. These suggest that OECs can handle oxidative stress better in ischaemic settings than mature endothelial cells, a prerequisite for their endothelium-reparative and cerebral barrier-restorative function. However, suppression of NOX2 in OECs by gp91ds-tat exacerbated the detrimental effect of OGD or OGD+R on integrity and function of the BBB where sharp increases in NADPH oxidase activity and O₂^{•-} availability appeared to play a crucial role. This might happen due to several reasons. Firstly, as stated above OECs possess low levels of NADPH oxidase activity and O₂^{•-} levels compared to HBMECs, so it is possible that treatments with gp91ds-tat may completely neutralise NADPH oxidase activity and ensuing O₂^{•-} generation and as a result trigger a negative feedback mechanism to compensate these decreases in both normal and ischaemic settings. Secondly, while NADPH oxidase represents the main enzymatic source of O₂^{•-} in HBMECs (48, 49), other pro-oxidant enzymes may also contribute to O₂^{•-} production in OECs and get activated by depletion of O₂^{•-}

via gp91ds-tat. Further studies specifically exploring these pathways in the OECs can (dis)prove the accuracy of this hypothesis. Sensitivity to agents manipulating NADPH oxidase activity has also been reported in haematopoietic stem cells which contain various self-fuelling mechanisms to maintain NADPH oxidase in an assembled state to continuously generate low levels of $O_2^{\bullet-}$ (50, 51).

Changes in cellular architecture, accompanied by stress fibre formation, during and after ischaemic insult also contribute to BBB damage. Compared to HBMECs, OECs has developed significantly lower numbers of stress fibres during OGD or OGD+R, another prerequisite that can be added to the OECs endothelium-reparative and cerebral barrier-restorative function. Co-application of gp91ds-tat with OGD or OGD+R attenuated stress fibre formation and restored cortical actin staining in HBMECs while greatly increasing their numbers in OECs subjected to OGD or OGD+R. Hypoxia-induced increases in the expression of Nox4 and Nox2 have previously been associated with the diminished proliferative, migratory, tubulogenic and adherence capability of EPCs (45). Contrary to this study, other studies have reported that hypoxia induces EPCs migration and re-endothelialisation selectively in wild type, but not NOX2 knock out, mouse model of carotid artery damage (43) and that inhibition of NOX4, a form of NADPH oxidase results in decreased proliferation, migration, and reduced survival of OECs (52). Collectively these data support the notion that a certain degree of $O_2^{\bullet-}$, derived from NOX2, is required for appropriate EPC function.

In conclusion, our study demonstrates that compared to HBMECs, OECs have higher antioxidant capacity, lower basal levels of NADPH oxidase activity and $O_2^{\bullet-}$ generation, and perform better during the reperfusion, supporting the idea that progenitor cells can work effectively in ischaemic environments and may therefore be reliable therapeutics to repair neurovascular damage. It also demonstrates that inhibition of NOX2 in microvascular

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

There are some limitations to this study. Firstly, given the cell-specific unique correlation between NOX2 inhibition and NADPH oxidase activity and $O_2^{\bullet -}$ generation, it would have been beneficial to study the total antioxidant capacity in HBMECs and OECs in the presence of gp91ds-tat to elucidate whether differences in total antioxidant capacity contribute to this phenomenon. Secondly, albeit the link between NADPH oxidase activity and the expression of its membrane-bound components, i.e. NOX2 and p22-phox, is well-established in endothelial cells, the analysis of NOX2 expression, particularly in OECs, would have been of value (29, 31, 53-55). Finally, considering that treatments with OECs or NOX2 inhibitor effectively restore ischaemia-evoked *in vitro* BBB damage, it might have been a good idea to assess whether attenuation of NOX2 during OGD and subsequent addition of OECs during reperfusion may have provided additional benefits. Future studies are needed to explore these points and are required to assess whether a therapeutic approach targeting NOX2 may be useful in an animal model of human transient ischaemic stroke.

Data availability statement

The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

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Figure Legends

Fig. 1. Morphological, tubulogenic features, immunocytochemical analyses of HBMECs and OECs. (A) HBMECs and OECs show cobblestone morphology (a and b) and form well-established tubules on Matrigel (c and d). (B) both cells stain positively with FITC-conjugated *Ulex europaeus* agglutinin (a and d) and endocytose Dil-labelled acetylated-low density lipoprotein (Dil-AcLDL) (b and e). Merged images reveal the pattern of staining in both cell types (c and f). Scale bars: 100 μ m.

Fig. 2. Effect of ischaemia-reperfusion injury on NADPH oxidase activity and superoxide generation in HBMECs and OECs

HBMECs or OECs were exposed to OGD (4 h) alone or followed by reperfusion (20 h) in the absence or presence of gp91ds-tat, given at the beginning of respective treatments. NADPH oxidase activity and superoxide anion levels were measured by lucigenin chemiluminescence and cytochrome C reduction assays, respectively (A and B). Data are illustrated as fold-difference to OEC controls which were regarded as “1”. Data are expressed as mean \pm SEM from three independent experiments. * $P < 0.05$ between any two groups, # $P < 0.05$, control vs gp91ds-tat group, + $P < 0.05$ vs corresponding HBMEC group. One-way analysis of variance followed by Tukey’s post hoc analysis were used to compare variations across different HBMEC or OEC experimental groups. Unpaired t-test was used to compare OEC groups to corresponding HBMEC groups. NS: not significant.

Fig. 3. Status of F-actin microfilament staining in HBMECs and OECs subjected to experimental ischaemia-reperfusion injury

HBMECs or OECs were exposed to OGD (4 h) alone or followed by reperfusion (20 h) in the absence or presence of gp91ds-tat, given at the beginning of respective treatments. Cells were fixed and permeabilised in 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS, respectively prior to staining with rhodamine phalloidin and visualisation by fluorescence microscopy (A). Data are expressed as mean \pm SEM from three independent experiments (B). *P<0.05 between any two groups, #P<0.05, control vs gp91ds-tat group, +P<0.05 vs corresponding HBMEC group. One-way analysis of variance followed by Tukey's post hoc analysis were used to compare variations across different HBMEC or OEC experimental groups. Unpaired t-test was used to compare OEC groups to corresponding HBMEC groups. NS: not significant. Stress fibres are indicated by white arrows. Scale bars: 20 μ m.

Fig. 4. Measurements of total antioxidant capacity

The total antioxidant capacity was measured in confluent HBMECs or OECs exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h). Data are expressed as mean \pm SEM from three independent experiments. Unpaired t-test was used to compare OEC groups to corresponding HBMEC groups *P<0.05 vs corresponding HBMEC group.

Fig. 5. Effects OECs therapy on ischaemic injury-evoked BBB damage

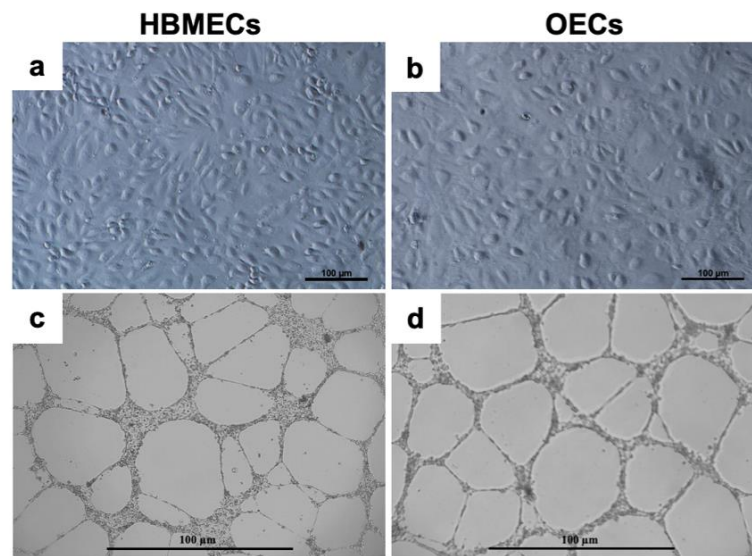
A triple culture model of human BBB composed of human astrocytes, pericytes and microvascular endothelial cells were exposed to 4 h of OGD followed by 20 h of reperfusion (OGD+R) in the absence or presence of OECs (10^4 cells). BBB integrity and function were assessed by measurements of transendothelial electrical resistance (TEER, A) and paracellular flux of sodium fluorescein (NaF, B), respectively. Data are expressed as mean \pm SEM from

three independent experiments. * $P < 0.05$ between groups. One-way analysis of variance followed by Tukey's post hoc analysis were used to compare differences amongst experimental groups. NS: not significant.

Fig. 6. Effect of NOX2 inhibition on BBB integrity and function in experimental ischaemia

BBB models, established by co-culture of human astrocytes and pericytes with HBMECs or OECs, were exposed to OGD (4 h) alone or followed by reperfusion (OGD+R, 20 h) in the absence or presence of gp91ds-tat, introduced at the beginning of each respective treatment. BBB integrity and function were assessed by measurement of transendothelial electrical resistance (TEER, A) and paracellular flux of NaF (B), respectively. Data are expressed as mean \pm SEM from three independent experiments. * $P < 0.05$ between any two groups, # $P < 0.05$, control vs gp91ds-tat group, + $P < 0.05$ vs corresponding HBMEC group. One-way analysis of variance followed by Tukey's post hoc analysis were used to compare variations across different HBMEC or OEC experimental groups. Unpaired t-test was used to compare OEC groups to corresponding HBMEC groups. NS: not significant.

Fig. 1 A



B

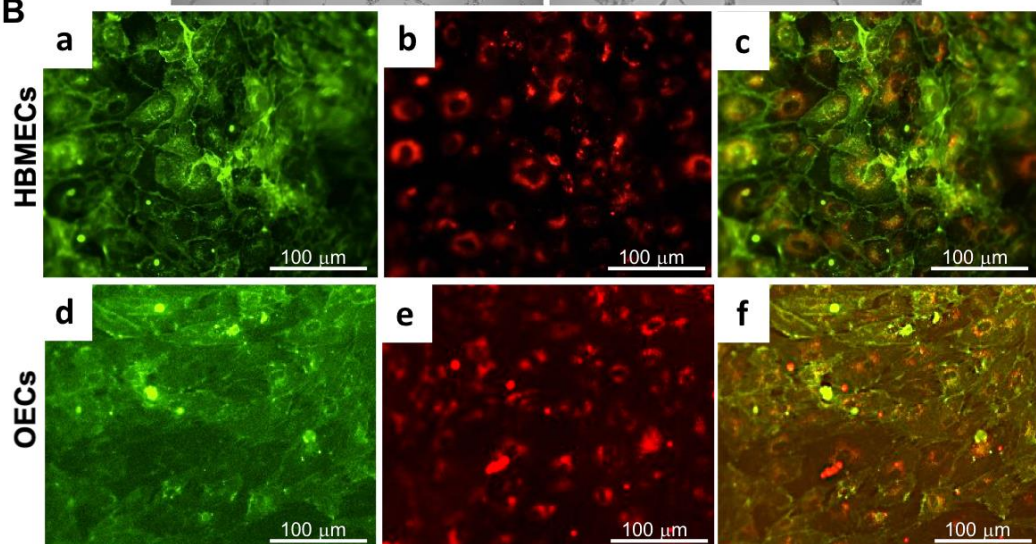


Fig. 2

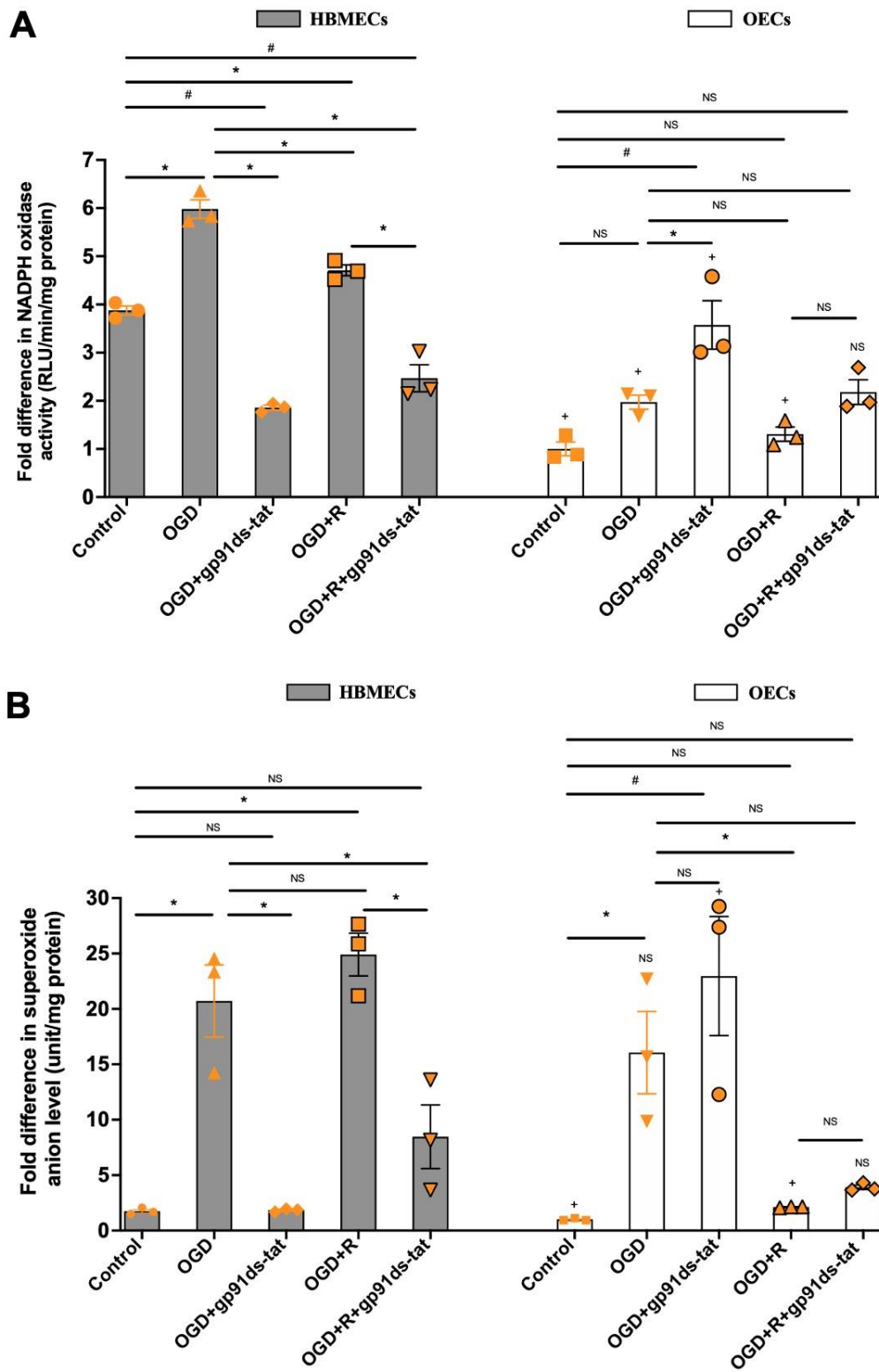
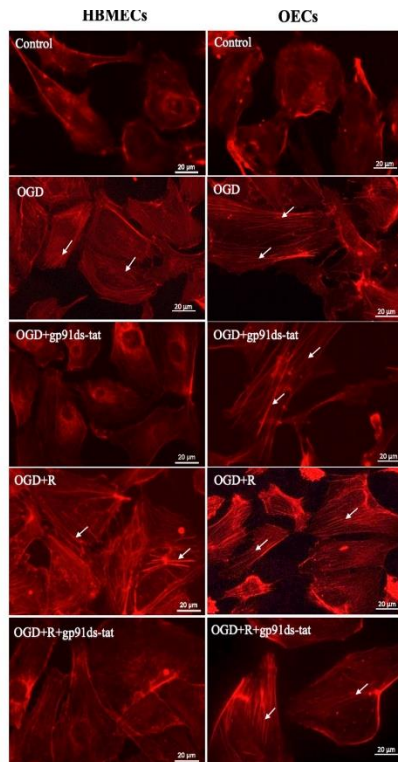


Fig. 3 A



B

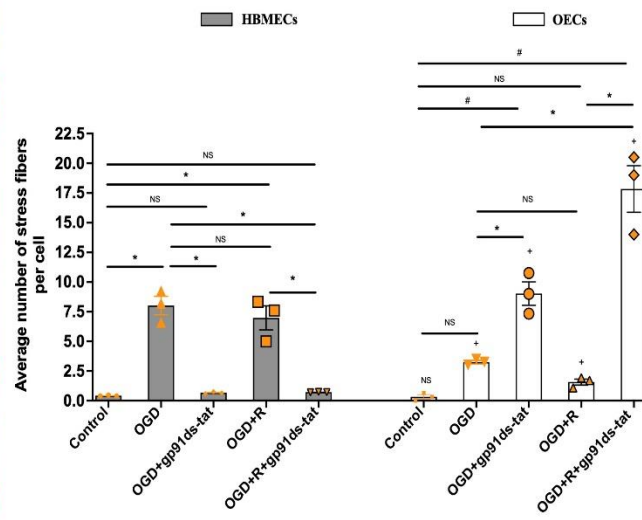


Fig. 4

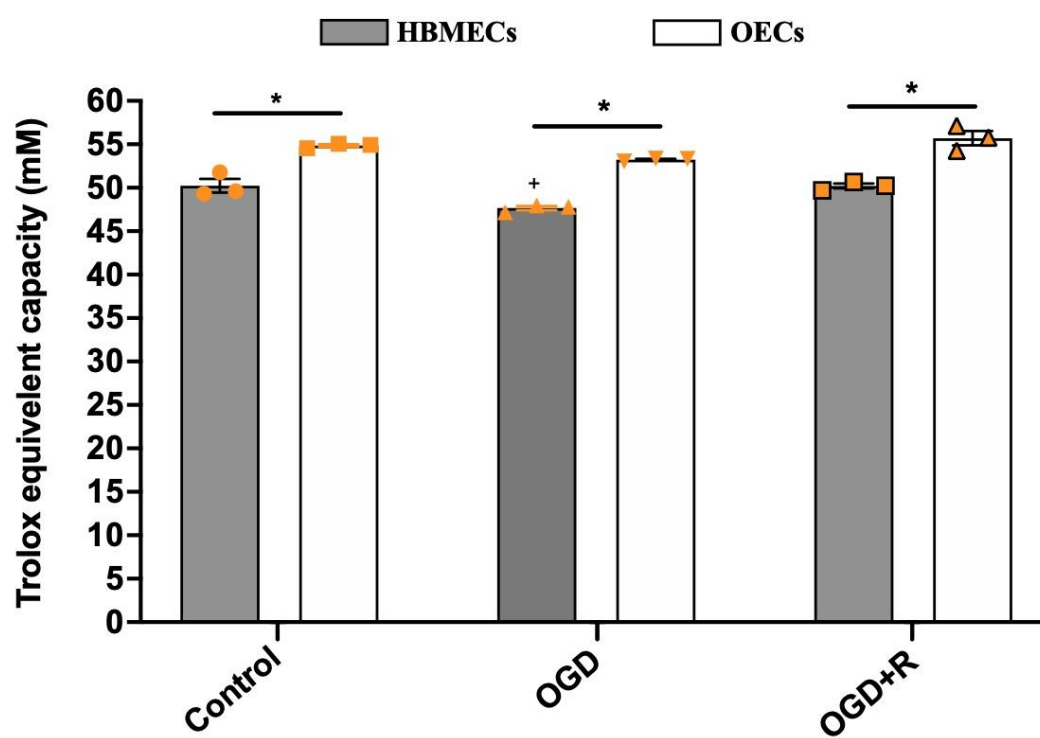


Fig. 5

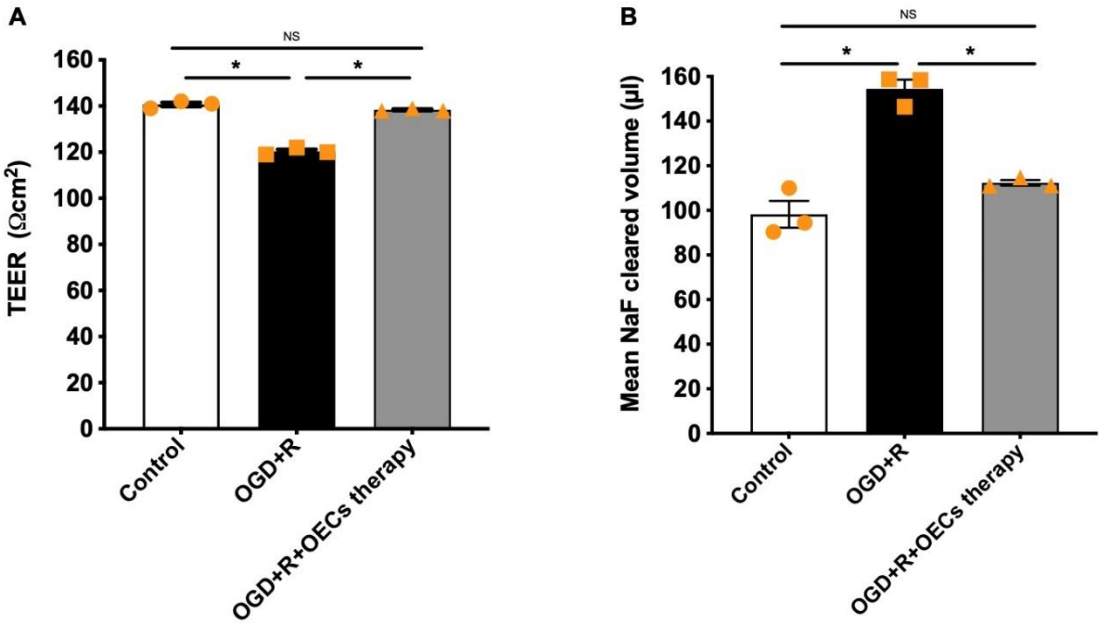


Fig. 6

