GAVIN PUBLISHERS

Journal of Neurology and Experimental Neural Science

Mathur M and Bayraktutan U, et al. J Neurol Exp Neural Sci 2016: JNENS-114.

DOI: 10.29011/JNNS-114. 100014

Research Article

Therapeutic Hypothermia Protects *In Vitro* Brain Barrier from Ischaemic Damage: Roles of Oxidative Stress, Rho-Kinase and PKC-β

Manik Mathur PhD, Ulvi Bayraktutan* PhD

Stroke, Division of Clinical Neuroscience, School of Medicine, University of Nottingham, UK.

***Corresponding author:** Dr Ulvi Bayraktutan, Associate Professor, Stroke, Division of Clinical Neuroscience, Clinical Sciences Building, University of Nottingham, Hucknall Road, Nottingham, NG5 1PB, United Kingdom. Tel: +44 (115)8231764; Fax: +44 (115)8231767; E-mail: Ulvi.Bayraktutan@nottingham.ac.uk.

Citation: Mathur M and Bayraktutan U (2016) Therapeutic Hypothermia Protects ***In Vitro*** Brain Barrier from Ischaemic Damage: Roles of Oxidative Stress, Rho-Kinase and PKC-β. J Neurol Exp Neural Sci 2016: JNENS-114. DOI: 10.29011/JNNS-114. 100014

Received Date: 23 November, 2016; **Accepted Date:** 23 December, 2016; **Published Date:** 30 December, 2016

Abstract

Aim: Ischaemic stroke develops through an interference with blood supply to the brain and is associated with oxidative stress-mediated damage to the blood-brain barrier (BBB). Accumulating evidence indicate that hypothermia may be of therapeutic value in minimising this damage.

Methods: An *in vitro* model of human BBB, composed of human brain microvascular endothelial cells, astrocytes and pericytes, was exposed to ischaemic settings mimicked by oxygen-glucose deprivation alone or followed by reperfusion (OGD±R) in the absence or presence of hypothermia (35°C). The integrity and function of the BBB were studied by measurements of transendothelial electrical resistance (TEER) and paracellular flux of Evan's blue-labelled albumin (EBA) across the barrier, respectively. Similar experiments were repeated in the presence of inhibitors for Rho kinase, NADPH oxidase and protein kinase C-β. Level and activity of superoxide anion and NADPH oxidase were assessed by cytochrome C reduction and lucigeninchemiluminescence assays, respectively.

Results: OGD significantly enhanced superoxide anion release and NADPH oxidase activity in all cells which were attenuated by hypothermia applied during or after ischaemic insult. OGD±R compromise BBB integrity and function as evidenced by decreases in TEER and increases in EBA flux respectively. Both intra- and post-ischaemic treatments with hypothermia attenuated the OGD±R-mediated damage on BBB and increased the activities of both CuZn- and Mn-containing superoxide dismutases in all cells.

Conclusion: Therapeutic hypothermia may protect neurovascular integrity during intra- and post-ischaemic injury by concomitantly reducing oxidative stress and enhancing antioxidant capacity.

Keywords: Hypothermia; Ischaemic Stroke; Oxidative Stress; Blood-Brain Barrier; NADPH Oxidase

Introduction

Ischaemic stroke develops through an interference with blood supply to the central nervous system and continues to be one of the leading causes of morbidity and mortality in the Western World. Disruption of the blood-brain barrier (BBB) integrity and ensuing formation of cerebral oedema account for most of the mortalities observed within the first week after an ischaemic stroke. Despite being a leading cause of cerebrovascular damage, thrombolysis with recombinant-tissue plasminogen activator (r-tPA) remains the only pharmacotherapy for this debilitating condition. However, due to a short therapeutic window, namely the first 4.5 h of stroke onset, only 3%-5% of ischaemic stroke patients receive this treatment. Hence, it is of pivotal importance to discover new therapeutic agents and/or approaches to treat ischaemic stroke beyond the acute phase of the disease. In this context, hypothermia has long been considered as an efficacious therapeutic option in cases of ischaemia caused by cardiac arrest or hypoxic-ischaemic

encephalopathy in neonates [1,2]. However, its efficacy in a longitudinal stroke study and the molecular mechanisms involved in putative barrier-protective effects remain largely unknown.

Accumulating evidence indicate that much of the ischaemic injury-evoked cellular damage occurs during restoration of blood flow to the hypo-perfused tissue in that oxidative stress plays a crucial role. Oxidative stress is defined by an imbalance between oxidants and the antioxidants and is characterized, at cellular level, by the excessive availability of superoxide anion (O_2^-) and other reactive oxygen species (ROS) which react with proteins, lipids and nucleic acids and thus compromise the integrity and function of the BBB [3,4]. NADPH oxidase represents the main enzymatic source of O_2^- in the vasculature and is closely implicated in breakdown of the BBB under experimental conditions that mimic ischaemic stroke like oxygen-glucose deprivation alone or followed by reperfusion (OGD±R) and in the presence of key pathologies associated with ischaemic injury e.g. hyperglycaemia and inflammatory cytokines [5-10]. Indeed, suppression of NADPH oxidase activity by apocynin in all these conditions has proven to be extremely efficacious in protecting BBB from damage or restoring its integrity and function, once the damage has occurred [10].

A decrease in ATP production emerging from a sudden decline in oxygen and glucose delivery alongside a concomitant increase in H^+ due to cellular acidification also adversely affect barrier integrity during the acute phase of an ischaemic stroke by impairing mitochondrial function and promoting glial cell death. Moreover, increased local availability of H^+ also triggers rapid dismutation of O_2^- into hydrogen peroxide (H_2O_2), another ROS, by a class of antioxidant enzymes called superoxide dismutases. Both enhanced formation of H_2O_2 and anomalies in the activity and/or expression of CuZn- and Mn-containing isoforms of SODs have previously been implicated in the pathogenesis of ischaemic stroke and the severity of brain oedema following an ischaemic insult [11,12].

The extent of barrier damage under ischaemic conditions may be determined by concurrent regulation of a series of distinct but interrelated signaling pathways in major cell lines that make up the BBB, notably brain microvascular endothelial cells, astrocytes and pericytes [13]. In this regard, Rho-kinase, the downstream effector of small GTPaseRhoA and protein kinase C-β (PKC-β) have attracted much of the attention in recent years [5]. Despite its regulatory involvement in cell proliferation, shape, motility and apoptosis, Rho-kinase has also been implicated in stress fibre formation, myosin light chain (MLC) phosphorylation and nitric oxide availability and consequent openings of interendothelial cell junctions [14,15]. Similarly, inhibition of PKC-β has been shown to prevent BBB damage through regulation of various downstream effectors including NADPH oxidase in brain microvascular endothelial cells [10]. Because the inhibition of RhoA/Rho kinase, NADPH oxidase or PKC-β negates the deleterious effects of ischaemic injury on the BBB [14,16], it is possible that their co-administration may augment the barrier-protective effect of hypothermia in ischaemic settings.

Material and Methods

Human cell culture

Human brain microvascular endothelial cells (HBMEC), human astrocytes (HA) and human pericytes (HP) were purchased from TCS Cellworks (Buckingham, UK) and cultured up to passage 7. While HBMEC and HA were grown in their respective specialised media (Sciencell, Caltag Systems, Buckingham, UK) and HP were cultured using Dulbecco's Modified Eagle's Medium (DMEM) in a humidified atmosphere (95% relative humidity) under normal conditions at 37°C with 5% CO_2.

Induction of oxygen-glucose deprivation

To study the effects of intra-ischaemic and post-ischaemic injury, the confluent cells were placed in a cell culture incubator (SANYO, MCO-18M Multi-gas cell) before exposure to 94% N_2, 1% O_2 and 5% CO_2 at 37ºC. In these experiments, the growth media were replaced with glucose-free RPMI-1640 media (Life Technologies, Paisley, UK). While cells subjected to 4 or 20 h of OGD in the absence or presence of hypothermia mimicked intra- ischaemic treatments, those exposed to 4 h of OGD followed by 20 h of hypothermia mimicked post-ischaemic treatment. Cells cultured under normal conditions served as controls.

In vitromodel of human blood-brain barrier

A triple culture model of human BBB composed of HBMEC, HA and HP were set up using transwell inserts (polyster membrane, 12 mm diameter, 0.4 μm pore size, Corning Costar, High Wycombe, UK). HA were seeded on the basolateral side of polyester membrane. Following overnight adherence, inserts were transferred to 12-well plates containing fresh media and cultured to about 90% confluence. Thereafter, the HBMEC were seeded onto the inner part of the same inserts. Once full confluence achieved in both cell layers, the inserts were then transferred to a fresh 12-well plate containing confluent HP to establish the triple culture model.

Measurement of BBB integrity and function

BBB integrity and function were studied by measurements of transendothelial electrical resistance (TEER) via an Ohm's meter and paracellular flux, respectively. To study permeability across the barrier, high molecular weight permeability marker (Evan's blue-labelled albumin, EBA: 67 kDa, 0.5 mL) was gently added to the inserts and incubated for 1 h at 37°C. Following incubation, samples were collected from the lateral and basolateral sides (400

µL). The concentration of EBA molecules in the samples was measured by spectrophotometry (BMG LABTECH Omega, 610 nm). The flux of EBA was calculated as cleared volume by the formula: Cleared volume (µL) = concentration (abluminal reading) × volume (abluminal) × concentration (luminal reading)$^{-1}$.

Similar experiments were carried out in the presence of inhibitors for NADPH oxidase (apocynin 1mmol/L; Calbiochem, Nottingham, UK), Rho-kinase (Y-27632 (2.5µmol/L, Calbiochem, Nottingham, UK) and PKC-β (LY333531, 0.05µmol/L; Alexis Biochemical; Bingham, UK).

Measurement of O_2^- generation

The levels of O_2^- were detected by cytochrome C reduction assay as previously described [6]. In brief, cell pellets were sonicated in cold lysis buffer containing 20 mmol/L HEPES buffer (pH 7.2), 1 mmol/L EGTA, 210 mmol/L mannitol and 70 mmol/L sucrose. Equal amounts of homogenate (50 µl) were then incubated with 50 µmol/L cytochrome C for 1 h at 37°C. Superoxide anion generation was measured as the reduction of cytochrome C and monitored as the change in absorbance at 550 nm using a FLUOstar Omega plate reader (BMG, Aylesbury, UK).

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured by lucigenin-chemiluminescence assay as before [9]. Briefly, cell homogenates (50 µl) were incubated at 37°C with assay buffer containing 50 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EGTA, 150 mmol/L sucrose, 5 µmol/L lucigenin and the specific inhibitors of enzymes known to generate O_2^-, namely nitric oxide synthase (L-NAME, 100 µmol/L), xanthine oxidase (allopurinol, 100 µmol/L), mitochondrial complex I (rotenone, 50 µmol/L) and cyclooxygenase (indomethacin, 50 µmol/L). After 15 min NADPH (100 µmol/L; Sigma-Aldrich, Poole, UK) was added into the mix to initiate the reaction. Readings obtained for blanks devoid of homogenates were subtracted from experimental readings before calculating relative luminescence unit by normalising the average luminescence values against "mg protein" levels in homogenates used.

Detection of superoxide dismutase activity

Superoxide dismutase activity was measured using a specific kit (Calbiochem, Nottingham, UK). Briefly, cell homogenates were diluted at a ratio of 1:20 with radical detector, provided in the kit containing 250 µl of tetrazolium salt and assayed in triplicate using a 96-well plate. Reactions were initiated by the addition of 20 µl diluted xanthine oxidase to generate O_2^-. The plates were then incubated on a shaker for 20 min at room temperature before reading absorbances at 450 nm. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the O_2^-. Mn-SOD activity was detected following inhibition of CuZn-SOD activity through incubation with 3 mmol/L potassium cyanide (BDH Chemicals Ltd, Dorset, UK), at room temperature for 45 min. CuZn-SOD activity was subsequently calculated by the subtraction of Mn-SOD activity from total SOD activities and normalising to "mg protein" levels in the cell homogenates used.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis were conducted using the IBM SPSS statistics 20.0 software package. Mean values were compared by student's two-tailed t-test or one-way ANOVA, where appropriate, followed by Dunnett's post-hoc testing. $P<0.05$ was considered significant.

Results

Effects of OGD on NADPH oxidase activity in the absence or presence of hypothermia

Compared to the respective control groups, exposures to 4 or 20 h of OGD significantly increased NADPH oxidase activity in HBMEC, HA and HP in a manner that was independent of cell type and OGD time. Concomitant exposure of cells to hypothermia during the ischaemic insult led to significant reductions in OGD-mediated increases in oxidase activity. Post-ischaemic application of hypothermia suppressed the level of NADPH oxidase in all cells similar to the levels obtained with 20 h of hypothermic treatment groups (Figure 1A-C).

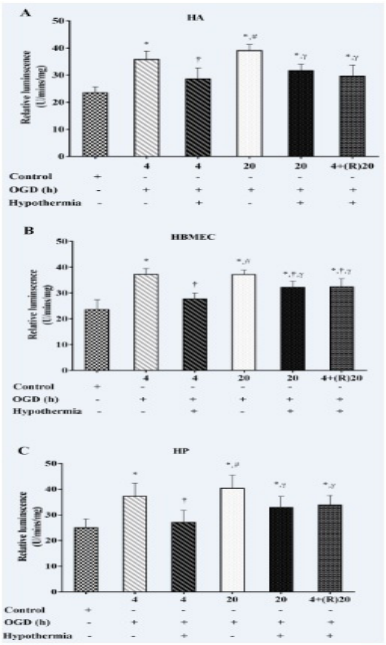

Figure 1: Effects of intra- and post-ischaemic treatments with hypothermia on NADPH-oxidase activity in human astrocytes (HA), human brain microvascular endothelial cells (HBMEC) and human pericytes (HP). Data are expressed as mean ± SEM from five independent experiments.

*P<0.05 versus controls, †P<0.05 versus 4 h OGD,#P<0.05 versus 4 h OGD + hypothermia and γP<0.05 versus 20 h OGD.

Effects of OGD on O_2^- levels in the absence or presence of hypothermia

Compared to the respective control groups, exposure of HBMEC, HA and HP to 4 or 20 h of OGD alone evoked substantial increases in O_2^- generation in all cells which were, similar to oxidase activity, not dictated by the duration of OGD and cell type. Concurrent application of intra-ischaemic or post-ischaemic hypothermia significantly reduced O_2^- generation in all cells. Post-ischaemic treatment with hypoxia normalised O_2^- levels in HA and HBMEC, an effect was also seen in HBMEC subjected to 20 h of intra-ischaemic hypothermia (Figure 2A-C).

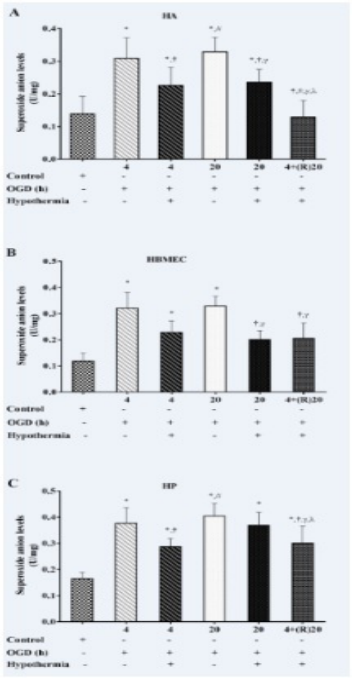

Figure 2: Effects of intra- and post-ischaemic treatments with hypothermia on superoxide anion levels in human astrocytes (HA), human brain microvascular endothelial cells (HBMEC) and human pericytes (HP). Data are expressed as mean ± SEM from six independent experiments. *P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + hypothermia, γP<0.05 vs 20 h OGD.

Effects of hypothermia on SOD activity in human astrocytes

Exposure to 4 or 20 h of OGD alone reduced both CuZn-SOD and Mn-SOD activities in HA by almost 50%. The magnitude of decreases were slightly but insignificantly greater in cells exposed to longer periods of OGD. Intra-ischaemic application of hypothermia attenuated the effect of OGD on SOD activities. Hypothermia almost completely neutralized the inhibitory effect of 4 h OGD on Mn-SOD. Again, post-ischaemic application of hypothermia prevented the effect of OGD on both SOD activities, more so in case of Mn-SOD (Figure 3A-B).

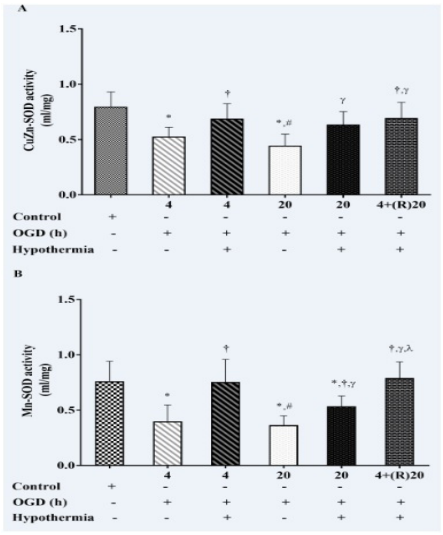

Figure 3: Effects of intra- and post-ischaemic treatments with hypothermia on CuZn-SOD and Mn-SOD activities in human astrocytes (HA). Data are expressed as mean ± SEM from six independent experiments. *P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + hypothermia, γP<0.05 vs 20 h OGD.

Effects of hypothermia on endothelial cell SOD activity

Exposure of HBMEC to OGD (4 or 20 h) produced significant decreases in CuZn-SOD and Mn-SOD activities compared to cells cultured under normal conditions. Despite level of decreases being greater in cells subjected to longer periods of OGD, only decreases in Mn-SOD activity were significantly lower compared

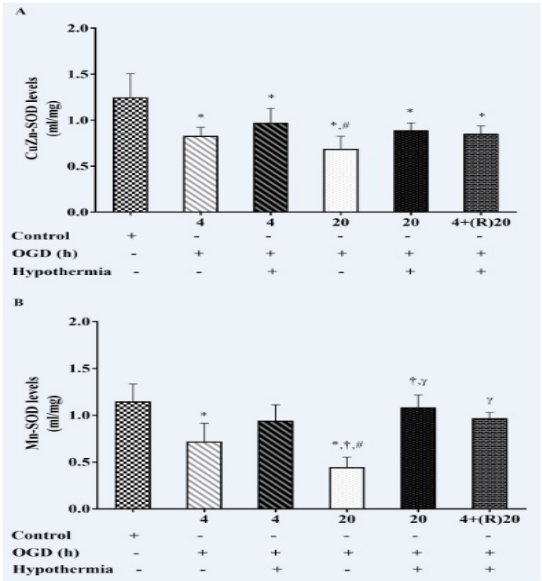

Figure 4: Effects of intra- and post-ischaemic treatments with hypothermia on CuZn-SOD and Mn-SOD activities in human brain microvascular endothelial cells (HBMEC). Data are expressed as mean ± SEM from six independent experiments.*P<0.05 vs controls, †P<0.05 vs 4 h OGD #P<0.05 vs 4 h OGD + hypothermia, γP<0.05 vs 20 h OGD.

to the corresponding 4 h OGD group. Concurrent application of hypothermia with OGD for 4 h did not greatly affect CuZn-SOD and Mn-SOD activities compared to OGD alone groups. In contrast, exposure to longer periods of hypothermia was only effective in normalising Mn-SOD activity. Similarly, post-ischaemic application of hypothermia only brought the levels of Mn-SOD activity closer to those seen in controls (Figure 4A-B).

Effects of hypothermia on SOD activity in human pericytes

Exposure of HP to 4 or 20 h of OGD led to marked decreases in Mn-SOD but not CuZn-SOD activities compared to their counterparts cultured under normal conditions. Co-application of hypothermia in intra-ischaemic settings negated the suppressive effects of OGD on CuZn-SOD and slightly but insignificantly enhanced that of Mn-SOD. Post-ischaemic treatment with hypothermia selectively potentiated the level of CuZn-SOD activity compared to those subjected to OGD alone (Figure 5A-B).

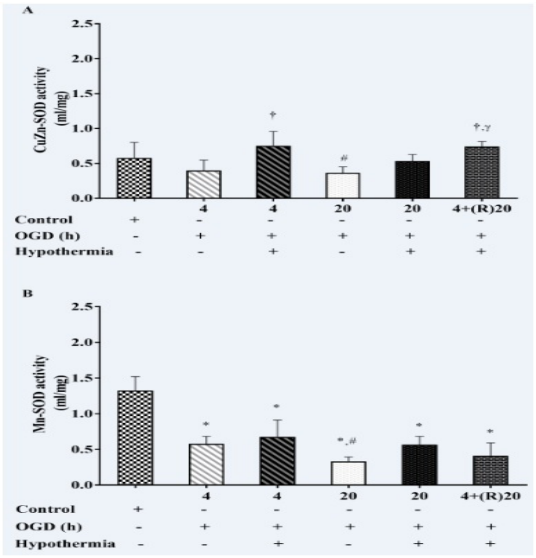

Figure 5: Effects of intra- and post-ischaemic treatments with hypothermia on CuZn-SOD and Mn-SOD activities in human pericytes (HP). Data are expressed as mean ± SEM from six independent experiments.*P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + hypothermia, ɣP<0.05 vs 20 h OGD.

Effects of hypothermia on the integrity and function of the in vitro cerebral barrier

Treatment of triple cultures with 4 h of OGD alone impaired BBB integrity and function as ascertained by significant decreases in TEER and a concomitant increase in EBA flux, respectively. Inhibition of NADPH oxidase by apocynin diminished the detrimental effects of OGD on barrier integrity and function. Combination of apocynin with hypothermia decreased the level of EBA flux below these seen in controls without further improving TEER values. The level of improvement in barrier integrity obtained with post-ischaemic hypothermia plus apocynin treatment was significantly less than corresponding treatment performed during ischaemic phase (Figure 6A-B).

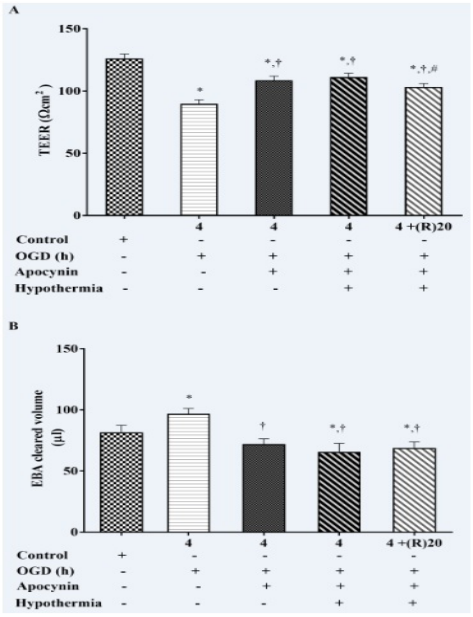

Figure 6: Effects of apocynin on blood-brain barrier integrity and function in the absence and presence of intra- and post-ischaemic hypothermia as assessed by transendothelial electrical resistance (TEER, A) and Evan's blue-labelled albuminflux (B). Data are expressed as mean ± SEM from three independent experiments.*P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + apocynin + hypothermia.

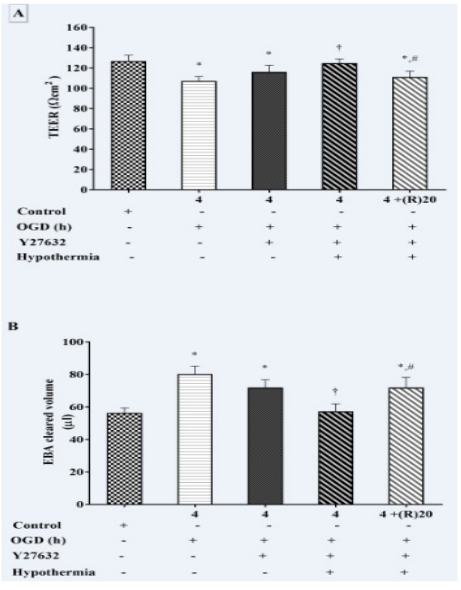

Figure 7: Effects of Y-27632 on blood-brain barrier integrity and function in the absence and presence of intra- and post-ischaemic hypothermia as assessed by transendothelial electrical resistance (TEER, A) and Evan's blue-labelled albuminflux (B). Data are expressed as mean ± SEM from three independent experiments.*P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + Y-27632 + hypothermia.

Despite attenuating the effects of OGD on barrier integrity and function, inhibition of Rho-kinase by Y-27632 alone failed to improve these parameters compared to OGD groups. However, combinatory approach with hypothermia improved both the barrier integrity and function as evidenced by further increases in TEER values and decreases in EBA flux, respectively. Post-ischaemic combinatory approach did not affect either parameter compared to OGD group (Figure 7A-B).

Treatment of triple cultures with OGD plus an inhibitor for PKC-β (LY333531) attenuated the deleterious effects of OGD on barrier which were slightly augmented by addition of hypothermia to the treatment regimen. Post-ischaemic application of hypothermia with LY333531 failed to improve barrier integrity and function compared to OGD alone group (Figure 8A-B).

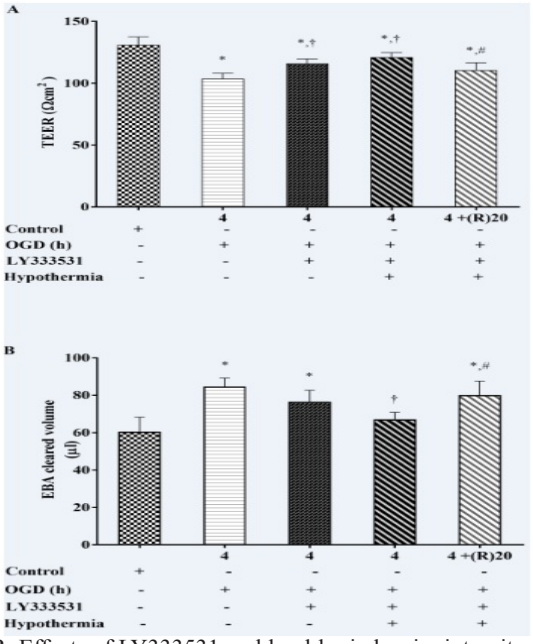

Figure 8: Effects of LY333531 on blood-brain barrier integrity and function in the absence and presence of intra- and post-ischaemic hypothermia as assessed by transendothelial electrical resistance (TEER, A) and Evan's blue-labelled albuminflux (B). Data are expressed as mean ± SEM from three independent experiments. *P<0.05 vs controls, †P<0.05 vs 4 h OGD, #P<0.05 vs 4 h OGD + LY333531 + hypothermia.

Discussion

The BBB represents a highly selective, but permeable, barrier between the systemic circulation and the brain. Various pathologies like ischaemic injury adversely affect the integrity of this barrier thereby triggering leakage of circulating components into the central nervous system and forming vasogenic oedema, the leading cause of death within the first week after an ischaemic stroke [17]. Since ischaemic strokes derive from an occlusion of blood vessels leading to or within the brain, thrombolysis with r-tPA represents the most immediate (and only available) form of pharmacotherapy at present. However, due to elevated risk of intracranial haemorrhage associated with late administration of r-tPA (beyond the first 4.5 h of an ischaemic stroke), about 3-5% of patients currently receive this therapy. Hence, discovery of novel therapeutic agents or approaches that can be applied beyond the acute phase of an ischaemic attack and (replace or) augment the efficacy of r-tPA is of critical importance. In this regard, therapeutic hypothermia, defined by a core body temperature ≤35ºC, has long been considered as an efficacious option in other ischaemic settings evoked by cardiac arrest or hypoxic-ischaemic encephalopathy where it improved both neurological and functional outcomes [1,2]. Using an in vitro model of human BBB composed of BMEC, astrocytes and pericytes, the current study assessed whether hypothermia (35ºC) could effectively preserve the integrity and function of cerebral barrier during or after an ischaemic attack, mimicked by OGD±R. Given that BMEC make up the main cellular component of the BBB and express/retain the key tight junction proteins, i.e. occludin and claudin-5, only when co-cultured with astrocytes and possibly pericytes [18], the use of triple culture model throughout this study was particularly important. Indeed, this model produced normoxic TEER values that were like those reported in related previous models and closely mimicked the characteristics of cerebral barrier, revealing profound structural and functional impairments when exposed to OGD±R as ascertained by dramatic decreases in TEER and increases in EBA flux, respectively [8,9].

Despite ambiguity of molecular mechanisms involved in ischaemic injury-induced BBB breakdown, accumulating incontrovertible evidence suggest that oxidative stress plays a crucial role in this phenomenon. Oxidative stressis defined by an imbalance between the synthesis and metabolism of ROS and may in part emerge from reintroduction of oxygenated blood to the hypoperfused tissue [8]. Although low-level generation of ROS is a prerequisite for neurovascular stability, their exaggerated release, especially by NADPH oxidase, is associated with cerebrovascular injury and haemorrhage [19,20]. Indeed, the critical role played by NADPH oxidase in modulating in vitro and in vivo cerebral barrier function under a variety of pathological phenomena including ischaemia is well documented [3,14]. Increases in certain NADPH oxidase subunit expressions and overall enzymatic activity have been shown in rats with transient middle cerebral artery occlusion, an animal model of human ischaemic stroke, and correlated with greater infarct size and oedema volume as well as severe neurological deficits [21,22]. Furthermore, reduced BBB damage and cerebral lesion volumes have been reported in NADPH oxidase-knockout (gp91-phox-/-) versus wild-type mice [22]. In this context, the current study also reveals significant increases in NADPH oxidase activity and O_2^- production in cerebral endothelial cells, astrocytes and pericytes subjected to 4 or 20 h of OGD and dem-

onstrates the barrier-protective effect of oxidase inhibition through use of apocynin. In support of these findings, apocynin has previously been shown to maintain BBB function [23] and reduce infarct size in rats and mice subjected to middle cerebral artery occlusion [24-27]. In addition to the above, this study also shows that exposure to hypothermia during and after ischaemic injury substantially reduces both oxidase activity and O_2^- release in all cell lines and therefore improves barrier integrity and function. A reduction in metabolic rate for oxygen alongside a depression in oxidase activity may be responsible for attenuated generation of O_2^- and protection of the BBB [28]. Although concurrent inhibition of leukocyte activity and adhesion to vascular wall by hypothermia may also account for attenuated O_2^- release in in vivo settings, unavailability of leukocytes in the current experimental settings rule out this possibility [29]. However, considering diminished generation of O_2^- in peri-infarct areas by hypothermia, it is possible that incomplete reduction of O_2 to O_2^- and H_2O_2 may be involved in the barrier-protective effect of hypothermia [30]. Nevertheless, acquisition of similar levels of TEER and paracellular flux with apocynin in the absence or presence of hypothermia attributes much of the ischaemia-induced barrier damage to NADPH oxidase overactivity and suggestneutralisation of this enzyme complex as an important therapeutic target.

Endogenous antioxidant enzymes, SODs, play an important role in keeping ROS within the physiological range. As deficiencies in Mn-SOD and CuZn-SOD may contribute to the enhanced oxidative stress status [31] the activities of both enzymes were explored in cells subjected to OGD (4 or 20 h) which reduced both enzyme activities in HBMEC and astrocytes and selectively suppressed Mn-SOD activity in pericytes. Interestingly, the regulatory effects of intra- and post-ischaemic treatments with hypothermia appeared to be cell- and SOD isoform-specific in that while consistently enhancing both enzyme activities in astrocytes, hypothermia selectively potentiated CuZn-SOD activity in pericytes and Mn-SOD activity in endothelial cells subjected to longer periods of hypothermia. Taken together these data imply that diminished activities of SODs in BBB-related cell lines may individually or collectively exacerbate the degree of ischaemia-induced oxidative stress and ensuing barrier failure. In consistent with this, others have also shown increases in specific SOD expressions after 6 h of hypothermic exposure [32,33]. Albeit helpful, level of increases attained in CuZn-SOD and/or Mn-SOD activities by hypothermia during or after an ischaemic insult may not be sufficient to quench all O_2^- generated, suggesting a requirement for greater levels of SOD activity to negate the deleterious effects of ischaemic attack. Of course, possible changes in expressions and activities of other key antioxidant enzymes, namely catalase and glutathione peroxidase which metabolise H_2O_2, cannot be dismissed in this context. Indeed, enhanced formation of H_2O_2 and aberrant SOD activities have previously been associated with the severity of ischaemic stroke-induced brain oedema [31]. Moreover, higher rates of increases in CuZn-SOD has been shown to diminish hyperglycaemia- and oxidative stress-mediated elevations in EBA leakage and brain oedema in heterozygous SOD1 Tg rats carrying human SOD1 gene to achieve four- to six-fold increase in CuZn-SOD [34]. Similarly, moderate hypothermia applied after traumatic brain injury has also shown selective effects on antioxidant enzyme activities in that it markedly reduced catalase and glutathione peroxidase activities while increasing that of SOD activity in hippocampus, suggesting the idea that hypothermia may also somewhat suppress the antioxidant response after ischaemic injury [35].

In addition to oxidative stress, accumulating evidence also reveals enhanced expression and activity of Rho-kinase as an important determinant of BBB permeability in experimental settings of ischaemic injury and hyperglycaemia [5, 14].Under normal conditions, Rho-kinase modulates vascular integrity and tone through its mediatory roles in cell growth, inflammation, stress fibre assembly, myosin light chain phosphorylation and nitric oxide bioavailability [36]. Using an animal model of human focal cerebral ischaemia, in a recent study we have shown that both early and slightly delayed inhibition of Rho-kinase during the acute phase of stroke (achieved by treatments with fasudil at the onset of reperfusion plus 4 h post-ischaemia and 4 h post-ischaemia alone, respectively) exert a neurovascular protection. For instance, both therapeutic approaches significantly improved functional outcome while concurrently reducing cerebral lesion and oedema volumes in fasudil- versus vehicle-treated animals [22]. In the same study, co-treatment of BMEC, subjected to OGD±R, with a Rho-kinase inhibitor also improved in vitro barrier function by downregulating both NADPH oxidase activity and O_2^- release, inferring that suppression of Rho-kinase activity after an acute ischaemic attack may protect BBB integrity in part through regulation of endothelial cell oxidative stress [14,22]. It is noteworthy here that suppression of MLC phosphorylation and cytoskeletal changes which normally result in interendothelial cell permeability via cell junction destabilisation and paracellular leakage via actin stress fibre formation, respectively may also be involved in barrier-stabilising effects exerted by Rho-kinase inhibition [14].

Co-application of hypothermia with Y-27632, a Rho-kinase inhibitor during or after an ischaemic injury in the current study has not dramatically improved barrier integrity and function compared to Y-27632 alone groups. As Y-27632 has been shown to suppress hypothermia in central nervous system pharmacologically induced by intracerebroventricular injection of bombesin, a 14-amino acid peptide, Y-27632 may also diminish the therapeutic impact of hypothermia in the current study settings [37]. Besides, accumulation of O_2^- deriving from inability of Y-27632 to enhance endothelial

cell CuZn-SOD activity as well as inhibition of nitric oxide and ensuing potentiation of hypothermia-mediated cellular constriction may also help explain these results [3,6,38]. Once accumulated in large quantities, O_2^- may compromise barrier integrity in a direct fashion and through induction of BBB-related cell apoptosis orchestrated in part by the components of plasminogen-plasmin system, matrix metalloproteinases and by induction of pro-apoptotic protein Bax [8,9,16].

Activation of PKC is regarded as another key step in cerebral vasculopathies where activation of different PKC isoforms, particularly that of PKC-β is coupled to endothelial dysfunction and neurovascular complications. Indeed, induction of PKC-β has been shown to perturb BBB function through stimulation of endothelial cell apoptosis by NADPH oxidase-mediated O_2^- generation [10] and an increased presence of PKC-β protein has also been documented in the brain infarcts of deceased ischemic stroke patients [39]. Considering this, the current study investigated whether and to what extent combination of hypothermia with an inhibitor for PKC-β (LY333531) could protect BBB from the disruptive effects of OGD±R. The results show that co-application of hypothermia and LY333531 during, but not after, the ischaemic insult significantly improves barrier integrity and function compared to OGD alone groups. Although the reasons for this remain unknown, concurrent inhibitions of MMP activities, tight junction protein dissolution and BMEC apoptosis during ischaemic phase may explain the barrier-protective effects of hypothermia thereby indicating the value of early treatments with hypothermia after an ischaemic attack [7, 10]. Indeed, by reducing the expression of pro-apoptotic PKC-δ and stimulating the preservation of the anti-apoptotic action of PKC-ε, hypothermia exerts a multifaceted role after cerebral ischaemia whereby maintains neurovascular unity [40]. Furthermore, through regulation of various PKC isoforms including PKC-α and PKC-β activity, hypothermia may attenuate the effects of inflammatory cytokines like TNF-α on BBB, reduce the infiltration of activated T cells to brain parenchyma and consequently protect neurovascular viability and function [8, 41].

In conclusion, when applied during or after an ischaemic attack, hypothermia may help maintain neurovascular unity by subsiding the extent of oxidative stress and augmenting the anti-oxidant capacity of cells that constitute the key elements of the BBB, astrocytes and endothelial cells. Combination of hypothermia with the specific inhibitors of molecular pathways that influence a wide range of pathophysiological phenomena during and after an ischaemicinsult may augment the benefits of either treatment.

References

1. Rees S, Harding R, Walker D (2011) The biological basis of injury and neuroprotection in the fetal and neonatal brain. Int J DevNeurosci 29: 551-563.
2. Holzer M and Behringer W (2008) Therapeutic hypothermia after cardiac arrest and myocardial infarction. Best Pract Res ClinAnaesthesiol 22: 711-728.
3. Allen CL and Bayraktutan U (2009) Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke 4: 461-470.
4. Pun PB, Lu J, Moochhala S (2009) Involvement of ROS in BBB dysfunction. Free Radic Res 43: 348-364.
5. Srivastava K, Shao B, Bayraktutan U (2013) PKC-exacerbates in vitro brain barrier damage in hyperglycemic settings via regulation of RhoA/Rho-kinase/MLC2 pathway. J Cereb Blood Flow Metab 33: 1928-1936.
6. Allen CL and Bayraktutan U (2009) Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood-brain barrier hyperpermeability. Diabetes ObesMetab 11: 480-490.
7. Shao B and Bayraktutan U (2013) Hyperglycaemia promotes cerebral barrier dysfunction through activation of protein kinase C-beta. Diabetes ObesMetab 15: 993-999.
8. Abdullah Z, Rakkar K, Bath PM, Bayraktutan U (2015) Inhibition of TNF-protects in vitro brain barrier from ischaemic damage. Mol Cell Neurosci 69: 65-79.
9. Abdullah Z and Bayraktutan U (2014) NADPH oxidase mediates TNF-alpha-evoked in vitro brain barrier dysfunction: roles of apoptosis and time. Mol Cell Neurosci 61: 72-84.
10. Shao B and Bayraktutan U (2014) Hyperglycaemia promotes human brain microvascular endothelial cell apoptosis via induction of protein kinase C and prooxidant enzyme NADPH oxidase. Redox Biol 2: 694-701.
11. Nemethova M, Danielisova V, Gottlieb M, Burda J (2008) Post-conditioning exacerbates the MnSOD immune-reactivity after experimental cerebral global ischemia and reperfusion in the rat brain hippocampus. Cell BiolInt 32: 128-135.
12. Lee JC and Won MH (2014) Neuroprotection of antioxidant enzymes against transient global cerebral ischemia in gerbils. Anat Cell Biol 47: 149-156.
13. Ktz D (2005) Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol 67: 225-257.
14. Allen C, Srivastava K, Bayraktutan U (2010) Small GTPaseRhoA and its effector rho kinase mediate oxygen glucose deprivation-evoked in vitro cerebral barrier dysfunction. Stroke 41: 2056-2063.
15. Riento K and Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4: 446-456.
16. RakkarK, Srivastava K, Bayraktutan U (2014) Attenuation of urokinase activity during experimental ischaemia protects the cerebral barrier from damage through regulation of matrix metalloproteinase-2 and NAD(P)H oxidase. Eur J Neurosci 39: 2119-2128.
17. Hayashi K, Nakao S, Nakaoke R, Nakagawa S, Kitagawa N et al. (2004) Effects of hypoxia on endothelial/pericytic co-culture model of the blood-brain barrier. RegulPept 123: 77-83.
18. Hayashi Y, Nomura M, Yamagishi S, Harada S, Yamashita J et al. (1997) Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. Glia 19: 13-26.
19. Crack PJ and Taylor JM (2005) Reactive oxygen species and the modulation of stroke. Free RadicBiol Med 38: 1433-1444.
20. Guzik TJ, West NEJ, Black E, McDonald D, Ratnatunga C et al. (2000)

Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res 86: E85-90.

21. Kusaka I, KusakaG, Zhou C, Ishikawa M, Nanda A et al. (2004) Role of AT1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. Am J Physiol Heart CircPhysiol 286: H2442-2451.
22. Gibson CL, Srivastava K, Sprigg N, Bath PMW, Bayraktutan U (2014) Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem 129: 816-826.
23. Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H et al. (2007) NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. Stroke 38: 3000-3006.
24. Chen H, Song YS, Chan PH (2009) Inhibition of NADPH oxidase is neuroprotective after ischemia-reperfusion. J Cereb Blood Flow Metab 29: 1262-1272.
25. Jackman KA, Miller AA, Silva TD, Crack PJ, Drummond GR (2009) Reduction of cerebral infarct volume by apocynin requires pretreatment and is absent in Nox2-deficient mice. Br J Pharmacol 156: 680-688.
26. Genovese T, Mazzon E, Paterniti I, Esposito E, Bramanti P, et al. (2011) Modulation of NADPH oxidase activation in cerebral ischemia/reperfusion injury in rats. Brain Res 1372: 92-102.
27. Kelly KA, Li X, Tan Z, VanGilder RL, Rosen CL, et al. (2009) NOX2 inhibition with apocynin worsens stroke outcome in aged rats. Brain Res 1292: 165-172.
28. Gordon CJ (2001) The therapeutic potential of regulated hypothermia. Emerg Med J 18: 81-89.
29. Sirimanne ES, Blumberg RM, Bossano D, Gunning M, Edwards AD (1996) The effect of prolonged modification of cerebral temperature on outcome after hypoxic-ischemic brain injury in the infant rat. Pediatr Res 39: 591-597.
30. Maier CM, Sun GH, Cheng D, Yenari MA, Chan PH et al. (2002) Effects of mild hypothermia on superoxide anion production, superoxide dismutase expression, and activity following transient focal cerebral ischemia. Neurobiol Dis 11: 28-42.
31. Dai DF, Chiao YA, Marcinek DJ, Szeto HH, Rabinovitch PS (2014) Mitochondrial oxidative stress in aging and healthspan. LongevHealthspan 3: 6.
32. Liu XH, Kato H, Nakata N, Kogure K, Kato K (1993) Animmunohistochemical study of copper/zinc superoxide dismutase and manganese superoxide dismutase in rat hippocampus after transient cerebral ischemia. Brain Res 625: 29-37.
33. Fukuhara T, Nishio S, Ono Y, Kawauchi M, Asari S et al. (1994) Induction of Cu,Zn-superoxide dismutase after cortical contusion injury during hypothermia. Brain Res 657: 333-336.
34. Kamada H, Yu F, Nito C, Chan PH (2007) Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction. Stroke 38: 1044-1049.
35. DeKosky ST, Abrahamson EE, Taffe KM, Dixon CE, Kochanek PM et al. (2004) Effects of post-injury hypothermia and nerve growth factor infusion on antioxidant enzyme activity in the rat: implications for clinical therapies. J Neurochem 90: 998-1004.
36. Van Aelst L and D'Souza-Schorey C (1997) Rho GTPases and signaling networks. Genes Dev 11: 2295-2322.
37. Tsushima H, Mori M, Fujiwara N, Moriyama A (2003) Pharmacological characteristics of bombesin receptor mediating hypothermia in the central nervous system of rats. Brain Res 969: 88-94.
38. Thompson-Torgerson C.S, Holowatz LA, Kenney WL (2008) Altered mechanisms of thermoregulatory vasoconstriction in aged human skin. Exerc Sport Sci Rev 36: 122-127.
39. Krupiski J, Slevin MA, Kumar P, Gaffney J, Kauza J (1998) Protein kinase C expression and activity in the human brain after ischaemic stroke. ActaNeurobiolExp (Wars) 58: 13-21.
40. Gonzalez-Ibarra FP, Varon J, Lopez-Meza EG (2011) Therapeutic hypothermia: critical review of the molecular mechanisms of action. Front Neurol2: p. 4.
41. Abdullah Z and Bayraktutan U (2016) Suppression of PKC-alpha attenuates TNF-alpha-evoked cerebral barrier breakdown via regulations of MMP-2 and plasminogen-plasmin system. BiochimBiophysActa 1862: 1354-1366.