INHIBITION OF OXIDATIVE STRESS DELAYS SENESCENCE AND AUGMENTS FUNCTIONAL CAPACITY OF ENDOTHELIAL PROGENITOR CELLS

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The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

The Dunhill Medical Trust EPC study protocol was reviewed and approved by West Midlands - Coventry & Warwickshire Research Ethics Committee (16/WM/0304).

Author contributions

RRAK and MA performed the experimental study. OAO and KR conducted the clinical study. RRAK analysed the data and wrote the original draft of the manuscript. NS and PB were the DMT grant co-applicants and performed manuscript revision. UB was the chief investigator of the DMT study. He designed the current study, interpreted the data, and wrote the manuscript.

Patient consent statement

All study participants provided written informed consent prior to enrolment.

Clinical trial registration

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Abstract

Ageing is characterised by a progressive loss of vascular endothelial function and integrity. Endothelial progenitor cells (EPCs) play an integral role in endothelial regeneration but are prone to age-dependent changes which may accelerate their senescence and diminish their availability and functionality. Considering these, we firstly investigated the quantity of circulating EPCs in older (73.3±7.2 years) and younger (40.2±14.3 years) healthy volunteers and showed sharp declines in the number of EPCs expressing stemness markers (CD34+ and/or CD133+) in older people. These coincided with the decreases in total anti-oxidant capacity (TAC) 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 functional subtype of EPCs, showed that chronological ageing, mimicked by replicative senescence, profoundly impaired proliferation, migration, tubulogenesis, and blood-brain barrier (BBB)-forming capacity of these cells. Similar to those seen in the clinical observational studies, senescent OECs also manifested decreased TAC and increased pro-oxidant NADPH oxidase activity and endostatin level. Suppressing oxidative stress level using structurally and functionally distinct anti-oxidants, namely vitamin C or VAS2870, an NADPH oxidase inhibitor, delayed OEC senescence and restored their tubulogenic and BBB-forming capacities. In conclusion, the enhanced oxidative stress level that develops during physiological ageing may promote EPC senescence and evoke endothelial dysfunction. Effective control of oxidative stress using either compound somewhat delays both phenomena and augments EPC functionality.

Keywords: Senescence – endothelial progenitor cells – oxidative stress – aging – blood-brain barrier

1. Introduction

Endothelial cells (ECs) cover the entire inner surface of all blood vessels and synthesise a large number of vasoactive substances, notably nitric oxide (NO), to control and maintain vascular tone and homeostasis at all times (Martinez-Majander et al. 2021). In some organs, ECs differentiate and develop into highly specialised barriers to regulate vascular permeability. One example is the blood-brain barrier (BBB) that monitors and regulates the selective passage of molecules between systemic circulation and the central nervous system (Segarra et al. 2021). Brain microvascular endothelial cells (BMEC) constitute the main cellular component of the BBB and form tight junctions with adjacent ECs to perform such critical tasks. Hence, it is of paramount importance to effectively detect and replace injured ECs at all times to preserve appropriate BBB function (Bayraktutan 2019).

Circulating endothelial progenitor cells (EPCs) attract much of the attention as an endogenous vascular repair system due their ability to differentiate into mature ECs and secrete a large number of trophic factors to help preserve the integrity and function of endothelial monolayer (Abdulkadir et al. 2020; Alwjwaj et al. 2021). However, the mobilisation, proliferation, migration, survival, and the reparative capacity of EPCs are adversely influenced by the ageing process in that age-related decline in the expression of various pro-angiogenic factors, including growth factors, cytokines and chemokines, appear to play an instrumental role (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 functional capacity is expected to contribute to the age-related progressive loss of cerebral endothelium and to the pathogenesis of several debilitating diseases, including stroke (Kukumberg et al. 2021). Hence, determination of molecular mechanisms involved in age-

mediated EPC senescence is of vital importance to develop novel strategies that can delay or prevent cerebrovascular dysfunction and improve lifelong health and wellbeing.

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 modulate the response of stem cells to metabolic and environmental signals by either 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 in them (Höhn et al. 2017; Wang et al. 2017). Hence, it is reasonable to suggest that suppression of oxidative stress may delay senescent phenotype and stem cell dysfunction and maintain cerebrovascular integrity. Indeed, treatments with wide-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 has explored 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 investigated the morphological, molecular, and functional changes occur in senescent EPCs and assessed 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 (Abdulkadir et al. 2020; Kadir et al. 2021).

2. Results

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

Investigation of the correlation between chronological ageing and the putative changes in circulating EPC numbers, peripheral blood samples obtained from 50 younger and 40 older healthy individuals were analysed by flow cytometry. This showed significant decreases in the numbers of undifferentiated cells (CD34+, CD133+, or CD34+CD133+) but not in endothelial-committed cells (KDR+, CD34+KDR+, CD133+KDR+, and CD34+CD133+KDR+) in older vs younger participants (Figure 1a – g).

The level of major elements known to affect mobilisation, number, and functionality of EPCs also appeared to be differentially affected by the ageing process in that while older individuals had significantly lower plasma total anti-oxidant capacity (TAC) compared to their younger counterparts, they had higher levels of inflammatory cytokine (TNF-α), chemokine (SDF-1), and angiogenic factors, endostatin and thrombospondin-1 (Figure 1h – l). In contrast, no marked differences were observed in plasma levels of other key pro-angiogenic (VEGF and PDGF-BB), anti-angiogenic (angiostatin and thrombopondin-2), and growth (G-CSF) factors between younger and older populations (Figure 1m – q).

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

Having ascertained the impact of chronological ageing on circulating EPC numbers, the subsequent laboratory studies examined whether or to what extent chronological ageing, induced by repetitive cell culture, might impact OEC morphological and molecular phenotype. These studies demonstrated that senescent OECs acquired bigger and flattened morphology and developed thick actin stress fibres traversing the cells (Figure 2a – c).

In addition to morphological changes, senescent cells also had significant increases in pro-oxidant NADPH oxidase activity and superoxide anion ($O_2^{\cdot -}$) production and concurrent decreases in total TAC and nitrite/nitrate (NOx) release thereby confirming the correlation

between cellular senescence and oxidative stress (Höhn et al. 2017). (Figure 2d – g). $O_2^{\cdot-}$ constitutes the foundation molecule of all ROS while NOx represent the final metabolites of NO, a short-lived anti-oxidant that scavenges $O_2^{\cdot-}$ and regulates vascular tone and homeostasis (Bayraktutan 2005).

Studies designed to provide further direct evidence for cellular senescence demonstrated that a significantly higher percentage of late-passage OECs stain positive for senescence-associated-β-galactosidase (SA-β-gal) compared to low-passage OECs (Figure 2h, i). However, as the increased 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 or 4,6-diamidino-2-phenylindole) showed that 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 2j – l). Taken together, these findings confirm that late-passage OECs possess a series of key characteristics of senescence, hereinafter described as senescent OECs.

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

As enhanced proliferative, migratory, and angiogenic capacities of OECs are essential prerequisites for their vasculo-reparative function, it was important to establish the impact of senescence on these crucial properties. Wound scratch assays, simultaneously assessing the proliferative and migratory potential of OECs, showed that while senescent OECs were unable to repair damage during the entire experimental period, i.e. 36 h, young OECs covered much of the scratch area within 12 h and completely repaired it within 24 h, proving the highly functional nature of young cells in the process (Figure 3a, b). In accordance with these findings,

significant reductions were also observed in proliferative and migratory rates of senescent versus young OECs in additional studies specifically looking into these features using a colorimetric assay and Transwell inserts, respectively (Figure 3c, d). Scrutiny of tubulogenic properties of OECs by matrigel assay, a reliable marker for angiogenesis *in vivo*, showed that senescent OECs formed considerably fewer number of tubes with shorter lengths and produced substantially higher levels of anti-angiogenic factor, endostatin, compared to young OECs (Figure 3e – h).

Through replacement of dead or dying ECs, 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 the young OECs. Indeed, unlike HBMECs and young OECs, failure of senescent OECs to form a tight and functional model of human BBB with astrocytes and pericytes supported this notion. In the study, the extent of changes in barrier integrity and function was determined by measurements of 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 3i – k).

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

Considering that OECs are in constant close contact with resident ECs *in vivo* and continuously replace them to maintain vascular homeostasis, it was important to probe the impact of young and senescent OECs on resident EC 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 impaired the integrity and function of the BBB (Figure 4a, b). Similarly, the presence of senescent OECs in a largely non-

senescent monolayer disrupted tight junction morphology, as evidenced by incomplete ZO-1 staining. In contrast, regeneration-competent young OECs successfully integrated with HBMECs and formed fully functional tight junctions (Figure 4c, d). In line with these findings, interaction with senescent OECs nullified the tubulogenic activity of HBMECs and substantially diminished the total number and length of tubes formed (Figure 4e – g).

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

Having established that senescent OECs possess an elevated NADPH oxidase activity and diminished TAC, in subsequent studies we investigated whether effective control of oxidative stress might somewhat delay OEC senescence and as a result improve functionality. As the overwhelming majority of OECs at passage 14 displayed the aforementioned signs of 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 5a). These studies showed that both treatment regimens significantly attenuated the level of NADPH oxidase activity and $O_{2}^{\cdot -}$ generation and decreased the number of OECs stained positive for SA-β-gal activity and DNA damage marker, γ-H2AX, by approximately 20% (Figure 5b – g).

Furthermore, both treatment regimens increased the cellular proliferation rate and tubulogenic activity coupled with increases in the number and length of tubules (Figure 6a – d). Considering the elevations observed in endostatin levels in both senescent OECs and older individuals alongside the previous studies attributing 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 6e). Finally, suppression of oxidative stress by either agent led to better integrations between senescent

OECs and HBMECs and improved the tightness and functionality of cerebral barrier, as evidenced by increases in TEER and decreases in paracellular flux of NaF (Figure 6f, g).

3. Discussion

Alterations in vascular *EC* 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 (Kadir et al. 2020; Martinez-Majander et al. 2021). Given that the number of circulating EPCs have been positively correlated with endothelial repair capacity, vasculogenesis, and overall vascular health (Chang et al. 2007; Kukumberg et al. 2021), it is possible that diminished availability of EPCs may be involved in age-dependent EC dysfunction and associated vascular abnormalities. Indeed, substantial decreases observed in the circulating number of EPCs expressing markers for stemness i.e. CD34+, CD133+, and CD34+CD133+ in otherwise healthy older (≥65 years) versus younger (18-64 years) individuals in this study verify this hypothesis and add further weight to those of the Framingham Heart Study, a large-scale prospective cohort study involving 1786 participants (Cohen et al. 2013).

Contrary to these results, the number of circulating endothelial-committed cells i.e. EPCs expressing an EC marker (namely KDR) alone or together with CD34 and/or CD133 (KDR+, CD34+KDR+, CD133+KDR+, CD34+CD133+KDR+) did not significantly change between younger and older healthy volunteers despite observing consistent decreases in older participants. Although somewhat larger standard deviations reported for these particular study groups may in part explain the dichotomy between endothelial-committed and undifferentiated cell groups, relevant previous studies reporting similar results to ours (Heiss et al. 2005; Kukumberg et al. 2021; Luo et al. 2018; Montenegro et al. 2018), attributed age-dependent EC dysfunction to diminished functional capacity, rather than numbers, of circulating endothelial-committed cells, implying the importance of functional assessments in age-related studies. As

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), cell culture remains the only option to produce large quantities of homogenous cells to perform these assays.

Based on adhesion of cells to specific substrates, such as collagen or fibronectin, EPC cultures generate two functionally and morphologically distinct cell subtypes, early EPCs (eEPCs) and outgrowth endothelial cells (OECs) (Bayraktutan 2019; Abdulkadir et al. 2020). While early EPCs are classified as haematopoietic cells (Medina et al. 2010), OECs express both endothelial and progenitor cell markers which naturally differentiate them from haematopoietic cells and circulating mature ECs. As OECs also display strong proliferative, migratory, and tubulogenic capacity *in vitro*, they are considered as the functional subtype of EPCs (Abdulkadir et al. 2020). Indeed, through their unique ability to differentiate into mature ECs and secrete various vasoactive compounds, notably stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and platelet-derived growth factor-BB (PDGF-BB) (Alwjwaj et al. 2021), OECs counteract EC dysfunction and help maintain vascular homeostasis in both physiological and pathological settings (Bayraktutan 2019; Abdulkadir et al. 2020). However, the capacity of OECs to restore endothelial integrity and function attenuate with age which inevitably increases the risk for vascular disease in ageing individuals (Xia et al. 2012; Kaur et al. 2018).

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, is removed (Arnson et al. 2010; Sanada et al. 2018). Increases observed in the circulating levels of TNF-α may be a key factor that shift the balance towards a pro-inflammatory state in older population (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. Despite increases in SDF-1, slight but consistent decreases observed in VEGF, PDGF-BB, and G-CSF plasma levels are likely to contribute to 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 EC proliferation, these anti-angiogenic factors prepare a fertile ground for senescence to flourish (Capillo et al. 2003; Meijles et al. 2017).

The oxidative stress theory of ageing postulates that age-related decline in physiological functions are triggered by a slow steady accumulation of oxidative damage to macromolecules, notably DNA, which increases with age (Höhn et al. 2017). At the cellular levels, increased ROS leads to senescence. Normally, EPCs are better protected against oxidative stress due to possession of greater TAC compared to mature (micro)vascular ECs (He et al. 2009). However, prolonged exposure to low level oxidative stress reduces EPC numbers and seriously damages their capacity to self-renew, proliferate, differentiate while evoking premature senescence (Höhn et al. 2017; Wang et al. 2017).

It is evident that almost all age-related changes in plasma induce EPC senescence as a final common pathway before affecting vascular endothelial function (Xia et al. 2012; Kukumberg et al. 2021). To scrutinise the impact of senescence on EPC morphological, molecular, and functional characteristics, we used a cell culture model of replicative senescence. It is acknowledged that such a procedure may not accurately represent chronological 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. Of course, caution needs to be exercised when translating *in vitro* findings to the entire organism (Cristofalo et al. 2003).

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. Block of senescent cells in late G1 may also contribute to these enlargements, where 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 characterised by elevations in NADPH oxidase activity and $O_2^{\cdot -}$ production and reductions in NOx availability and TAC. Taken together, with the decreased TAC in older versus younger individuals, these data confirm the seminal role of oxidative stress in both cellular senescence and chronological ageing.

$O_2^{\cdot -}$ 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, $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$ has long been regarded as a key therapeutic target in the modulation of senescence (van der Loo et al. 2000). Indeed, suppression of NADPH oxidase activity and $O_2^{\cdot -}$ 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. Taken together, these findings support the previous data indicating that modulation of redox state with vitamin C or an NADPH oxidase inhibitor (DPI or apocynin) can effectively alleviate 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 remain as one of the most critical functions of OECs *in vivo* (Bayraktutan 2019; Abdulkadir et al. 2020). 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 above, the present study demonstrates that the presence of senescent OECs drive their microenvironment, formed in large part by resident microvascular ECs, into a senescent

state and impair the overall barrier integrity and function, as 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 ECs (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 similar degree of HBMEC dysfunctionality with comparatively lower quantities of senescent OECs highlights the highly disruptive nature of senescent cells in a model of cerebral barrier.

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 the adjacent cells and surrounding tissue (da Silva et al. 2019). Alternatively, senescent cells may influence the neighbouring cells through a cell contact mechanism mediated by NOTCH/JAG1 signalling pathway or via direct intercellular protein transfer, which occurs through the formation of cytoplasmic bridge (Biran et al. 2015; Hoare et al. 2016). 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 both 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.

Contribution to angiogenesis, a process in that new blood vessels develop from the existing ones to help deliver oxygen and nutrients to tissues, is another mechanism by which OECs contribute to vascular homeostasis (Medina et al. 2010). Advancing age adversely affects the function of a series of components, including E(P)Cs and aforementioned growth factors and angiogenic factors, involved in neovascular process and therefore exacerbates 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, a reliable marker for angiogenesis

in vivo. 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 anti-angiogenic factor, endostatin, appeared to fluctuate the most, suggesting a prominent role for this factor for the initiation and progression of age-related vascular problems (Ärnlöv et al. 2013). In line with the tubulogenesis studies above, suppression of oxidative stress in senescent OECs significantly attenuated endostatin levels and confirmed the modulatory role of oxidative stress signalling pathway in angiogenesis.

4. Conclusion

In conclusion, the current study demonstrates that OEC senescence constitutes a key phenomenon in age-related vascular dysfunction. Targeting oxidative stress via 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.

5. Materials and methods

5.1. Circulating stem cells, flow cytometry, and biochemical measurement

The peripheral blood samples were obtained from younger (18 – 64 years old) and older (≥ 65 years old) healthy volunteers recruited for The Dunhill Medical Trust EPC study (DMT EPC study, NCT02980354). Table 1 documents the baseline characteristics of the individuals who participated in our study. The DMT EPC study is a single-centre, prospective, observational, case-controlled study designed, in part, to investigate the quantity of circulating EPCs and the major inflammatory and angiogenic elements that profoundly affect EPC characteristics in the context of chronological ageing. The study protocol was reviewed and approved by West Midlands - Coventry & Warwickshire Research Ethics Committee (16/WM/0304). Details of

the study rationale, design, and inclusion/exclusion criteria have already been reported elsewhere (Rakkar et al. 2020).

The quantities of nonhaematopoietic cells (CD45-) expressing markers for stemness (CD34), immaturity (CD133) and/or endothelial maturity (KDR) were detected in peripheral blood by flow cytometry in that fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ, USA), phycoeryhtrin-cyanine7- (BD Biosciences), allophycocyanin- (Miltenyi Biotech), and phycoeryhtrin- (R&D Systems) labelled antibodies were used. The changes in plasma total anti-oxidant capacity (Abcam) as well as TNF-α (R&D Systems), SDF-1 (R&D Systems), G-CSF (R&D Systems), VEGF (R&D Systems), PDGF-BB (R&D Systems), endostatin (R&D Systems), angiostatin (Abcam), thrombospondin-1 (R&D Systems), and thrombospondin-2 (R&D Systems) levels were assessed using the specific ELISA kits *as per* the manufacturers' instructions.

5.2. Cell culture

Human brain microvascular endothelial cells (HBMECs), astrocytes, and pericytes, purchased from TCS CellWorks Ltd. (Buckingham, UK), were cultured with their respective specialised media (Sciencell, Caltag Systems, Buckingham, UK) in a humidified atmosphere consisting of 75% N_2, 20% O_2 and 5% CO_2. OECs were obtained through cultured mononuclear cells isolated from peripheral blood sample according to a previous protocol (Abdulkadir et al. 2020). The outgrowth endothelial cells (OECs), the functional subtypes of EPCs, were cultured at 37°C with EBM-2 medium (Lonza) containing 20% FBS and all required supplements (provided with the media). OECs from passage 4 to passage 7 were used as young OECs. Repetitive cultures were performed up to passage 14 to acquire the senescent OECs. In some experiments, OECs at passage 12 were treated either with VAS2870 (5 μM) or vitamin C (0.5 μM), with the media changed every other day up to passage 14.

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

Triple cell co-culture models of human BBB were used throughout the study. To set up the models, astrocytes ($7.5x10^4$) were first seeded onto the outer side of polyester Transwell inserts (0.4 µm pore size, 12 mm diameter polyester membrane, High Wycombe, UK). Following overnight adherence, the inserts were inverted the correct way and placed into 12-well dishes containing fresh medium to grow to about 80% confluence. HBMECs, young OECs or senescent OECs (~$5x10^4$ cells) were then added onto the inner part of the inserts, and both cell layers were left to grow to full confluence. In some experiments, HBMECs were mixed with young or senescent OECs at a ratio of 2:1. To set up the triple-culture model, these Transwell inserts were subsequently transferred to 12-well plate containing confluent pericytes.

5.4. Assessment of BBB characteristics

BBB integrity and function were assessed as previously by measurements of transendothelial electrical resistance (TEER, World Precision Instruments, Hertfordshire, UK) and paracellular flux of low molecular weight permeability marker (sodium fluorescein, NaF, 376Da), respectively (Kadir et al. 2021).

5.5. Senescence-associated-β-galactosidase staining

A specific kit was used for this (Abcam Inc., Cambridge, MA). First, the cells were fixed in SA-β-gal fixative solution for 15 min and subsequently stained overnight at 37°C with staining solution mix, containing staining solution, staining supplement, and X-gal. Cells were then visualised under light microscope and the numbers of SA-β-gal-positive cells (blue staining) were counted in four random fields in each of the triplicate wells.

5.6. Immunocytochemistry

For these experiments, cells exposed to different experimental conditions were fixed and permeabilised with 4% paraformaldehyde/PBS for 20 minutes, and with 0.1% Triton X-100/PBS for 15 min, respectively. To detect γ-H2AX (Abcam, Cambridge, UK) and ZO-1

(Abcam, Cambridge, UK), the cells were successively incubated with the relevant primary antibody (overnight at 4^{o}C) and the appropriate secondary antibody. Nuclei were detected by 4,6-diamidino-2-phenylindole staining (DAPI, Sigma) before visualising the cells by fluorescence microscopy (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK). In experiments where HBMECs were co-cultured with OECs (at a ratio of 2:1), the latter was pre-labelled with Dil-Ac-LDL for 4 h at 37°C, prior to establishment of co-cultures. To evaluate the status of actin cytoskeleton, cells grown to 90% confluence, were fixed and permeabilised as above before staining with 1× rhodamine phalloidin for 60 minutes (Abcam, Cambridge, UK). The coverslips were mounted on glass slides and visualised under fluorescence microscopy (Zeiss Axio Observer, Carl Zeiss Ltd, Cambridge, UK). The number of stress fibres and γ-H2AX-postive cells were manually counted in a blinded fashion.

5.7. Wound scratch assay

Cells were seeded in 6-well plates ($1.5x10^5$ cells/well) and grown to full confluence before scratching the monolayers with a p1000 micropipette tip to induce a wound. The cells were then washed with PBS to remove the debris. The wound closure rates were quantified as the percentage of difference in scratch area at the beginning (immediately after scratch) and at the indicated time points (12, 24, and 36 h) using ImageJ software (version 1.52k, NIH, Maryland, USA).

5.8. Proliferation assay

The cell proliferation rate was studied using a colorimetric assay quantifying the transformation of water-soluble tetrazolium salt (WST-1, Roche, Mannheim, Germany) to formazan in viable cells. After exposure to the respective treatments, an equal number of cells ($5x10^3$) was seeded in the 96-well plate and cultured for 48 h. The medium was then replaced with 100 µL of fresh medium containing 10 µL of WST-1, and incubated for 2 h at 37°C. The absorbance (450 nm) was then read using a FLUOstar Omega plate reader (BMG Labtech Ltd., UK).

5.9. Migration assay

The migratory capacity of the cells was analysed using Transwell inserts (4.0 μm pore size, Corning) in 24-well plates. The cells were cultured in T25 flasks until reaching 90% confluence. The medium was then replaced with migration medium (EBM-2 medium without FBS and supplements) containing 10 μg/mL Calcein AM (Calbiochem). After 2 h of incubation, the cells (5 x 10^4) were trypsinised and seeded in the upper chamber and cultured with migration medium. EBM-2 medium containing all the supplements and 5 μL/mL VEGF (Fisher Scientific, Loughborough, UK) was added to the lower chamber. The plates were read after 18 h incubation at 37ºC using luminometer at excitation of 485 nm and emission of 520 nm. The final reading was obtained after subtracting with the blanks.

5.10. Tubulogenic assay

Cells (9 × 10^4 cells) were seeded onto 48-well plates pre-coated with Matrigel (150 μl/well; BD Biosciences) and cultured in EBM-2 media at 37°C for 8 h. Tube formation in each well was viewed using light microscope (50x magnification). The total number and length of tubules network, defined by the sum of number or length of segments, isolated elements and branches detected in the analysed area, were assessed using ImageJ software (version 1.52k, NIH, Maryland, USA) (Chevalier et al. 2014).

5.11. Measurement of NADPH oxidase activity and superoxide anion generation

NADPH oxidase activity and superoxide anion production were detected as before using the lucigenin chemiluminescence and cytochrome-*C* reduction assays, respectively (Kadir et al. 2021).

5.12. Total anti-oxidant capacity

Total anti-oxidant capacity was measured using a Total Anti-Oxidant Capacity assay kit (Abcam, Cambridge, UK). In brief, the cells were homogenised with 100 μL of ddH_2O and centrifuged at 10.000 rpm at 4ºC for 5 minutes. The supernatant was subsequently collected

and transferred to a 96-well plate. A Cu^{2+} solution was added to sample wells prior to being incubated at room temperature for 90 minutes. The plates were then measured in microplate reader at absorbance of 570 nm.

5.13. Nitric oxide measurement

A Nitric Oxide Assay Kit was used to measure the total nitrate/nitrite (NOx) generation (Abcam, Cambridge, UK). For this, the cells were homogenised with 500 μL of assay buffer and centrifuged 10.000 rpm at 4^{o}C for 5 minutes. The supernatant was collected and transferred to a provided microtiter plate. Nitrate reductase and enhancer was added to the well plate and incubated for 4 h and 30 min, respectively. Fluorescent probe DAN (2,3 diaminonaphthalene) was concomitantly added and followed by incubation for 10 min. NaOH was then added and incubated for 10 min prior to measuring the fluorescent intensity in microplate reader at excitation of 360nm and emission of 450nm.

5.14. Detection of Endostatin levels

Endostatin human ELISA Kit (ab100508) was used for this. Briefly, cell supernatants were added to the pre-coated 96-well plates provided. Following an overnight incubation, the supernatants were replaced with biotinylated endostatin detection antibody for 1 h. This was followed by the successive addition of HRP-streptavidin solution for 1h and TMB one-step substrate reagent for 30 min. The stop solution was then added to the wells before reading the absorbance (450nm) in a plate reader.

5.15. Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 8.0 statistical software package (GraphPad Software Inc). Data were analysed by *t*-test, Mann-Whitney test, chi-square, or one-way analysis of variance (ANOVA), followed by a Tukey post hoc analysis. $P<0.05$ was considered as significant.

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7. Figure legends

Figure 1. Analyses of plasma levels of circulating EPCs and major elements known to affect their number and function in young and healthy volunteers by flow cytometry and specific ELISAs. **(a – c)** Compared to younger individuals, older volunteers had lower number of undifferentiated cells expressing CD34+ (a), CD133+ (b), and CD34+CD133+ (c). **(d – g)** The number of cells expressing KDR+ (d), CD34+KDR+ (e), CD133+KDR+ (f) or CD34+CD133+KDR+ (g) did not change between older and younger healthy volunteers. **(h – l)** Compared to their younger counterparts, the older volunteers possess significantly lower total anti-oxidant capacity (h) but higher inflammatory and anti-angiogenic factors, namely TNF-α (i), SDF-1 (j), endostatin (k) and thrombospondin-1 (l). **(m – q)** In contrast, the levels of pro-angiogenic factors, VEGF (m) and PDGF-BB (n), anti-angiogenic factors, angiostatin (o) and thrombopondin-2 (p), and growth factor, G-CSF (q), were not statistically different between the two groups. Data are expressed as mean±SEM. $*P < 0.05$ compared to young volunteers.

Figure 2. Exposure of OECs to repetitive cell culture, an *in vitro* model of chronological ageing, induces profound alterations in morphology, oxidative stress status, and senescence-related marker expression. **(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 (b) and number of stress fibres (c) in early-passage (passage 7) vs late-passage OECs. **(d – g)** Late-passage OECs possessed substantially higher NADPH oxidase activity (d) and superoxide anion production (e) and lower total anti-oxidant capacity (f) and nitric oxide generation (g). **(h - i)** The activity (h) of senescence-associated-β-galactosidase (SA-β-gal) and the number of SA-β-gal-positive cells were higher in late-passage OECs. **(j)** Late-passage OECs showed consistent increases in γ-H2AX activity compared to early-passage OECs. **(k)** The quantification of γ-H2AX-positive

cells. **(l)** Late-passage OECs displayed significantly greater nuclear size compared to early-passage OECs. Scale bars: 50 μm. Data are expressed as mean±SEM from three independent experiments. *$P < 0.05$ compared to early-passage OECs.

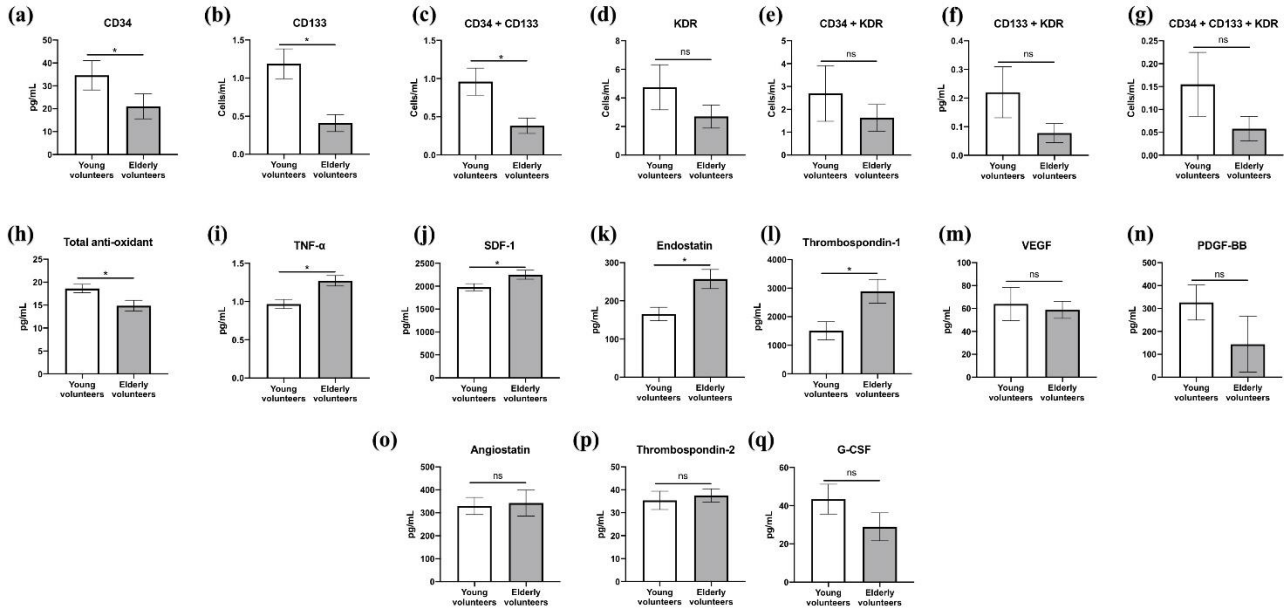
Figure 3. Senescent OECs display limited regenerative and BBB-forming capacity. **(a, b)** Time-course assessment of wound-reparative capacity of young vs senescent OECs (a) and the quantification of wound healing speed in both cells (b). (**c, d)** Assessment on the cell migratory (c) and proliferative (d) capacity via Transwell insert and WST-1 assay, respectively. **(e)** Senescent OECs form less organised tubule network on Matrigel. **(f, g)** The quantification of number (f) and length (g) of tubule network in both cells. **(h)** Level of anti-angiogenic factor endostatin in young and senescent OECs. **(i, j)** When co-cultured with astrocytes and pericytes to establish an *in vitro* model of human BBB, senescent OECs fail to form tight and functional BBB, as observed by the decreases in TEER (i) and concomitant increase in sodium fluorescein flux (j). **(k)** Senescent OECs show an interrupted distribution of ZO-1. Data are expressed as mean±SEM from three independent experiments. Scale bars: 50 μm. *$P < 0.05$ compared to young OECs, #$P < 0.05$ compared to HBMECs.

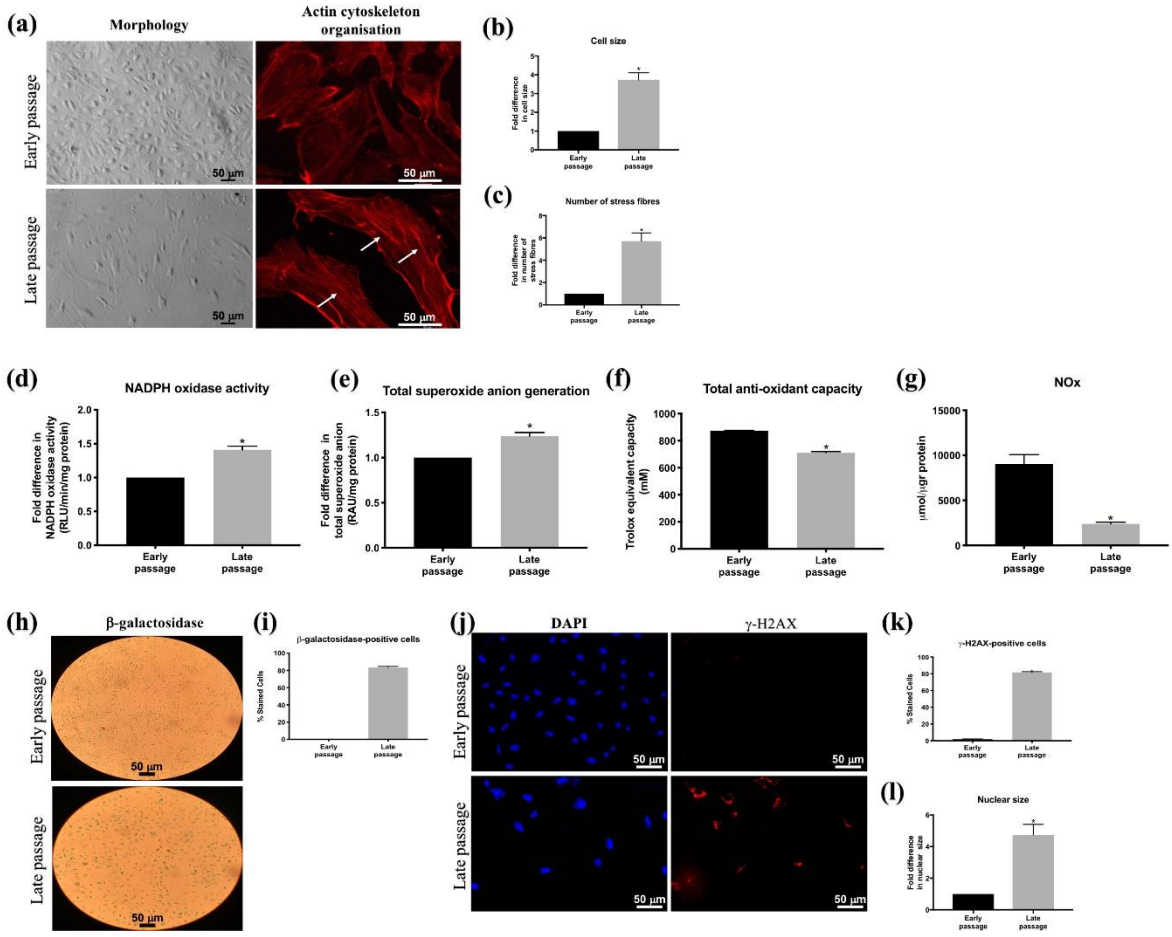
Figure 4. The presence of senescent OECs adversely affect endothelial monolayer and blood-brain barrier integrity. **(a, b)** Co-culture of young HBMECs and senescent OECs in a ratio of 2:1 in an *in vitro* model of human BBB decrease barrier integrity (a) and function (b) as evidenced by changes in TEER and paracellular flux of permeability marker, sodium fluorescein. **(c, d)** Senescent, but not young, OECs impair the distribution of ZO-1 in young HBMECs. **(e)** Co-culture of HBMECs with senescent OECs, but not young OECs, impair appropriate tubule formation. **(f, g)** The quantification of total number (f) and length (g) of tubule networks in HBMEC and young or senescent OEC co-cultures. Data are expressed as

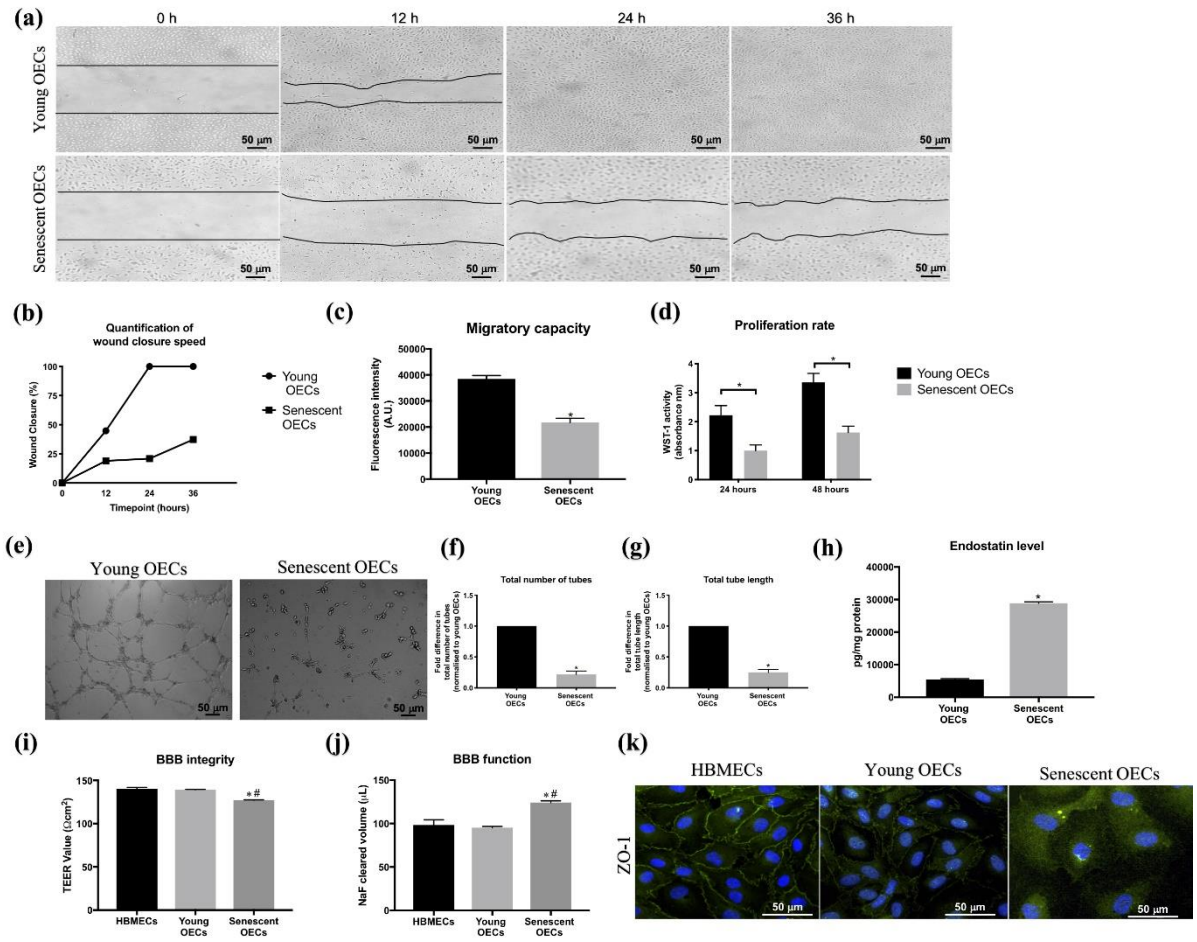
mean±SEM from three independent experiments. Scale bars: 50 μm. *P < 0.05 compared to HBMECs + young OECs.

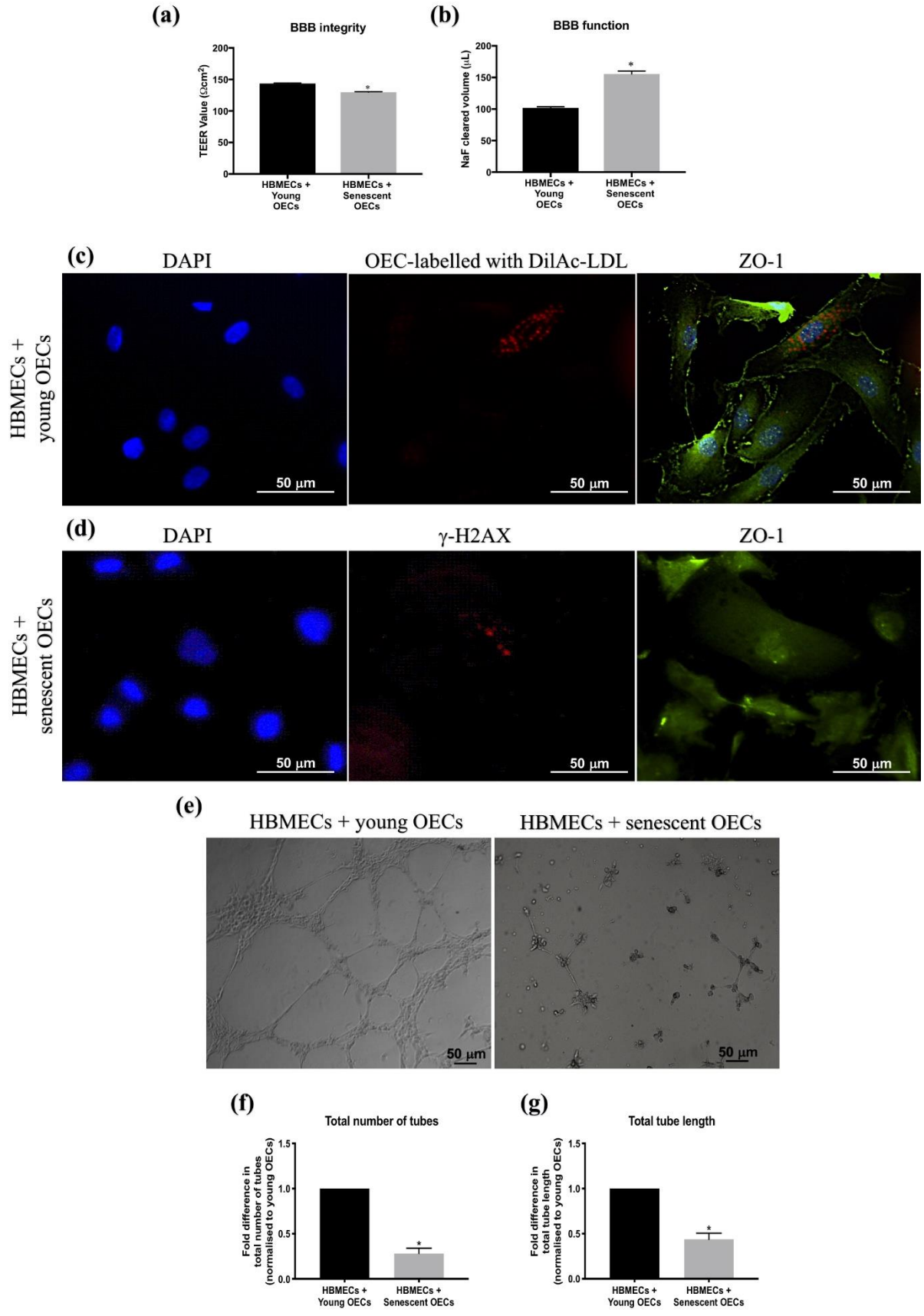
Figure 5. Suppression of oxidative stress delay OEC senescence. **(a)** Schematic diagram showing the treatment protocol with vitamin C or VAS2870, an inhibitor of NADPH oxidase. **(b, c)** The presence of VAS2870 (5 μM) or vitamin C (0.5 μM) inhibit NADPH oxidase activity (b) and superoxide anion production (c). **(d, e)** Treatment with either agent decrease the number of senescence-associated-β-galactosidase (SA-β-gal)- and γ-H2AX-positive cells. **(f, g)** The quantification of cells stained by SA-β-gal and γ-H2AX. Data are expressed as mean±SEM from three independent experiments. Scale bars: 50 μm. *P < 0.05 compared to passage 12, #P < 0.05 compared to passage 14.

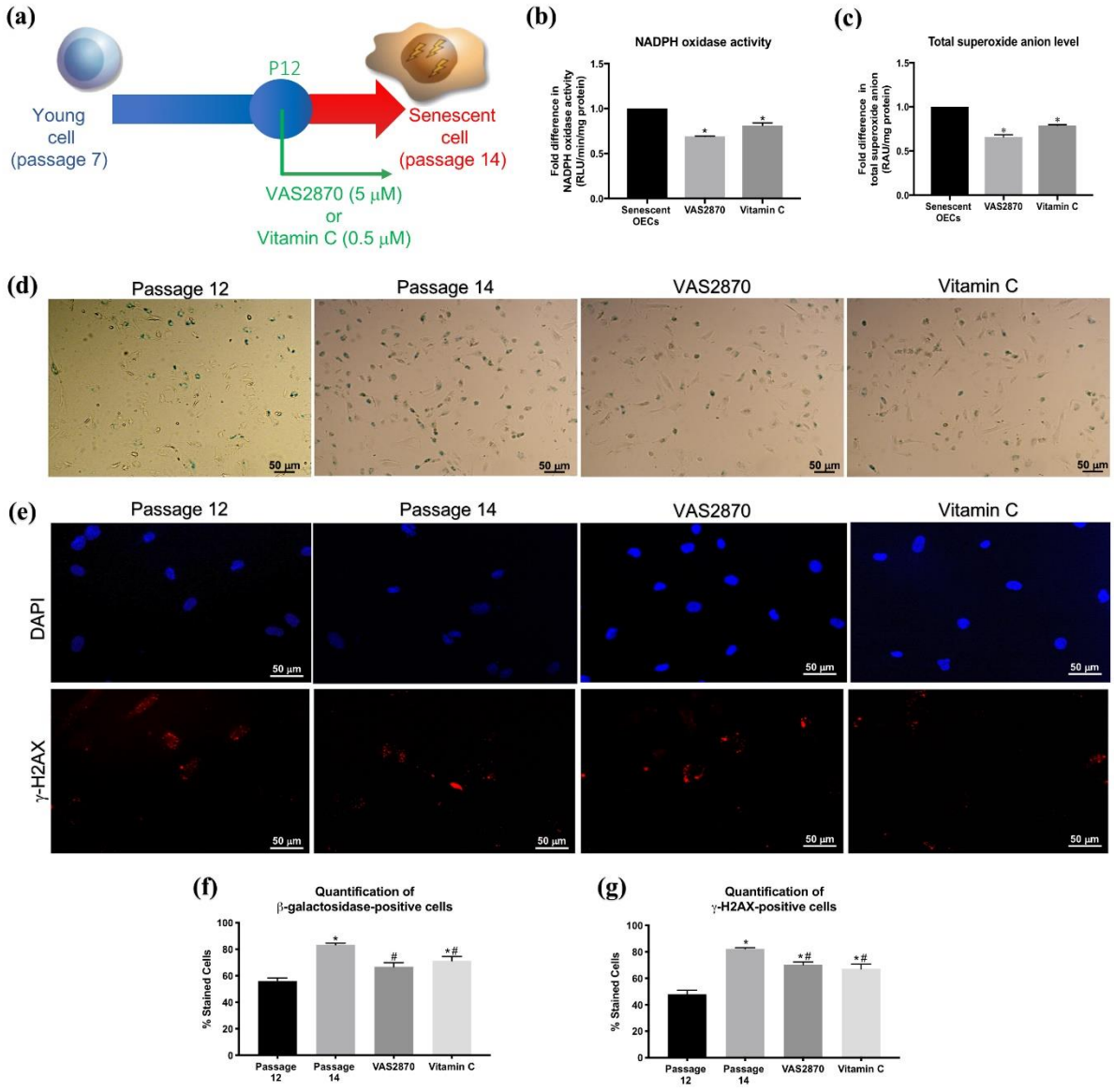
Figure 6. Suppression of oxidative stress restore the angiogenic and cerebral barrier-forming capacity of senescent OECs. **(a – c)** The presence of VAS2870 (5 μM) or vitamin C (0.5 μM) restore the angiogenic properties of senescent OECs (a), as indicated by the higher number (b) and total length (c) of tubule networks formed on Matrigel. **(d, e)** Treatment with either compound also decrease the level of angiogenic inhibitor, endostatin (d) and increase the proliferation (e) of senescent OECs. **(f, g)** Treatment with VAS2870 or vitamin C enhance the integration between senescent OECs and HBMECs, leading to the improvements in BBB tightness and functionality, as ascertained by the increases in TEER (f) and decreases in paracellular flux of NaF (g). Data are expressed as mean±SEM from three independent experiments. Scale bars: 50 μm. *P < 0.05 compared to senescent OECs, #P < 0.05 compared to VAS2870.

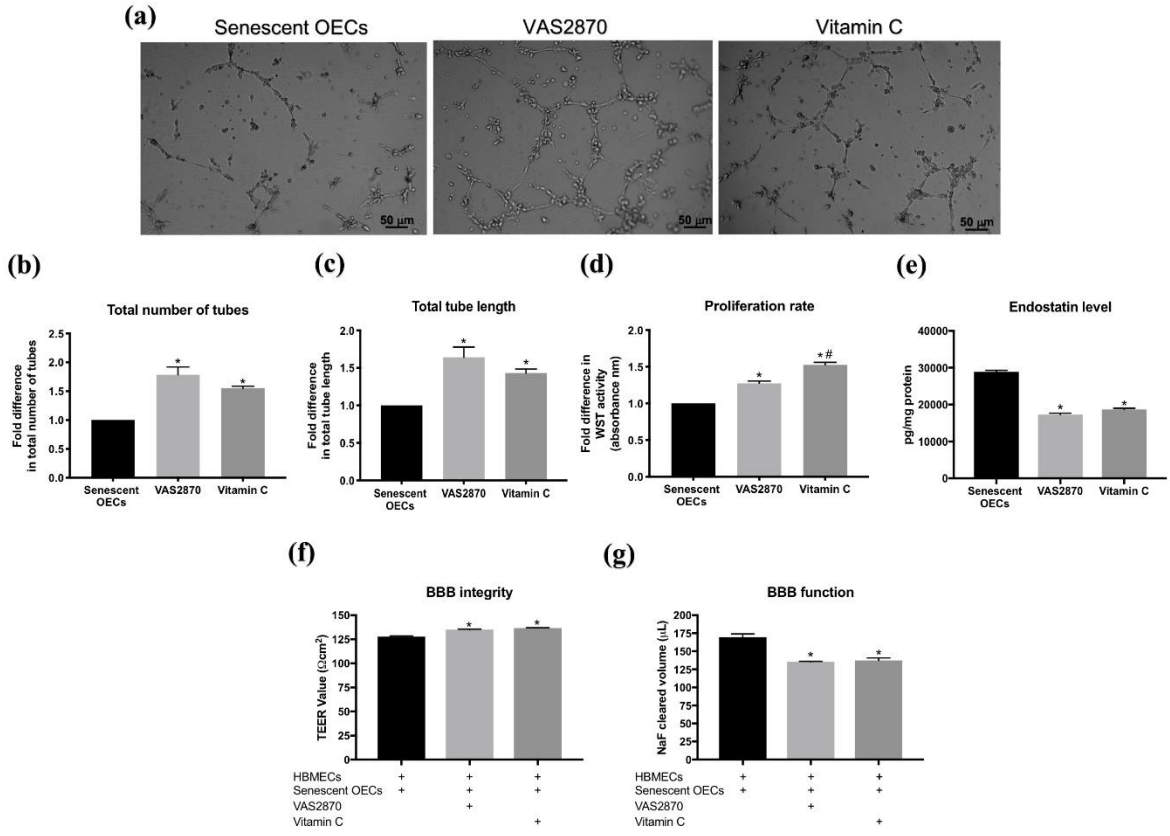

(a)
CD34
pg/mL
*
Young volunteers
Elderly volunteers
(b)
CD133
Cells/mL
*
Young volunteers
Elderly volunteers
(c)
CD34 + CD133
Cells/mL
*
Young volunteers
Elderly volunteers
(d)
KDR
Cells/mL
ns
Young volunteers
Elderly volunteers
(e)
CD34 + KDR
Cells/mL
ns
Young volunteers
Elderly volunteers
(f)
CD133 + KDR
pg/mL
ns
Young volunteers
Elderly volunteers
(g)
CD34 + CD133 + KDR
Cells/mL
ns
Young volunteers
Elderly volunteers
(h)
Total anti-oxidant
pg/mL
*
Young volunteers
Elderly volunteers
(i)
TNF-α
pg/mL
*
Young volunteers
Elderly volunteers
(j)
SDF-1
pg/mL
*
Young volunteers
Elderly volunteers
(k)
Endostatin
pg/mL
*
Young volunteers
Elderly volunteers
(l)
Thrombospondin-1
pg/mL
*
Young volunteers
Elderly volunteers
(m)
VEGF
pg/mL
ns
Young volunteers
Elderly volunteers
(n)
PDGF-BB
pg/mL
ns
Young volunteers
Elderly volunteers
(o)
Angiostatin
pg/mL
ns
Young volunteers
Elderly volunteers
(p)
Thrombospondin-2
pg/mL
ns
Young volunteers
Elderly volunteers
(q)
G-CSF
pg/mL
ns
Young volunteers
Elderly volunteers


(a)
Morphology
Actin cytoskeleton organisation
Early passage
Late passage
50 μm
(b)
Cell size
Fold difference in cell size
Early passage
Late passage
(c)
Number of stress fibres
Fold difference in number of stress fibres
(d)
NADPH oxidase activity
Fold difference in NADPH oxidase activity (RLU/min/mg protein)
(e)
Total superoxide anion generation
Fold difference in total superoxide anion (RAU/mg protein)
(f)
Total anti-oxidant capacity
Trolox equivalent capacity (mM)
(g)
NOx
μmol/μgr protein
(h)
β-galactosidase
(i)
β-galactosidase-positive cells
% Stained Cells
(j)
DAPI
γ-H2AX
(k)
γ-H2AX-positive cells
(l)
Nuclear size
Fold difference in nuclear size


(a)
0 h
12 h
24 h
36 h
Young OECs
Senescent OECs
50 µm
(b)
Quantification of wound closure speed
Wound Closure (%)
Timepoint (hours)
Young OECs
Senescent OECs
(c)
Migratory capacity
Fluorescence intensity (A.U.)
Young OECs
Senescent OECs
(d)
Proliferation rate
WST-1 activity (absorbance nm)
24 hours
48 hours
Young OECs
Senescent OECs
(e)
Young OECs
Senescent OECs
50 µm
(f)
Total number of tubes
Fold difference in total number of tubes (normalised to young OECs)
Young OECs
Senescent OECs
(g)
Total tube length
Fold difference in total tube length (normalised to young OECs)
Young OECs
Senescent OECs
(h)
Endostatin level
pg/mg protein
Young OECs
Senescent OECs
(i)
BBB integrity
TEER Value (Ωcm²)
HBMECs
Young OECs
Senescent OECs
(j)
BBB function
NaF cleared volume (µL)
HBMECs
Young OECs
Senescent OECs
(k)
HBMECs
Young OECs
Senescent OECs
ZO-1
50 µm


(a)
BBB integrity
TEER Value (Ωcm²)
200
150
100
50
0
*
HBMECs + Young OECs
HBMECs + Senescent OECs
(b)
BBB function
NaF cleared volume (µL)
200
150
100
50
0
*
HBMECs + Young OECs
HBMECs + Senescent OECs
(c)
DAPI
OEC-labelled with DilAc-LDL
ZO-1
HBMECs + young OECs
50 µm
50 µm
50 µm
(d)
DAPI
γ-H2AX
ZO-1
HBMECs + senescent OECs
50 µm
50 µm
50 µm
(e)
HBMECs + young OECs
HBMECs + senescent OECs
50 µm
50 µm
(f)
Total number of tubes
Fold difference in total number of tubes (normalised to young OECs)
1.5
1.0
0.5
0.0
*
HBMECs + Young OECs
HBMECs + Senescent OECs
(g)
Total tube length
Fold difference in total tube length (normalised to young OECs)
1.5
1.0
0.5
0.0
*
HBMECs + Young OECs
HBMECs + Senescent OECs


(a)
Young cell (passage 7)
P12
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)
Senescent OECs
VAS2870
Vitamin C
(c)
Total superoxide anion level
Fold difference in total superoxide anion (RAU/mg protein)
Senescent OECs
VAS2870
Vitamin C
(d)
Passage 12
Passage 14
VAS2870
Vitamin C
50 μm
(e)
Passage 12
Passage 14
VAS2870
Vitamin C
DAPI
γ-H2AX
50 μm
(f)
Quantification of β-galactosidase-positive cells
% Stained Cells
Passage 12
Passage 14
VAS2870
Vitamin C
(g)
Quantification of γ-H2AX-positive cells
% Stained Cells
Passage 12
Passage 14
VAS2870
Vitamin C


(a)
Senescent OECs
VAS2870
Vitamin C
50 μm
(b)
Total number of tubes
Fold difference in total number of tubes
Senescent OECs
VAS2870
Vitamin C
(c)
Total tube length
Fold difference in total tube length
(d)
Proliferation rate
Fold difference in WST activity (absorbance nm)
(e)
Endostatin level
pg/mg protein
(f)
BBB integrity
TEER Value (Ωcm²)
HBMECs
Senescent OECs
VAS2870
Vitamin C
(g)
BBB function
NaF cleared volume (μL)