Medizinische Fakultät
der
Universität Duisburg-Essen

Aus der Klinik für Neurologie

Hypoxic preconditioning combined with acid sphingomyelinase deactivation augments the proangiogenic effects of mesenchymal stromal cell-derived small extracellular vesicles

I n a u g u r a l d i s s e r t a t i o n
zur
Erlangung des Doktorgrades der Medizin
durch die Medizinische Fakultät
der Universität Duisburg-Essen

vorgelegt von

Mertcan Usluer

aus Düren

2024

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Tag der mündlichen Prüfung: 24. Oktober 2024

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1 INTRODUCTION

1.1 Stroke

Stroke is a leading cause of major disability and death among adults worldwide (Johnson et al., 2019, Katan and Luft, 2018). Ischemic stroke accounts for the majority of stroke events and is caused by compromised cerebral blood flow, resulting from a thromboembolic occlusion of brain-supplying arteries. In most cases either an embolus or a local thrombosis cause the arterial blockage (Dirnagl et al., 1999). Impaired cerebral blood flow deprives the brain tissue of oxygen and glucose as well as nutrients that are essential for brain function and consequently leads to focal brain ischemia (Hata et al., 2000). The most crucial pathophysiologic mechanisms are comprised of peri-infarct depolarization, excitotoxicity, inflammation and cell death, leading to neuronal injury as well as neurodegeneration and brain atrophy (Dirnagl et al., 1999). These consequences are directly initiated by cerebral hypoperfusion. Insufficient supply of oxygen and glucose leads to dysfunctional mitochondria and a lack of adenosine triphosphate (ATP), the main source of energy for our cells. This impairs the function of Na/K-ATPases and ultimately terminates in ischemia-induced cell death (Song and Yu, 2014). Ischemia-induced cell death can result in severe cerebral atrophy and neurological damage. Acute vessel blockage results in the formation of structurally intact, but functionally compromised tissue in close proximity to the ischemic core. This area is termed the ischemic penumbra and is the main target point of interventional therapies, aiming to enhance neurological recovery (Hata et al., 2000, Dirnagl et al., 1999). Currently available treatment options for ischemic stroke are divided into two main principles: mechanical thrombectomy and pharmacological thrombolysis. Both techniques aim to restore cerebral blood flow by dissolving (thrombolysis) or removing (thrombectomy) the thromboembolic occlusion. Intravenous medication for thrombolysis currently consists of recombinant tissue plasminogen activator (rtPA) – also called alteplase. It was approved by the FDA in 1996 and is the gold standard treatment for ischemic stroke. Although rtPA can reduce disability and mortality in stroke patients, the main disadvantage is that pharmacological thrombolysis has a short time window. rtPA has to be delivered within 4.5 hours after ischemic stroke (Hacke et al., 2008, National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

Mechanical removal of thromboembolic occlusions by means of thrombectomy was approved by the FDA in 2004. Obstructed cerebral arteries are recanalized by means of clot extraction. The operative procedure needs to be initiated within 6 hours of ischemic stroke incidents, as suggested by the American Heart/American Stroke Association. (Powers et al., 2019).

There is an ongoing effort to find novel treatment options for ischemic stroke, targeting, inter alia, thrombotic cascades and neuroprotection. Although calcium antagonists, N-methyl-D-aspartate (NMDA) receptor antagonists, intercellular adhesion molecule 1 (ICAM-1) antibodies and neuroprotective agents including nitric oxide inhibitors, gamma-aminobutyric acid (GABA) agonists or sodium channel blockers seemed to be promising approaches but all failed to improve outcome in clinical trials (O'Collins et al., 2006).

There is an evident demand for stroke therapy that targets neuronal recovery beyond the time window for acute interventions.

1.2 Angiogenesis

It is crucial that cells are provided with sufficient blood flow. Therefore, they need to be located within 100 to 200 μm of blood vessels. Recruitment and development of larger and denser blood vessels through vasculogenesis and angiogenesis can provide a dense blood supply architecture for multicellular organisms to grow and mature. The formation and expansion of novel blood vessels from preexisting vascular structures is termed angiogenesis. Angiogenesis is imperative for physiological mechanisms and further occurs during pathologic conditions such as cancer and psoriasis (Carmeliet and Jain, 2000). Under physiological conditions angiogenesis involves several steps including initiation, formation, maturation, remodeling and relapse according to the demands of the tissue (Staton et al., 2009).

Many signaling pathways are involved in regulating angiogenesis such as hypoxia, inflammation, and tumor cell signals. Pro-angiogenic signals include vascular endothelial growth factor (VEGF), angiopoietin-2 (ANG-2), fibroblast growth factors (FGFs), endothelial nitric oxide synthases (eNOS) and Ang-1/Tie2. The permeability of the vessel is increased through endothelial cells loosening their junctions in order to extravasate proteins that are needed for the formation of new vessels. This frame of extracellular matrix (ECM) is strengthened by structural proteins as well as new endothelial cells migrating onto its surface (Carmeliet and Jain, 2011, Ruan et al., 2015). One of the strongest stimuli for inducing angiogenesis is hypoxia. Numerous angiogenic factors are activated resulting from hypoxic conditions, the most prominent ones being hypoxia-inducible factors (HIFs) and their subsequently initiated pathways (Carmeliet and Jain, 2000).

In synergy with neurogenesis, angiogenesis allows functional and symptomatic rehabilitation post-stroke. As a means of reestablishing blood flow by virtue of collateral circulation in damaged brain tissue, angiogenesis sets forth the foundation for various healing pathways (Ruan et al., 2015). Angiogenesis, alongside neurogenesis, has been reported to positively correlate with patient survival and is one of the key research fields in investigating potential therapeutic applications for stroke patients (Krupinski et al., 1994).

1.3 Blood-brain-barrier

Over a century ago Paul Ehrlich, Edwin Goldmann and Lena Stern, described the molecular and morphological characteristics of the blood-brain barrier (BBB) (Bradbury, 1979). The BBB strictly regulates which blood components can enter the brain and which molecules or cells have to be excluded from moving across the BBB, in order to maintain the homeostasis of the central nervous system (CNS) and establish proper neuronal function. The restrictive structure of the BBB displays an obstacle for many components of the blood (Daneman and Prat, 2015).

The BBB mainly consists of endothelial cells, astrocytes and pericytes. The endothelial cells provide the structural basis for the walls of the blood vessel and interact with surrounding mural cells, immune cells, glial cells and neural cells. They strictly govern the movement of ions, molecules and cells form the blood to the brain and back. It is crucial for the integrity of the BBB to sustain cell-cell connections through tight junctions. These tight junctions as well as adherens junctions consist of claudin-5 and occludin proteins. (Blanchette and Daneman, 2015)

To further the stabilize the BBB, endothelial cells are located on top of a basal membrane which is mainly composed of collagen IV, laminin, fibronectin, and proteoglycans. Pericytes that surround this basal membrane additionally regulate the permeability of cerebral blood vessels by way of coordinating endothelial proliferation, angiogenesis and response to inflammation. Apart from endothelial cells and pericytes, which mainly construct the vasculature of the brain, other cells native to the brain tissue also help to moderate properties of the BBB. Astrocytes excrete regulatory factors such as transforming growth factor-β (TGF- β), glial cell line-derived neurotrophic factor (GDNF), FGF and interleukins, whereas neurons play a secondary role in the regulation of the BBB (Wilhelm et al., 2011).

Dysfunction of the BBB is involved in neurological diseases and cerebral pathologies including stroke. Disruption of endothelial cell-cell connections and loss of BBB integrity can lead to shifted homeostasis and dysregulation of ion, cell and drug movement along the BBB. Cerebral ischemia severely damages the BBB. The resulting hypoxic condition can lead to inflammation, functional impairment and alteration of cellular interaction (Blanchette and Daneman, 2015). This destruction of the BBB occurs in the first hours of ischemic stroke and can lead to damage of brain tissue, neuro-inflammation and loss of function (Liebner et al., 2018).

1.4 hCMEC/D3 cell line

Over the years, several *in vitro* cell lines have been investigated to model angiogenesis and mimic the BBB (Staton et al., 2009). Using primary human cerebral endothelial

cells is unfeasible in scaled research mainly because of the scarceness of fresh human brain tissue. Therefore immortalized cell lines have been developed that can be grown efficiently and successfully mimic the BBB (Weksler et al., 2013).

Considering the vast heterogeneity in endothelial cell lines obtained from different tissues of the body, the widely used and well-established brain microvascular endothelial derived cell line hCMEC/D3 was used in this study. Not long ago the most prevalent cell line for *in vitro* BBB research was amongst others human umbilical vein endothelial cells (HUVEC), which are isolated from umbilical veins. hCMEC/D3 were specifically developed for studying the physiology and pathology of microvascular rather than macrovascular brain vessels, making them more suitable for angiogenesis research (Staton et al., 2009).

The hCMEC/D3 cell line originates from human temporal lobe microvasculature that was extracted amid epilepsy surgery. By transducing a lentiviral vector, the cells were initially immortalized with the human telomerase (hTERT) and SV40 T antigen. After maturation as well as culturing, the clones were isolated and analyzed for characteristics fitting cerebral endothelial cells. These endothelial markers include CD34, CD31, CD40, CD105, CD144 (VE-cadherin) and von Willebrand factor (Weksler et al., 2013).

In vitro, hCMEC/D3 behave similarly to inherent cerebral endothelial cells and preserve distinct receptors and transporters on their surface including multidrug resistance mutation 1 (MDR1), breast cancer resistance protein (BCRP), multidrug resistance protein 4 (MRP4), transferrin and insulin receptors and glucose transporter 1 (GLUT-1). They furthermore display strict and defined permeability through expression of junctional proteins amongst others adherens and tight junctions. Similarly to *in vivo* endothelial cells, they retain structural proteins such as VE-cadherin, claudin-3 and -5 as well as occludin and catenin (Weksler et al., 2013).

The hCMEC/D3 cell line is a suitable and stable *in vitro* model of the BBB that is widely available and reproducible, while maintaining physiological BBB properties. It is especially beneficial for understanding endothelial processes of various cerebral pathologies most notably involving angiogenesis. The hCMEC/D3 cell line is broadly

adapted in angiogenesis research, assessing the cells proliferation, migration, viability and angiogenesis *in vitro.*

1.5 The acid sphingomyelinase / ceramide system

The cell membrane mainly consists of sphingolipids, cholesterol, and phospholipids. Sphingolipids, such as sphingomyelin, make up the lipid component of the plasma membrane as well as intracellular membranes of organelles. They not only provide structural but also regulate signaling processes to initiate and drive numerous cellular pathways including growth, senescence, migration, adhesion, inflammation and cell death (Verderio et al., 2018). Ceramide plays a key role in these processes. It consists of a sphingosine and fatty acid linked by an amid bond. Hydrophilia of the ceramide molecule results in interactions of sphingolipids with each other and with cholesterol. Ceramide forms microdomains and interconnects into large ceramide-enriched membrane platforms. These platforms reach diameters of 200 nanometers up to several micrometers. Research suggests that they play a key role in signaling pathways for apoptosis and growth inhibition besides others (Stancevic and Kolesnick, 2010, Zhang et al., 2009).

A key enzyme for the formation and regulation of membrane lipids, especially of sphingomyelin and ceramide, is the acid sphingomyelinase (ASM). The sphingomyelin phosphodiesterase gene (SMPD1) encodes the ASM, a lysosomal phosphodiesterase. It is expressed on chromosome 11p15.1-p15.4 (Schuchman et al., 1992). The enzyme is initially synthesized as a pre-pro-enzyme with N-glycosylation. After cleavage of its N-terminal peptide the pro-enzyme is transported into the Golgi complex and glycosylated post-translationally. The Golgi secretory pathway releases the ASM in its functional form (Wan and Schuchman, 1995).

When cell signals such as stress stimuli, irradiation and inflammation activate the ASM, it relocates to the outer cell membrane and produces ceramide and phosphorylcholine by hydrolyzing sphingomyelin (Wong et al., 2000).

Studies in which cerebral artery occlusion was induced in mice, rats, and gerbils showed correlation of ischemic stroke and the ASM/ceramide system (Mohamud Yusuf A, 2019). Synthesis of ceramide through ASM-induced cleavage of sphingomyelin seems to play a major role in injury-response of ischemic stroke processes. Transient focal cerebral ischemia induces activation of ASM and increases levels of ceramide in mice. Whereas ASM deficiency showed suppression of ischemia-induced ceramide levels. The ASM deficiency also showed reduction of neurological deficits and infarct size as well as inhibition of cytokines associated to inflammation and prevention of apoptosis (Yu et al., 2000). Due to the beneficial post-stroke outcome of ASM inhibition that results in decreased ceramide levels, drugs targeting this inhibition are regarded as a promising pathway in stroke treatment.

1.6 Functional inhibitors of acid sphingomyelinase

Functional inhibitors of acid sphingomyelinase (FIASMA) are a group of drugs that pharmacologically suppress ASM activity. Antidepressants including amitriptyline, nortriptyline and fluoxetine play a decisive role in the ASM/ceramide system (Kornhuber et al., 2010, Gulbins et al., 2013). In addition to the widespread theory that regulation of neurotransmitters in the synaptic cleft is the main effector of antidepressants, there are suggestions that the ASM/ceramide system plays a central role in treating depression (Gulbins et al., 2013). Fluoxetine is a selective serotonin reuptake inhibitor (SSRI). The tricyclic antidepressants nortriptyline and amitriptyline inhibit serotonin reuptake or noradrenalin reuptake respectively (Sánchez and Hyttel, 1999). FIASMAs passively enter the BBB and interfere with ASM function. ASM resides on intra-lysosomal membranes, which protect the enzyme from being proteolytically inactivated. FIASMAs disrupt the enzyme's functionality by accumulating in lysosomes, which causes a detachment of the ASM from the membrane

and leads to its degradation by lysosomal proteases (Kornhuber et al., 2010, Kornhuber et al., 2011).

Beneficial post stroke behavioral outcome and reduction of infarct size, as well as induction of neurological recovery have been demonstrated in several experimental stroke studies after administration of fluoxetine (Lim et al., 2009, Chollet et al., 2011).

Promising pre-clinical data, showing that drugs inhibiting ASM activity may induce beneficial effects following ischemic stroke, lead to the initiation of clinical trials. Although some trials have not been successful in regards to neurological outcome for stroke patients, several studies suggest a favorable influence of FIASMAs on post stroke enhancement of motor recovery, pain relief and increased probability of survival (Ricardo E. Jorge et al., 2003, Chollet et al., 2011, Dennis et al., 2019).

1.7 Mesenchymal stromal cells

Mesenchymal stromal cells (MCSs) are defined as pluripotent cells, that are able to differentiate into a variety of cell types. Their ubiquitous presence in the human body makes them an essential component in physiologic and pathologic response to trauma, disease, inflammation and aging (Pittenger et al., 1999). To date, clinically used MSCs are mainly derived from the bone marrow. They can also be isolated from a variety of embryonic tissues like skin, dental pulp, adipose tissue, amnionic fluid and blood. MSCs are recognized as a promising therapeutic agent due to their plasticity and immunomodulary characteristics, as well as their limited side effect profile (Heldring, 2015). Insight into the detailed mechanisms of MSC effects and their fate after clinical application is insufficient. It is suggested that they are safe for clinical use, due to their reduced inherent immunogenicity and apparent absence in affected tissues and circulation after 60 min of administration (Caplan and Dennis, 2006). However, some evidence challenges the safety of MSCs because of their ability to reside as tumor-associated cells, influencing the tumor microenvironment (Barcellos-de-Souza et al., 2013). Although MSCs seem to generally take on cancer-restraining roles, both promotion and inhibition in tumor contexts could be shown depending on the

experimental design. Other studies suggest that MSCs display a protumor function through secretion of soluble and insoluble factors that impact cancer cell proliferation and migration (Karnoub et al., 2007).

Extensive research in clinical trials shows a broad potential of MSCs for different diseases including hematological, neurological, cartilage and bone, cardiovascular, lung, kidney, liver, organ transplantation and autoimmune diseases (Heldring, 2015). Their mode of action is suggested to involve paracrine pathways (Caplan and Dennis, 2006). It is suggested that bioactive components secreted by MSCs are widely involved in suppressing local immune processes and inhibiting fibrosis, scaring and apoptosis. Further effects include increased angiogenesis, mitotic activity and differentiation of supportive stem cells and reparative tissue, including regeneration of functional neurological pathways such as synaptogenesis and neurogenesis (Caplan and Dennis, 2006). This regenerative responses, in addition to their angiogenesis stimulation, indicate that MSCs may be promising agents in ischemic stroke studies. Their angiogenic potency and effects in post stroke animal models as well as preclinical ischemic disease research is a topic of interest (Dao et al., 2013).

As MSCs are solely applicable after invasive isolation procedures from bone marrow biopsy or liposuction, their widespread use in clinical scenarios is limited. Furthermore insufficient proliferation and a potential functional decline, as well as the risk of cellular transfer rejection and inconsistent quality control in large-scale cell production portray their limitations and disadvantages (Xia et al., 2020). In order to overcome these constraints, the search for active secretory components of MSCs began. It is suggested that the influence of MSCs on the previously listed pathways might be associated or even ascribed to their paracrine activity, as their efficacy does not seem to depend on the proximity of application to the targeted tissue. Secretory extracellular vesicles (EVs) are proposed to mediate the effects of MSCs (Almeria et al., 2019). A variety of studies provided evidence that extracellular vesicles showed comparable therapeutic effects as their parental MSCs (Doeppner et al., 2015, Heldring, 2015).

1.8 Extracellular vesicles

1.8.1 What are EVs?

It is suggested that the various effects of MSCs can be attributed to their released extracellular vesicles (EVs), which are nanosized particles (50-150nm) that are incapable of replicating (Théry et al., 2018). EVs are surrounded by a lipid bilayer that is enriched in sphingolipids and gangliosides. Their content consists of proteins, nucleic acids and lipids, which are transported in EVs for intercellular communication and mediation of a wide range of cellular processes and signaling pathways. Subtypes of EVs include microvesicles, exosomes and apoptotic bodies, and show large heterogeneity in their size, content, function and biogenesis (Zaborowski et al., 2015). Due to this parity and overlapping in structure as well as biogenesis among different kinds of EVs, the International Society for Extracellular Vesicles (ISEV) suggested to define them as small extracellular vesicles (sEVs < 200nm) and medium or large extracellular vesicles (EVs > 200nm) (Théry et al., 2018).

The acknowledged consensus is that biogenesis of sEVs takes place in a “reverse endocytic” manner. They are secreted through invagination of the endosomal membrane and are secreted via fusing with the plasma membrane (Lai et al., 2015). The production of sEVs is based on endosomal networks, which serve to sort different intraluminal vesicles in membranous compartments and transport them to lysosomes or the cell membrane. The following fate of these vesicles includes destruction, recycling and exocytosis (Akers et al., 2013).

As a first step of sEV biogenesis endocytic vesicles release their content by fusing with early endosomes. This process is marked by the formation of multiple small interluminal vesicles (ILVS). A main point of this formation is that endosomal membrane proteins reorganize into tetraspanin-enriched microdomains. The reorganized early endosomes show a great affluence of phosphatidylinositol 3-phosphate (PIP3), which in combination with ubiquinated cargos and the specific curvature of endosomal membranes leads to the induction of endosomal sorting complexes (ESCRT) required for transport: ESCRT-0 initiates ubiquitin-dependent cargo clustering whereas ESCRT-I and ESCRT-II complexes promote and ESCRT-III completes membrane budding and

exocytosis (Babst, 2011). Finally, the fusion of the plasma membrane with the endosomes resolves in the secretion of sEVs into the extracellular space. The bilayered fusion of the plasma membrane results in asymmetric distribution of phospholipids: the inner layer is phosphatidylserine and phosphatidylethanolamine enriched, whereas outer layer predominantly consists of phosphatidylcholine and sphingomyelin (Zwaal and Schroit, 1997).

The rate of sEV release and their cargo seems to be associated to ESCRTs as well. Alteration of the ESCRT system has been shown to alter sEV content, including Alix and tumor susceptibility gene 101 (TSG101), that are themselves ESCRT components and are found enriched in sEVs and targeted as identification markers (Trajkovic et al., 2008). According to new insights ESCRT-associated proteins, such as Alix, induce vesicle budding and sEV biogenesis upon Syntenin interaction. Syntenin is a cytoplasmic adaptor of heparan sulphate proteoglycan receptors and is commonly used in identification and classification of sEVs as well (Kowal et al., 2014). As of now not one surface marker has stood out to independently define exosomes. For example, though CD63 and many others can reliably help identifying sEVs, they are also involved in various other processes and structures including apoptosis (Akers et al., 2013).

Although ESCRT was proven to be a key way in sEV biogenesis, ESCRT-independent pathways have been reported in genesis and formation of sEVs: these pathways involve tetraspanins, phospholipases, lipids and ceramide. Induced cholesterol upregulation was shown to increase the secretion of sEVs containing Flotillin-2, ALIX, CD63 and cholesterol (Strauss et al., 2010). Phospholipase D2 (PLD2) through phosphatidylcholine hydrolysis to phosphatidic acid (PA) induces inward curvature of membranes and was shown to induce sEV secretion, and was also shown to be enriched in sEVs (Laulagnier et al., 2004).

For the secretion of sEVs, certain proteins of the tetraspanin family have recently been studied. Tetraspanins, also referred to as transmembrane 4 superfamily (TM4SF) proteins, seem to be found in large amounts in sEVs. Some of them showed induction of sEV secretion and change in their protein content (Kowal et al., 2014). The most

recognized tetraspanins, CD9, CD63 and CD81, are ordinarily used in identification, selective isolation, and description of sEVs (Pols and Klumperman, 2009).

It is also suggested that the ASM/ceramide system plays a key role in biogenesis and surface budding of sEVs. Due to its inherent effect on lipid metabolism, ASM can alter cell membrane fluidity and curvature. Ceramide was shown to increase formation of vesicles and secretion by establishing membrane curvature and blebbing of the sEVs (Verderio et al., 2018). A study from 2008 suggest that SM hydrolysis and ceramide formation stimulate biogenesis of intraluminal vesicles, that are then released as sEVs, independently from inherent endosomal complexes (ESCRT-independent) (Trajkovic et al., 2008). They observed high enrichment of ceramide in certain sEV subpopulations. Trajkovic et. al. propose that sEVs with high ceramide concentrations are progressively formed at SL enriched membrane domains through NSM mediated ceramide generation. Inhibition of sphingomyelinases caused reduction of their release through impaired ceramide biogenesis.

Another study suggests that inhibition of sphingomyelinases regulates microvesicular ectosome (MV) and sEV secretion differently. Pharmacological and genetic inhibitors of sphingomyelinases were shown to mediate an increase in MV and a decrease in sEV budding at the plasma membrane (Menck et al., 2017).

1.8.2 Role of sEVs in intercellular communication

sEV secretion is ubiquitous in the human body. sEVs have been detected in serum, plasma, saliva, tears, cerebral spinal fluid, breast milk, urine, semen, amniotic fluid, synovial fluid, gastric acid, and bile. They serve as a means of intercellular communication for a wide range of signaling pathways (Doyle and Wang, 2019). Their complex cargo consisting of lipids including sphingolipids and ceramide, nucleic acids, enzymes, and other proteins makes them a vehicle for various molecules. sEVs express a unique proficiency of packaging this active cargo and dispatching it to the intended cells. By this way sEVs can alter the targeted cell's function and mediate cell signaling. As encapsulated particles, sEVs are easily capable of being transported through bodily

fluids and being taken up via endocytosis (Lai et al., 2015). Therefore, their function is not limited to paracrine activity but comprises a variety of cellular processes in both adjacent and distant areas of the body. Analysis of sEV-biodistribution showed an uptake of red blood cell-derived sEVs by the liver (44.9%), bone (22.5%), skin (9.7%), muscle (5.8%), spleen (3.4%), kidney (2.7%) and lung (1.8%) (Willekens et al., 2005).

sEVs effect their targeted cells through direct and indirect cellular interaction and mediate changes in the expression of target cell proteins. This is achieved through ligand-to-receptor interaction. For example major histocompatibility complex (MHC) I and II (Simons and Raposo, 2009), and tetraspanins (Nazarenko et al., 2010) are targeted pathways and can provoke integrin- and calcium-signaling (Clayton et al., 2004) as well as mitogen-activated protein kinase (MAPK) activation (Calzolari et al., 2006) among others.

Noteworthy membrane receptors include CD14, CD91, Toll-like receptor (TLR)-2, TLR-4 and lectin-like oxidized low-density lipoprotein receptor (LOX-1) as well as CD94/CD56 (Macario et al., 2010).

Some of the biological processes sEVs are involved in include the induction of proliferation, migration, and invasion as well as angiogenesis. (Zaborowski et al., 2015). By inducing the secretion of anti-inflammatory cytokines, sEVs also have immunomodulary purposes (Doeppner et al., 2015). sEVs can also boost restorative processes as a response to injury (Zhang et al., 2019). It was also shown that both induction as well as suppression of cancer-linked angiogenesis can occur depending on MSC-sEV preparation, sources, and tumor environment (De Palma et al., 2017).

Though their exact molecular function and their involvement in various signaling processes are yet to be fully understood, sEVs have been a topic of research for numerous therapeutic approaches including drug delivery, pathogen vaccination and anti-tumor -, immunomodulatory and regenerative therapies (Lener et al., 2015). In this study we mainly focus on MSC derived sEVs that are similarly beneficial in therapeutic use as their parental MSCs (Doeppner et al., 2015). Most comprehensive pre-clinical models of MSC-sEVs to date show beneficial effects of sEVs in liver and lung injuries, acute kidney injury (AKI), myocardial infarction (MI) and stroke. In these settings sEVs

showed promising effects including tissue repair, promotion and support of hematopoiesis and angiogenesis, as well as immune modulation (Heldring, 2015).

1.8.3 sEVs in stroke

MSCs have shown favorable effects in numerous animal models of ischemic stroke and a unique potential of inducing angiogenesis-related pathways in preclinical stroke studies (Dao et al., 2013). Due to these promising properties, MSC derived sEVs, as potential mediators of these effects, have been a novel focus of stroke research. Due to their involvement ins intercellular communication and their ability to serve as cargo transportation vehicles EVs are potential candidates for stroke treatment (Saint-Pol et al., 2020). Systemic application of MSC-sEVs has been shown to induce recovery and neuroprotection, as well as neurogenesis and angiogenesis. Through immunomodulation, MSC-sEVs could limit infectious processes and post-ischemic injury in the stroke recovery phase in mice (Doeppner et al., 2015). As MSC-sEVs have shown similar effects of increasing post-ischemic angioneurogenesis and neuroprotection, it is suggested that they themselves mediate the involved signaling pathways of MSCs (Doeppner et al., 2015). A main center of attraction in MSC-sEV research is based upon their pro-angiogenetic properties. It is suggested that the angiogenesis inducing sEV effects in post-stroke environments, in combination with neuroprotective and immunomodulatory tendencies, play a crucial role in recovery. In concordance with this premise, MSC-sEVs were found to reduce infarct volume and enhance long-term neurological deficits in post-stroke rat models, as well as increase cell migration and tube formation of cerebral endothelial cells in vitro (Xia et al., 2020). These pro-angiogenic effects are considered to be conveyed via sEV-cargo, especially VEGF, transforming growth factor-beta (TGFB1), transcription factors for example STAT3/STAT5 and microRNAs (miRNAs). Studies suggest that these cargo components play a role in activating signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), phosphoinositide 3-kinases (PI3K), and extracellular signal-regulated kinases (ERK1/2) (Todorova et al., 2017).

What exactly promotes these effects and how the underlying pathways can be regulated is yet to be determined. Some studies suggest that hypoxia-inducible factor-1α (HIF-1α) dependent processes mediate the increased tube formation and proliferation of cultured human umbilical vein endothelial cells (HUVECs) after treatment with sEVs. (Gonzalez-King et al., 2017). Other studies highlight the role of miRNAs and sphingolipids. MicroRNA is a kind of non-coding RNA that is located in nuclei. It consists of 22-25 nucleotides and can effect a wide range of cellular processes through intercellular communication and regulation of gene expression (Kim, 2005). Some of these effects include proliferation, differentiation, metabolism, immunomodulation and angioneurogenesis in stroke (Rahmani et al., 2020). While some studies suggest, that sEVs may also play a role in regulating expression and circulation of miRNAs in physiological and pathological circumstances (Chen et al., 2015), other studies suggest miRNAs play a less important role than initially assumed (Chevillet et al., 2014).

Another widely discussed factor in the intercellular activity of sEVs are sphingolipids (SL). Though the specific pathways of sEV involvement are not yet determined, research indicates that sEVs can act through regulation of SL metabolism in targeted cells by acting as transport vehicles to provide them with key SL metabolic enzymes (Verderio et al., 2018). The exact molecular pathways of sEVs and their involvement in various signaling processes are yet to be fully understood.

1.8.4 Regulation and alteration of sEVs

Due to the promising efficacy of MSC-sEVs in stimulating beneficial pathways in various pathological conditions, interest has grown to further enhance their potency. The main factors of achieving increased sEV effectiveness have been shown to be higher secretory concentrations and alteration of sEV content. sEV production and secretion can be stimulated using different biochemical or biophysical strategies. Patel et. al. suggest that the release and effectiveness of sEVs highly depends on cell culture parameters. Increased biogenesis and secretion of sEVs per MSC could be demonstrated through a higher frequency of sEV collection, as well as a decrease in seeding density.

Negative effects on MSC-sEV potency was observed with increasing culture passages (Patel et al., 2017).

Not only cell culture parameters can affect MSC-sEV yield. Alteration of MSC culturing parameters (e.g., oxygen and glucose levels) and of associated biochemical pathways suggests high fluctuation and variability in sEV release and composition. The pretreatment during MSC growth can include pharmacological agents (such as vitamins, hormones, and several clinical drugs), cytokines and physical factors (such as low-level lasers, electromagnetic fields, and Silica) (Hu and Li, 2018). There is little knowledge about the influence of the ASM/ ceramide system on EV content and EV functionality. Although the interconnection between lipid metabolism and sEV production are still being investigated, some studies showed involvement of ASM pathways in sEV release, cargo and effects (Trajkovic et al., 2008). Regulation of the ASM/ceramide system may portray a promising interconnection between sEV biogenesis and ASM related pathologies like stroke.

According to Verderio et. al. sEV release can be enhanced through ASM activation at the plasma membrane via surface receptors. This underlines the role of ASM in increased sEV secretion as a response to cell damage, stressful membrane processes like sickle cell disease, or danger signals like ATP and TRPV1 agonists (Verderio et al., 2018). ASM inhibition on the other hand, caused by amitriptyline treatment, decreased generation of sEVs in red blood cells (RBCs) and prevented lung inflammation (Jenkins et al., 2009, Hoehn et al., 2017). Certain types of sEVs, especially generated as a response to plasma membrane stress, can cause inflammatory processes (Awojoodu et al., 2014). sEV composition and concentration seem to have ambiguous effects depending on distinct pathologies.

It is suggested that sEVs themselves can mediate their effects through inducing and altering SL metabolism in targeted cells. sEVs seem to achieve this by providing the addressed cells with SLs and key metabolic enzymes and receptors and also by directly activating contact-mediated SL enzymes, like ASM (Verderio et al., 2018). Some studies underline the involvement of miRNA cargo inside the sEVs in regulation of SL metabolic pathways (Schatz et al., 2017). It is not fully understood whether increased

sEV efficacy in pathologic conditions is induced via greater secretion of the vesicles, the alteration of their content, their ability to interact with target cell metabolism, or a combination of all. It is necessary to understand the exact mechanisms and interconnections of sEVs in cellular pathways and targeted cells. In this study we aim to focus on pro-angiogenic effects of sEVs and their alteration as a target for stroke models.

Several studies link an increased sEV secretion by MSCs and a change in the content of their sEVs as a response to danger signals like hypoxia. It is further observed that hypoxic preconditioning of MSCs affect the angiogenic tendencies and immunomodulary as well as restorative characteristics of their sEVs through regulation of their vesicle content (Bian et al., 2014). Hypoxia (1-2% O2) induced enhanced production of sEVs by MSCs. As an consequence, increased angiogenesis, immunomodulation, neuroprotection and cardioprotection were observed (Cui et al., 2018). A recent study analyzed the angiogenic potency of sEVs derived from hypoxia pretreated MSCs *in vitro* (Almeria et al., 2019). They could show that *in vitro* indicators of angiogenesis, such as proliferation, migration and tube formation were significantly increased in sEVs derived from hypoxic MSCs compared sEVs from MSCs.

2 AIM

The aim of this study is to investigate how preconditioning mesenchymal stromal cells influences the effect of secreted sEVs on angiogenesis in cerebral endothelial cells. We hypothesized that, combining the beneficial culture conditions stated above, i.e. hypoxia and ASM inhibition, could potentially maximize favorable sEV effects. After isolation and characterization of MSC-sEVs we aimed to determine their effect on angiogenesis in hCMEC/D3. These assays serve to investigate proliferation and migration, as well as cell viability and effects on tube formation. Thereby, we aimed to investigate the therapeutic potential of MSC-EVs derived from pre-conditioned cells.

3 MATERIAL AND METHODS

3.1 Experimental design

In this study we looked at angiogenesis inducing effects of sEVs. We constructed a model of 8 experimental conditions. These include a control condition with human platelet lysate (hPL), as MSC culture media requires PL which themselves contain sEVs that are indistinguishable from cell-secreted sEVs. As a positive control amitriptyline was used at a concentration of 50µM, which was previously shown to induce angiogenesis. The other experimental groups were 50 µg/mL sEVs isolated from the supernatant of MSCs exposed to the following conditions: (1) sEVs derived from regular 'normoxic' MSC supernatants (21% O2; $sEV_{normoxic}$), (2) sEVs derived from amitriptyline treated 'normoxic' MSC supernatants ($sEV_{normoxic+ami}$) (3) sEVs derived from supernatants of MSCs cultured under hypoxic conditions for 48 hours (1% O2; $sEV_{hypoxic}$), (4) sEVs derived from supernatants of MSCs cultured under hypoxic conditions for 48 hours (1% O2; $sEV_{hypoxic}$) and treated with amitriptyline, ($sEV_{hypoxic+ami}$), (5) sEVs derived from MSC exposed to 48 hours hypoxia followed by 6 hours reoxygenation ($sEV_{reoxygenated}$) or (6) sEVs derived from MSC exposed to 48 hours hypoxia followed by 6 hours reoxygenation and treated with amitriptyline ($sEV_{reoxygenated+ami}$).

3.2 Cell culture

3.2.1 hCMEC/D3

As reviewed in several papers the brain microvascular endothelial cell line hCMEC/D3 has been established as a suitable representation of the BBB. (Weksler et al., 2013)

The procedure of cultivating the human temporal lobe derived immortalized cells was according to the manufacturer protocols (hCMEC/D3 cell line, Merck KGaA Darmstadt, Germany catal. no. 9QQ0M9). The culturing medium consists of Endothelial Cell Growth Basal Medium-2 (EBM-2; Lonza) containing 5% fetal bovine serum (FBS) (Gibco/Life Technologies), 1% penicillin/streptomycin solution (Gibco/ Life Technologies), 1% lipid concentrate (Gibco/Life Technologies), 1% Hepes buffer (10 mM, Gibco/Life Technologies), 0.5% ascorbic acid (5 µg/mL) and 0,1% hydrocortisone (1.4 µM, Sigma-Aldrich). The cells from passages 28 to 34 were

incubated at 37°C in a 5% CO_2 humified incubator containing 21% O2 in T75 tissue culture flasks (New Brunswick, Galaxy 170 S).

3.2.2 Mesenchymal stromal cells

MSCs from previously harvested bone marrow aspirates of healthy individuals (kindly provided by the Institute for Transfusion Medicine at the University Hospital Essen (MSC 41.5 P4)) were cultured following previously established protocols (Kordelas et al., 2014). The cultivation medium contained low-glucose DMEM (Pan Biotech, cat. no. P04-01500), 10% human platelet lysate (hPL; Institute for Transfusion Medicine at the University Hospital Essen in house production; available also from Macopharma and PL Bioscience), 100 U/ml, penicillin-streptomycin-glutamine (Life Technologies, cat. no. 10378016), 5 IU/ml heparin (Ratiopharm, cat. no. N68743.06). hPL was used as opposed to fetal bovine serum (FBS) to keep preparations xeno-free and in order to allow potential clinical translation.

At ~80% confluency cells were detached with trypsin/EDTA and centrifuged for 5 min at 900 × g, at 4°C. We then counted and resuspended the cells in corresponding volumes of culture medium (CM), for passaging and separation into different culturing processes.

The CM from the differently preconditioned MSCs were collected and separately centrifuged for 15 min at 2000 x g, at 4°C. The supernatant was transferred into new 50 mL Falcon tubes stored at -20°C until future processing for sEV isolation.

3.3 sEV isolation and analysis

In order to isolate MSC-derived sEVs we used a combination of ultracentrifugation and PEG precipitation, as described in (Börger et al., 2020).

CM were firstly centrifuged for 45 min at 10,000 x g, at 4°C (Avanti J26XP with rotor JS-5.3, Beckman Coulter). After discarding the pellet, we filtered the supernatant using 0.22 μm filters (Nalgene, cat. no. 595-4520) and added polyethylene glycol (PEG 6000,

50% weight/volume) and 3.75 M NaCl (Sigma Aldrich, cat. no. 71376) to a final concentration of 10% or 75 mM, respectively.

CM-supernatant were supplemented with PEG and NaCl and incubated overnight at 4°C. Following another centrifugation in 500-ml centrifuge tubes (Corning, cat. no. 431123) for 30 min at 1500 x g, at 4°C (Avanti J26XP with rotor JS-5.3, Beckman Coulter), the pellet was resuspended in 10 mL 0.9% NaCl (B. Braun, cat. no. 151072). For ultracentrifugation the CM was transferred to polycarbonate ultracentrifuge tubes (Beckman Coulter cat. no. 355622) and 0.9% NaCl was added. The samples were ultracentrifuged for 130 min at 100,000 x g, at 4°C (L7-65 with rotor Ti45, Beckman Coulter). The supernatant was removed with a pipette, while the tubes were placed on ice, and pellet was resuspended in 10 mM HEPES buffer (Gibco, cat. no. 15630049; filtered using a 0.22 μm bottle-top filter; Nalgene, cat. no. 595-4520).

The yielded sEV solutions were stored in low-retention tubes (Kisker, cat. no. G017) at -80°C. We characterized all MSC-sEVs preparations according to currently established minimal recommendations of the International Society of Extracellular Vesicles (ISEV).

3.3.1 Bicinchoninic acid analysis (BCA)

The protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermofischer, cat. no. 23227) according to the manufacturer's recommendations.

3.3.2 Nanoparticle tracking analysis

To further analyze the physical properties of our sEVs, we used nanoparticle tracking analysis (NTA) which allowed us to evaluate particle size and concentration. The analysis was performed via a Nanoparticle Tracking - Video Microscope (ZetaView PMX-420, Particle Metrix, Meerbusch, Germany). Based on the Brownian motion of particles and their distinct patterns of scattering light into the microscope, particle size and concentration were calculated by the software and the 50% median value (D50) as well as the standard deviation were noted.

3.3.3 Western blot

MSC-sEV preparations of 30 µg in NP40 cell lysis buffer (Thermo Fisher, catal. no. FNN0021) were solubilized with 10% Laemmli sample buffer containing dithiothreitol (DTT; AppliChem, Darmstadt, Germany) for Western blots. The sample-proteins were loaded on a 10% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated through electrophoresis. Polyvinylidene fluoride membranes (PVDF; Millipore, Darmstadt, Germany) were used for transfer purposes. After the transfer process we washed the membranes in 0.1 M phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 0.1 M Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) respectively. The membranes were blocked using 5% (w/v) skim milk powder (Sigma-Aldrich, Deisenhofen, Germany). Primary antibody incubation was performed overnight with anti-calnexin (ab10286; Abcam) and anti-syntenin (clone EPR8102; Abcam, Cambridge, U.K.) antibodies. We also used anti-CD81 (clone JS-81; BD Biosciences, San Jose, CA, U.S.A.) antibodies as common sEV markers. Counterstaining was established through appropriate mouse or rabbit secondary antibodies (Dianova, Hamburg, Germany).

3.3.4 Imaging flow cytometry

CD9+, CD59+, CD63+ and CD81+ expression were evaluated in the different sEVs by imaging flow cytometry (IFCM) using the AMNIS ImageStreamX Mark II Flow Cytometer (Luminex, Seattle, WA, USA). For labeling of sEVs potentially derived from human platelet lysate, CD41 was used as a negative marker. IFCM was performed, as described before (Tertel et al., 2020).

All antibodies were centrifuged for 10 min at 17,000 × *g* before they were applied to sEV samples. The following antibodies were used: CD9-PE (Exbio, clone MEM-61), CD63-PE (Exbio, clone MEM-259) and CD81-FITC (Beckman Coulter, clone B25329). 1h incubation with antibodies was performed at room temperature. sEVs were then stained and diluted with PBS to avoid swarm detection. They were analyzed through auto sampling on 96-well round-bottom plates at an acquisition time of 5 min per well. For analysis of 60 × magnified data the IDEAS software (version 6.2) was as

previously described (Tertel et al., 2020). Fluorescent events and average concentration data were collected.

3.3.5 Transmission electron microscopy

For transmission electron microscopy analysis of the sEVs samples were diluted in 10mM HEPES buffer. Each sample was placed on a 200 mesh hydrophilized copper grid covered with a carbon coated formvar film (Plano, Wetzlar, Germany). The 0.5 µl droplets were then contrasted with 1.5 % phosphotungstic acid (PTA), as a staining component, in aqueous solution. Afterwards adherence of sEVs to the film surface was established through drying the samples at room temperature. A JEM 1400Plus electron microscope (JEOL, Tokyo, Japan) with a 4096 x 4096 pixel CMOS camera (TemCam-F416; TVIPS, Gauting, Germany) and 120 kV settings was used for imaging. 16 bit images were taken with EMMENU (Version 4.09.83) image acquisition software. Postprocessing was established through ImageJ (Version 1.52b; National Institutes of Health, Bethesda, MD, U.S.A.) software.

3.4 Angiogenesis assays

3.4.1 Proliferation

After seeding $3*10^5$ cells per well on a 6-well plate in 1 mL of the cultivation medium (pre-warmed to 37°C), sEVs were added (50 µg/mL) and incubated for 72 h, at 37°C (New Brunswick, Galaxy 170 S). Next, the cells were washed twice with PBS (Gibco/Life Technologies) and detached by Trypsin/EDTA (Thermo Fisher Scientific**)**. Cells were counted using an automatic cell counter (EVE Automatic Cell Counter; NanoEnTek, Waltham, MA, USA).

3.4.2 Migration

The effect of MSC-sEVs on the transmigration of hCMEC/D3s was measured over a fetal bovine serum (FBS) gradient. Transfilter assays, as a modification of the Boyden chamber (Boyden 1962), are commonly used to investigate the physiological migration of endothelial cells (Staton et al., 2009).

Firstly, $3*10^4$ hCMEC/D3 cells were seeded in cultivation medium containing 1,25 % FBS in the upper compartment of a transwell insert (polycarbonate membrane insert with 8.0µm pores; GE Healthcare Life Sciences, Chicago, IL, USA) in a 24 well plate. Then MSC-sEVs (50 µg/mL) were added in a solution of 400 µL cultivation medium containing 5 % FBS into the lower compartment. Following an incubation for 24 h, at 37°C (New Brunswick, Galaxy 170 S), we removed the unmigrated cells from the top of the filter with a PBS-soaked cotton swab and washed both compartments 3 times with PBS. After this we fixed the cells with 4% Paraformaldehyde (PFA) for 20 min and subsequently washed the cells twice with PBS. In order to count the migrated cells, hCMEC/D3 nuclei were stained with Hoechst 33342 (20mM) (Thermo Fisher Scientific). Thereafter glass slides and fluoromount medium (Thermo Fisher Scientific, Waltham, MA, USA) were used for imaging. The cells were counted with a fluorescence microscope (10 x Zoom; EVOS fl, AMG, Bothell, WA, USA) on at least 8 regions of interest (ROI) per filter measuring 600 × 400 µm. Cell numbers were quantified using the analytic toolsets in Image J (NIH, Baltimore, MD, USA).

3.4.3 Viability

Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. In this assay, formation of formazan crystals, as a result of cellular MTT metabolization is quantified and correlates to the number of vial cells (Denizot and Lang, 1986).

$2*10^4$ hCMEC/D3s were seeded per well of a 96 well plate including control wells with just medium. After incubation overnight at 37°C (New Brunswick, Galaxy 170 S), we added our treatment compounds respectively. sEV samples were added at a concentration of 50 µg/mL and incubated overnight at 37°C. Following this, we removed the medium and added 100 µL of 90% cultivation medium + 10% 600 µg/ml MTT. After incubating the cells for another 2 h at 37°C, we removed the solution and added 100 µL DMSO (Sigma-Aldrich) to dissolve formazan crystal formations and fixed the cells. To quantify the cell viability through absorbance of formazan crystals produced by vial cells, we measured absorbance at 570 nm with a microplate absorbance reader (iMarkTM; Bio-Rad Laboratories, Hercules, CA, USA).

As a second variation of the MTT assay, we were interested in seeing the effects of the different MSC-sEVs on hCMEC/D3s exposed to hypoxia. For this iteration of the experiment, we subjected the cells to 24 h oxygen-deprivation in a hypoxia chamber and subsequently reoxygenated them for 6 h, at 37°C in a 5% CO2 humified incubator (New Brunswick, Galaxy 170 S) during which the cells were incubated with sEVs derived from MSCs exposed to the depicted treatment conditions. Viability was again measured by the absorbance of formazan crystals at 570 nm.

3.4.4 Tube formation

Tube formation assays mimic the development of capillary-like tubes and models early phases of vessel construction (Staton et al., 2009).

60 µl matrigel per well was pipetted into 96-well plates incubated at 37°C until solidified (~ 30 min) (New Brunswick, Galaxy 170 S. We detached the cells and seeded them in fresh cultivation medium at a concentration of $3*10^4$ cells per 100 µL. After addition of the different conditions of sEVs (50 µg/mL) we plated 100 µL per well of the cell-EV-solution onto the solidified Matrigel layer and incubated the plates for 20 h, at 37°C (New Brunswick, Galaxy 170 S). Tube formation was quantified using Image J (NIH, Baltimore, MD, USA).

3.5 Statistics

Statistical analysis was executed with GraphPad Prism Macintosh Version 8.2.1 (279) (MacKie, La Jolla, CA, USA) using two-way analysis of variance (ANOVA). Mean ± standard deviation (SD) values (angiogenesis assays) or median values ± interquartile ranges (IQR) with maximum and minimum data as whiskers were used to illustrate results. Significance was defined by p values < 0.05.

4 RESULTS

Isolated MSC-sEV samples showed *bona fide* sEV characteristics

Transmission electron microscopy (TEM) analysis confirmed that sEVs in all preparations had a double-layered membrane structure **(Fig. 1)**. The flow cytometric characterization showed that sEVs expressed CD9, CD41, CD59, CD63 and CD81. No significant changes were noted in the sEVs derived from MSCs exposed to different experimental conditions **(Fig. 2)**. Nanoparticle tracking and bicinchoninic acid assays (BCA) results demonstrated that the isolated sEVs correspond to sEV size and protein concentration characteristics **(Tab. 1)**.

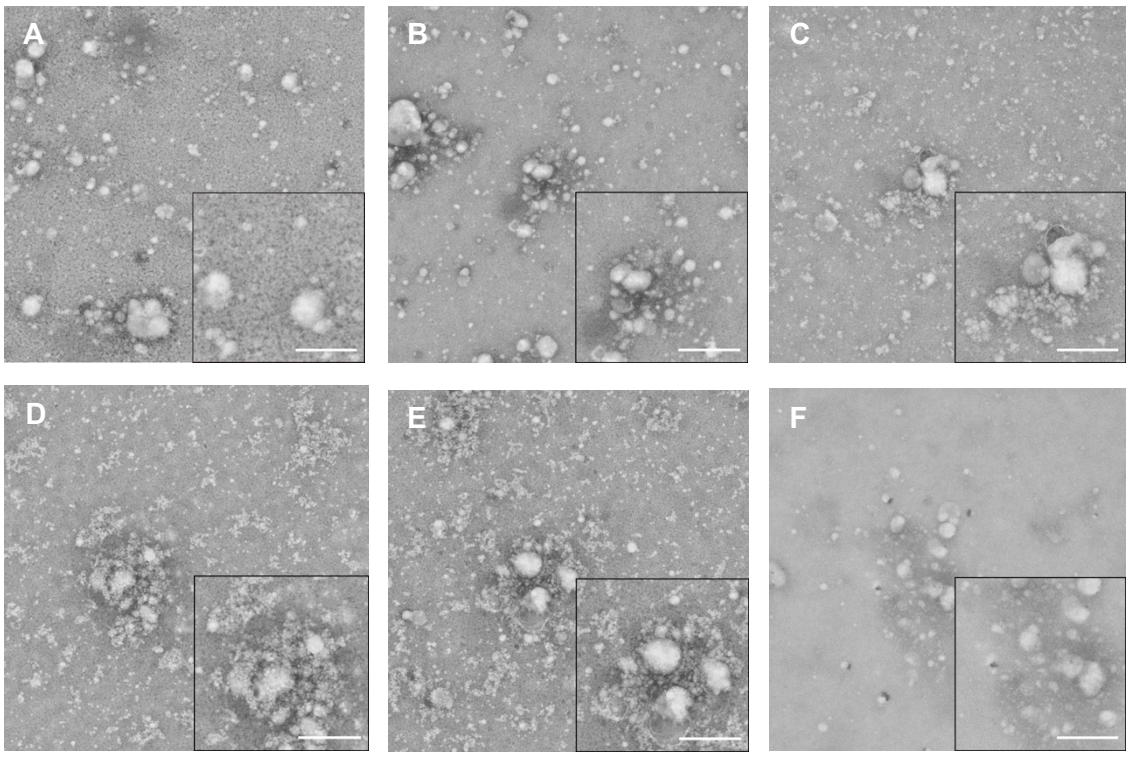

Figure 1: Transmission electron microscopy (TEM) image of representative mesenchymal stromal cell derived small extracellular vesicles (sEVs). sEVs presence was confirmed for all preparations through transmission electron microscopy (TEM) and showed double membrane-like configuration as well as vesicular structures which correspond to the size and appearance of sEVs. (A) $sEV_{normoxic}$ (B) $sEV_{normoxic+ami}$ (C) $sEV_{hypoxic}$ (D) $sEV_{hypoxic+ami}$ (E) $sEV_{reoxygenated}$ (F) $sEV_{reoxygenated+ami}$. Scale bar: 300 nm.

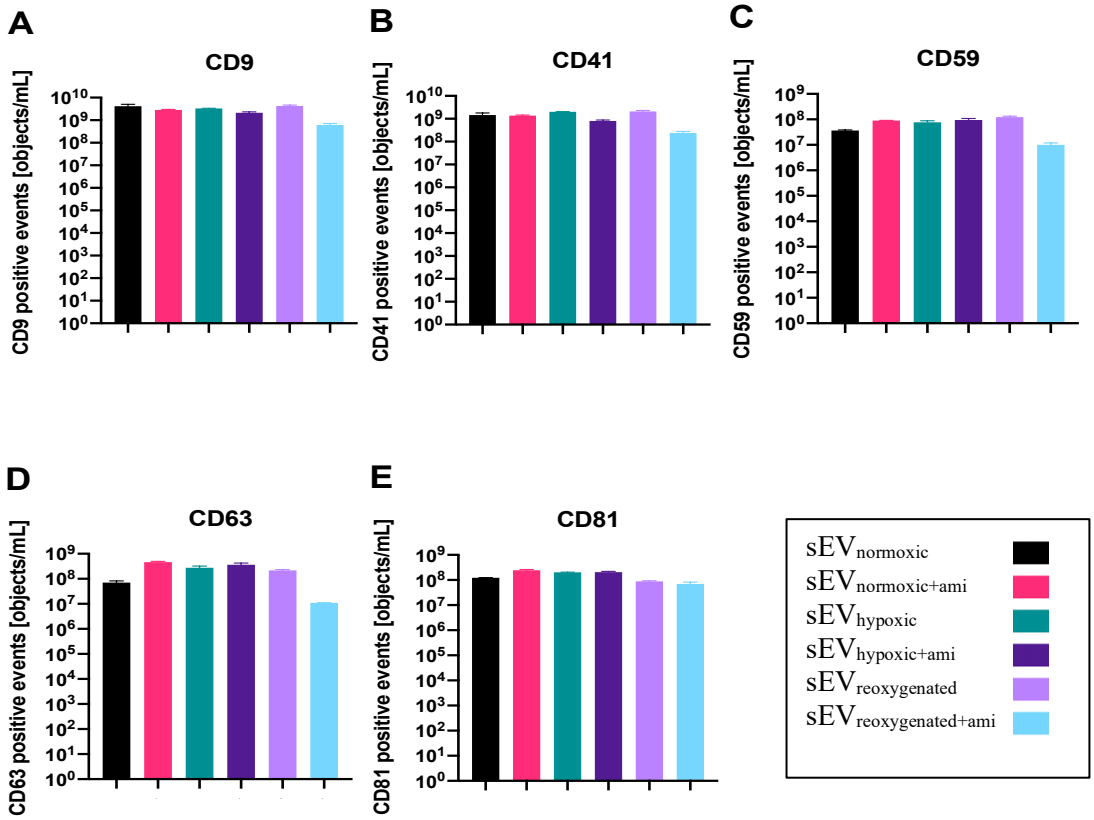

Figure 2: Characterization of MSC-derived small extracellular vesicles (sEV) through imaging flow cytometry (IFC). (**A**) CD9+, (**B**) DC41+, (**C**) CD59+, (**D**) CD63+ and (**E**) CD81+ expression was confirmed different sEV preparation shown by imaging flow cytometry using the AMNIS ImageStreamX Mark II Flow Cytometer (Luminex, Seattle, WA, USA). Data are mean ± SD values.

Table 1: Characterization of MSC-sEV preparations by nanoparticle tracking analysis (NTA) and bicinchoninic acid assay (BCA). Data are mean ± SD values.

MSC-sEV preparation	Particle concentration [particles/ml]	Paticle size [nm]	Protein concentration [µg/µl]	Purity [particles/mg protein]
$sEV_{normoxic}$	8.3 x10^{10}	113	1.29	1.1 x10^{8}
$sEV_{normoxic+ami}$	1.6 x10^{10}	110.1	2.78	4.5 x10^{7}
$sEV_{hypoxic}$	1.6 x10^{10}	108.3	3.65	5.8 x10^{7}
$sEV_{hypoxic+ami}$	1.3 x10^{11}	109.3	4.42	5.7 x10^{8}
$sEV_{reoxygenated}$	9.4 x10^{8}	85.4	4.86	4.6 x10^{6}
$sEV_{reoxygenated+ami}$	2.3 x10^{8}	100.2	3.59	8.3 x10^{5}

Characterization by Western Blots also showed the presence of exosomal markers CD81 and Syntenin in all MSC-sEV preparations and the absence of the contamination marker Calnexin (**Figure 3).**

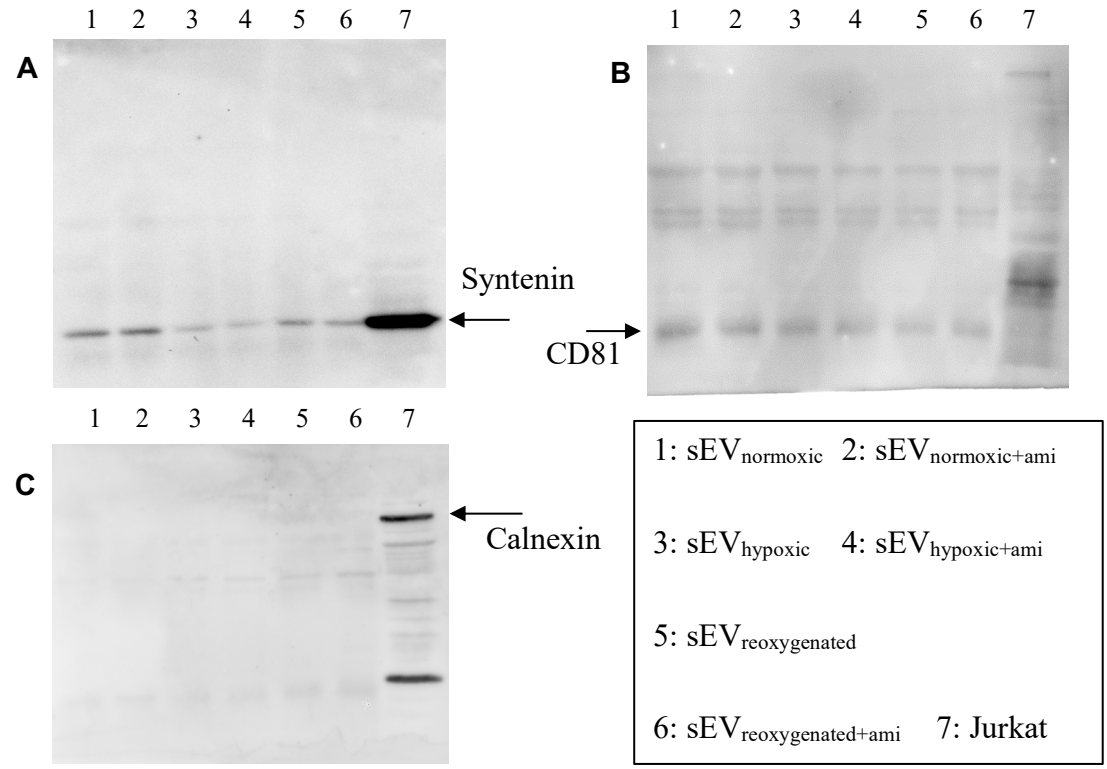

Figure 3: Characterization of MSC-derived small extracellular vesicles (sEV) by Western Blots. Exosomal marker proteins Syntenin **(A)** and CD81 **(B)** were detected in all MSC-sEV preparations used within this study (1-6). In contrast, the cytosolic contamination marker Calnexin **(C)** is not expressed in any of the sEV samples but the positive control containing cell lysate (7). Blots where sequentially probed with anti-syntenin and anti-calnexin antibodies or with anti-CD81 antibodies, respectively.

ASM-inhibition induced by amitriptyline treatment increased angiogenesis

Compared to the control condition with platelet lysate, amitriptyline at a concentration of 50 µM, at which it was previously shown to induce angiogenesis, increased tube formation and cell viability while decreasing proliferation (**Fig. 4)**.

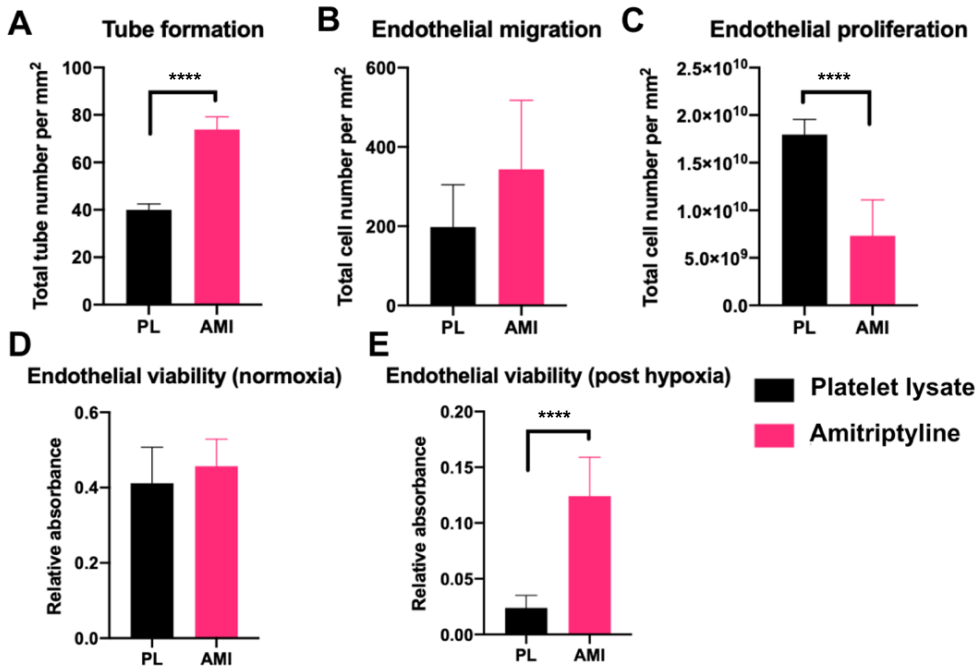

Figure 9: ASM inhibition induced by amitriptyline treatment of hCMEC/D3 increased tube formation and cell viability, while decreasing proliferation. Angiogenesis assays were done in hCMEC/D3 cells exposed to either platelet lysate (PL) or platelet lysate containing amitriptyline (AMI, 50 µM). Amitriptyline treatment increased the number of tubes **(A)**. Endothelial cell viability was tested via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Compared to PL alone, amitriptyline increased cell viability in cells exposed to 24 h hypoxia (1% O2) followed by 6 h reoxygenation (21% O2) **(E)**, while no change could be shown for endothelial cells cultured under 'normoxic' conditions (21% O2) **(D).** Amitriptyline treatment of hCMEC/D3 significantly decreased endothelial proliferation **(C)**. There was no significant change in migration of amitriptyline treated hCMEC/D3 cells, compared to platelet lysate samples (PL) **(B)**. Data are mean ± SD values (n = 8 independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001 compared with PL.

Preconditioning mesenchymal stromal cells modified the effect of their secreted sEVs on angiogenesis-like processes in cerebral endothelial cells

Treatment of hCMEC/D3 with sEVs isolated from MSCs exposed to normoxia, hypoxia or hypoxia followed by reoxygenation and treated with or without amitriptyline induced no significant change of cell proliferation **(Fig. 5)**. Cell migration was also not significantly influenced by sEV treatment **(Fig. 6)**.

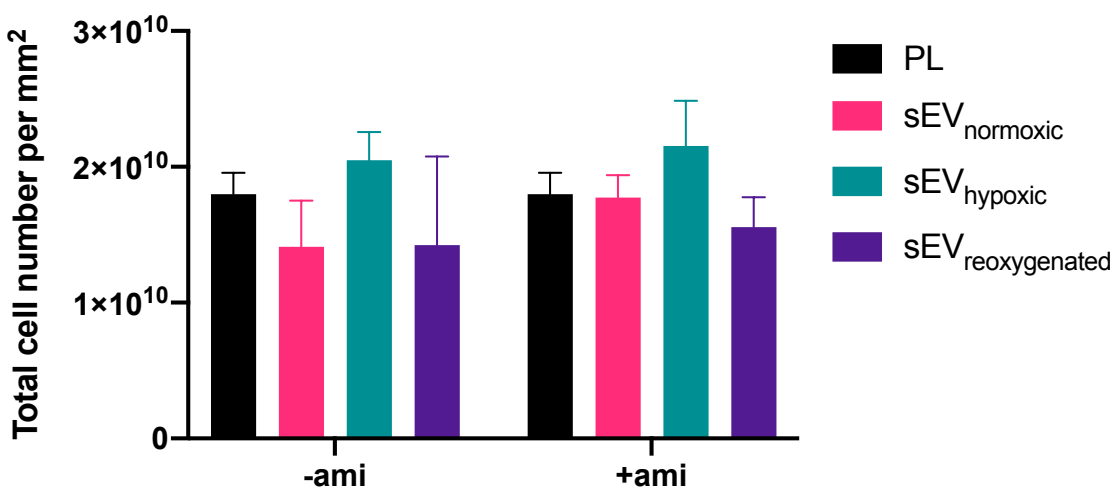

Figure 5: MSC-sEV preparations did not influence cerebral microvascular endothelial cell proliferation, when MSCs were exposed to amitriptyline.

sEVs obtained from MSCs cultured under different conditions neither increase nor decrease cerebral microvascular endothelial cell proliferation significantly. Amitriptyline treatment of MSCs before sEV isolation did not influence proliferation of the relative number of human microvascular endothelial cells (hCMEC/ D3). Oxygen alteration of MSC culturing conditions (21% O2; $sEV_{normoxic}$ vs 1% O2; $sEV_{hypoxic}$) as well as reoxygenation after 1% O2 hypoxia ($sEV_{reoxygenated}$) did not influence hCMEC/D3 proliferation. Data are mean ± SD values (n = 7 independent experiments).

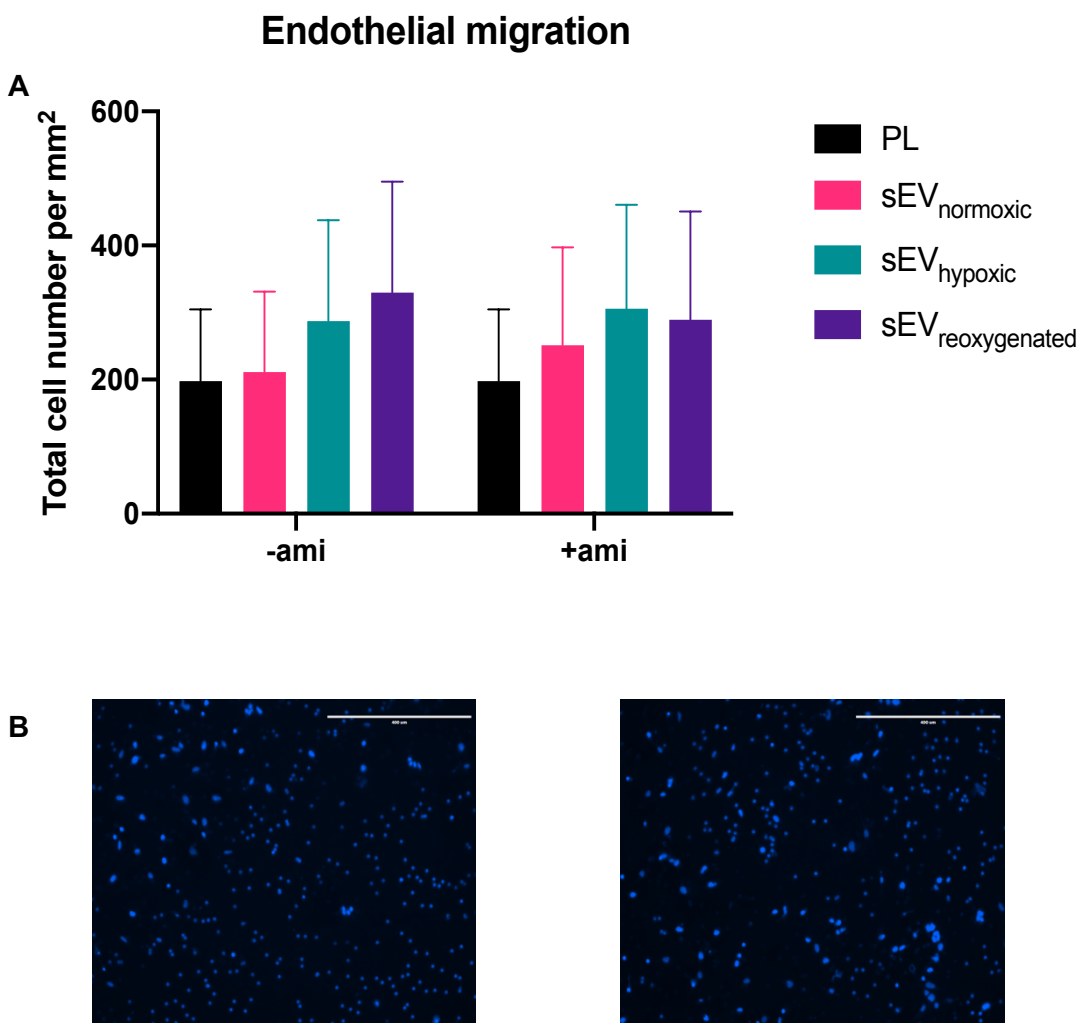

Figure 6: MSC-derived sEVs did not influence cerebral microvascular endothelial cell migration, when MSCs were exposed to amitriptyline.

The transwell migration of hCMEC/D3 treated with different sEV preparations is depicted. **(A)**. sEVs obtained from MSC sources cultured under 'normoxic' conditions (21% O2; $sEV_{normoxic}$) or hypoxic conditions (1% O2; $sEV_{hypoxic}$) or hypoxia followed by reoxygenation ($sEV_{reoxygenated}$) with or without amitriptyline treatment did not influence migration of hCMEC/D3 cells, compared to platelet lysate samples (PL). Representative microphotographic pictures for hCMEC/D3 cells exposed to control condition or sEVs are shown **(B)**. Data are mean ± SD values (n = 6 independent experiments). Scale bars: 400 µm in B.

Under normoxic conditions, viability of hCMEC/D3 cells decreased, when treated with sEVs derived from MSCs exposed to hypoxia and reoxygenation ($sEV_{reoxygenated}$) as well as amitriptyline treated MSCs exposed to hypoxia ($sEV_{hypoxic}$ +ami) and subsequent reoxygenation ($sEV_{reoxygenated}$ +ami), in comparison to $sEV_{normoxic}$-treatment **(Fig. 7A)**. Post hypoxia MTT-assays showed significant increase in cell viability after treatment with $sEV_{hypoxic}$ +ami or $sEV_{reoxygenated}$ +ami **(Fig. 7B)**

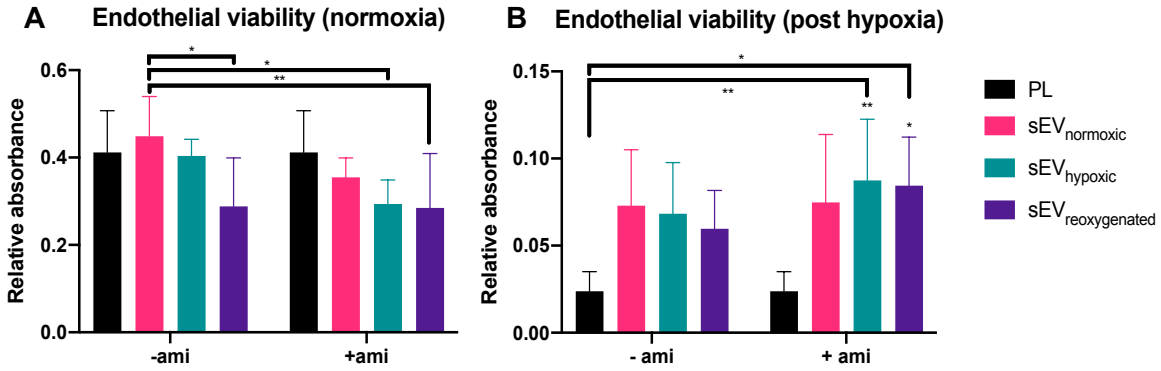

Figure 7: MSC-sEVs decreased the survival of cerebral microvascular endothelial cells under normoxic assay conditions and increased endothelial viability following oxygen–deprivation independent of MSC exposure by amitriptyline.

Cell viability of hCMEC/ D3 cultured under **(A)** 'normoxic' conditions (21% O2) or **(B)** 24 h hypoxia (1% O2) followed by 6 h reoxygenation (21% O2) measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 'Reoxygenated' sEVs as well as amitriptyline treated 'hypoxic' and 'reoxygenated' sEVs decreased cell viability in comparison to $sEV_{normoxic}$-treatment **(A)**. After oxygen–deprivation**,** $sEV_{hypoxic}$ +ami and $sEV_{reoxygenated}$ +ami increased survival of endothelial cells compared to both native as well as amitriptyline treated PL **(B)**. Data are mean ± SD values (n = 8 independent experiments [in A], 6 independent experiments [in B]). *$p < 0.05$, **$p < 0.01$ compared with PL.

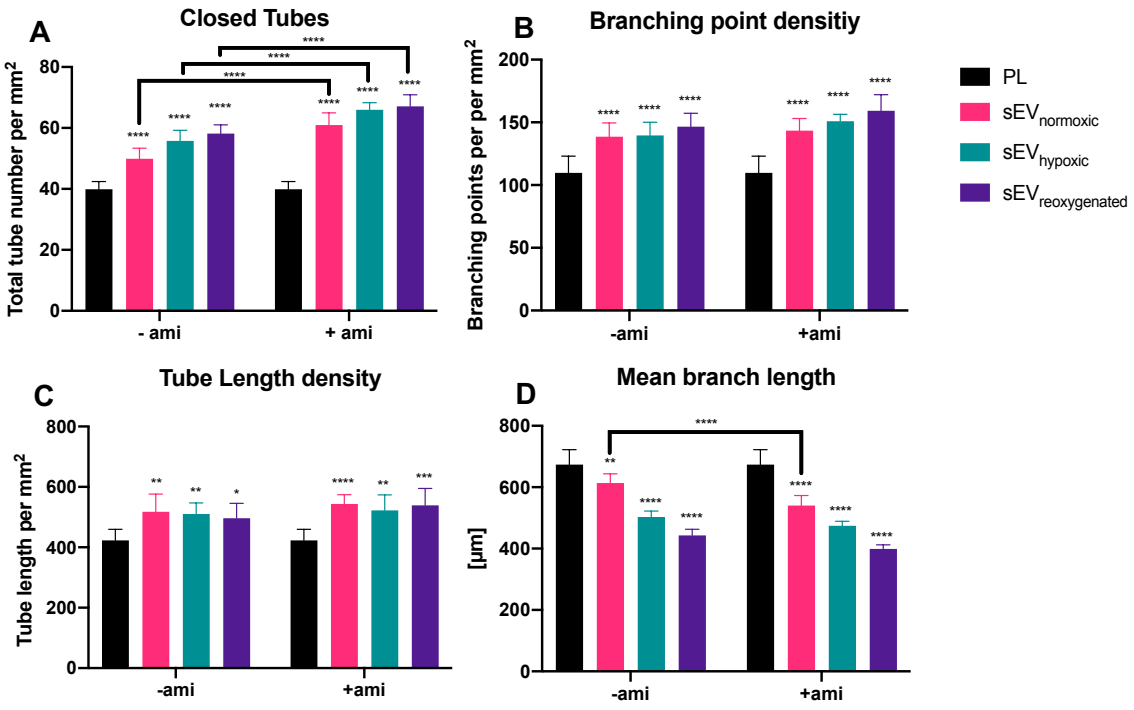

Figure 8: MSC preconditioning by amitriptyline augments the effects of MSC-sEVs on cerebral microvascular endothelial tube formation.

Matrigel-based tube formation assays were used to evaluate capillary-like structures formed by hCMEC/D3 exposed to different sEV samples. All sEVs increased the number of tubes **(A)**, branching points **(B)** as well as tube length density **(C)**, in comparison to treatment with platelet lysate (PL). sEVs isolated from MSCs cultured under 'hypoxic' conditions (1% O2) and subsequently reoxygenated at 21% O2 (sEVreoxygenated), increased the number of closed tubes and branching point density the most, in comparison to 21% O2 sEVnormoxic or sEVs obtained from MSCs just exposed to hypoxia (sEVhypoxic) **(A, B)**. amitriptyline treatment of MSCs while in culture resulted in the secretion of sEVs (+ami) that induce a higher increase of closed tubes than their respective untreated MSC-sEVs (-ami) **(A)**. sEVs significantly increased tube length density and decreased the mean branch length between two branching points **(C, D)**. This decrease is further progressive in sEVs obtained from amitriptyline treated MSCs (sEVnormoxic; +ami). Data are mean ± SD values (n = 8 independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001 compared with PL.

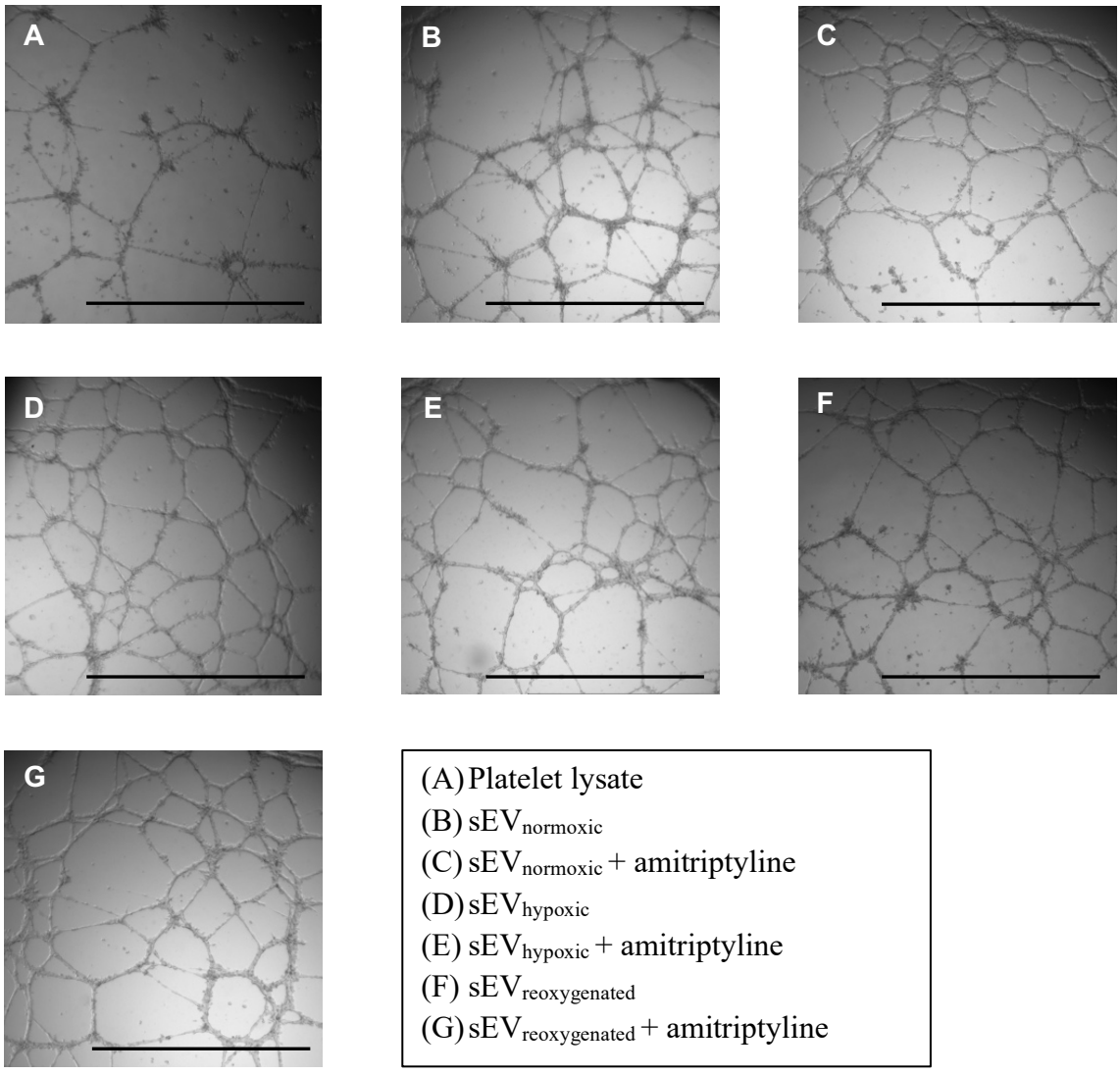

Fig. 9: Representative microphotographs of tube formation assays

Representative microphotographs of hCMEC/ D3 cells exposed to PL (A) or sEV preparations (B-G) are shown. Scale bars: 2000 µm

All sEV preparations stimulated a significant increase in tube formation and the number of branching points **(Fig. 8 A, B)**. A significant increase of tube length density was shown as well as a decrease of mean branch length between two branching points **(Fig. 8 C, D)**. Amitriptyline pretreatment of MSCs increased the angiogenic activity of

released sEVs, as revealed by a higher tube number. Representative pictures of tube formation assays are shown in **Fig. 9**.

5 DISCUSSION

There is an evident need for stroke therapies that targets angiogenesis as a means of cerebral tissue recovery. By reestablishment of blood flow and circulation in damaged brain tissue, angiogenesis lays the foundation for post-stroke remodeling and patient survival (Ruan et al., 2015, Krupinski et al., 1994). Stem cell-based approaches have shown promising efficacy ischemic stroke studies (Dao et al., 2013). Due to their solely invasive means of isolation from bone marrow biopsy or liposuction, as well as functional decline, cellular rejection risk and inconsistent quality in large-scale, widespread use of MSCs in clinical scenarios is limited (Xia et al., 2020). In accordance to established analysis and characterization methods, we showed that the sEVs used in this study possess *bona fide* sEV characteristics. sEV concentration, size, and surface signature did not differ significantly between conditions.

sEVs derived from MSCs stimulate comparable therapeutic effects as their parental cells (Doeppner et al., 2015, Heldring, 2015). In an attempt to maximize these sEV effects, we altered culture conditions of MSCs by exposure to hypoxia and drug induced ASM-inhibition. Our results show that the impact of MSC-sEVs on angiogenesis is influenced by pre-treatments of sEV donor cells.

We have previously shown that sEVs isolated from MSCs under hypoxic (1% O2), but not normoxic (21% O2) conditions, dose-dependently promoted endothelial proliferation, migration, and tube formation and increased post-ischemic endothelial survival (Gregorius et al., 2021). In the present study we obtained similar results in regards to tube formation as well as cell viability. Hereinafter underlying mechanisms and potential causes of our results are discussed.

It is recognized that the cargo incorporated into sEVs is regulated by hypoxic preconditioning, which ultimately affects their angiogenic potential, as well as their

immunomodulatory and regenerative properties (Bian et al., 2014). Unfortunately, it is mostly unknown which specific molecules in MSC-sEVs promote this effects. Hypoxia-inducible factor (HIF) was suggested to be one of the reasons for increased angiogenesis through higher sEV release (Bian et al., 2014). The signaling pathway for HIF is activated by hypoxia. There are studies showing that the activation of HIF may have an immediate impact on sEV biogenesis pathways. The HIF-induced recruitment of the membrane-anchored Ras superfamily of small G proteins (RABs) for membrane budding and fusion events is one of the recognized pathways for sEV formation. This underlying connection of hypoxia and the release of sEVs was demonstrated through abolishment of sEV release when HIF1 or 2 were silenced (Wang et al., 2014). In order to account for quantitative changes in sEV effects, we used a fix concentration of sEVs for our angiogenesis assays. It was further shown that not just the amount of released sEVs but also the expression of angiogenic factors is induced by HIF1A and HIF2A (Pulido-Escribano et al., 2022). Prolyl-4-hydroxylases (PHDs) quickly hydroxylate HIF-α in normoxic circumstances and route it to proteosomal breakdown. However, when there is hypoxia, this process of degradation is stopped, and the HIF-α subunits translocate into the nucleus where they attach to HIF-1β. After locating the hypoxia-responsive elements (HREs), the heterodimeric complex activates more than 100 target genes to support tissue survival (Pugh and Ratcliffe, 2003). The expression of target genes encodes additional angiogenic factors such as VEGF, stromal cell-derived factor 1 (SDF-1A), C-X-C chemokine receptor type 4 (CXCR4), angiopoietin-2 (ANG-2), PDGF, and TGFB. These elements stimulate endothelial cell migration, differentiation, and proliferation for the formation of vessels (Pulido-Escribano et al., 2022). The Notch ligand Jagged-1 was reported to be present in greater amounts in sEVs of MSCs that overexpress HIF1. In Matrigel-based tube formation experiments, sEVs expressing Jagged-1 induced enhanced angiogenesis. Prior incubation of sEVs with an anti-Jagged-1 antibody prevented these effects. As a result, it was shown that hypoxia can boost MSC-sEVs' ability to produce angiogenic factors by increasing the amount of Jagged1 cargo (Gonzalez-King et al., 2017). These factors in sEV composition can explain the favorable results obtained in this study regarding cell viability and tube formation assays. Several proteins, nucleic acids (including miRNA), and growth factors that are associated with tissue regeneration and angiogenesis were previously shown to be

enhanced in sEVs produced under hypoxia (Pulido-Escribano et al., 2022). One study demonstrated that MSCs cultured for 72 hours at 1% oxygen produced sEVs with pro-angiogenic effects by overexpressing the genes for urokinase receptor, angiogenin (ANG), VEGF, insulin-like growth factor (IGF), angiopoietin receptor tyrosine kinase with immunoglobulin-like and epidermal growth factor (EGF)-like domains 2 (Tie-2) and also IL-6 (Chen et al., 2014).

The above listed transcription elements and signaling pathways such as HIF allow cells to adapt to hypoxia. One cause of our differing results in migration and proliferation assays may lay in the response of MSCs to hypoxia and hypoxia-related conditions such as low pH and oxidative stress. None of the MSC-sEV preparations in our study showed significant alteration of cerebral microvascular endothelial cell proliferation or changes in migration of hCMEC/D3 , although previous studies suggested dose-dependent promotion of endothelial proliferation and migration (Gregorius et al., 2021). Cell migration and proliferation are induced by tissue regeneration as opposed to solely angiogenesis. Both migration and proliferation are affected by HIF pathways. Cell-cycle progression and proliferation can be affected through HIF activation. Interestingly, HIF2A upregulates cellular myelocytomatosis (c-MYC) expression, encouraging cell-cycle progression and proliferation, whereas HIF1A downregulates c-MYC expression and causes cell-cycle arrest. HIF also plays a role in migration. HIF pathways lay the foundation for a balanced control of genes for integrin-alpha and beta-1, 3 and 5 (ITGA1, ITGA5, ITGAV, ITGB3, and ITGB5, respectively), as well as matrix metalloproteinases MMP2, MMP7, and MMP9, which are crucial for cell migration (Pulido-Escribano et al., 2022). It is possible that sEV cargo in our experiments did not affect HIF pathways in the same way as previous experiments. The lack of HIF activation could explain why our sEVs have not shown promotion of endothelial migration or proliferation, and needs to be further investigated.

A further point of interest in current studies of sEVs that is suggested to alter their angiogenic potential is their miRNA cargo. miRNAs have recently been shown to be decisively involved in regulating proliferation, differentiation, survival and function of MSCs and their isolates (Clark et al., 2014). In addition to our analysis of morphology, size, protein content and markers, miRNA analysis could be an important way to further

characterize sEVs and their cargo. It was previously shown that sEVs derived from hypoxia pretreated MSCs were found to regulate a set of miRNAs in hCMEC/D3 cells, which were either upregulated (miR-126-3p, let-7c-5p, miR-140-5p) or downregulated (miR-186-5p, miR-370-3p, miR-409-3p) (Gregorius et al., 2021). It has been reported that MSC-derived $sEV_{hypoxic}$ had higher levels of miR-126 than $sEV_{normoxic}$. It was stated that HIF-1 was required for miR-126 synthesis and angiogenic effects of $sEV_{hypoxic}$ were provided by the raised miR-126 levels. In fact, miR-126 levels in MSCs and MSC-sEVs were lowered by HIF-1 knockdown, eliminating their angiogenic capabilities (Liu et al., 2020). As demonstrated in research using matrigel-based tube formation studies, hypoxia-responsive miRNAs can enhance angiogenesis in an HIF1 and argonaute-1 (AGO1)-dependent manner in HUVECs (Chen et al., 2013). A recent study found that hypoxic preconditioning upregulated the expression of miR-210 in MSC-sEVs, which promoted angiogenesis in vitro and in vivo. They also found that treatment with hypoxia preconditioned MSC-sEVs significantly increased tube formation and migration of hCMEC/D3, and that these effects were mediated by miR-210 (Zhuang et al., 2022). In fact, VEGF and VEGFR2 production, which promote angiogenesis, was shown to be driven by miR-210 (Liu et al., 2012). Studies have shown reduced cell migration of glioma and osteosarcoma cells after sEV treatment by miR-124 and miR-145, and miR-143 (Lee et al., 2013, Shimbo et al., 2014). HUVECs that were subjected to low oxygen levels, resulted in decreased miR-140-5p cell levels while increasing VEGFA expression. According to their findings, miR140p-5p prevented angiogenesis by reducing cell proliferation, migration, and tube formation (Sun et al., 2016). In previous studies of our group, miR140p-5p was upregulated in hCMEC/D3 by sEVs derived from hypoxia pretreated MSCs (Gregorius et al., 2021). It is possible that the $sEV_{hypoxic}$ in our current study similarly upregulated miR140p-5p in hCMEC/D3.

These alterations of miRNA cargo in sEVs could explain lacking promotion of cell proliferation and migration in our assays. It is yet to be clearly analyzed how exactly promotion of angiogenesis through sEV-miRNA is affected by quantity and compilation of different miRNAs. There is no thorough understanding how the angiogenic factors listed above are passed on from MSCs to their sEVs. In our study, it is possible that sEV cargo that is essential for promotion of tube formation was passed on to the sEVs but the transfer of migration and proliferation promoting cargo did not fully occur.

Differences in MSC source, cultivation, preconditioning, hypoxia duration and intensity as well as sEV isolation, purity and cargo may play a role in the cargo composition. Analysis and investigation of these cargo compounds as well as their passing on to sEVs is an important step for future studies.

We could confirm results of a previous study showing that hypoxia conditioned MSC-derived sEVs were more potent than normoxic MSC-sEVs regarding their induction of angiogenesis (Almeria et al., 2019). Similar to these findings, hypoxia treated MSC-sEVs showed increased numbers of closed tubes in our experiments. Our new approach to reoxygenate MSCs previously exposed to hypoxia resulted in isolated sEVs showing higher tube formation than normoxic and hypoxic sEVs. We hereby provide a novel way of promoting pro-angiogenic potency of sEVs. It was previously shown that cells respond to reoxygenation by influx of oxygen and restoration of extracellular pH (Kalogeris et al., 2012). Depending on the severity and duration of ischemia, ROS generation, damage of lipids, DNA and proteins, activation of inflammation, angiogenesis and thrombogenesis, cytokine release and apoptosis occur.,. Tissue response to reoxygenation after hypoxia seems to be bimodal. While long duration of ischemia and reperfusion leads to cell injury and death, short duration can lead to adaptive responses and activation of survival programs (Kalogeris et al., 2012). Studies analyzing the effects of hypoxia followed by reoxygenation on MSCs and their sEVs are rare. One study analyzed the effect of microvesicles derived from HUVEC exposed to hypoxia followed by reoxygenation on cardiomyocytes. The cardiomyocytes showed decreased cell viability, increased cell apoptosis, and increased generation of reactive oxygen species (ROS) through phosphorylation of p38 and c-Jun N-terminal kinases (JNK1/2) after treatment with the microvesicles (Zhang et al., 2016). Though it is important to recognize that not every organ exhibits the same level of ischemia vulnerability. It is also important to consider miRNA changes resulting from reoxygenation. It is evident, that hypoxia-induced changes in miRNA cargo of sEVs depend on the duration, the cell type and cultivation of paternal cells. Studies have shown that also reoxygenation can influence miRNA expression in a complex and highly variable way. This includes miR-125a-5p, miR-125b-5p, and miR-143-3p. Moreover, hypoxia and reperfusion caused damage led to downregulation of miR-134 and expression of HIF1, which acts pro-angiogenic as stated above (Zhang et al., 2020).

More research is needed to investigate release and cargo of sEVs derived from cells subjected to hypoxia and subsequent reoxygenation.

In our current study, in addition to hypoxia and reperfusion pretreatment, we analyzed sEVs derived from MSCs pretreated with ASM-inhibition through amitriptyline. Recent studies showed that amitriptyline lowers the intracellular formation of ceramide-rich vesicles induced by ischemia/reperfusion (I/R) and reduces the ASM activity of human cerebral microvascular endothelial cells *in vitro* (Mohamud Yusuf et al., 2022). Furthermore, amitriptyline boosted hCMEC/D3 tube formation and transwell migration, as well as the amount of VEGFR2 on the cell surface and the concentration of VEGF in supernatants They could show that subsequently endothelial cells released higher numbers of CD9+ and CD63+ sEVs upon amitriptyline treatment plus hypoxia or I/R exposure (Mohamud Yusuf et al., 2022). This ASM-dependency of sEV release was also shown in our study. sEV preparations derived from hypoxia and amitriptyline pretreated MSCs showed the highest particle concentration as well as protein concentration and purity. This study further demonstrated similar angiogenic activity of sEVs derived from endothelial cell lines when exposed to an ASM-inhibitor, as sEVs released during I/R. sEVs from cells that were pretreated with amitriptyline and exposed to ischemia and reoxygenation increased tube formation, migration and VEGF secretion of hCMEC/D3 (Mohamud Yusuf et al., 2022). We herein reproduced this previously established role of ASM-inhibition and reoxygenation. In our study sEVs increased the quantity of closed tubes, branching points and tube length density in tube formation assays when parental MSCs were, additionally to hypoxia and reperfusion, treated with amitriptyline. The observed alteration of angiogenesis of different sEV preparations is most likely caused by variations in their cargo since hCMEC/D3 were treated with equal amounts of sEV protein. In light of the tube formation promoting potency of our sEVs, it is likely that sphingomyelin alterations resulting from ASM-inhibition and hypoxia (and reoxygenation) of MSCs contributed to enhanced production of sEVs that provide pro-angiogenic function through their distinct cargo.

Apart from tube formation, other indicators of physiological angiogenesis that were assessed through assays did not yield similarly clear results in the present study. Regarding post-ischemic endothelial survival that was previously shown to be increased

dose-dependently with hypoxic sEVs, these present results show promising similarities (Gregorius et al., 2021). The $sEV_{hypoxic}$ correspondingly increased cell viability in oxygen-deprivation assays. In the present study we showed that in oxygen–deprivation assays**,** $sEV_{hypoxic}$ +ami and $sEV_{reoxygenated}$ +ami increased the survival of endothelial cells compared to PL. One explanation might be the synergy of increased viability through sEVs and the pro-angiogenic potency of hCMEC/D3 in hypoxic environments. One study underlining this hypothesis demonstrated that hypoxia promoted a pro-angiogenic phenotype in brain microvascular endothelial cells. The findings demonstrated that hypoxia generated expression of HIF-1, VEGF, Endothelin-1 and heme oxygenase-1, as well as a reduction in endothelial nitric oxide synthase (Luo et al., 2012). Under normoxic assay conditions sEVs did not increase cell viability, which conforms previous findings (Gregorius et al., 2021). Furthermore, sEVs obtained from amitriptyline treated MSCs exposed to hypoxia as well as hypoxia and reoxygenation decreased the survival of cerebral microvascular endothelial cells under normoxic experiment conditions. One explanation for this decrease may be oxidative stress induced by the differing O2 environments. One study suggests HIF1 to be a factor. By boosting the expression of Bcl-2 binding proteins (BNIP3 and NIX), which inhibits Bcl-2's antiapoptotic action, HIF1 can trigger hypoxia-mediated apoptosis (Greijer and van der Wall, 2004). The potential of yielding angiogenesis promotion from hypoxic states needs to be further investigated for it to result in maximum benefits without sacrificing cell viability. New research analyzing sEV cargo in differing hypoxic conditions and durations as well as a variance of O2 environments for angiogenesis assays could yield new insights.

Our experimental setup regarding amitriptyline effects on sEV angiogenesis promotion is not entirely flawless. MSC culture media requires platelet lysate or serum which themselves produce sEVs. These sEVs are hardly distinguishable from cell-secreted sEVs, making it conceivable that sEVs released by endothelial cells were diluted by platelet sEVs. Migration and proliferation might have not been influenced due to sEV-dilution from PL. Interestingly, amitriptyline in combination with MSC sEVs induced an enhancement of angiogenesis. This synergistic effect was shown in endothelial viability post hypoxia and tube formation assays and is consistent with previous data

(Mohamud Yusuf et al., 2022). Underlying mechanisms could be the enhancement of sEV-uptake through ASM-inhibition.

In this study we focused on sEVs as a means to stimulate post-stroke revascularization. *In vitro* assays are useful for studying these approaches. They provide quick results due to their speed and ease of quantification. Though we showed promising results, the validity of cultured cells mimicking physiological functions *in vivo* is criticized as being too different from the natural cellular environment. In previous studies it was shown that MSC-sEV samples significantly reduced neurological deficits and decreased infarct volume as well as neuronal injury in a stroke mouse model. Neuroprotective sEV effects were associated with decreased brain infiltration of leukocytes, monocytes/macrophages, and lymphocytes (Wang et al., 2020). Hypoxic preconditioning enhanced the restorative effects of MSC-sEVs. Post-MCAO, sEVs from hypoxic MSCs increased microvascular length and branching point density in previously ischemic tissue assessed by 3D light sheet microscopy over up to 56 days, reduced delayed neuronal degeneration and brain atrophy, and enhanced neurological recovery (Gregorius et al., 2021).

Our data underlines the importance of MSC pretreatment for sEV efficacy in *in vitro* angiogenesis models. Hypoxia and ASM-inhibition in MSC cultures might also affect different pathways that are typically increased through the use of MSC-sEVs from hypoxic conditions, such as chondrogenesis (Malladi et al., 2006), immunosuppressive properties (Roemeling-van Rhijn et al., 2013), or the expression of regenerative growth factors (Wei et al., 2012). While our data show that ASM-inhibition and hypoxic pretreatment of MSCs can enhance the ability of sEVs to induce vascular tube formation, different types of preconditioning remain to be analyzed. Further investigation on various pretreatment methods during MSC culturing could include pharmacological agents (such as vitamins, hormones, and clinical drugs), cytokines and physical factors (such as low-level lasers and electromagnetic fields) in order to find different combinations of pretreatment parameters that lead to the secretion of beneficial sEVs for the respective pathologies. In finding these parameters, modification and specific engineering of sEVs could pave the way for targeted therapies at maximum efficacy (Rahmani et al., 2020).

6 SUMMARY

Stem cell-based treatments have been suggested as promising candidates to meet the demand for post-stroke therapy targeting cerebral tissue recovery. Recently, mesenchymal stem cells (MSCs) have been discovered to help the reestablishment of blood flow through angiogenesis, paving the way for less invasive and time restricted interventions. Their disadvantage lies in their solely invasive means of isolation from bone marrow biopsy or liposuction, as well as their functional decline, cellular rejection risk and inconsistent quality in large-scale. Recent attention was given to their paracrine actions. Small extracellular vesicles (sEVs) secreted by MSCs show promising potential to serve as non-replicating and non-toxic delivery vehicles. By using sEVs as excretory cargo transportation vehicles to achieve comparable therapeutic effects as their parental MSCs, we investigated their angiogenetic potential in *in vitro* assays. We preconditioned the cell cultures by MSC exposure to hypoxia (and reoxygenation) and drug-induced acid sphingomyelinase (ASM) inhibition. With our present study we are first to evaluate and clearly display that the pretreatment of MSCs with hypoxia and ASM-inhibition amplifies the pro-angiogenic effects of their secreted sEVs. Furthermore, sEVs obtained from amitriptyline treated MSCs exposed to hypoxia as well as hypoxia and reoxygenation decreased the survival of cerebral microvascular endothelial cells under normoxic experiment conditions but increased endothelial cell viability in hypoxic assay conditions. None of the MSC-sEV preparations induced significant alterations of cerebral microvascular endothelial cell proliferation. In conclusion, our findings highlight the importance of culture conditions for the production and efficacy of MSC-derived sEVs.

ZUSAMMENFASSUNG

Behandlungen auf der Grundlage von Stammzellen wurden als vielversprechende Kandidaten für die Nachfrage nach Therapien nach einem Schlaganfall vorgeschlagen, die auf die Erholung des Hirngewebes abzielen. Kürzlich wurde entdeckt, dass mesenchymale Stammzellen (MSZ) die Wiederherstellung des Blutflusses durch Angiogenese unterstützen, was den Weg für weniger invasive und zeitlich begrenzte Eingriffe ebnet. Ihr Nachteil besteht in ihrer ausschließlich invasiven Isolierung aus einer Knochenmarksbiopsie oder Fettabsaugung sowie ihres Funktionsverlusts, des Risikos der Zellabstoßung und der uneinheitlichen Qualität in großem Maßstab. In jüngster Zeit rückte ihre parakrine Wirkung in den Vordergrund. Kleine extrazelluläre Vesikel (sEVs), die von MSZ sezerniert werden, haben ein vielversprechendes Potenzial, als nicht replizierende und nicht toxische Transportmittel zu dienen. Wir haben sEVs als Transportvehikel verwendet, um vergleichbare therapeutische Wirkungen wie die elterlichen MSZs zu erzielen, und haben ihren Einfluss auf die Angiogenese in *in-vitro*-Tests untersucht. Wir modifizierten die Kulturbedingungen von MSZ durch Präkonditionierung durch Hypoxie (+Reoxygenierung) und medikamentöse Hemmung der sauren Sphingomyelinase (ASM). Mit der vorliegenden Studie haben wir zum ersten Mal gezeigt, dass die Kombination einer hypoxischen Präkonditionierung mit ASM-Inhibition in der Lage ist, die sEV-induzierte Angiogenese zu verstärken. Wir konnten bestätigen, dass unter Hypoxie konditionierte MSZ-sEVs in Bezug auf die Förderung der Gefäßbildung wirksamer waren als normoxische MSZ-sEVs. Unsere Daten zeigen, dass sEVs die Anzahl geschlossener Tubes in Tube-Formation Assays stärker erhöhten, wenn die elterlichen MSZs zusätzlich zur hypoxischen Vorkonditionierung mit Amitriptylin behandelt wurden. Das Überleben zerebraler mikrovaskulärer Endothelzellen unter normoxischen Versuchsbedingungen wurde durch sEVs verringert, welche aus Amitriptylin behandelten und Hypoxie (+Reoxygenierung) ausgesetzten MSZs gewonnen wurden. Selbige sEVs erhöhten die Lebensfähigkeit der Endothelzellen unter hypoxischen Versuchsbedingungen. MSZ-sEV-Präparate induzierten keine signifikante Veränderung der zerebralen mikrovaskulären Endothelzellproliferation oder Veränderungen der Zellmigrationsraten. Zusammenfassend unterstreichen unsere Ergebnisse die Bedeutung der Kulturbedingungen für die Produktion und Wirksamkeit von MSZ-sEVs.

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7 ATTACHMENT

7.1 List of abbreviations

ANG	angiopoietin
ANOVA	analysis of variance
ASM	acid sphingomyelinase
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCRP	breast Cancer Resistance
CNS	central nervous system
CSE	cystathionine γ-lyase
DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ESCRT	endosomal sorting complexes
EVs	extracellular vesicles

FDA	Food and Drug Administration
FGFs	fibroblast growth factors
FIASMA	functional inhibitors of Acid sphingomyelinase
GABA	γ-Aminobutyric acid
GDNF	glial cell line-derived
GvHD	graft-versus-host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia-inducible factor
hPL	human platelet lysate
ICAM-1	intercellular Adhesion Molecule 1
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MDR	multidrug resistance mutation protein
miRNA	micro Ribonucleic acid
MRP	multidrug resistance protein
MSCs	mesenchymal stem/stromal cells
MSC-sEVs	mesenchymal stromal cell-derived small extracellular vesicles
MVBs	multivesicular bodies
NF-kB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NTA	nanoparticle tracking analysis
O2	oxygen
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with Tween-20

PEG	polyethylene glycol
PIP3	phosphatidylinositol 3-phosphate
rtPA	recombinant tissue plasminogen activator
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SL	sphingolipid
SMPD	sphingomyelin phosphodiesterase gene
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20
TEM	transmission electron microscopy
TLR	toll-like receptor
VEGF	vascular endothelial growth factor

7.2 List of tables

7.3 List of figures

8 ACKNOWLEDGEMENT

I'd like to express great gratitude to my supervisor, Prof. Dirk Hermann, for his help throughout my doctoral study at NeuroScienceLab. I would like to further thank AG Giebel at the Institute for Transfusion Medicine in University Hospital Essen for their collaboration and support regarding my experiments on extracellular vesicles (EVs). Especially, I thank Prof. Bernd Giebel, Dr. Verena Börger, and their team for sharing their knowledge and resources, and always being kind and thoughtful.

I also could not have successfully finished my studies without my supervisors and colleagues at NeuroScienceLab, who generously provided knowledge and expertise as well as their invaluable patience and feedback. In particular, this endeavor would not have been possible without the generous support from: Nina Hagemann, Ayan Mohamud Yusuf, Egor Dzyubenko, Britta Kaltwasser and Tanja Hussner.

I am also grateful to my university friends for their understanding and moral support.

Lastly, I must mention my family, especially my parents and my sister. Their belief in me and support of all my choices has kept motivation high during this process. I would like to thank and be reminiscent of my two late grandfathers. One of them who taught me about work ethic, discipline, and kindness; and the other being the reason I was able to grow up and study in Germany.

9 CURRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.