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Stem Cells Translational Medicine, 2023, **12**, 707–713
https://doi.org/10.1093/stcltm/szad055
Advance access publication 11 September 2023
Perspective

Preconditioning Concepts for the Therapeutic Use of Extracellular Vesicles Against Stroke

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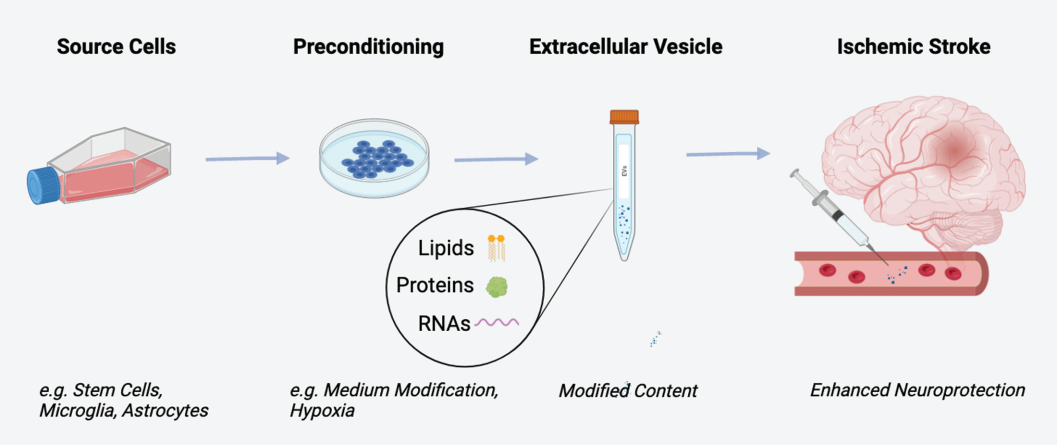
Abstract

Various preclinical stroke models have demonstrated the neuroprotective effects of extracellular vesicles (EVs) obtained from several types of cells, including neurons, astrocytes, microglia, neuronal progenitor cells, bone marrow stem cells, and mesenchymal stem cells. EVs interfere with key mechanisms in stroke pathophysiology such as cell death, neuroinflammation, autophagy, and angiogenesis. The mode of action and efficacy depend on the specific EV content, including miRNAs, proteins, and lipids, which can be modified through (I) bioengineering methods, (II) choice of source cells, and (III) modification of the source cell environment. Indeed, modifying the environment by preconditioning the EV-secreting cells with oxygen-glucose deprivation or medium modification revealed superior neuroprotective effects in stroke models. Although the concept of preconditioned EVs is relatively novel, it holds promise for the future treatment of ischemic stroke. Here, we give a brief overview about the main mechanisms of EV-induced neuroprotection and discuss the current status of preconditioning concepts for EV-treatment of ischemic stroke.

Key words: stroke; neuroprotection; extracellular vesicle; preconditioning; neuroinflammation.

Graphical Abstract



Significance Statement

The role of extracellular vesicles (EVs) derived from various cell types has emerged as neuroprotectants in preclinical stroke models. Interestingly, the efficacy of these EVs appears to be enhanced when source cells are preconditioned with oxygen-glucose deprivation or medium modifications, indicating a potential novel strategy in stroke treatment. Although being a relatively new scientific field, the concept of preconditioned EVs could significantly influence future methodologies for treating ischemic stroke, a topic that will be elaborated on in this perspective article.

Received: 19 May 2023; Accepted: 2 August 2023.

Introduction

Current standard procedures of care for ischemic stroke treatment focus on recanalizing concepts, for which only a minority of all stroke patients qualify. Despite the development of numerous neuroprotective strategies over the last few decades, none have yet been successfully implemented in clinical settings. In recent years, there has been increasing interest in using extracellular vesicles (EVs) obtained from various types of cells, including neurons, astrocytes, neuronal progenitor cells (NPCs), bone marrow stem cells (BMSCs), and mesenchymal stem cells (MSCs) as both as a neuroprotective strategy and as diagnostic markers.[1,2] EVs are membrane-bound structures released by cells into the extracellular space, composed of a lipid bilayer structure with a diameter between 30 and 1000 nm.[3] They play a crucial role in intercellular communication processes and contain various molecular components, such as proteins, lipids, and microRNA. There are several subtypes of EVs, including exosomes, microvesicles, and apoptotic bodies, each with different biological functions.[3] Exosomes, typically having a diameter ranging from 30 to 150 nm and originating from the late endosomal trafficking machinery, are of significant interest for therapeutic strategies.[4] EVs are characterized by low immunogenicity, high transportation efficiency, low tumorigenicity, high stability, and the ability to cross the blood-brain barrier (BBB).[5] Several preclinical studies have demonstrated both the neuroprotective and neuroregenerative potential of EVs in different stroke models. Importantly, the concept of preconditioning, also known as priming, of EVs has been shown to improve the therapeutic potential through modification of the EV content. Therefore, the use of preconditioned EVs, depending on their individual application purpose, appear as a groundbreaking approach in the field of neuroprotection.

In this article, the main mechanisms of EV-induced neuroprotection will be discussed, and an overview of preconditioning concepts for such EVs will be presented, thereafter.

Main Mechanisms of EV-Mediated Neuroprotection

Systemic administration of EVs between 24 hours and 48 hours after stroke induction reduces the infarct volume, improve the functional recovery, and enhances the neuronal plasticity.[2] In addition, the delayed administration of EVs, for instance, 14 days after the onset of a stroke, led to an improvement in neuroglial recovery.[6] This was evidenced by enhanced functional recovery and an increase in oligodendrogenesis,[6] depending on the specific type of source EVs modulating distinct key factors of stroke pathophysiology. Although, a complete understanding of the underlying mechanisms is still lacking, several specific mechanisms involving inflammation, apoptosis, angiogenesis, and neurogenesis have been identified. The mechanisms are discussed briefly in the following section and are also visualized in Fig. 1.

Inflammation

EVs have immune modulating effects under ischemic stroke conditions. They can inhibit the proliferation, activation, and secretion of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in various immune cells, including natural killer cells, macrophages, and T and B cells.[7] Importantly, MSC-derived extracellular vesicles successfully reverse poststroke lymphopenia in the peripheral blood, in which polymorphonuclear neutrophils play a predominant role.[8,9] In addition, EVs play a role in regulating microglia activation and microglia phenotype shifting.[6] For example, a recent study by Hu et al using adipose-derived stem cell (ADSC)-derived exosomes showed ameliorated cerebral injury by promoting the M2 phenotype and decreasing the M1 phenotype of residing microglia.[10] The authors demonstrated that the upregulation of miRNAs involved in microglial polarization is a primary mechanism underlying the observed effect. Specifically, the expression of proteins such as the signal transducer and activator of transcription 1 (STAT1) and PTEN appears to be negatively regulated by these miRNAs. Both proteins play crucial roles in microglial polarization: STAT1 acts as a transcription factor that is essential in the microglial proinflammatory pathway and is closely related to the expression of pro-inflammatory cytokines, while PTEN is a main responding transcription factor mediating inflammatory signaling in microglia. Furthermore, treatment with ADSC-EVs resulted in enhanced post-ischemic angiogenesis, evidenced by an increased number of newly proliferated endothelial cells and a larger quantity of microvesicles observed 14 days after MACO. In addition, it has been demonstrated that EVs derived from MSCs can reverse the M1 polarization of microglia, mediated by the downregulation of the Cysteinyl leukotrienes (CysLTs) member $CysLT_2R$ in stroke-induced rats.[11] CysLTs are inflammatory mediators that are highly expressed in microglia within ischemic brain tissue. This expression is suggested to result in enhanced M1 microglial polarization, an effect that has been shown to be reversible following treatment with MSC-derived EVs.[11]

Apoptosis

Apoptosis is a crucial factor that contributes to the loss of hypoxic tissue. Recent studies suggest that regulation of cell death is an important mechanism of EV-mediated neuroprotection.[12] For instance, in an oxygen-glucose-deprivation (OGD) model, Xiao et al showed that BMSC-derived EVs were able to negatively regulate the caspase-8-dependent apoptosis pathway through miR-134.[13] Pei et al demonstrate that astrocyte-derived EVs inhibited OGD-induced apoptosis by reduced expressions of caspase-3 and Bax.[7] Furthermore, Seifali et a demonstrated that human umbilical vein endothelial cell-derived EVs could reduce the levels of apoptotic-related proteins Bax/Bcl in a rodent stroke model. This resulted in improved sensorimotor function and reduced neuronal death.[14] However, concrete mechanisms underlying the observed EV-mediated anti-apoptotic effects are missing in these studies.

Angiogenesis

Angiogenesis is critical recovery after brain injury, as it facilitates the delivery of blood flow and metabolic nutrients to the injured regions, promoting neural tissue repair.[15] In their study, Xin et al demonstrated that EVs derived from MSCs promote neurite remodeling, neurogenesis, and angiogenesis in the ischemic boundary zone following ischemic stroke.[16] Next, Hu et al demonstrated that EVs derived from pluripotent stem cells induced a significant increase in microvessel density and blood perfusion in mouse ischemic limbs. Consistent with these findings, in vitro studies revealed that EVs induced cell migration, proliferation, and tube formation, as well as

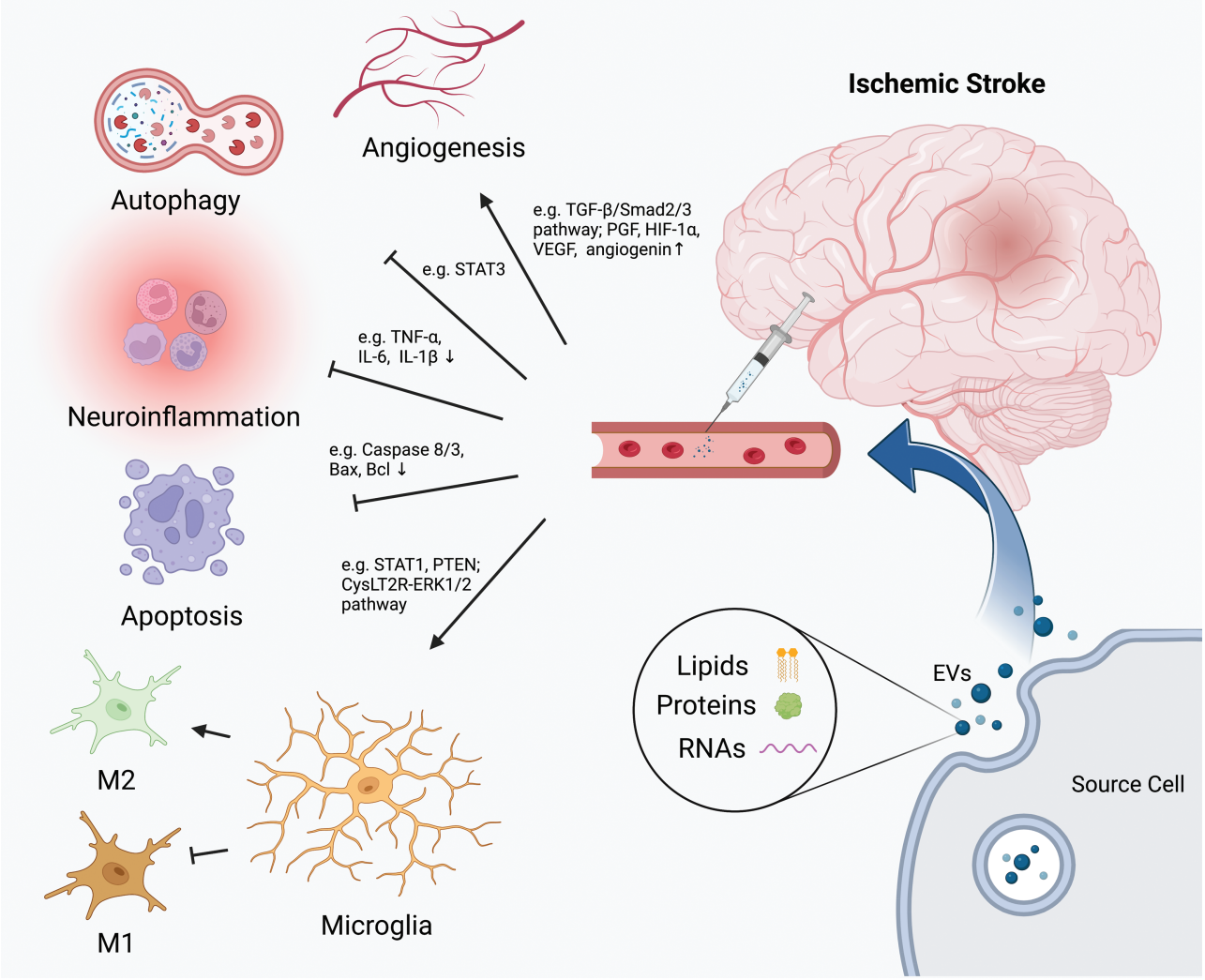


Figure 1. Important mechanisms of EV-mediated neuroprotection in stroke models.

enhanced expression of angiogenesis-related proteins such as placental growth factor (PGF), hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor B (VEGFB), vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGFB1), and Angiogenin.[17] In a recent study conducted by our workgroup, we were able to show that the TGF-β/Smad2/3 pathway plays a crucial role in mediating the EV-mediated angiogenic effects.[18]

Autophagy

Autophagy is suggested to play a dual role in ischemic stroke by initially promoting cell survival through the removal of damaged cellular components, but prolonged activation can lead to cell death.[19] Pei et al. reported that in a rodent stroke model, EVs derived from astrocytes ameliorated neuronal damage by suppressing autophagy activity.[7] This was indicated by the quantification of typical autophagy markers such as Beclin-1, LC3-I, LC3-II, and P62. In addition, Xia et al discovered that EVs derived from human-induced pluripotent stem cells (iMSC) activate signal STAT3, resulting in the suppression of autophagy and the facilitation of angiogenesis both in vivo and in vitro.[20] Interestingly, the suppression of autophagy and promotion of angiogenesis induced by iMSC-EVs were reversed upon the suppression of STAT3, highlighting the crucial role of STAT3-mediated autophagy regulation in this process. Next, a recent study by Gao et al utilized neural stem/progenitor cell-derived EVs in a rodent stroke model.[21] The researchers observed a decrease in apoptotic activity and an enhancement in neurogenesis. In addition, their research demonstrated an activation of the MEK-ERK pathway in mice with induced stroke, driven by increased phosphorylation through EV treatment. This finding is significant as the MEK-ERK signaling pathway plays critical roles in neurogenesis, inflammation, and apoptosis, providing a potential mechanistic understanding.

Concepts of EV Preconditioning

EVs derived from various native cell sources provide significant neuroprotective effects in preclinical ischemic models. The differences in EV content based on their source may be a critical factor in their varying effectiveness. In addition, EVs can serve as delivery vehicles loaded with specific content through bioengineering methods such as miRNAs, which highlights the importance of their individual composition.[22] Importantly, cells respond differently based on

their microenvironment. Therefore, it is expected that the content of EVs and their resulting therapeutic potential can be modulated by modifying their source cell microenvironment.[23] In the following sections, various preconditioning concepts for the therapeutic use in the context of ischemic stroke will be discussed. A graphical summary is also given in Fig. 2.

Hypoxia

Several studies have demonstrated that EVs derived from cells subjected to hypoxic conditions exhibit a superior neuroprotective effect in stroke and oxygen-glucose deprivation (OGD) models compared to EVs derived from native cells. However, 2 studies showed contrary results. An overview of studies discussed in the following is given in Table 1.

The researchers Yu et al recently conducted a study showing that EVs obtained from BMSCs can significantly reduce the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), CD86, and inducible nitric oxide synthase while increasing the levels of anti-inflammatory cytokine IL-10, CD206, and Arginase-1 in microglia that were exposed to OGD.[24] This led to a shift toward a more protective state, with lower levels of M1 and higher levels of anti-inflammatory M2 microglia polarization. The researchers also compared EVs from native BMSCs with EVs obtained from BMSCs that were exposed to low oxygen conditions for 24 hours. They found that the EVs obtained from the hypoxia-preconditioned BMSCs were more effective in protecting microglia against OGD-induced cell damage and apoptosis, and in promoting M2 polarization of microglia than the EVs from normoxic BMSCs. In addition, the total protein level of the hypoxic preconditioned EVs was much higher than that from

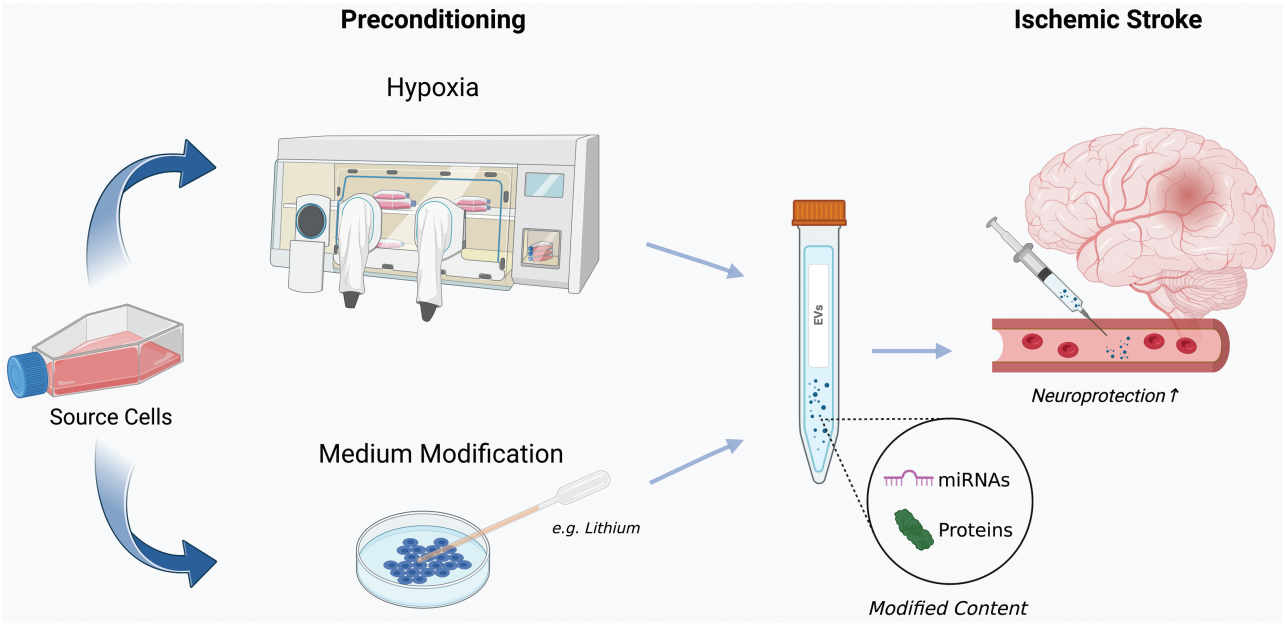


Figure 2. Concepts of extracellular vesicle preconditioning to enhance the neuroprotective effect.

Table 1. In vitro and in vivo studies using EVs derived from hypoxic preconditioned cells.

Authors	Year	Cell line	Main results	Mechanism
Gregorius et al.	2021	MSC	Angiogenesis ↑, brain atrophy ↓, neurological recovery ↑	Modulated miRNA expression, including miR-126
Yu et al.	2021	MSC	Apoptosis ↓, M2 polarization of microglia ↑	Total protein level ↑
Yang et al.	2022	ADSC	Neuronal damage ↓, M1 to M2 shift of microglia ↑,	Enhanced circ-Rps5 with downstream effects on SIRT7 and miR-124-3p
Ye et al.	2022	UCMSC	Infarct size ↓, cerebrovascular remodeling ↑, neurological function ↑	Modified expression of 236 miRNAs
Zhang et al.	2021	microglia	Angiogenesis ↑, apoptosis ↓, M1 to M2 shift of microglia ↑,	TGF-β1 enrichment, activated Smad2/3 signaling pathway
Chiang et al.	2021	neurons	Cell viability ↓, neurite outgrowth ↓,	Modified expression levels of 45 miRNAs
Xie et al.	2020	microglia	Cell damage ↑, BBB integrity ↓	miR-424-5p ↑, modulated FGF2/STAT3 pathway

Abbreviations: ADSC: adipose-derived stem cells; BBB: blood-brain barrier; MSC: mesenchymal stem cells; TGF-β1: transforming growth factor beta 1; UCMSC: umbilical cord mesenchymal stem cells.

normoxic EVs, suggesting that hypoxia preconditioning could induce more secretion of EVs from BMSCs. However, the study did not analyze the specific changes in the content of the EVs.[24] Next, Yang et al investigated the potential mechanisms by which EVs, derived from adipose-derived stem cells preconditioned with hypoxia, provide neuroprotection.[25] The researchers found that these EVs can promote the shift of microglia from M1 to M2. Mechanistically, the authors analyzed differences in the content of circular RNA (circRNA) between EVs from preconditioned sources and those from native sources. CircRNAs are a class of highly conserved non-coding RNAs with a closed-loop structure. They are involved in the pathophysiological processes underlying ischemic stroke, and emerging evidence points to their potential as therapeutic targets and clinical biomarkers.[26] Indeed, in EVs derived from preconditioned sources, the researchers found an enhanced expression of the specific circRNA, circ-Rps5. They further elucidated the mechanistic pathway by which circ-Rps5 acts through downstream effects on SIRT7 and miR-124-3p, thereby modulating the shift in microglial phenotype.[25] Our research group conducted a study analyzing both in vitro and in vivo stroke models to investigate the therapeutic potential of EVs derived from OGD-preconditioned microglia, specifically with respect to their impact on angiogenesis, anti-apoptosis, and immuno-modulation.[27] Our findings indicate that OGD preconditioning of microglia leads to an enrichment of TGF-β1 protein in the produced EVs. Size and morphology analysis showed no significant differences between EVs obtained from the preconditioned group compared to EVs derived from native microglia. However, these TGF-β1-enriched EVs promote the phenotype shift of microglia from M1 to M2 state within the ischemic area, leading to reduced apoptosis activity and improved neurological recovery in both in vitro and in vivo settings. At the signaling level, TGF-β1 activates the Smad2/3 signaling pathway contributing to the observed effects. Likewise, Ye et al. demonstrated that EVs derived from umbilical cord mesenchymal stem cells (UCMSC) that were pretreated with OGD had superior effects on reducing infarct size and promoting cerebrovascular remodeling, as well as improving neurological function, compared to EVs from native UCMSC.[28] In addition, these researchers discovered that 236 miRNAs were differentially expressed in EVs from pretreated UCMSC compared to EVs from native one, and a gene ontology and KEGG enrichment analysis suggested that preconditioned exosomes regulated cell proliferation, endocytosis, and glucose metabolism. Moreover, the target genes were highly enriched in the phosphatidylinositol 3-kinase-Akt (PI3K-Akt), mitogen-activated protein kinase (MAPK), and mechanistic target of rapamycin (mTOR) pathways. Gregorius et al evaluated the effects of EVs obtained from OGD pretreated MSCs on cerebral angiogenesis.[29] The results showed that the EVs obtained from pretreated MSCs promoted the growth of endothelial cells, cell migration, tube formation, and cell survival in vitro. Moreover, the EVs obtained from pretreated MSCs modulated angiogenesis, as demonstrated by the analysis of miRNAs, which revealed the modulation of specific miRNAs associated with focal adhesion, VEGF signaling, leukocyte migration, and adherent junctions. Among these miRNAs, the analysis demonstrated higher levels of miR-126, which is associated with the angiogenesis factor HIF-1α. Additional in vivo studies revealed superior effects of EVs from preconditioned MSCs in terms of reduced delayed neuronal degeneration, brain atrophy, and enhanced neurological recovery. In addition, angiogenesis dependent on the presence of polymorphonuclear neutrophils was significantly higher in EVs from pretreated MSCs compared to native.

Conversely, while pretreated EVs are suggested to have superior protective potential (see above), a study by Xie et al found that EVs derived from microglia treated with OGD had detrimental effects.[30] In their study, the authors compared non-pretreated EVs with OGD-treated EVs derived from microglia. OGD-preconditioning of EVs resulted in a remarkable upregulation of 127 miRNAs, defined as a change of more than 2-fold according to an miRNA microarray analysis. The authors specifically focused on miR-424-5p, which is known to play a role in mediating central nervous system disease by alleviating OGD-induced neuronal injury.[30] In vitro results of the study revealed that EVs from OGD-activated microglia induced a significant cell damage in which upregulated miR-424-5p and modulation of the FGF2/STAT3 pathway play an important role. Additional in vivo experiments indicated that inhibition of miR-424-5p mitigated neurological dysfunction and endothelial cell damage in mice subjected to middle cerebral artery occlusion. However, this was an indirect observation, as EVs were not administered directly. Beyond this limitation of demonstrating the main outcomes solely in vitro, differences in the duration of OGD incubation and reoxygenation, compared with the studies previously mentioned, could result in variances in the EV content. These factors could be contributing to the contrasting findings observed. Next, Chiang et al conducted a study on EVs derived from neurons subjected to OGD treatment.[31] EVs from neurons exposed to OGD significantly impaired neuronal cell viability and neurite outgrowth in terms of primary and total neurite numbers as well as primary neurite length compared to EVs from normoxic conditions. In a subsequent analysis, the authors compared the EV contents and found that the expression levels of 45 miRNAs were significantly different between normoxic and OGD conditions. Dysregulated miRNAs were also identified through a bioinformatic analysis, revealing multiple pathways involved in cell survival and death processes and neuronal signaling, including rat sarcoma (RAS), MAPK, forkhead box O (FoxO), mTOR, Wnt, and Ras-related protein 1 (Rap1) signaling pathways.[31]

Medium Modification

Lithium has been demonstrated to have neuroprotective effects in several preclinical studies using different stroke models. It achieves this by modulating key players in stroke pathophysiology, such as cell death, neuroinflammation, and BBB integrity.[32] In a recent study by our workgroup, we investigated for the first time whether incubation of MSCs with lithium-conditioned medium (I) modulates the content of secreted EVs and (II) results in inferior neuroprotective effects compared to EVs obtained from native MSCs.[33] Importantly, in contrast to the studies discussed in the previous paragraph, the EVs were not exposed to OGD. The study revealed that preconditioning MSCs with lithium resulted in EVs that significantly improved the resistance of cultured astrocytes, microglia, and neurons against hypoxic injury. These effects were more pronounced when compared to EVs derived from non-preconditioned MSCs. In a stroke mouse model, mice treated with intravenous delivery of lithium-derived EVs showed greater neurological recovery and neuroregeneration, compared to mice treated with

native EVs. Importantly, preconditioning MSCs with lithium altered the secretion pattern of EVs, leading to a modified content of various miRNAs, including higher levels of miR-1906, which has been shown to regulate toll-like receptor 4 (TLR4) signaling. EVs from lithium-treated MSCs reduced postischemic TLR4 abundance, which resulted in inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, decreased proteasomal activity, and lowered the expression of both inducible NO synthase and cyclooxygenase-2. Such actions ultimately resulted in reduced levels of poststroke cerebral inflammation. Notably, compared to native EVs, EVs obtained from lithium preconditioned MSCs had a more significant impact on both peripheral and cerebral immune responses. Lithium is known to modulate several major signaling pathways such as glycogen synthase kinase 3-beta and MAPK; however, the mechanisms by which lithium leads to altered content of MSC-derived EVs remain unclear to date. Nevertheless, this study provides evidence that the therapeutic potential of EVs can be increased by enriching the incubation medium of the source cells with specific substances. This point is further emphasized by the fact that preconditioning stem cells with specific substances, such as IL-6, minocycline, and doxycycline, exhibit superior effects compared to their native state.[34,35] However, while it is known that stem cells secrete EVs, the studies primarily investigated stem cells, not specifically their EVs.

Conclusion and Perspective

EVs obtained from different cell sources gained interest as a neuroprotective strategy in stroke, as they have demonstrated in most studies superior neuroprotective effects compared to stem cells themselves. The EV-induced neuroprotection relies on modulation of different key players in stroke pathophysiology, based on their individual content, including miRNAs and proteins. On one hand, the EV content is dependent on their individual EV source. On the other hand, the content can be influenced by both cargo loading through bioengineering methods and specific preconditioning of the cells that secrete the EVs. For the latter, hypoxic preconditioning, as well as medium modification with lithium, has shown significantly enhanced efficacy of EV treatment. Genomic analysis of the EV content has revealed various modifications in numerous miRNAs, which seem to play a pivotal role in EV-induced neuroprotection. However, the exact mechanisms and key players in cell preconditioning are largely elusive to date. In the future, EV preconditioning has the potential to be a groundbreaking neuroprotective concept, even though a more thorough understanding is necessary to choose specific preconditioning methods for individual use cases.

Acknowledgments

The images are created with BioRender.com.

Funding

None declared.

Conflict of Interest

The authors declared no potential conflict of interest.

Author Contributions

M.H.: Conception and design, manuscript writing, visualization, final approval of manuscript. S.T.G., H.B.H., T.R.D: Conception and design, manuscript writing, final approval of manuscript.

Data Availability

No new data were generated or analyzed in support of this research.

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