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Exosomes in Acquired Neurological Disorders: New Insights into Pathophysiology and Treatment

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Abstract
Exosomes are endogenous nanovesicles that play critical roles in intercellular signaling by conveying functional genetic information and proteins between cells. Exosomes readily cross the blood-brain barrier and have promise as therapeutic delivery vehicles that have the potential to specifically deliver molecules to the central nervous system (CNS). This unique feature also makes exosomes attractive as biomarkers in diagnostics, prognostics, and therapeutics in the context of multiple significant public health conditions, including acquired neurological disorders. The purpose of this review is to summarize the state of the science surrounding the relevance of extracellular vesicles (EVs), particularly exosomes, to acquire neurological disorders, specifically traumatic brain injury (TBI), spinal cord injury (SCI), and ischemic stroke. In total, ten research articles were identified that examined exosomes in the context of TBI, SCI, or stroke; these manuscripts were reviewed and synthesized to further understand the current role of exosomes in the context of acquired neurological disorders. Of the ten published studies, four focused exclusively on TBI, one on both TBI and SCI, and five on ischemic stroke; notably, eight of the ten studies were limited to pre-clinical samples. The present review is the first to discuss the current body of knowledge surrounding the role of exosomes in the pathophysiology, diagnosis, and prognosis, as well as promising therapeutic strategies in TBI, SCI, and stroke research.

Keywords Acquired neurological disorders · Traumatic brain injury (TBI) · Stroke · Spinal cord injury (SCI) · Exosomes · Extracellular vesicles

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Introduction

Extracellular Vesicles and Exosomes

What They Are and Where They Come From

The term extracellular vesicle (EV) has yet to be definitively standardized [1]. Classification is most often dependent on several key characteristics including the biogenesis, size, composition, and cargo of each vesicle type (Table 1). Broadly, EV refers to a collection of structures (e.g., microvesicles, exosomes) that are formed by, and obtain their membrane from, the plasma membranes of cells. EVs are highly heterogeneous because they are derived from several cell types leading to variation in the markers on the outer membrane as well as the cargo contained within [4]. Likewise, depending on the

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Table 1 Extracellular vesicle types and their corresponding characteristics

EV subtype	Biogenesis	Size	Lipid content	Cargo type/use
Apoptotic bodies	Plasma membrane (blebbing of membrane on a cell undergoing apoptotic cell death)	~500–2000 nm	Phosphatidylserine	Fragmented DNA; cellular organelles; released by cells undergoing programmed cell death
Exosomes	Multivesicular body (MVB), also known as a "late endosome" (fuses with cell membrane to release exosomes)	~30–100 nm	Ceramide	DNA, RNA (mRNA, miRNA, non-coding RNA), and proteins; cargo is transferred horizontally between cells to affect the recipient cell.
Microvesicles (i.e., Ectosomes)	Plasma membrane (outward budding of the membrane)	~50–1000 nm	Phosphatidylserine	DNA, RNA (mRNA, miRNA, non-coding RNA), and proteins; cargo is transferred horizontally between cells to affect the recipient cell
Exosome-like vesicles	Unknown (possibly MVB from other organelles)	~20–50 nm	No lipid rafts	DNA, RNA (mRNA, miRNA, non-coding RNA), and proteins; their cargo is transported to affect various cell types

Comparison of exosomes to other type of extracellular vesicles based on how they are made, their physical properties (size; lipid content), cargo type, and what it is used for. Information in the table is based off information from the following sources: [2] and [3]

cell type from which they are formed and the surrounding microenvironment, exosomes can be detected in various human secretions [5–9]. In addition to the shared mechanism by which EVs are formed, these structures are thought to share a role in cell-to-cell communication by facilitating exchange of DNA, RNA, and proteins between cells.

Exosomes are one type of EVs that are relatively well studied compared to other EV subtypes [1, 10, 11]. Exosomes form when an endocytic, multivesicular body (MVB) fuses with the plasma membrane, and the MVB's contents (*exosomes*) are exocytosed [2]. Fusion with the plasma membrane results in protein markers from the cell of origin integrating into the membrane of the EVs which is useful for determining the source of the exosome [12]. After release into the extracellular milieu, exosomes fuse with other cells, and their cargo (e.g., RNA, enzymes, peptides) is transferred to the recipient cell, where it can participate in signaling processes, thereby orchestrating cellular response [13]. In addition to their characteristic biogenesis, exosomes are distinguished from other types of EVs based on their physical properties (e.g., size, lipid content), as well as their cargo (Table 1).

Exosomal samples can be enriched for exosome-specific protein markers to increase the specificity, as well as the certainty that the exosomes are not contaminated with other EVs or cellular materials. Exosomal protein markers most commonly reported in the acquired neurological disorder literature are ALIX [14], CD9 [15–18], CD63 [18, 19], CD81 [17, 18, 20, 21], and TSG101 [20, 21]; a less commonly reported marker is HSP70 [15]. A variety of standard methods can be used for detection of these marker proteins, including western blot analysis, ELISA, and ultra-sensitive protein quantification techniques. In addition to the common exosomal markers described above, enrichment for certain markers can also be used to isolate exosomes from specific cell types. This is useful when studying acquired neurological disorders, as it is often the goal to identify exosomes secreted from a specific cell type or those generally of central origin. For example, L1CAM, a nerve cell marker, has been used in Alzheimer's disease (AD) research [22]. While this marker is capable of detecting exosomes of nerve-cell origin, it is unable to distinguish between centrally and peripherally derived exosomes since peripheral nerve cells also express L1CAM [22, 23], as do cells of the kidney and soft tissue [24]. More recently, the ionotropic glutamate receptor, GluR2—also referred to as GRIA2 or AMPA2—has been used as an exosomal surface marker for determining central origin in a pre-clinical TBI study [25]. GluR2 is widely expressed within the brain in both neurons and developing oligodendrocytes, with only low levels of expression reported in other tissue types [24]. Another option in histological studies is to use co-staining techniques for specific cell type markers to identify where the cargo is being expressed as a possible source of the exosomal contents. One study that examined miRNA

expression within exosomes after TBI found miR-21 was highly expressed in neuronal cell bodies based on MAP2 co-localization. In this study, miR-21 was not expressed in microglia, based on a lack of co-localization with Iba-1 [15].

A key feature of exosomes and other EV is their small size. A general way to isolate and detect exosomes from a biological specimen is to use one of a variety of size-exclusion methods. For example, electron microscopy can be used to screen particles by size [14], which is often followed up by enriching for an exosomal marker. Other options include a combination of differential centrifugation and filtration [23], or sucrose gradient centrifugation, which separates vesicles based on flotation densities [26]. The abovementioned techniques can be used alone or in combination with commercially available kits that facilitate exosomal isolation. Several commercially available kits have been used in the literature and there is no established gold-standard approach to exosomal isolation in the research community [3, 14, 16–18, 26–31]. However, the methods available for isolating exosomes are rapidly improving.

Why They Are Being Increasingly Researched

EVs and other nanoparticles are increasingly being studied for their potential to improve diagnosis, prognosis, and treatment of various diseases, including acquired neurological disorders [32]. The secretion of EVs occurs across species, suggesting that EV-mediated communication is an evolutionarily conserved process [33–36]; however, the study of EVs is a relatively new area of scientific inquiry, especially as they relate to human health. A recent review found that between 2006 and 2016, there was a tenfold increase in the number of peer-reviewed exosome research publications [2]. However, there remains a great deal to understand, specifically related to acquired neurological disorders. Despite their discovery and characterization in the 1970s and 1980s [37, 38], exosomes have remained largely understudied for decades, in part due to an inability to accurately characterize their activity. Since their initial characterization, several key advances in the scientific understanding of exosomes occurred. Exosomes and other EVs have been isolated from numerous accessible human biological fluids, including blood [5], saliva [7], urine [8], stool [39], semen [40], breastmilk [9], and cerebrospinal fluid [6], making it feasible to study exosomes in patients with a variety of disorders. RNAs and proteins packaged within exosomes are stable because they are protected from nucleases and proteinases found in plasma and other biological tissues; thus, they can be readily assayed in stored samples. In 2007, it was shown for the first time that messenger RNA (mRNA) and microRNA (miRNA) could be transferred between cells using exosome-mediated mechanisms, indicating that genomic signaling occurs from cell to cell in part through exosome activity [28]. The relevance of this RNA transfer was demonstrated in 2008, with the finding that tumor growth in glioblastoma depends in part on exosome-mediated transport of RNA and proteins between cells; this study linked exosomes to the neuropathology associated with cancer progression [29]. Despite this increased interest, the mechanisms by which exosomes are produced and the consequences of exosome-mediated information transfer remain poorly understood. Consequently, the translation of exosome research into clinical research and practice has been limited [2, 11].

This gap in knowledge is especially evident in the context of acquired neurological disorders, a field where biomarker research has lagged behind compared to monogenic or other heritable disorders. Moreover, the inaccessibility of brain tissue for histological testing, and high cost of neurological imaging, results in a dire need for reliable circulating biomarkers for acquired neurological disorders. While all acquired neurological conditions are poorly understood, the decision was made to focus this review on traumatic brain injury (TBI), spinal cord injury (SCI), and stroke, which are especially underrepresented in the EV literature. Further rationale for the decision to focus the discussion to TBI, SCI, and stoke is that all three conditions are characterized by a primary neurological insult, followed by a sustained pattern of secondary injury cascades. Moreover, there is overlap in the types of secondary injury mechanisms triggered by all three conditions, such as inflammation, oxidative stress, and cellular death/regeneration [41–45].

Acquired Neurological Disorders

Acquired neurological disorders represent injuries that affect the central nervous system (CNS) in the form of one or more diverse insults to the brain or spinal cord. Since the CNS controls the functionality of other organs, an array of symptoms and deficits can result, including cognitive, motor, and emotion/behavior issues [46]. Many of these symptoms and deficits are ultimately associated with poorer health and quality of life [47–49], which may influence the ability to return to normal roles (e.g., work, family, athletics) [50, 51]. Considered together, acquired neurological disorders are one of the leading causes of disability. Progress in developing diagnostic tools and effective therapies has been limited by the absence of biomarkers measurable in accessible biological fluids that reflect the pathology in CNS tissue. Since it is rarely practical to biopsy CNS tissues, it has been difficult to assess the relationship between molecules expressed in neural tissues and peripheral biomarker levels. This is of critical importance, since identifying biomarkers of central origin that can be detected peripherally would facilitate a better understanding of the CNS microenvironment in acquired neurological disorders. This would be especially helpful for patients who do not require neurosurgical interventions or shunts, which provide direct access to neural tissue and CSF. Detecting

exosomes in peripheral blood is both practical and clinically relevant. Exosomes are known to readily pass from the brain, through the blood-brain barrier (BBB), and into the peripheral circulation. One study suggests that BBB-derived exosomes associated with amyloid beta (Aβ) can contribute to the Aβ pathology and deposition seen in neurodegenerative diseases such as AD [27]. More recently, efforts have been made to exploit the natural transport mechanisms of exosomes as potential therapeutic delivery vesicles. A 2011 study demonstrated that intravenously administered exosomes containing siRNA could be delivered to mouse brain by crossing the BBB [52]. Thus, it is possible to deliver exosomes to the brain and distribute cargo proven to be a useful therapy to combat the consequences of acquired neurological disorders. Moreover, the ability of exosomes to cross the BBB makes them relevant to conditions other than TBI, SCI, and stroke, though this is beyond of the scope of this review.

This information could guide the development and testing of therapeutics, and may also be relevant to precision medicine initiatives aimed at personalizing therapy based on individual characteristics. Ultimately, the evaluation of exosomal cargo could inform the choice of therapy. In addition, exosomes themselves could be administered therapeutically, since in pre-clinical studies, exogenous administration of exosomes results in beneficial effects on physiological and behavioral endpoints [14, 19].

Exosomes are released from all types of brain cells (Fig. 1) [54], but remain understudied in the context of acquired neurological disorders. Still, exosomes are worth pursuing considering the promising evidence in neurodegenerative conditions which share important features with acquired neurological disorders. Thus, diagnostic or therapeutic approaches addressing neurodegenerative pathologies may be of benefit for TBI, SCI, and stroke patients. For example, exosomes have shown promise in the contexts of AD/dementia [55–57], Parkinson's disease [58–60], amyotrophic lateral sclerosis [61–63], and Huntington's disease [64–66]. Not only does building evidence related to exosomes in TBI, SCI, and stroke adds to the evidence gleaned using traditional biomarkers (e.g., DNA, RNA, protein), it also offers some advantages over these more well-studied alternatives. For example, circulating pro-inflammatory cytokines in serum after brain injury may be peripheral in origin due to the confounding effects of polytrauma or other factors (e.g., exercise) [67, 68]. Likewise, circulating RNA may be of peripheral origin, making it potentially less useful as a diagnostic or prognostic indicator. Double-stranded DNA as well as mitochondrial and chromosomal DNA has also been identified within exosomes [69, 70]. Thus, by identifying exosomes with centrally derived markers on the outside, any protein, RNA, or DNA contained within can be more reliably considered indicative of the CNS microenvironment. Since in CNS disorders, only certain cell types may be affected, identifying evidence of damaged cells peripherally that contain signature cargo reflecting the acquired neurological disorder is promising. Still, there may be diffuse effects that alter exosomes beyond the site of injury. For example, a pre-clinical TBI study using a unilateral CCI model found differentially expressed exosomal miRNA mostly on the ipsilateral (i.e., injured) side of the brain, with only miR-146 dysregulated bilaterally [15].

The purpose of this review is to capture the state-of-the-science surrounding exosomes in the context of acquired neurological disorders. Due to the low number of published studies, each will be summarized and followed by a synopsis of the existing evidence. This review will also suggest areas for future research to improve the clinical treatment of these devastating conditions; but first, an introduction to the acquired neurological disorders of interest in this review (TBI, SCI, and stroke) is provided.

Traumatic Brain Injury

Blunt traumatic brain injury (TBI) is a form of acquired injury to the brain due to an object contacting the skull or sometimes brain tissue with a rotational injury, axonal injury, or a combination. There are many causes of TBI; more common are falls, motor vehicle accidents, assaults, and sports-related head impacts [71]. Typically, a TBI is classified based on its severity (e.g., mild, moderate, severe), as well as the mechanism of injury (e.g., blunt-force, penetrating). TBIs affect individuals across the life span, and represent a significant cause of death and disability worldwide [72]. In the United States (U.S.) alone, there are approximately 2.8 million emergency department visits, 282,000 inpatient hospitalizations, and 56,000 deaths attributable to TBIs each year [73, 74]. In addition to the high prevalence, TBIs are associated with very high direct and indirect costs associated with death, healthcare costs, and the consequences of disability [75]. Moreover, despite high health care utilization, a recent estimate shows that disability after TBI remains common, with 3.2 million individuals living with one or more TBI-related disability in North America alone [76]. Overall, TBI affects countless individuals worldwide every year, many of whom go without diagnosis or treatment. Since some individuals are largely asymptomatic and/or underreport their symptoms, objective markers of injury to support accurate diagnosis and prognosis are needed; exosomes represent a promising avenue for biomarker discovery, as described in detail later. There are no FDA-approved interventions to mitigate TBI pathology and subsequent symptoms. Moreover, given the high degree of variability in TBI symptoms and recovery profiles, exosomes may provide insights regarding which patients to follow more closely as well as inform clinical management to attenuate symptoms and deficits.

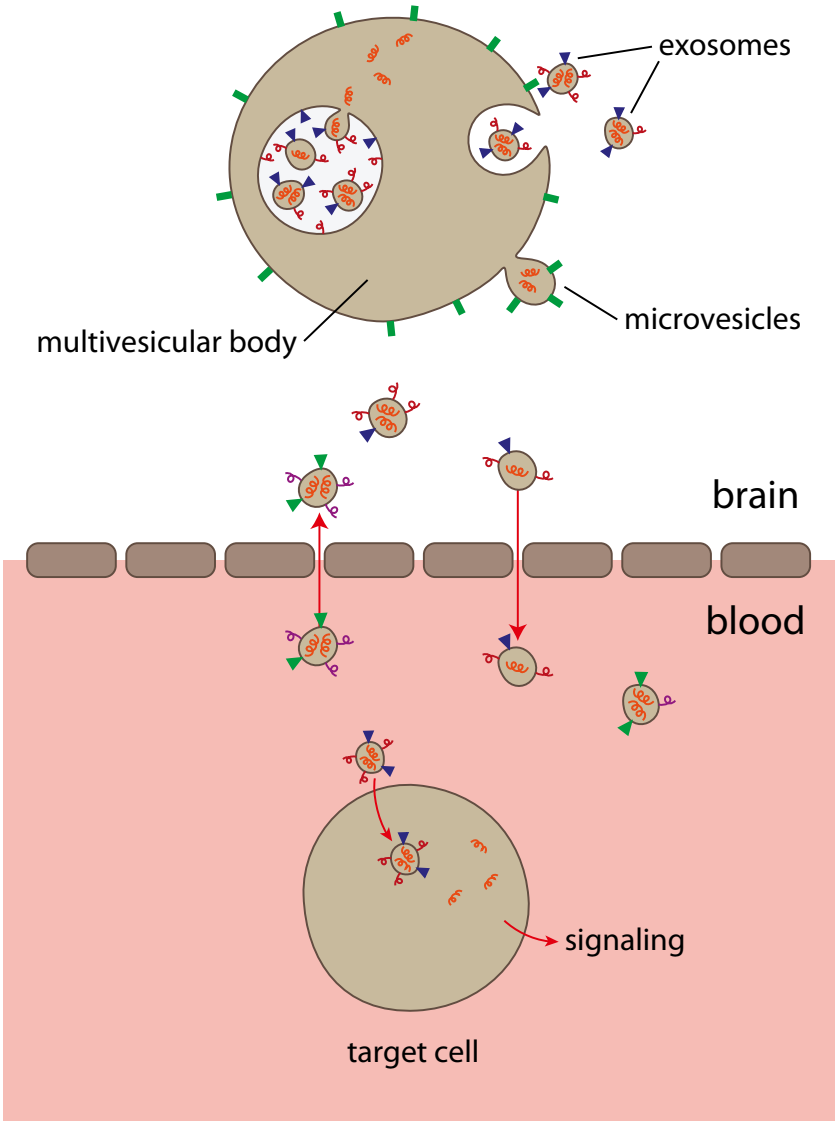

Fig. 1 This figure depicts key features of exosomes, including their release following fusion of a multivesicular body with the plasma membrane (vs. microvesicles which bleb directly off the membrane), ability to cross the blood-brain barrier, and role in cellular signaling. This figure was generated by Nicole Osier and Michael Farmer using Adobe Illustrator based on information from the following sources: [2, 25, 53]

Spinal Cord Injury

Spinal cord injury (SCI) is defined as a form of acquired injury to the spinal cord, which often results in serious disruptions to normal sensorimotor and autonomic functions [77]. SCIs occur due to many of the same causes as TBIs including, but not limited to, motor vehicle accidents, falls, assaults, and sports-related traumas [78]. According to the National SCI Statistical Center, there are approximately 17,000 new SCI cases in the U.S. each year [79]. Subsequently, SCI is associated with significant healthcare costs, the average lifetime expenditure for treating a patient with a SCI ranges between $500,000–$2 million USD [80]. The classification of SCIs is typically based on the location of the injury [the cervical (C), thoracic (T), or lumbar (L) vertebrae affected], as well as the neurological and functional impairments that arise as the result of injury. SCI severity is commonly graded using the guidelines outlined in the American Spinal Injury Association (ASIA) impairment scale [81]. The chronic complications of SCI often include dysfunction in the respiratory, cardiovascular, genitourinary, and gastrointestinal systems, as well as increased spasticity of motor neurons throughout the body. Following SCI, up to 80% of patients report musculoskeletal, visceral, and/or neuropathic pain, which often persist chronically and may require long-term pharmacological and psychotherapeutic intervention [82]. Exosomes may represent an avenue for development of therapeutics capable of improving outcomes of SCI.

Stroke

Stroke occurs when blood flow through a vessel to or within the brain is interrupted, by a blockage or bleed, resulting in dysfunction and death of the affected brain cells. Resulting secondary injury processes can

compromise more distal cells [83]. The American Heart Association reports that approximately 795,000 U.S. citizens suffer a stroke annually, resulting in significant morbidity and mortality [84]; indeed, stroke is the fifth leading cause of death in the U.S. The total direct cost of stroke care in the U.S., including inpatient/outpatient health care services, medications, and home health care, is estimated annually at over $193 billion dollars, over twice the direct costs of cancer [84]. Complications following stroke include decreased mobility and cognitive ability, aphasia/dysarthria, and anxiety and depressive symptoms; complications limit social interactions for survivors [85] and have long-term detrimental consequences on quality of life [86]. There are two major types of stroke [83, 87]: (1) hemorrhagic stroke, which results from bleeding in the brain often caused by a weakened arterial wall (i.e., aneurysm) [87]; and (2) ischemic strokes which lead to brain tissue death caused by a blockage, often due to atherosclerosis or a clot in a cerebral blood vessel supplying oxygen and nutrients to the brain [83]. Ischemic strokes account for the most common type (87%) of strokes [88] and are further subdivided into large vessel disease, small vessel disease, and cardioembolism [89]. As with TBI and SCI, exosomes may represent a promising prognostic biomarker and therapeutic avenue for stroke [90, 91].

Methods

Between October 18, 2016 and April 27, 2016, primary literature searches were conducted using the following online databases: PubMed, PubMed Central (PMC), Google Scholar, and The Cochrane Database. The following search terms and truncations (*) were used, alone, or in combination with standard Boolean operators (AND; OR; NOT): exosomes; exosom*; traumatic brain injury; TBI; brain injury; brain trauma; stroke; ischemia; ischem*; spinal cord injury; SCI; neurological injury; neurodegeneration; neurodegenerat*; cell-free; extracellular vesicles; vesicles. The following Medical Subject Heading (MeSH) terms were also added to the searches: exosomes; brain injuries; neurodegenerative diseases; stroke; cerebrovascular accident; apoplexy; brain ischemia; cerebral ischemia. Secondary literature searches were performed using the bibliographies of relevant manuscripts identified during primary searches. Following preliminary screening of the title and abstract for relevance, over 100 full-text articles were assessed for the following inclusion criteria: (1) studied TBI, SCI, or stroke in either humans or animals; (2) either isolated and assessed exosomes or tested their therapeutic potential; and (3) articles that were originally written in English or subsequently had an English translation published. In total, ten articles were identified that met the abovementioned inclusion criteria, four of which focused exclusive on TBI, one on both TBI and SCI, and five on stroke.

Results

Exosomes in Traumatic Brain Injuries

To date, four studies were published that examined exosomes in the context of TBI. All four were animal studies; one study was performed with rats [14, 25] and three were performed with mice [15, 19]. Published studies differed with respect to methodological considerations, including the method of inducing the TBI, techniques used for exosomal isolation/enrichment, and study goals. Only two studies tested the therapeutic potential of exosomes in the context of TBIs [14, 19]; both showed beneficial effects of EV therapy, including improved cellular outcomes (e.g., attenuated inflammation, increased generation of both newly formed endothelial cells and neurons) [14] and attenuation of post-injury cognitive deficits (e.g., reduced sensorimotor deficits on the foot fault test, improved spatial learning on the Morris water maze) [14, 19].

One study exposed C57BL/6 mice to TBI modeled using the controlled cortical impact (CCI) model or sham (control) surgery resulting in what would be considered to be a moderate to severe brain injury; mice were sacrificed 7 days after surgery and exosomes were isolated from the cerebellum, brain stem, and both hemispheres based on the presence of exosomal markers (CD9, CD63, CD81, HSP70, and TSG101) [15]. This study was the first to profile miRNA in exosomes isolated from harvested brain tissue after a TBI [15]. Specifically, RNA-sequencing revealed miR-212 was downregulated, whereas miR-21, 146, 7a and 7b were upregulated, with the largest fold increase being in miR-21 after CCI [15]. The authors of this study were especially interested in the upregulation of miR-21 which has known neuroprotective roles. A key finding of this study was that microglia may be activated by the entry of neuronally derived exosomal cargo [15]; this finding warrants ongoing inquiry to better understand the origins and consequences of exosomes.

A second mouse study sought to test a novel point-of-care tool to isolate and detect brain-derived exosomes with a smartphone-based μ MED chip [25]. In this study, exosomes were obtained from three sources: (1) a cell culture model of murine cortical neurons, (2) a pre-clinical model of mice exposed to mild TBI induced using CCI (or sham control), and (3) a pre-clinical model of mice exposed to mild TBI induced using blast (or sham control) [25]. In cell culture, a stretch model of injury demonstrated increased levels of GluR2+ exosomes in injured cortical neurons, as compared to non-injured neurons ($p = 0.003$). The smartphone-based tool was effective at isolating and detecting exosomes, which were enriched for Glutamate receptor 2 (GluR2), a protein primarily

expressed in the brain making it a good central marker [25]. This protein was also found to be endocytosed after brain trauma leading to further cellular injury; thus, this protein is implicated in TBI pathology [92, 93]. Exosomes from the two mouse models were isolated from serum; GluR2+ exosomes were elevated in both injury models compared to the respective sham control groups [25]. In addition to being the first to study exosomal GluR2+ levels, this study was strengthened by its methodological advancements. Exosome sample preparation typically takes over 24 h using standard methods; however, the point-of-care tool tested in this study reduces the wait time to less than 1 h from the time of sample collection until the time of obtaining results [25]. Monitoring the counts and cargo of GluR2-containing exosomes may provide insights into the acute and chronic pathology associated with TBIs [25]. Future directions include expanding the platform to examine exosomal cargo and potential opportunities for clinical translation [25].

The therapeutic potential of exosomes has also been explored in the context of TBIs. A third study induced TBI in Wistar rats using the CCI model and examined the therapeutic effects of cell-free exosomes on outcomes related to neurovascular remodeling and functional recovery [14]. The TBI-exposed group was further subdivided based on whether they were administered exosomes derived from mesenchymal stem cells (MSC) or phosphate buffered saline (PBS) control solution 24 h after CCI or sham induction [14]. The key findings in this study were that the administration of MSC-derived exosomes led to physiological changes, including increased angiogenesis, vascular density, and neurogenesis within the dentate gyrus [14]. EV therapy also leads to decreased neuroinflammation; however, there was no effect of exosomes on the cortical lesion volume, compared to PBS control [14]. The physiological changes in the exosome-treated group were associated with enhanced spatial learning on the Morris water maze and better sensorimotor outcomes assessed using the neurological severity score [14]. These findings may lay the foundation for development of novel therapeutic approaches for treatment of TBIs. Future studies should attempt to identify the specific constellation of miRNAs and growth factors that contribute to the therapeutic benefits. Considerations for individualizing exosomal therapies and/or tailoring the therapeutic regimen should also be explored.

The final TBI study identified was the second to test MSC-derived EVs as a TBI therapy (vs. control solution) in mice. A novel in vitro protocol, capable of producing large numbers of EVs with anti-inflammatory properties, was developed and the therapeutic EVs were tested in an in vivo CCI model using 7–8-week-old male C57BL/6J mice [19]. In this study, MSC-EVs from the bone marrow of a human donor (or PBS control solution) were administered 1 h after injury (or sham control). Among mice exposed to TBI, EV therapy was associated with reduced neuroinflammation when assessed 12-h post-injury, in a dose-dependent manner. This was evidenced by progressively lower levels of interleukin (IL)-1β in brain tissue with increased dose of MSC-EVs [19]. Moreover, EV therapy improved cognitive function (e.g., spatial learning and pattern separation) after TBI, compared to the control solution [19]. This study addressed limitations of previous studies including the development of novel protocols that facilitated the production and isolation of high volumes of EVs from bone marrow and evaluation of dose-response patterns [19].

Exosomes also hold promise for individuals with multiple sub-concussive hits, and the subsequent risk of chronic traumatic encephalopathy (CTE). This is a timely application for exosomes, since CTE has become an area of increased research emphasis due to its link with repeated head traumas in athletes [94]. In the context of National Football League players with CTE (vs. healthy controls), elevated levels of tau-positive, exosomal concentrations, suggest that exosomal tau in peripheral blood samples may serve as a clinically available, diagnostic biomarker for CTE [95].

Exosomes in Spinal Cord Injuries

Only a single published study was identified that examined exosomes in the context of SCI. This study examined exosomes isolated from the CSF of human SCI patients as well as exosomes in rats exposed to SCI. Notably, the sample used to address the clinical aim also included a small number of individuals with TBI, as well as uninjured controls [16]. Thus, this study also makes some contribution to the TBI knowledge base, though it was not summarized above. Additional pre-clinical and clinical studies are needed to garner further evidence of the potential for exosomes to guide care and subsequently improve outcomes of SCI.

The single published SCI study examined the clinical occurrence of inflammasomes in SCI/TBI patients, followed by a pre-clinical examination of the effects of therapeutic exosomes targeted against inflammasomes in a rat SCI model [16]. First, in the clinical portion of the study, post-injury spinal cord motor and cortical neurons from nine banked human tissue samples were demonstrated to have elevated levels of nucleotide-binding and oligomerization domain (NOD)-like receptor protein-1 (NLRP1) inflammasome (which regulates caspase 1 activation and processing of IL-1β and IL-18), caspase 1, and a caspase recruitment domain (ASC) as well as the presence of NLRP1 within human CSF exosomes versus uninjured controls. Thus, the clinical portion of the study demonstrated the elevation of inflammasomes within human CSF exosomes following CNS injury. Second, in the pre-clinical portion of the study, a rat model of moderate contusive SCI using adult female Fischer rats evaluated the use of exosomes to target this CNS inflammasome activation as compared to sham control rats. The exosomes were isolated from cultured rat embryonic cortical neurons. Two types of exosomes were

compared: therapeutic exosomes were siRNA labeled using green fluorescent protein against ASC, versus unmodified exosomes, which had scrambled siRNA [16]. In vitro, therapeutic exosomes were found to successfully deliver their cargo, resulting in blocked activation of inflammasome signaling. In vivo, exosomes loaded with siRNA against ASC protein resulted in lower ASC expression (76%), in addition to significantly lower caspase 1 activation and IL-1β processing, when compared to SCI rats treated with unmodified exosomes [16].

Exosomes in Stroke

A total of five studies were identified that examined exosomes in the context of ischemic stroke; no published studies examined exosomes in hemorrhagic stroke. Of these, one used clinical data, while four used pre-clinical models. Among the pre-clinical studies, the models and methods varied and included but were not limited to middle cerebral artery occlusion [20], carotid artery occlusion [17], and umbilical cord occlusion for in utero modeling of stroke [21].

One study used in vitro and pre-clinical methods to study stroke [23]. Wistar rats were exposed to a focal cerebral ischemia model that used intraluminal occlusions, either transiently or permanently, to the middle cerebral artery [23]. In this study, cultured human brain endothelial cells were also used to isolate exosomes. In culture, oxygen-glucose deprivation resulted in lower exosomal miR-126 levels [23]. In the rat model, the results suggested that exosomal miR-126 obtained from peripheral blood was more sensitive to the effects of cerebral ischemia, responding to both mild and severe ischemic episodes. However, total serum miR-126 may be a more specific indicator of the severity of ischemia [23]. Taken together, the study suggests that while both blood and exosomal miR-126 levels are informative for detecting cerebral ischemia, serum levels increased sensitivity to qualifying the severity of ischemia [23]. Future directions include exploring central and peripheral miRNA signaling, determining the source of miR-126 which may be vascular or non-vascular, and extending this work to more diverse samples [23].

Another study examined induced stroke in C57BL/6 mice using a model of hypoxic ischemic brain injury via cerebral artery occlusion [20]. This study examined the therapeutic effects of EVs derived from human bone marrow MSCs and found their therapeutic effect to be comparable to MSCs alone [20]. Only MSC-EVs were found to attenuate post-ischemic peripheral immune responses (B and T cell activity); however, infiltration of immune cells into the cerebral tissue was not modulated by MSC-EVs [20]. The finding that MSCs and MSC-EVs comparably promoted neurogenesis and angiogenesis post-stroke might suggest that the active component of MSC therapy is due in part to the administration of exosomes, though this remains to be empirically established [20].

A third study tested the therapeutic effects of exosomes in the context of hypoxic-ischemic injury in pre-term ovine brains modeled via umbilical occlusion [21]. In this study, EVs were derived from human bone marrow MSCs and administered on the day of umbilical cord occlusion (day 0) and again on day 4 [21]. In this study of pre-term brains, the therapeutic administration of MSC-EVs reduced the number and duration of seizures; MSC-EVs also preserved the sensitivity of the baroreceptor reflex, which was associated with an observed tendency to prevent hypo-methylation. There was no effect of MSC-EV therapy on apoptosis or neuroinflammation [21]. Future directions to build on this work include evaluation of the temporal effects of MSC-EV therapy by including additional endpoints and comparing administration of MSC-EVs to MSC alone, given the results of the abovementioned study [21].

The fourth study tested the therapeutic effects of MSC-derived exosomes obtained from stromal cells in the bone marrow on stroke outcomes modeled using both an endothelial cell culture model and a bilateral carotid artery ligation model of ischemic-reperfusion injury in adult Sprague-Dawley rats [17]. In this study, two types of MSC-derived exosomes were tested for their therapeutic potential: exosomes treated with 500 μL/mL Buyang Huanwu decoction (BYHWD) versus untreated exosomes [17]. A commercially available kit was used to isolate exosomes and exosomal markers CD9 and CD81 via western blot analysis; exosomes were visualized using electron microscopy. A key finding of this study is that BYHWD-treated exosomes resulted in higher expression of angiogenic miRNA in cell culture; in the rat model, expression of vascular endothelial growth factor (VEGF) and Ki-67 (also known as MKI67) was increased, which was associated with augmented vascular density after stroke [17].

The fifth and final study examined exosomes using 65 acute ischemic stroke patients and 66 healthy volunteers who did not have a history of stroke [18]. Patients provided serum samples which were used to isolate exosomes, and western blot analysis was used to assess levels of established exosomal markers (CD9, CD63, and CD81) [18]. When compared to controls, individuals with stroke had significantly higher concentrations of exosomes in serum, as well as significantly (all p's < 0.01) higher median levels of miR-9 and miR-124, two micro-RNAs implicated in regulation of gene expression [18]. A second key finding was that exosomal levels of both miR-9 and miR-124 were positively correlated with total score on the National Institutes of Health Stroke Scale and were also correlated with the overall volume of the infarct as well as the concentration of the inflammatory biomarker interleukin (IL)-6 in serum [18]. Overall, this study suggested that exosomes obtained from serum samples are helpful in identifying patients with acute ischemic stroke and can be used to gain insights into the likely extent of damage [18].

Pre-clinical models suggest that exosomes may have applicability as indicators of ischemic stroke injury severity [23] as well as delivery of potential therapeutics [17, 21] such as mitigation of the immune response [20]. Similarly, the clinical study suggests clinical utility of CNS-derived exosomes as markers of acute ischemic stroke, including injury severity [18]. Future studies should enrich for exosomes of central or at least nerve-cell origin to enhance the quality of the evidence. Continued exploration of the therapeutic effects of exosomes in the context of pre-clinical stroke models is needed, and, if warranted, translation of exosomal therapies to clinical trials should be pursued.

Discussion

Exosomes As Clinically Relevant Biomarkers

Compared to traditional biomarkers, the ability to localize the cell type of exosome origin enhances their diagnostic, prognostic, and pharmacodynamic utility. Exosomes are derived from a variety of tissues; thus, their cargo represents the microenvironment of the cell type from which they originated. Within the context of acquired neurological disorders, peripheral markers are needed that are indicative of central changes. By tailoring the isolation and enrichment methods, exosomes offer information on the nature and degree of CNS damage and the sites or cell types affected. In this way, exosomes provide a critical advantage over traditional systemic, peripheral biomarkers (e.g., levels of protein in serum or plasma). There are also several practical issues of peripheral biomarkers that can be mitigated by using peripherally obtained, but centrally derived, exosomes. For example, proteins and nucleic acids in the peripheral circulation are relatively unstable due to the abundance of proteinases and nucleases in plasma, whereas exosomes have known stability, due in part to their structure which protects their cargo from degradation and preserves their biological activity [96].

Some potential directions for studies of exosomal biomarkers include those involved in inflammation, neurodegeneration, and other pathological cascades activated in acquired neurological disorders. Past studies have implicated inflammatory biomarkers that feed into apoptotic pathways such as TNFα, IL-6, and IL-10 using peripheral samples, and ASC, NALP-1 using CSF samples to study the pathology associated with TBI [97, 98], stroke [99–104], and SCI [105, 106]. Alternatively, proteins traditionally used as markers of neurodegenerative diseases including those related to amyloid plaques and neurofibrillary tangles, such as Aβ-40, Aβ-42, and tau, which have been implicated in TBI [107, 108], stroke [109–111], and SCI [112].

Exosomes as Therapy

The therapeutic applications of exosomes are of great interest with many efforts underway. Three features of exosomes make them an excellent therapeutic agent, namely, they can effectively deliver functional molecules (e.g., siRNA, MSCs, miRNA) to target cells [113–116], their ability to rapidly pass through the BBB [117], and their known low immunogenicity [118]. Sources of exosomes administered therapeutically to date include MSCs [119] and induced pluripotent stem cells [120], though the therapeutic effects remain to be clarified. In these studies, the ultimate goal is to modulate intercellular communication networks and improve outcomes for patients with acquired neurological disorders [121]. Efforts to engineer the cargo are underway with publications to date exploring let-7 [122], miR-9 [123], miR-124a [124], and miR-204-5p [98]. Likewise, efforts to engineer targets are also underway, with published literature exploring connexin 26 [125], EGFR [126], notch [127], and tenasin [128]. Several delivery mechanisms of therapeutic exosomes have been explored including both intranasal [129, 130] and systemic routes [131–133]. In addition to being used as a therapy, studying sequential samples of exosomes may prove useful for monitoring the effects of therapies on pathophysiological processes. It may also be the case that therapies can alter endogenous exosomes, leading to subsequent improvements in downstream activities. One study found that microenvironmental enrichment was associated with generation of miR-219-containing exosomes which were associated with increased CNS myelination, and reduced oxidative stress [134].

Remaining Gaps in Knowledge and Future Directions

Reliance on pre-clinical methods is a limitation of most of the studies examining exosomes in the context of acquired neurological disorders. There remains a substantial gap in the clinical knowledge base surrounding the role of exosomes in clinical cases of TBI, SCI, and stroke. Many studies are also limited by small, homogenous samples, requiring validation in larger cohorts. For example, females are underrepresented in many of the pre-clinical and clinical studies, which limit the generalizability of the findings [14, 15, 23]. Further pre-clinical and clinical research will be required to supplement the current state-of-the-science. Ensuring that the exosomes/EVs examined are derived from the desired population of EVs is important. A small proportion of non-target EVs have been reported in some studies [19]. Future clinical studies are needed to fully understand how exosomal biomarkers can be used for diagnostic, prognostic, and pharmacodynamic purposes.

For studies exploring therapeutic effects of exosomes, dose-response considerations should be explored, as should the distribution of therapeutic exosomes, their cargo, and how to increase the specificity to target cells in the CNS

[14]. Moreover, the specific mechanism by which exosomes pass through the BBB should be further examined to increase the efficacy of key therapies. Some future directions include further exploration of exosomal miRNAs and growth factors and their effects on recovery [14].

Conclusion

Exosomal release is highly specific to the microenvironment from which it originates. For these reasons, exosomal cargo is an ideal biomarker to better understand the mechanisms underlying TBI, SCI, and stroke pathology. The stability of exomes in peripheral circulation suggests that they could be used both acutely and chronically and may be useful indicators of recovery. Exosomes can also transfer their contents to recipient cells, making them candidates for the therapeutic administration of key proteins or drugs. The methodological isolation and profiling of cargo in circulating exosomes can provide novel, objective diagnostic biomarkers for acquired neurological disorders. This field of research, especially within the context of acquired neurological disorders such as those described in this review, remains in the early stages. Further research is required to optimize and improve isolation techniques for greater CNS specificity and to understand fundamental exosome biology, in relation to these disorders, prior to larger clinical studies. Altogether, recent studies investigating the multiple roles of exosomes shed light on an opportunity to improve diagnostic and prognostic methods, and ultimately patient outcomes, in the clinical setting following a TBI, SCI, or stroke.

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