Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect the Fetal Brain After Hypoxia-Ischemia

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**ABSTRACT**

Preterm neonates are susceptible to perinatal hypoxic-ischemic brain injury, for which no treatment is available. In a preclinical animal model of hypoxic-ischemic brain injury in ovine fetuses, we have demonstrated the neuroprotective potential of systemically administered mesenchymal stromal cells (MSCs). The mechanism of MSC treatment is unclear but suggested to be paracrine, through secretion of extracellular vesicles (EVs). Therefore, we investigated in this study the protective effects of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in a preclinical model of preterm hypoxic-ischemic brain injury. Ovine fetuses were subjected to global hypoxia-ischemia by transient umbilical cord occlusion, followed by in utero intravenous administration of MSC-EVs. The therapeutic effects of MSC-EV administration were assessed by analysis of electrophysiological parameters and histology of the brain. Systemic administration of MSC-EVs improved brain function by reducing the total number and duration of seizures, and by preserving baroreceptor reflex sensitivity. These functional protections were accompanied by a tendency to prevent hypomyelination. Cerebral inflammation remained unaffected by the MSC-EV treatment. Our data demonstrate that MSC-EV treatment might provide a novel strategy to reduce the neurological sequelae following hypoxic-ischemic injury of the preterm brain. Our study results suggest that a cell-free preparation comprising neuroprotective MSC-EVs could substitute MSCs in the treatment of preterm neonates with hypoxic-ischemic brain injury, thereby circumventing the potential risks of systemic administration of living cells.

**SIGNIFICANCE**

Bone marrow-derived mesenchymal stromal cells (MSCs) show promise in treating hypoxic-ischemic injury of the preterm brain. Study results suggest administration of extracellular vesicles, rather than intact MSCs, is sufficient to exert therapeutic effects and avoids potential concerns associated with administration of living cells. The therapeutic efficacy of systemically administered mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) on hypoxia-ischemia-induced injury was assessed in the preterm ovine brain. Impaired function and structural injury of the fetal brain was improved following global hypoxia-ischemia. A cell-free preparation of MSC-EVs could substitute for the cellular counterpart in the treatment of preterm neonates with hypoxic-ischemic brain injury. This may open new clinical applications for “off-the-shelf” interventions with MSC-EVs.

**INTRODUCTION**

Hypoxia-ischemia (HI) in the developing brain has been strongly correlated with morbidity and mortality in premature and full-term infants [1]. One of the induced pathologies is hypoxic-ischemic encephalopathy (HIE), which is associated with adverse neurodevelopmental outcomes, resulting in enormous physical, psychological, and economic burdens [1, 2]. Currently, therapeutic intervention strategies for HIE are limited. Term and late-preterm neonates with moderate or severe HIE are eligible for 72 hours of therapeutic cooling, which has been shown to improve outcomes [1, 3, 4]. Preterm neonates born before 35 weeks’ gestational age, however, are still excluded from this therapy [5]. Thus, new treatment strategies are urgently needed.
In a preclinical animal model of HIE, we have demonstrated that systemic administration of mesenchymal stromal cells (MSCs) promoted functional recovery and prevented structural injury in the preterm brain after global hypoxia-ischemia [6]. These effects were, in part, attributable to reduction of neuroinflammatory processes coordinated by a splenic response [6]. However, the exact mechanisms of action of the exogenously delivered MSCs are not clear.

Initially, MSCs were considered to home to affected tissues and substitute lost cell types. Increasing evidence, however, indicates that, instead, MSCs exert their therapeutic effects in a paracrine manner via secretion of small extracellular vesicles (EVs) (i.e., exosomes 70–150 nm in diameter) and microvesicles (100–1,000 nm) [7–14], suggesting that administration of intact MSCs is not mandated to exert therapeutic effects. In comparison with MSCs, mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) have been shown to elicit similar biological effects after administration in various preclinical disease models, including models of kidney, cardiac, and brain injury [9, 15–21]. Recently, a therapy-refractory patient with graft-versus-host disease was successfully treated with allogeneic MSC-EVs with no side effects were assessed using histological detection of microglia infiltration of T lymphocytes.

Thus, the aim of this study was to assess the therapeutic efficacy of MSC-EVs in preterm brain injury. We hypothesized that systemic administration of MSC-EVs, the paracrine mediators of MSCs, would be neuroprotective in hypoxic-ischemic injury in the preterm brain. We tested this hypothesis in a well-established preclinical animal model in which hypoxic-ischemic brain injury was induced by transient umbilical cord occlusion (UCO) in the preterm ovine fetus [23]. The therapeutic effects of systemic administration of MSC-EVs on brain function were determined by evaluation of seizure burden, a measure for cortical function, and by calculating baroreflex-mediated heart rate response (baroreceptor reflex sensitivity), which is needed to regulate blood pressure fluctuations, as a measurement for function of the brainstem. Structural injury was investigated by histological examination of the subcortical white matter. Anti-inflammatory effects were assessed using histological detection of microglia in the hippocampus and subcortical white matter, and of cerebral infiltration of T lymphocytes.

Study Approval
Experimental procedures and the study design were in line with institutional guidelines for animal experiments and approved by the Animal Ethics Committee of Maastricht University, The Netherlands.

Randomization and Blinding
Singleton fetuses (n = 24) of time-mated Texel ewes were randomly assigned to 4 experimental groups: (a) sham umbilical cord occlusion, saline treatment (sham-SAL; n = 6), (b) sham umbilical cord occlusion, MSC-EV treatment (sham-MSC-EV; n = 6), (c) umbilical cord occlusion, saline treatment (HI-SAL; n = 6), and (d) umbilical cord occlusion, MSC-EV treatment (HI-MSC-EV; n = 6) (Fig. 1). The investigator performing the umbilical cord occlusions, including the sham occlusions, was blinded to treatment allocation.

Tissue sampling and postmortem analyses were conducted in a blinded fashion.

A dropout of 16% (n = 4) was observed which was primarily restricted to the sham-MSC-EV group: (i.e., 3 animals of the sham-MSC-EV group and 1 animal of the HI-MSC-EV). Importantly, autopsy of these animals revealed that fetal death was exclusively caused by a single technical reason namely arterial catheter not in situ resulting in exsanguination and subsequent fetal death, thereby excluding any other cause.

Animals and Surgery
Singleton fetuses were surgically instrumented at 102 days of gestational age (term is approximately 147 days), as described previously [23] (Fig. 1). In short, fetuses were exposed through a midline laparotomy of the ewe. Umbilical vessel catheters (1.2 mm; Medtronic, Mansfield, MA, http://www.medtronic.com/covidien) were inserted in the femoral artery and brachial vein for blood pressure recordings, blood sampling, and administration of MSC-EVs. Three electrocardiogram (ECG) electrodes were attached to the fetal chest for cardiologic monitoring. Two pairs of custom-made, shielded, silver-tipped electroencephalogram (EEG) electrodes (Cooner Wire Co., Chatsworth, CA, http://www.coonewire.com/) were placed bilaterally on the dura over the parasagittal cortex. An additional reference electrode was placed in the neck [23]. An inflatable vascular occluder (OCD16HD, 16 mm; InVivoMetric, Healdsburg, CA, http://www.inivometric.com) was placed around the umbilical cord for induction of transient global HI.

An additional catheter was placed in the amniotic sac for recordings of amniotic pressure. All leads were exteriorized through a trocar hole in the maternal flank. Postoperatively, the sheep were housed individually with access to food and water ad libitum.

Experimental Design
After a 4-day recovery period, the fetuses (gestational age: 106 days; experimental day 0) were subjected to 25 minutes of sham or actual umbilical cord occlusion by rapid inflation of the vascular occluder with a predefined volume of sterile saline (Fig. 1).

Occlusion was confirmed by an acute drop in heart rate and a gradual decline of blood pressure. Furthermore, global hypoxia-ischemia was monitored with subsequent arterial blood gas analysis (data not shown).

Animals that were randomized to receive MSC-EV treatment received 1 aliquot of 4.0 × 10^7 cell equivalents divided into two boluses of 2.0 × 10^7 cell equivalents. The first bolus of 2.0 × 10^7 cell equivalents was administered 1 hour following sham or actual umbilical cord occlusion (UCO). The second bolus was administered 4 days after the hypoxic-ischemic insult. Control animals received an equal volume of sterile 0.9% NaCl intravenously at the designated time points. The fetuses were killed 7 days after sham or actual UCO and prepared for tissue sampling.

Data Acquisition and Analysis
Blood pressure, amniotic pressure, ECG, and EEG data were acquired and digitized by a custom-made Maastricht-Programmable Acquisition System unit (Maastricht Instruments BV, Maastricht, The Netherlands, http://www.maastrichtinstruments.nl) with
media were passed through a 0.22-μm filter membrane. Changes were performed every 48 hours. MSC-conditioned media from passage 3 onward, as described previously [22]. Briefly, media exchanged were every 48 hours. MSC-conditioned media were passed through a 0.22-μm filter membrane.

**Figure 1.** Study design. Fetuses were instrumented at gestational age 102 days. After a recovery period of 4 days, fetuses were subjected to 25 minutes of umbilical cord occlusion or sham occlusion (d0). One hour and 4 days (d4) after umbilical cord occlusion or sham occlusion, fetuses received either intravenous MSC-EVs (2.0 x 10^7 cell equivalents; closed arrow) or saline 0.9% (open arrow). After a 7-day reperfusion period, brain tissue was collected. Abbreviations: d, day; END, end of experiment; GA, gestational age; HI, hypoxia-ischemia; IN, instrumentation; MSC-EV, mesenchymal stem cell-derived extracellular vesicle; UCO, umbilical cord occlusion.

IDEQ software (Maastricht Instruments) and stored for off-line analysis, as described in detail previously [6, 23]. Briefly, following detection and removal of high-voltage (EEG signal > 1,000 μV) and flat-line artifacts, raw signals were converted into amplitude-integrated EEG (aEEG) traces. The aEEG processing include an asymmetric band-pass filter that strongly attenuates activity below 2 Hz and above 15 Hz, semilogarithmic amplitude compression, and time compression. As in the neonatal intensive care unit, these aEEG traces were used to detect electrographic seizure activity, which is characterized by an abrupt rise in the lower and upper margin amplitude of the aEEG. Electrographic seizure activity with a duration of at least 10 seconds was annotated using aEEG/EEG traces and were performed by a neonatologist experienced in neonatal aEEG interpretation.

To quantify baroreflex-mediated heart rate response (baroreceptor reflex sensitivity), the ratio between the standard deviation of the mean heart rate variability and that of the mean systolic blood pressure was calculated, as reported previously [24, 25].

**MSC-EVs**

Human bone marrow-derived MSCs were raised from bone marrow from donors after informed consent according to the Declaration of Helsinki, as described previously [22]. Briefly, MSCs were expanded in MSC basal media (Pan Biotech, Aidenbach, Germany, http://www.pan-biotech.de) supplemented with 10% human thrombocyte lysate, 1% glutamine, and 1% penicillin-streptomycin (Thermo Fisher Scientific, Darmstadt, Germany, https://www.thermofisher.com). Their MSC nature was confirmed by flow cytometry with fluorescent labeled anti-CD44, anti-CD73, anti-CD90, anti-CD105, and anti-CD146 antibodies as positive markers, as well as with by anti-CD14, anti-CD31, and anti-CD45 antibodies as negative controls. In addition, their osteogenic and adipogenic differentiation potential was confirmed in conventional MSC differentiation assays. MSC-EVs were harvested from MSC-conditioned media from passage 3 onward, as described previously [22]. Briefly, media exchanges were performed every 48 hours. MSC-conditioned media were passed through a 0.22-μm filter membrane (TPP, Trasadingen, Switzerland, http://www.tpp.ch) and stored at −20°C until use. Upon processing, all conditioned media samples where thawed and processed at once.

After adding polyethylene glycol (PEG) and NaCl to a final concentration of 10% PEG 6000 volume per volume and 75 mM NaCl overnight incubation at 4°C, MSC-EVs were concentrated by low-speed centrifugation (30 minutes at 1,500 g). Pellets were washed in 0.9% NaCl twice and resuspended in 0.9% NaCl. MSC-EVs were stored as 1-ml aliquots, each containing the MSC-EV equivalents harvested from the 48-hour conditioned media of 4 x 10^7 cells. Aliquots were stored at −80°C until use. The obtained MSC-EV fraction was characterized as presented by Kordelas et al. [22] by nanoparticle tracking analyses (NTA) [26] and Western blot analysis (supplemental online Fig. 1), as described previously [22]. Briefly, protein concentrations were determined by the micro-BCA assay (Thermo Fisher Scientific). Concentrated MSC-EVs (5 μg) were treated with sample buffer (dithiothreitol, 0.1% SDS, 0.1 M Tris HCl, pH 7.0) and boiled for 5 minutes at 95°C. Samples were separated on 12% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany, http://www.merckmillipore.com). Membranes were blocked in 5% skim milk in phosphate-buffered saline (PBS) followed by incubation with antibodies recognizing Tsg101 (Sigma-Aldrich, St. Louis, MO, http://www.sigmaalrich.com) or CD81 (Becton Dickinson, Franklin Lakes, NJ, http://www.bdb.com). Subsequently, membranes were incubated with appropriate horseradish-peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany, http://www.dianova.com). Visualization was accomplished by enhanced chemiluminescence (Thermo Fisher Scientific). Furthermore, test results showed MSC-EVs were negative for the presence of bacteria, viruses, and endotoxins.

**Immunohistochemistry in the Brain**

Right-side cerebral hemispheres underwent immersion fixation in 4% paraformaldehyde and subsequently were embedded in gelatin. Serial coronal sections (50 μm) were cut on a Leica VT 1200S vibrating microtome (Leica Biosystems, Nussloch, Germany, http://www.leicabiosystems.com). Free-floating sections...
Seizure data, cumulative throughout the entire experiment, showed right-skewness due to the absence of seizures in non-HI groups that could not be corrected by log-transformation. Therefore, group comparisons were performed with Mann-Whitney tests. Seizure burden is represented as median and interquartile range (IQR). A value of \( p < .05 \) was considered statistically significant. All statistical analyses were performed with SPSS Statistics version 22 (IBM Corp., Armonk, NY, http://www-01.ibm.com).

Statistical analysis of baroreflex sensitivity was performed using a Bayesian multilevel model [29]. Statistical time series analysis was conducted using the Stan for R package, version 2.6.0, in R version 3.1.1 (https://www.r-project.org).

**RESULTS**

**Animal Characteristics**

To assess the neuroprotective capacities of MSC-EVs, we randomized 24 preterm ovine fetuses in different experimental groups (Fig. 1). Fetuses were subjected to 25 minutes of global HI through UCO or sham UCO. MSC-EVs were administered 1 hour and 4 days after UCO (Fig. 1).

Brain to body weight ratios did not differ significantly between the experimental groups. Splenic weight relative to body weight, indicative of activation of the splenic inflammatory response [23], was significantly reduced after global HI (sham-SAL vs. HI-SAL; \(< .05 \)) and indicative of splenic involution, which is an indication of activation of the peripheral immune system [23, 30]. MSC-EVs prevented HI-induced splenic involution (HI-SAL vs. HI-MSC-EV; \(< .05 \)) (Table 1).

**MSC-EVs Prevent Loss of Cortical Function and Baroreflex Sensitivity After Global HI**

Electrographic seizure burden was assessed as a measure of cortical function and was investigated by aEEG for total numbers of seizures (Fig. 2A) and total amount of time-of-seizure activity (Fig. 2B). Global HI induced a profound increase in the cumulative number of seizures (sham-SAL vs. HI-SAL; \( p = .003 \)) and the cumulative duration (seconds) of seizure activity (sham-SAL vs. HI-SAL; \( p = .002 \), which were reduced by MSC-EV treatment (cumulative number: HI-SAL vs. HI-MSC-EV; \( p = .003 \)) and the cumulative duration (seconds) of seizure activity (sham-SAL vs. HI-SAL; \( p = .002 \)), which were reduced by MSC-EV treatment (cumulative number: HI-SAL vs. HI-MSC-EV; \( p = .021 \); cumulative duration: HI-SAL vs. HI-MSC-EV; \( p = .029 \)). No differences in seizure burden between the sham conditions could by detected.

Brain stem function was assessed by analysis of baroreflex sensitivity (Fig. 3). The baroreflex is a vital part of the vascular autoregulatory system, ensuring adequate perfusion of the fetal brain upon disturbance of homeostasis [24]. Baroreceptor reflex sensitivity was reduced at experimental day 4 following global HI when compared with controls and remained compromised throughout the experiment. MSC-EV treatment prevented HI-induced compromise of the baroreceptor reflex sensitivity from experimental day 3 onward. Surprisingly, MSC-EV treatment significantly reduced baroreflex sensitivity in healthy controls.

**MSC-EVs Partially Protect Against HI-Induced Hypomyelination, but Not Against Apoptosis**

MBP immunoreactivity, which was analyzed to determine white matter injury, was assessed in the SCWM (Fig. 4A, 4B). Global HI
resulted in marked hypomyelination in the SCWM, as indicated by a significant decrease of MBP immunoreactivity (sham-SAL vs. HI-SAL; \( p = .001 \)). MSC-EV treatment showed a mild trend toward protection against hypomyelination; however, statistical significance was not reached (HI-SAL vs. HI-MSC-EV; \( p = .100 \)).

Assessment of cleaved caspase-3 in the SCWM was performed as a marker for apoptotic cell death. The numbers of caspase-3-positive cells after global HI tended to be higher at 7 days in comparison with controls (\( p = .070 \)). There was no effect on the numbers of caspase-3-positive cells after hypoxia-ischemia by MSC-EV treatment (HI-SAL vs. HI-MSC-EV; \( p = .457 \)).

MSC-EVs Did Not Protect Against HI-Induced Neuroinflammation

We assessed neuroinflammation in the SCWM (Fig. 5A–5C) and the hippocampus (Fig. 5B–5D) by analyzing immunoreactivity of IBA-1. Global HI induced a marked increase of IBA-1 immunoreactivity in the SCWM (sham-SAL vs. HI-SAL; \( p = .001 \)) and the hippocampus (sham-SAL vs. HI-SAL; \( p < .0001 \)), indicative of profound microglial activation and proliferation. MSC-EV treatment had no effects on hippocampal IBA-1 immunoreactivity (HI-SAL vs. HI-MSC-EV; \( p = .398 \)) but increased IBA-1 immunoreactivity in the SCWM (HI-SAL vs. HI-MSC-EV; \( p = .041 \)). MSC-EV treatment had no effects on IBA-1 immunoreactivity in either the SCWM or hippocampus in sham-occluded animals compared with controls.

Finally, we assessed the influx of T lymphocytes in the fetal brain by immunohistochemical analysis for CD3 in the SCWM (Fig. 6). Cerebral influx of CD3-positive cells tended to increase following global HI (sham-SAL vs. HI-SAL; \( p = .078 \)). Remarkably, MSC-EV treatment resulted in an increase of CD3-positive cells in the SCWM of sham-occluded animals (sham-SAL vs. sham-MSC-EV; \( p = .013 \)).

**DISCUSSION**

In this study, we showed that intravenous administration of MSC-EVs prevented functional impairment and showed a tendency to protect against structural injury of the preterm brain after global HI, apparently without reducing cerebral inflammation. These findings are clinically highly relevant because postischemic seizure activity is associated with adverse neurodevelopmental outcomes [31–34]. The baroreceptor reflex buffers short-term changes in blood pressure by adapting systemic vascular resistance, myocardial contractility, and heart rate. The clinical relevance of an impaired baroreceptor reflex function relates to the increased risk of developing additional brain injury by exposing the vulnerable developing cerebral vascular network to large fluctuations in blood pressure.

In preterm infants, fluctuations associated with high blood pressure may disrupt the cerebral capillaries in the germinal matrix, whereas periods of low blood pressure may cause localized hypoxia-ischemia of the watershed areas [35]. Moreover, preterm infants display fluctuating pressure passivity between systemic blood pressure and cerebral blood flow, representing a considerably increased risk for cerebral hemorrhage or hypoxia [36]. We have shown that HI reduces the baroreceptor reflex-mediated heart rate response [24].

In this report, we demonstrate for the first time that MSC-EV treatment results in preservation of baroreceptor reflex sensitivity after exposure to global HI. Our data suggest a widespread...
functional protection by MSC-EVs of the central nervous system after global HI. Remarkably, we found that MSC-EV treatment reduced baroreceptor reflex sensitivity in healthy controls, which suggests that MSC-EV treatment should be given with care to avoid adverse effects and warrants further analyses of the effects of MSC-EVs on the developing brain in preclinical animal models before introducing MSC-EVs into clinical trials. However, clinical data in an adult patient with graft-versus-host...
disease demonstrated safe intravenous administration of MSC-EVs without adverse cardiovascular and hemodynamic outcomes [22].

Consistent with improved electrocortical function and preservation of baroreceptor reflex sensitivity, we demonstrated that intravenous administration of MSC-EVs tended to protect against HI-induced white matter injury, which is the clinical hallmark of neonatal HI brain injury [37]. This study suggests that the functional neuroprotective effects of MSC-EVs are not primarily caused by anti-inflammatory mechanisms. This is in contrast to our previous results in which the neuroprotective effects of intravenously administered MSCs could be, at least in part, attributed to anti-inflammatory capacities of MSCs [6]. Although prevention of splenic involution [6] might indicate inhibition of systemic immune activation [30], this effect was not paralleled by anti-inflammatory modifications of the ischemic fetal brain. This latter finding is in contrast to results from our previous study in which splenic involution was associated with prevention of cerebral inflammation after systemic MSC administration. This difference in anti-inflammatory properties of MSCs-EVs in the current study and of MSCs in the previous study could be explained by two reasons: First, the immune modulatory properties of MSCs and their EVs differ between independent MSC preparations [22]. Second, in contrast to MSC-EVs, MSCs can sense microenvironmental conditions to which they are exposed (e.g., licensing); in a proinflammatory environment, MSCs stimulate polarization toward an anti-inflammatory phenotype, whereas alternative stimulation has been reported to propagate a proinflammatory phenotype instead [38–45]. This may affect their secretion of therapeutically active immune modulatory components. However, we compared the therapeutic effect of MSC-EVs and that of corresponding MSCs in a murine ischemic stroke model and did not recognize any differences [21]. Although, such a side-by-side comparison was not performed in our model, this latter study suggests that differences between the two studies cannot be attributed to licensing.

The concept of inflammation-independent effects as neuroprotective mechanisms of MSC-EVs therapy is supported by several studies showing that MSC-EVs can prevent apoptosis and stimulate angiogenesis after hypoxia-ischemia [16, 20, 46–50]. However, no effects on angiogenic markers (mRNA levels of vascular endothelial growth factor [VEGF]-A and VEGF-receptor 2; data not shown) or on the number of apoptotic...
cells after global HI with MSC-EV treatment were found in our study, suggesting that the neuroprotective potential of MSC-EVs cannot be attributed to antiapoptotic or angiogenic properties.

Despite the identified discrepancies in therapeutic effects between MSCs and MSC-EVs used in this study, the application of EVs offers several advantages over the administration of MSCs: (a) The risk for malignant transformation is greatly reduced because EVs are nonself-replicating [51]. (b) Lacking an own metabolism, the EVs’ activity can hardly be influenced by the in vivo environment in patients, thus allowing for a much better characterization of their functional properties. (c) Owing to their small size, EVs are less likely to generate emboli upon intravenous administration, as may be the case with MSCs. (d) In addition, EVs can be sterilized by filtration. Thus, from a regulatory point of view, the production, and especially the quality control of EV fractions for clinical treatment application, is less complicated than for a cellular therapeutic of in vitro expanded cells [52]. (e) Last but not least, EVs can be developed independently of the original donor because they are derived from MSCs from unrelated donors and thus offer the opportunity to be turned into an “off-the-shelf” product.

We have chosen this well-established preclinical sheep model because it enables us to accurately mimic the etiology of hypoxic-ischemic injury of the developing preterm brain, including continuous registration of clinical parameters with strong clinical relevance and predictive values [6, 53, 54]. Nevertheless, our study has several shortcomings. We have no information on the dynamics of the effects of MSC-EVs nor have we studied different doses. Time-course information is thus desirable in our model. In future experiments, back-to-back comparison of MSCs and their corresponding EV fraction will provide crucial mechanistic insights into the mode of action of MSCs and their EVs. The scope of this study, however, was assessing the feasibility of MSC-EV treatment to improve brain function after hypoxia-ischemia.

CONCLUSION

We have demonstrated in a preclinical animal model that MSC-EVs harbor neuroprotective potential as shown by improved functional and structural outcomes of the preterm brain following global HI, which is in line with previous reports [16, 17, 19, 22, 55, 56]. Future studies to determine the optimal EV contents and dosing strategy should be performed to establish a clinically safe, cell-free therapy for preterm babies after hypoxia-ischemia.

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AUTHOR CONTRIBUTIONS

D.R.M.G.O.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, animal
experiments, final approval of manuscript; T.G.A.M.W., R.K.J., and B.W.K.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript; A.Z.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; P.A. and T.D.: data analysis and interpretation, manuscript writing, final approval of manuscript; A.-K.L. and S.R.: provision of study material or patients, final approval of manuscript; V.P. and L.I.: collection and/or assembly of data, final approval of manuscript; B.G.: conception and design, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
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