Human Neural Stem Cell Extracellular Vesicles Improve Recovery in a Porcine Model of Ischemic Stroke

Robin L. Webb, PhD*; Erin E. Kaiser, BSA*; Brian J. Jurgielewicz, MS; Samantha Spellicy, BS; Shelley L. Scoville, BS; Tyler A. Thompson, MS; Raymond L. Swetenburg, PhD; David C. Hess, MD; Franklin D. West, PhD; Steven L. Stice, PhD

Background and Purpose—Recent work from our group suggests that human neural stem cell–derived extracellular vesicle (NSC EV) treatment improves both tissue and sensorimotor function in a preclinical thromboembolic mouse model of stroke. In this study, NSC EVs were evaluated in a pig ischemic stroke model, where clinically relevant end points were used to assess recovery in a more translational large animal model.

Methods—Ischemic stroke was induced by permanent middle cerebral artery occlusion (MCAO), and either NSC EV or PBS treatment was administered intravenously at 2, 14, and 24 hours post-MCAO. NSC EV effects on tissue level recovery were evaluated via magnetic resonance imaging at 1 and 84 days post-MCAO. Effects on functional recovery were also assessed through longitudinal behavior and gait analysis testing.

Results—NSC EV treatment was neuroprotective and led to significant improvements at the tissue and functional levels in stroke-damaged pigs. NSC EV treatment eliminated intracranial hemorrhage in ischemic lesions in NSC EV pigs (0 of 7) versus control pigs (7 of 8). NSC EV–treated pigs exhibited a significant decrease in cerebral lesion volume and decreased brain swelling relative to control pigs 1-day post-MCAO. NSC EVs significantly reduced edema in treated pigs relative to control pigs, as assessed by improved diffusivity through apparent diffusion coefficient maps. NSC EVs preserved white matter integrity with increased corpus callosum fractional anisotropy values 84 days post-MCAO. Behavior and mobility improvements paralleled structural changes as NSC EV–treated pigs exhibited improved outcomes, including increased exploratory behavior and faster restoration of spatiotemporal gait parameters.

Conclusions—This study demonstrated for the first time that in a large animal model novel NSC EVs significantly improved neural tissue preservation and functional levels post-MCAO, suggesting NSC EVs may be a paradigm changing stroke therapeutic.

Visual Overview—An online visual overview is available for this article. (Stroke. 2018;49:00-00. DOI: 10.1161/STROKEAHA.117.020353.)

Key Words: brain ischemia ■ extracellular vesicles ■ magnetic resonance imaging ■ stroke ■ white matter
Neuroprotective and regenerative properties of NSC EVs versus isogenically derived MSC EVs in a mouse thromboembolic stroke model. MSC EV treatments trended significantly decreased lesion size, preserved motor function, and improved episodic memory. These findings collectively warrant further rigorous testing of NSC EVs in a secondary pig ischemic stroke model.

Following the Stem Cell Emerging Paradigm in Stroke and Stroke Therapy Academic Industry Roundtable committees’ recommendations, NSC EV therapeutic benefits should be extensively tested using clinically relevant routes of administration, treatment regimen, and end points in a large animal model of ischemic stroke. The porcine permanent middle cerebral artery occlusion (MCAO) model possesses several advantages, including brain anatomy and physiology comparable to humans. Both human and porcine brains are gyrencephalic and are composed of >60% white matter (WM) while rodent brains are lissencephalic and are composed of <10% WM. These similar attributes in cytoarchitecture are critically important as WM is highly vulnerable to pathological processes that follow ischemic stroke. Because pigs are of similar body size to humans and their brains are only 7.5x smaller than human brains, compared with the 650x smaller rodent brain, pigs are a more direct assessment of dosing in a preclinical model. These similarities in brain composition, cytoarchitecture, and size collectively support the use of a pig ischemic stroke model to better predict outcomes between preclinical rodent models and human clinical trials.

The objectives of this study were to evaluate the therapeutic potential of NSC EVs through magnetic resonance imaging (MRI) at 1 and 84 days post-MCAO and to longitudinally assess changes in motor function via gait analysis and open field testing. In this study, we present for the first time, evidence NSC EVs promote extensive tissue and functional level recovery in a large animal preclinical stroke model.

Materials and Methods
Data that support the findings of this study are available from the corresponding author on reasonable request.

Study Design
The overarching aim of these studies were to evaluate NSC EV efficacy as a potential acute stroke therapy in a preclinical, biologically relevant porcine MCAO model of ischemic stroke. End points were selected to evaluate tissue and functional level changes in response to treatment. We used a split plot experimental design, where all treatment groups were conducted within 1 day to control for and reduce experimental variation. The sample size for this study was determined by a power calculation based on our previously published work using the pig MCAO model with lesion volume changes by MRI being the primary end point. The power analysis was calculated using a 2-tailed ANOVA test, α=0.05, and an 80% power of detection, effect size of 1.19, and a SD of 44.63. Initially, 14 pigs were randomly assigned to the treated and control groups. However, because of high mortality rates within the control group, 2 additional pigs were added to the control group for a total 9 pigs in the control group and 7 pigs in the treated group (physiological data and mortality information included in Tables I and II in the online-only Data Supplement, respectively). Although a greater percentage of NSC EV pigs survived relative to control pigs, there were no statistically significant survival rate differences between treatment groups (Figure I in the online-only Data Supplement). Ischemic stroke was induced by a blinded surgeon, and EVs were delivered as single use aliquots by investigators. To control for potential day effects, 1 treated and 1 control pig were assigned to each surgical day except for 1 surgical day in which the 2 additional control pig surgeries were performed. Because of the timing of the first treatment, it was not possible to show proof of identical lesion sizes before NSC EV administration or account for progression rate of lesions. However, a 1-way ANOVA and post hoc Tukey–Kramer pair-wise test comparing the lesion volumes of pigs within each treatment group between the first and second half of the study resulted in no significant difference (treated P=0.9994, nontreated P=0.7804).

This consistency in lesion volumes suggests that there was no significant difference in time-dependent variables, including the effect of surgical procedures during the course of the study. All end points and functional measurements were prospectively planned and underwent unblinded analysis. Predefined exclusion criteria from all end points included instances of infection in the injury site, self-inflicted injuries that required euthanasia, inability to thermoregulate, uncontrolled seizure activity, and respiratory distress. One control pig was excluded from MRI collection because of postoperative complications and premature death (Table II in the online-only Data Supplement). Data collection from 1 treated pig was retrospectively excluded from all assessments because of a Trueperella (Arcanobacterium) pyogenes abscess and was determined to be the result of the surgery by pathologists and veterinarians. No outliers were removed from the data.

Results
NSC EV Manufacture Consistently Produced Biologically Active and Reproducible Vesicles
EVs were harvested from NSC basal culture medium according to standard production protocol and with reproducible size profile with >90% of EVs under 200 nm in diameter as determined by Nanosight (methods in the online-only Data Supplement). To determine cellular uptake of NSC EVs, a critical component of EV function, uptake of Dil-labeled NSC EVs was evaluated using an interferometric technique known as spatial light interference microscopy. Time lapse imaging (18-hour time point shown) indicated NSC EVs were taken up by cells and were visualized while being transported within the cell (Figure 1A–1C; Movie I in the online-only Data Supplement). NSC EVs may ultimately exert their efficacy through uptake by various cell types when in circulation. NSC EVs were analyzed using a commercially available MACSPlex exosome kit and displayed a consistent EV marker profile (Figure 1D). Along with the recently published physical size evaluation, these data supported a consistent profile and bioactivity of NSC EVs derived from separate purifications.

NSC EVs Decreased Lesion Volume and Mitigated Cerebral Swelling 1-Day Post-MCAO
To confirm ischemic stroke 1-day post-MCAO, MRI T2-weighted fluid-attenuated inversion recovery and diffusion-weighted imaging sequences were assessed and exhibited territorial hyperintense lesions characteristic of an...
edematous injury (Figure 2A, white arrows). Hypointense lesions observed on corresponding apparent diffusion coefficient (ADC) maps confirmed areas of restricted diffusion indicative of cytotoxic edema (Figure 2A, white arrows), thus confirming permanent cauterization of the middle cerebral artery resulted in ischemic stroke. T2-weighted sequences at 1-day post-MCAO revealed characteristic hyperintense lesions indicative of acute ischemic stroke (Figure 2B). To account for the space-occupying effect of brain edema, edema-corrected lesion volume was calculated using T2-weighted and corresponding ADC maps revealing a significant (P<0.05) decrease in edema-corrected lesion volume in NSC EV–treated pigs when compared with controls (6.0±1.4 versus 10.7±1.4 cm³, respectively, Figure 2C). T2-weighted–based results also indicated significantly (P≤0.01) decreased swelling of the affected ipsilateral hemisphere resulting in a less pronounced midline shift in NSC EV–treated pigs relative to control pigs 1-day post-MCAO (113.7±2.6% versus 126.8±3.4%, respectively; Figure 2B and 2D). Despite these acute changes, there were no significant differences in lesion volume or brain atrophy between treatment groups 84 days post-MCAO (Figure III in the online-only Data Supplement). The occurrence of intracranial hemorrhage (ICH) was also substantially reduced in NSC EV–treated pigs relative to controls (0 of 7 and 7 of 8 pigs, respectively, Figure 2B, white arrows; Figure II in the online-only Data Supplement).

NSC EVs Promoted Increased Diffusivity and WM Integrity 1 and 84 Days Post-MCAO

Cerebral diffusivity was evaluated using diffusion-weighted imaging sequences and derived ADC maps. Signal void, consistent with restricted diffusion and indicative of cytotoxic edema, was quantified (Figure 3A, white arrows). Mean ADC values in the affected ipsilateral hemisphere were compared with the contralateral hemisphere with calculated percent changes closer to zero being more similar to normal tissue. NSC EV–treated pigs exhibited a significantly (P<0.01) reduced percent change in ADC values when compared with control pigs 1-day post-MCAO (−18.7%±2.6% versus −32.3%±1.5%, respectively; Figure 3C). To assess long-term changes in WM integrity, the corpus callosum was examined 84 days post-MCAO. Changes in fractional anisotropy in the affected ipsilateral hemisphere were again compared with the contralateral hemisphere. Fractional anisotropy maps depicted
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Figure 2. Neural stem cell–derived extracellular vesicle (NSC EV) treatment decreases intracranial hemorrhage, lesion volume, and hemispheric swelling 1-day post-middle cerebral artery occlusion (MCAO). T2-weighted (T2W) and diffusion-weighted imaging (DWI) sequences revealed territorial hyperintense lesions characteristic of an edematous injury (A, white arrows). Hypointense lesions observed on corresponding apparent diffusion coefficient (ADC) maps confirmed areas of restricted diffusion indicative of cytotoxic edema (A, white arrow). These resulting hallmarks demonstrated permanent cauterization of the ventral aspect of the middle cerebral artery resulted in bona fide, repeatable ischemic stroke in all pigs. NSC EV–treated pigs exhibited a reduced incidence of intracranial hemorrhage (B, white arrows). NSC EV–treated pigs also demonstrated a significant (P<0.05) decrease in edema-corrected lesion volume when compared with control pigs at 1-day post-MCAO (6.0±1.4 vs 10.7±1.4 cm³, respectively; C) and a significantly (P<0.01) lower percent increase in hemisphere volume resulting in a less pronounced midline shift relative to control pigs at 1-day post-MCAO (113.77%±2.571% vs 126.83%±3.41% respectively; D). *Significant difference between treatment groups.

Figure 3D). Collectively, MRI results offered compelling evidence NSC EV treatment provided neuroprotection and promoted tissue level recovery by decreasing cerebral lesion volume, swelling, incidence of ICH, and preserving diffusivity and WM integrity.

a decrease in the corpus callosum of the ipsilateral hemisphere of control pigs 84 days post-MCAO (Figure 3B, white arrow) while NSC EV–treated pigs exhibited a significantly (P<0.01) lower percent decrease in fractional anisotropy values (−13.9%±3.2% versus −43.3%±6.7%, respectively; Figure 3D). Collectively, MRI results offered compelling evidence NSC EV treatment provided neuroprotection and promoted tissue level recovery by decreasing cerebral lesion volume, swelling, incidence of ICH, and preserving diffusivity and WM integrity.
NSC EVs Resulted in Increased Motor Activity and Exploratory Behavior

Explanatory behavior and motor activity pre- and post-MCAO were assessed by open field testing. NSC EV–treated pigs did not significantly decrease their distance traveled while control pigs were less active, compared with pre-MCAO time points (113.6±12.0 versus 42.0±12.7 m; P<0.01; Figure 4A). Interestingly, longitudinal analysis at 84 days post-MCAO revealed NSC EV–treated pigs exhibited a significant increase in distance traveled compared with their pre-MCAO time points (107.3±9.9 versus 217.0±29.6 m; P<0.05). However, this trend was not observed in control pigs. Together, these findings suggest NSC EVs preserved normal exploratory behaviors and motor activity post-MCAO.

NSC EV Treatment Led to Faster and Improved Recovery of Spatiotemporal Gait Parameters

In addition to exploratory activity, there were several key differences in measured gait parameters between treatment groups. Velocity (distance traveled/second), cadence (strides/min), and swing percent of cycle (percentage of 1 full gait cycle in which the contralateral hindlimb was in the non-contact phase) significantly decreased 1-day post-MCAO for both NSC EV–treated and control pigs. However, by 7 days post-MCAO, NSC EV–treated pigs recovered when compared with pre-MCAO performance (Figure 4B). In contrast, control pigs’ deficits in velocity, cadence, and swing percent of cycle persisted through 7 days post-MCAO. By 28 days post-MCAO, NSC EV–treated pigs exhibited a significant increase in temporal gait parameters relative to control pigs, thus demonstrating substantial improvement.

Similar functional outcomes in spatial gait parameters were also observed. Stride length (distance between consecutive hoof prints of the contralateral forelimb), hoof print area (measured by the number of activated sensors of the contralateral forelimb), and total scaled pressure (the sum of peak pressure values recorded from each activated sensor by a hoof during contact) decreased similarly in both groups 1-day post-MCAO (Figure 4C). However, NSC EV–treated pigs recovered by 7 days post-MCAO while control pigs remained significantly impaired at the same time points for these spatial parameters, indicating faster recovery.

Discussion

This pivotal study presents the first experimental evidence that intravenous administration of NSC EVs improved tissue...
and functional level outcomes in a translational porcine ischemic stroke model while adhering to the Stem Cell Emerging Paradigm in Stroke and Stroke Therapy Academic Industry Roundtable committee recommendations for developing and testing novel stroke therapeutics.3–6,25,26 NSC EV intervention led to significant decreases in lesion volume, which has never been observed before in EV-related neural injury studies and has been considered a key biomarker for recovery.12,13,27,28

Figure 4. Neural stem cell–derived extracellular vesicle (NSC EV) treatment results in increased motor activity and improved recovery of spatiotemporal gait parameters. Ethovision XT tracking software was used during open-field testing to automatically assess differences in distance traveled between treatment groups; representative 10-min movement tracings shown for control (A, blue) and NSC EV treated (A, green) pigs. Control pigs experienced a significant decrease in distance traveled at 7-days post-middle cerebral artery occlusion (MCAO) while treated pigs did not. Both groups increased distance traveled over 28 days; however, treated pigs traveled significantly further than their pre-MCAO distance while control pigs did not. At 1-day post-MCAO, NSC EV–treated and control pigs exhibited significant decreases in temporal gait parameters, including velocity, cadence, and swing percent pigs (B). By 7-days post-MCAO, NSC EV–treated pigs recovered these parameters while control pigs did not recover until 28 days. At 28 days, the NSC EV–treated pigs performed significantly better in velocity, cadence, and swing percent than control pigs. Differences in spatial gait parameters were also noted between NSC EV–treated and control pigs in terms of stride length, hoof print area, and relative pressure (C). By 7-days post-MCAO, NSC EV–treated pigs had recovered from deficits in stride length, hoof print area, and relative pressure, whereas control pigs remained impaired. In addition, NSC EV–treated pigs performed significantly better in terms of stride length when compared with control pigs at the same time point. *, #Significant (P<0.01) difference between pre- and post-MCAO time points. a indicates significant (P<0.01) difference between treatment groups.
Although EVs were harvested from human NSC EVs, no overt negative immune responses were detected in the porcine model. These data support our recently published data in a thromboembolic mouse model where the injury response to stroke was dampened while augmenting a reparative systemic response favoring macrophage polarization toward anti-inflammatory M2 cells, increasing regulatory T cells (Treg) cells, and decreasing proinflammatory T helper 17 (TH17) cells. In addition, NSC EV therapy led to preserved diffusivity and sustained WM integrity, which strongly correlates with improvements in executive function, cognitive decline, and sensorimotor deterioration, as well as decreased hemispheric swelling and ICH incidence, which are intimately associated with stroke patient morbidity.

Significant decreases in hemispheric swelling and decreased incidence of ICH indicated NSC EV treatment not only preserved cellular integrity in the ischemic site but also preserved the integrity of microvessels and associated capillary beds 1-day post-MCAO. A recent study of MSC EVs post-MCAO reported increased vascular remodeling in the ischemic boundary zone of rats. The NSC EV marker profile (Figure 1D) indicated consistent presence of integrins, including integrin $\alpha$-1 (CD29) and integrin $\alpha$ 2b (CD41b). Integrin $\beta$-1 is known to mediate cell-to-cell and cell-to-matrix interactions and regulate cell migration. Similarly, integrin $\alpha$-2b is a receptor known to bind a variety of ligands leading to rapid platelet aggregation, as well as regulation of leukocyte migration and megakaryocyte differentiation. In addition, blockade of integrin $\alpha$-2b (CD41) increases ICH incidence and mortality after transient MCAO in a dose-dependent manner. By altering the processes of coagulation and vascular function, intravenously administered NSC EVs may protect the integrity of the blood–brain barrier through inherent intercellular signaling components. Although the exact molecular mechanism of action is currently unknown, whether dependent on one or multiple EV components or direct action at the systemic level or on the brain directly, these data, in addition to our published rodent study, support that NSC EVs are biologically active and elicit a positive neuroprotective response in vivo in both rodent and large animal preclinical stroke models.

A frequently used predictive indicator of patient prognosis is acute lesion volume because of the high correlation between neurological deficits and long-term functional outcomes. Although multiple MSC EV–related rodent models of stroke have observed improvements in tissue and functional recovery, the extent of neural protection seen with NSC EV treatment is unprecedented. Previous rodent stroke studies assessing the efficacy of MSC EVs showed no changes in lesion volume. In comparison, our recently published data indicated an $\approx$35% reduction in lesion volume in the mouse thromboembolic stroke model. Comparatively, our data in the porcine model possessed a significant 44% decrease in lesion volume at 1-day post-MCAO, suggesting NSC EVs are potentially more protective and thus more therapeutically relevant than MSC EVs.

Restoring motor function in patients with stroke is critical for improvement in quality of life and is a robust measure of therapeutic potential. Most patients with stroke exhibit hemiparesis with correlative asymmetries, decreased velocity, stride length, and other spatiotemporal parameters, therefore it was vital to determine whether these cellular benefits resulted in functional benefits at the organismal level. To date, no exosome efficacy study has performed a comprehensive assessment of changes in gait function poststroke. Previous studies have relied on gross measurements (foot fault tests, rotordor), which do not account for fine motor changes in gait as do relative pressure, swing percent, and stride length. In this study, we found significant changes and decreased recovery time in these and other translational parameters that are critical readouts for human patients. The pig is also likely a more representative model of human gait changes poststroke when compared with rodents, despite being a quadraped, as weight (pigs in this study were between 72 and 104 kg), limb, and body length are more comparable to humans and are more similarly affected by biomechanical forces generated during normal movement.

In this study, we have demonstrated that NSC EVs are a potent biological treatment that positively impact both molecular and functional outcomes poststroke while abiding by Stem Cell Emerging Paradigm in Stroke and Stroke Therapy Academic Industry Roundtable committee recommendations for rigorously developing and testing therapeutics. NSC EVs in our porcine ischemic stroke model exhibited a multifactorial effect leading to decreased lesion volume, hemispheric swelling, and ICH while also promoting diffusivity, WM integrity, and functional performance in a large animal model with similar cerebral architecture and WM composition to humans. As an effective treatment in both rodent and porcine stroke models, NSC EVs possess inherent biological characteristics suitable for translation into human stroke therapeutics.

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Disclosures

Drs Webb and Stice have submitted a patent filing on the neural stem cell–derived extracellular vesicles, and this technology is licensed from the University of Georgia Research Foundation by Aruna Biomedical, Inc. All authors affiliated with Aruna Biomedical, Inc own equity in the company. The other authors report no conflicts.

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Human Neural Stem Cell Extracellular Vesicles Improve Recovery in a Porcine Model of Ischemic Stroke
Robin L. Webb, Erin E. Kaiser, Brian J. Jurgielewicz, Samantha Spellicy, Shelley L. Scoville, Tyler A. Thompson, Raymond L. Swetenburg, David C. Hess, Franklin D. West and Steven L. Stice

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SUPPLEMENTAL MATERIAL

Materials and Methods

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Materials and Methods

Animals and Housing

All work performed in this study was approved by the University of Georgia Institutional Animal Care and Use Committee guidelines. Sexually mature, castrated male Landrace pigs, 5-6 months old and 72-104 kg were obtained from the biosecure University of Georgia Swine farm, original seed stock was from one commercial entity. Young, healthy male pigs were used in accordance with the STAIR guidelines that suggests initial therapeutic evaluations should be performed with young, healthy male animals, and further studies should be performed in females, aged animals, and animals with co-morbidities such as hypertension and diabetes. Pigs were individually housed at a room temperature of 27ºC with a 12 hour light/dark cycle. All pigs were fed standard grower diets ad libitum prior to stroke induction, and only feed restricted prior to anesthetic events.

Middle Cerebral Artery Occlusion (MCAO) surgical procedure

MCAO was induced as previously described with minor adjustments. The day of surgery pigs were administered antibiotics (Ceftiofur sodium (Naxcel®; 4 mg/kg IM) and non-steroidal anti-inflammatory Flunixin Meglumine (Banamine-S; 2.2mg/kg IM). Pre-induction analgesia and sedation was achieved using xylazine (7 mg/kg IM), butorphanol (0.3 mg/kg IM) and midazolam (0.3 mg/kg IM). Anesthesia was induced with IV propofol to effect, and prophylactic lidocaine (0.5 to 1.0 mL of 2% lidocaine) was administered topically to the laryngeal folds to facilitate intubation. Anesthesia was maintained with 1.5% inhalational isoflurane (Abbott Laboratories) in oxygen.

As previously described, neural injury was induced by making a curvilinear skin incision extending superiorly from the right orbit to an area rostral to the auricle. A portion of zygomatic arch was resected with the rostral aspect extended from the insertion point of the orbital ligament caudally 3-4 cm. The temporal fascia and muscle were elevated and a craniectomy was generated exposing the local dura mater. The distal middle cerebral artery (MCA) and associated branches were permanently occluded using bipolar cautery forceps thus resulting in ischemic infarction spanning the most caudal aspect of the frontal lobe, significant areas of the temporal lobe, and portions of the parietal and occipital lobes. The exposed brain was covered with a sterile biograft made of porcine
small intestine submucosa (MatriStem, ACell) and the temporalis muscle was routinely reattached along the temporalis line and the skin was routinely reapposed.

Anesthesia was discontinued and pigs were returned to their pens upon extubation and monitored every 4 hours for the next 24 hours. Heart rate, respiratory rate, and temperature were recorded at each time point. Banamine (2.2 mg/kg) was administered IM for postoperative pain, acute inflammation, and fever management every 12 hours for the first 24 hours, and every 24 hours for 3 days post-MCAO. Naxcel (4 mg/kg) was administered IM as an antibiotic every 24 hours for 3 days post-MCAO.

**Cell Culture, EV enrichment, and characterization**

H9 cells were differentiated into NSCs using standard operating procedures previously published. Media was harvested off NSC cultures when cells reached ~80% confluence. Media was filtered through a 0.22 µm filter and further enriched by ultrafiltration using a 100 kDa regenerated cellulose Amicon or Centricon ultra-centrifugal filter units or the Amicon stirred cell system, and washed twice with PBS. Enriched EVs were stored in single use aliquots 52 ml for pigs (2.7 x 10¹⁰ ± 10% vesicles/kg) and stored at -20°C. Labeled EVs were incubated with 10 uM DiI for 30 minutes before washes. DiI labeled EVs were applied to differentiated NSCs or MSCs and visualized by SLIM as previously described.

**NSC EV and PBS +/- Administration**

A total of 50 mLs of either PBS with calcium and magnesium (PBS+/+) or NSC EVs suspended in PBS +/- IV access were administered via peripheral ear vein. PBS +/- or NSC EVs treatment was administered 2, 14, and 24 hours post-MCAO.

**MRI Acquisition and Analysis**

MRI was performed 1 and 84 days post-MCAO on a Siemens 3.0 Tesla Magnetom Avanto MRI system. Utilizing the previously described surgical anesthesia protocol, MRI of the cranium was performed using a 12 channel head coil, 25 cm in diameter with the pig positioned in supine recumbency. Standard multiplanar magnetic resonance (MR) brain imaging sequences were acquired including T2FLAIR, T2W, DWI, and DTI. T2FLAIR, T2W, DWI, and ADC maps were analyzed using Osirix software whereas DTI and computed FA values were analyzed using ImageJ software. Cytotoxic edema consistent with ischemic stroke was confirmed at 1 day post-MCAO by comparing corresponding hyperintense regions in T2FLAIR and DWI sequences, and hypointense regions in ADC maps. To control for the space-occupying effect of brain edema, hemisphere volumes were calculated utilizing T2W sequences while ischemic lesion volumes were calculated via ADC maps as previously described by Gerriets et al. Corrected lesion volumes were calculated according to the following formula modified from Loubinoux et al. where LVc and LVu indicate corrected and uncorrected lesion volume, respectively, and HVc and HVi indicate volume of the contralateral and ipsilateral hemisphere, respectively.

\[
LV^c = HV_C + HV_i - \left( \frac{HV_C + HV_i - LV^u}{2HV_C} \right) \cdot HV_C + HV_i
\]

DWI sequences were utilized to identify hypointense regions of interest (ROI) in the ipsilateral hemisphere and directly compared to identical ROIs in the contralateral hemisphere at each coronal plane. Average ADC values were calculated for each coronal slice, and changes in mean ADC value of the ipsilateral hemisphere were expressed as a percentage change relative to the contralateral hemisphere. DTI was utilized to generate FA values in the corpus callosum and was expressed as a percent change in the ipsilateral hemisphere relative to the contralateral hemisphere.
Behavior Assessment and Analysis

Open field testing occurred pre-MCAO, 1, 7, and 21 days post-MCAO. Pigs were permitted to enter the open field arena via two starting gates according to a predetermined pseudorandomized pattern. Pigs were recorded utilizing Ethovision™ XT tracking software (Noldus) while exploring the novel 14ft x 16ft open field arena for 10 minutes. All surfaces of the open field arena were cleaned thoroughly with ethanol between pigs.

Gait data was collected pre-MCAO, 1, 7, and 28 days post-MCAO. Analysis was performed using an automated computer software program (GaitFour 4.9x9i, GaitRite, New Jersey) to objectively evaluate multiple spatiotemporal gait parameters 8. Predefined inclusion criteria included a consistent gait with less than a 10% velocity variation, a minimum of 12 consecutive footfalls or 3 gait cycles, and no external distractions within each individual trial. The surfaces of the gait track were cleaned thoroughly with ethanol between pigs.

Statistical Analysis

All quantitative data was analyzed with SAS version 9.3 (Cary, NC) and statistical significances between groups were determined by one-way analysis of variance and post-hoc Tukey-Kramer Pair-Wise comparisons. Treatments where p-values were ≤ 0.05 were considered significantly different.

Supplement Table I: Physiological data. There were no statistical differences in any physiological parameters between groups.

|                  | Control | NSC EV |
|------------------|---------|--------|
|                  | HR (bpm)| Temp (°F) | RR (bpm) | HR (bpm) | Temp (°F) | RR (bpm) |
| Average 0 hours  | 73.78   | 97.90  | 25.33    | 65.33    | 96.92    | 28.00    |
| Standard Deviation 0 hours | 36.99 | 1.09  | 8.94     | 17.28    | 1.65     | 6.69     |
| Average 13 hours | 100.44  | 100.68| 36.78    | 92.67    | 100.68   | 35.33    |
| Standard Deviation 13 hours | 26.64 | 1.85  | 33.00    | 35.09    | 1.50     | 14.18    |

Supplement Table II: Death summary.

| Pig # | Treatment Group | Survival Post-MCAO | Cause of Death                      |
|-------|-----------------|--------------------|------------------------------------|
| 4     | Control         | 1 day              | Seizure                            |
| 5     | NSC EV treated  | 7 days             | Non-stroke related post-operative injury; broken leg |
| 6     | Control         | 3 days             | Seizure                            |
| 8     | Control         | 0 days             | Non-stroke related post-operative complication |
| 11    | NSC EV treated  | 21 days            | Idiopathic; possibly endocarditis  |
| 13    | Control         | 2 days             | Seizure                            |
Supplement Table III: Checklist of Methodological and Reporting Aspects

| Methodological and Reporting Aspects                  | Description of Procedures                                                                                                                                                                                                 |
|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Experimental groups and study timeline              | - The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.                                                                                           |
|                                                      | - An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.                                                        |
|                                                      | - An overall study timeline is provided.                                                                                                                                                                                  |
| Inclusion and exclusion criteria                     | - A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.                                                                                                         |
| Randomization                                        | - Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. |
|                                                      | - Type and methods of randomization have been described.                                                                                                                                                                   |
|                                                      | - Methods used for allocation concealment have been reported.                                                                                                                                                            |
| Blinding                                             | - Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. |
|                                                      | - Blinding procedures have been described with regard to masking of group assignment during outcome assessment.                                                                                                        |
| Sample size and power calculations                   | - Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.       |
| Data reporting and statistical methods               | - Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. |
|                                                      | - Baseline data on assessed outcome(s) for all experimental groups have been reported.                                                                                                                                   |
|                                                      | - Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.                                                                              |
|                                                      | - Statistical methods used have been reported.                                                                                                                                                                            |
|                                                      | - Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.                                                                    |
| Experimental details, ethics, and funding statements | - Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. |
|                                                      | - Different sex animals have been used. If not, the reason/justification is provided.                                                                                                                                   |
|                                                      | - Statements on approval by ethics boards and ethical conduct of studies have been provided.                                                                                                                             |
|                                                      | - Statements on funding and conflicts of interests have been provided.                                                                                                                                                    |
Supplement Figure I: NSC EVs do not alter post-stroke survival rate. Although a greater percentage of treated pigs survived to the endpoint, there were not statistically significant differences in survival rate between groups.
Supplement Figure II: NSC EVs resulted in decreased ICH. T2W sequences revealed characteristic hyperintense lesions indicative of acute ischemic stroke. Control pigs exhibited significantly (p=0.0100) greater hemorrhage indicated by hypointense areas in the infarct region relative to NSC EV treated pigs at 1 day post-MCAO.
Supplement Figure III: NSC EVs do not alter lesion volume and brain atrophy at 84 days post-MCAO. There were no significant differences in lesion volume or brain atrophy between groups 84 days post-MCAO.
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