SUPPLEMENTAL MATERIAL

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 [1]. 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 [2]. 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 [3-5]. 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 ($2.7 \times 10^{10} \pm 10\%$ 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 [6]. 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 [7].

$$LV^c = HV_C + HV_i - (HV_C + HV_i - LV^u) \cdot \frac{HV_C + HV_i}{2HV_C}$$

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.

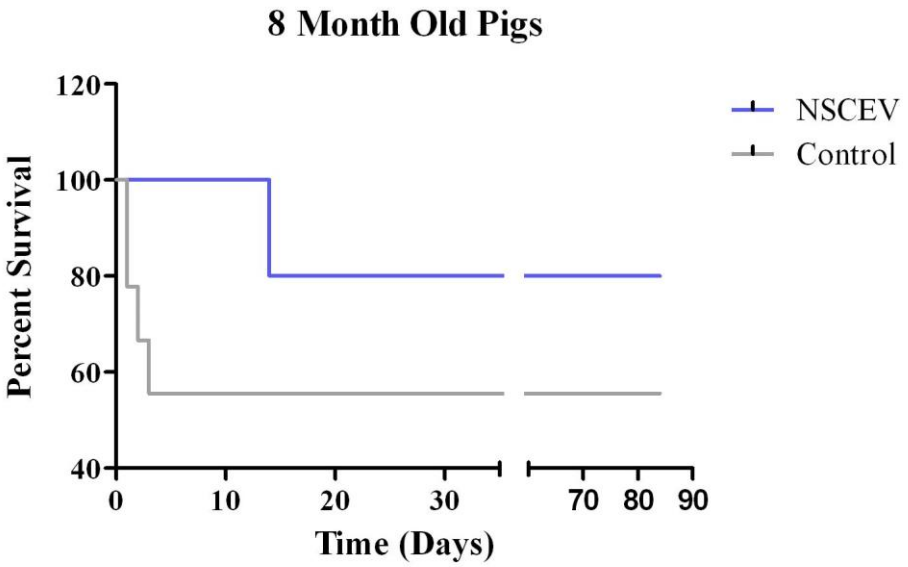
	Control			NSC EV		
	HR (bpm)	Temp (°F)	RR (bpm)	HR (bpm)	Temp (°F)	RR (bpm)
Average 0 hours	73.78	97.90	25.33	65.33	96.92	28.00
Standard Deviation 0 hours	36.99	1.09	8.94	17.28	1.65	6.69
Average 13 hours	100.44	100.68	36.78	92.67	100.68	35.33
Standard Deviation 13 hours	26.64	1.85	33.00	35.09	1.50	14.18

Supplement Table II: Death summary.

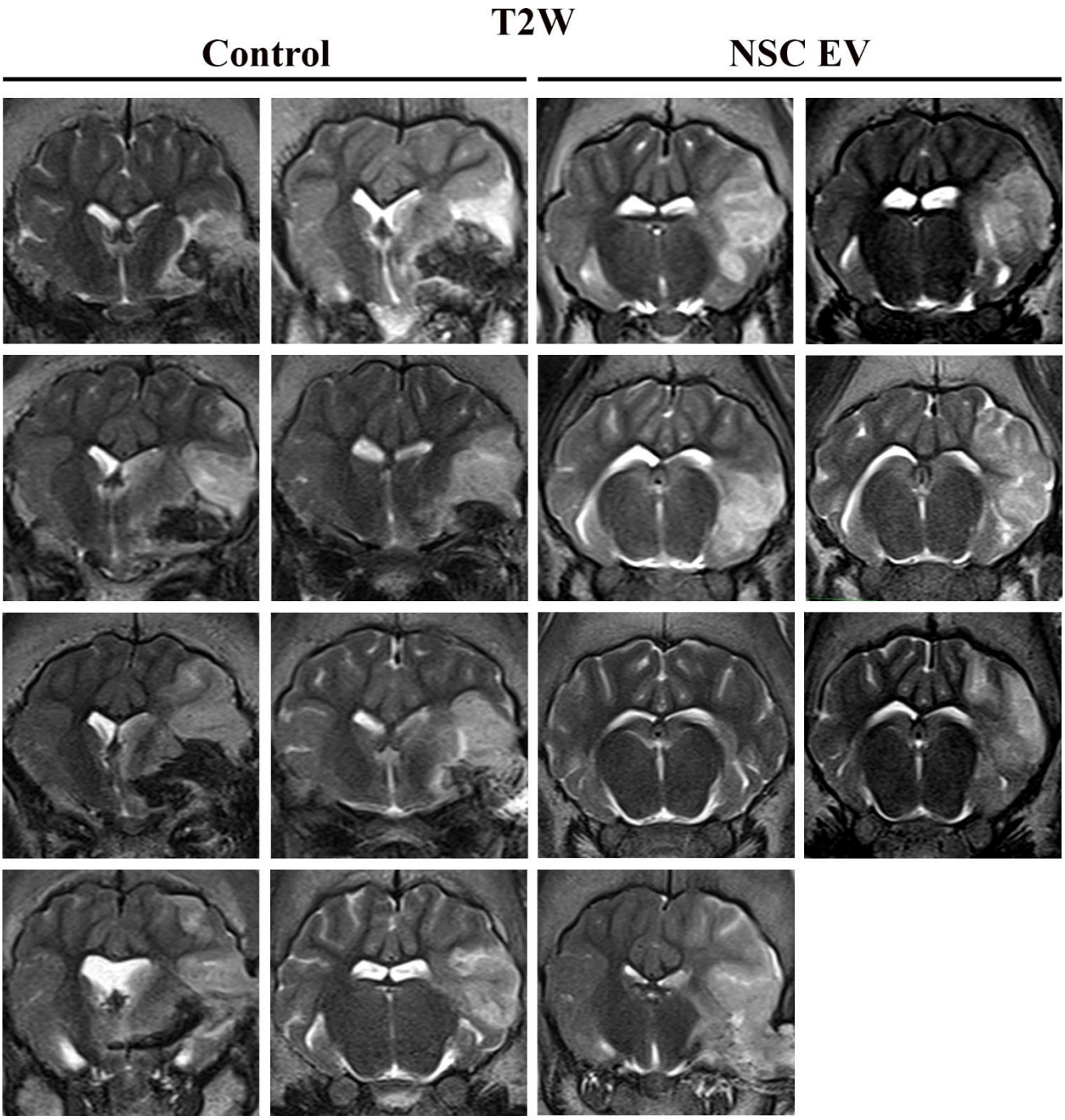
Pig #	**Treatment Group**	**Survival Post-MCAO**	**Cause of Death**
4	Control	1 day	Seizure
5	NSC EV treated	7 days	Non-stroke related post-operative injury; broken leg
6	Control	3 days	Seizure
8	Control	0 days	Non-stroke related post-operative complication
11	NSC EV treated	21 days	Idiopathic; possibly endocarditis
13	Control	2 days	Seizure

Supplement Table III: Checklist of Methodological and Reporting Aspects

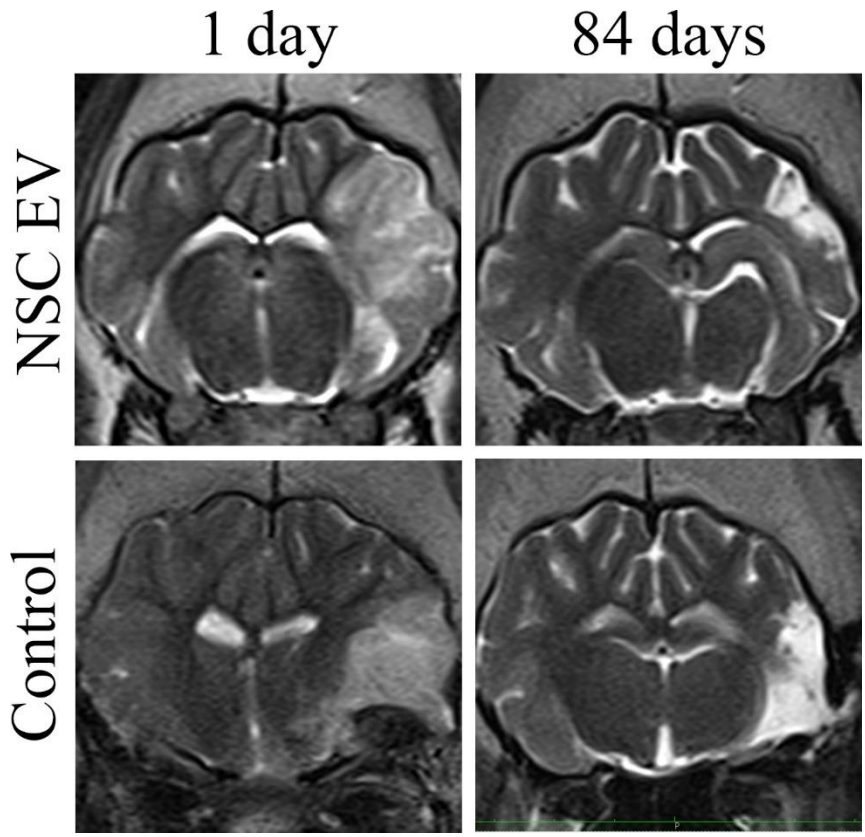
Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals 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.

Supplemental references

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