This is the peer-reviewed accepted version of the following article: [Diekhorst L, Gómez-de Frutos MC, Laso-García F, Otero-Ortega L, Fuentes B, Jolkkonen J, Detante O, Moisan A, Leyva L, Martínez-Arroyo A, Díez-Tejedor E, Gutiérrez-Fernández M; RESSTORE Consortium. **Mesenchymal Stem Cells From Adipose Tissue Do not Improve Functional Recovery After Ischemic Stroke in Hypertensive Rats**. ***Stroke. 2020 Jan;51(1):342-346***. *Epub 2019 Nov 7. PMID: 31694504*], which has been published in final form at [**doi: 10.1161/STROKEAHA.119.027133**]
The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.119.027133.

Visual Overview—An online visual overview is available for this article. (Stroke. 2020; 51:342-346. DOI: 10.1161/STROKEAHA.119.027133.)

Title**: Mesenchymal Stem Cells From Adipose Tissue Do not Improve Functional Recovery After Ischemic Stroke in Hypertensive Rats**

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## Abstract

***Background and Purpose***—Hypertension is the most frequent comorbidity in stroke. The purpose of this study was to evaluate whether hypertension alters the response to treatment with adipose tissue-derived mesenchymal stem cells (ADMSCs) after an ischemic stroke in rats.

***Methods***—Ischemic stroke was induced in male normotensive or hypertensive rats. Either vehicle or $1\times10^6$ ADMSC was intravenously administered at 48 hours poststroke. Functional outcome, lesion size and volume, and markers of brain repair (GFAP [glial fibrillary acidic protein], doublecortin, CD-31, α-smooth muscle actin) were evaluated.

***Results***—Hypertensive rats had larger lesions, higher apparent diffusion coefficients (ADC) and worse functional outcomes than normotensive rats. Hypertension increased GFAP and vascular markers (CD-31 and α-smooth muscle actin). The hypertensive rats treated with ADMSC did not show any significant improvement in functional recovery, lesion size, ADC values, or histological markers compared with those which received the vehicle.

***Conclusions***—ADMSC did not reverse the hypertension-induced increase in lesion severity or functional impairment. Gliosis, neurogenesis, or vascular markers were not affected by ADMSC in hypertensive rats. Hypertension has a negative impact on the therapeutic effect of ADMSC after an ischemic stroke.

**Key Words:** brain; comorbidity; hypertension; neurogenesis; stem cells

## Introduction

Hypertension is considered one of the most common and important vascular risk factors for stroke, affecting >80% of those patients.[1]

Cell therapy is a promising treatment for stroke, and studies have shown that the administration of adipose tissue-derived mesenchymal stem cells (ADMSC) can lead to improved functional recovery and brain repair after a stroke.[2]

Because of the high percentage of patients with comorbidities such as hypertension, stroke research recommendations consider comorbidities to be a key element to be studied to successfully translate the experimental data to the clinic.[3,4]

The aim of this study was to assess the impact of hypertension on stroke in rats. It also evaluated the effect of hypertension on the therapeutic response to the intravenous administration of human ADMSC (hADMSC) in an experimental stroke model.

## Methods

The original data are available from the author for correspondence upon request.

## Ethics Statement

Animal care and experimental procedures were performed in strict compliance with the Guide for the Care and Use of Laboratory Animals, and the study was approved by La Paz University Hospital's Ethics Committee, according to the Spanish and European Union rules (86/609/CEE and RD53/2013). Experiments were conducted according to the stroke therapy academic industry roundtable[3] and ARRIVE (Animal Research: Reporting of In Vivo Experiments)[5] guidelines in terms of randomization, blinding, and statistical power.

## Animals, Hypertension, and Surgery

These experiments were conducted on adult (9–10 weeks old) male spontaneously hypertensive rats (SHR) and normotensive Wistar rats weighing 200 to 250 g. The rats were randomly divided into 5 groups of 10 rats each: (1) sham group: rats subjected to surgery without infarction; (2) vehicle-normotensive group: normotensive rats subjected to a permanent middle cerebral artery occlusion and vehicle; (3) vehicle-hypertensive group: SHR subjected to a permanent middle cerebral artery occlusion and vehicle; (4) hADMSC-normotensive group: normotensive rats subjected to a permanent middle cerebral artery occlusion and hADMSC administration; and (5) hADMSC-hypertensive group: SHR subjected to a permanent middle cerebral artery occlusion and hADMSC administration. Vehicle or $1\times10^6$ hADMSC in 1 mL of 0.9% NaCl was administered via the tail vein 48 hours after surgery. Rats were euthanized for histology at 6 weeks poststroke (Figure 1). See the Methods section in the online-only Data Supplement for more information.

## Results

### Hypertension-Induced Impairments in Motor and Sensory Functions Which Could Not Be Reversed With hADMSC Treatment

See the Results section in the online-only Data Supplement for more information.

The vehicle-hypertensive group exhibited more severe neurological deficits compared with the vehicle-normotensive group as measured by the beam-walking test at 7 days (P=0.031), 3 weeks (P=0.014), and 6 weeks (P=0.001) and by the adhesive-removal test at 7 days (P=0.034). hADMSC resulted in a significant improvement in normotensive animals compared with their vehicle in Rogers test at 3 weeks (P=0.011) and 6 weeks (P=0.0001; Figure 2A and 2B), hADMSC-hypertensive rats did not show an improved functional outcome compared to the vehicle-hypertensive group either in Rogers test (P=0.095), or in the beam-walking test (P=0.356) and the adhesive-removal test (P=0.408) at 6 weeks. hADMSC-normotensive rats showed significant recovery in Rogers test at 6 weeks (P=0.028), in the beam-walking test at 3 (P=0.008) and 6 weeks (P=0.043); and in the adhesive removal test at 7 days (P=0.017) compared with hADMSC hypertensive rats (Figure 2A and 2B).

### Hypertension Increased Brain Damage and hADMSC Treatment Did Not Reduce the Infarct Size

The vehicle-hypertensive rats had larger lesions compared with the vehicle-normotensive rats at 24 hours (P=0.0001) and at 6 weeks (P=0.0001). hADMSC treatment did not decrease lesion size either in hypertensive or in normotensive rats compared with their respective vehicle groups (P=0.156 and P=0.86) after 6 weeks. hADMSC-normotensive

rats had significantly smaller lesions compared with hADMSC-hypertensive rats after 6 weeks (P=0.01; Figure 2C).

After 6 weeks, the vehicle-hypertensive rats had significantly higher diffusion coefficient values compared with the vehicle-normotensive rats (P=0.0001). hADMSC-normotensive and hADMSC-hypertensive rats did not show significant differences in relative apparent diffusion coefficient values compared with their respective vehicle groups (P=0.114 and P=0.211, respectively). hADMSC-normotensive rats showed significantly lower relative apparent diffusion coefficient values compared with hADMSC-hypertensive rats after 6 weeks (P=0.0001; Figure 2C).

After 6 weeks, vehicle-hypertensive rats showed no significant differences in the number of cortical motor neurons compared with vehicle-normotensive rats (P=0.694). The hADMSC increased the number of motor neurons in the normotensive group (P=0.0001) but not in the hypertensive group (P=0.398) compared with their controls. The hADMSC-treated rats in the normotensive group showed higher numbers of motor neurons compared with hADMSC-treated rats in the hypertensive group (P=0.008; Figure 2D).

**Hypertension Increased Astrocyte Marker Levels and hADMSC Treatment Did Not Reverse It**

There was an increase in the GFAP signal in the vehicle hypertensive group compared with vehicle-normotensive rats (P=0.021). Treatment with hADMSC decreased GFAP signal in hADMSC-normotensive rats compared with their vehicle control (P=0.041). We did not observe any changes in the GFAP signal between vehicle- and hADMSC-hypertensive rats (P=0.498). A significantly higher GFAP signal was found in the hADMSC-hypertensive group compared with the hADMSC-normotensive group (P=0.006; Figure 3).

**Hypertension Had No Effect on Neurogenesis, and This Was Not Affected by hADMSC Treatment**

We found no differences in the doublecortin signal between vehicle-hypertensive and vehicle-normotensive animals ($P>0.05$). hADMSC increased the doublecortin signal in normotensive rats ($P=0.005$) but not in hypertensive rats ($P=0.631$). No significant differences were found between the treatment groups ($P=0.839$; Figure 3).

**Hypertension Increased CD-31 and α-Smooth Muscle Actin Signals and hADMSC Had No Effect on These Vascular Proteins**

Increased CD-31 and α-smooth muscle actin (α-SMA) signals were found in the vehicle-hypertensive animals compared with the vehicle-normotensive group ($P=0.0001$).

Although treatment with hADMSC decreased the α-SMA signal in normotensive rats compared with their vehicle control ($P=0.001$), there were no differences in CD-31 and α-SMA signal between the hADMSC- and the vehicle-hypertensive groups ($P=0.56$ and $P=1.00$, respectively). hADMSC-hypertensive rats showed higher CD-31 and α-SMA signals than hADMSC-normotensive animals ($P=0.048$ and $P=0.0001$, respectively; Figure 3).

## Discussion

hADMSC treatment has demonstrated efficacy and safety in the treatment of stroke in animal models.2 The results of our previous studies showed that ADMSC administration increased the levels of brain repair markers associated with improved functional recovery in normotensive stroke animals.[6]

Comorbidities may exert a detrimental impact on treatment efficacy.[7] However, the influence of hypertension on the response to hADMSC treatment has not been thoroughly explored in ischemic stroke.

The present study provides evidence that hADMSC had no beneficial effects on hypertensive rats poststroke. The results showed that hypertension increased the lesion volume and rADC values, which may inhibit behavioral recovery. ADC modifications could be related to the relative increase in water content of the tissue as has been reported in SHR.[8] This worse preservation of the tissue could be partly a reason why hADMSC had no beneficial effects on hypertensive stroke rats.

Our results suggest that hypertensive rats with an ischemic stroke showed increased astrocytic activation. Astrogliosis has been associated with behavioral impairment in the SHR model.[9] Treatment with hADMSC was not able to reverse astrogliosis. The exacerbation of astrogliosis mediated by hypertension may be one of the reasons for the absence of any positive effects from hADMSC treatment in hypertensive rats with ischemic stroke. After stroke, the endogenous brain repair is activated.[10] Our results demonstrated that the therapeutic modulation of neurogenesis can be limited by hypertension. Another possible explanation may be an exhausted neurogenic reserve.[11]

## Conclusions

Our data show that hypertension increased lesion size and behavioral impairment in an animal model of stroke. The administration of hADMSC did not reduce lesion volume or functional deficits and had no effect on gliosis, neurogenesis, or vascular marker levels in hypertensive rats. These results suggest a negative impact of hypertension on the therapeutic effect of hADMSC after an ischemic stroke. Hypertension may be one of the reasons for the unsuccessful translation of experimental stroke therapies to the clinic.

**Acknowledgments**

We appreciate Morote traducciones S.L support for editing assistance.

**Sources of Funding**

This work was sponsored by the REgenerative Stem cell therapy for STroke in Europe (RESSTORE) project (www.resstore.eu) funded by the European Commission under the Horizon2020 program (grant number 681044), Miguel Servet (CP15/00069, María Gutiérrez-Fernández), predoctoral fellowship (FI17/00188, Mari Carmen Gómez-de Frutos), Spanish Research Network on Cerebrovascular Diseases (INVICTUS PLUS-RD16/0019/0005) of the Carlos III Health Institute, Ministry of Science and Innovation of Spain and the European Regional Development Fund (FEDER Funding) and Juan de la Cierva fellowship (IJCI-2017-33505,Laura Otero-Ortega), (http://ec.europa.eu/

esf). Blanca Fuentes: Financial activities outside the submitted work: Novo Nordisk, Bayer, BMS-Pzifer, Novartis, and Daiichi Sankyo.

**Disclosures**

None.

**References**

1. Sare GM, Ali M, Shuaib A, Bath PM; VISTA Collaboration. Relationship between hyperacute blood pressure and outcome after ischemic stroke: data from the VISTA collaboration. Stroke. 2009;40:2098–2103. doi:10.1161/STROKEAHA.108.539155

2. Gutiérrez-Fernández M, Otero-Ortega L, Ramos-Cejudo J, Rodríguez-Frutos B, Fuentes B, Díez-Tejedor E. Adipose tissue-derived mesenchymal stem cells as a strategy to improve recovery after stroke. Expert Opin Biol Ther. 2015;15:873–881. doi: 10.1517/14712598.2015.1040386

3. Development RD. Stroke Therapy Academic Industry Roundtable (STAIR). Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke. 1999;30:2752–2758.

4. Savitz SI, Chopp M, Deans R, Carmichael T, Phinney D, Wechsler L; STEPS Participants. Stem Cell Therapy as an Emerging Paradigm for Stroke (STEPS) II. Stroke. 2011;42:825–829. doi: 10.1161/STROKEAHA.110.601914

5. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG; NC3Rs Reporting Guidelines Working Group. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol. 2010;160:1577–1579. doi: 10.1111/j.1476-5381.2010.00872.x

6. Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Teresa Vallejo-Cremades M, Fuentes B, Cerdán S, et al. Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. Stem Cell Res. Ther.2013;4:11.

7. Cui LL, Golubczyk D, Tolppanen AM, Boltze J, Jolkkonen J. Cell therapy for ischemic stroke: are differences in preclinical and clinical study design responsible for the translational loss of efficacy? Ann Neurol. 2019;86:5–16. doi: 10.1002/ana.25493

8. Ménard B, Chazalviel L, Roussel S, Bernaudin M, Touzani O. Two-kidney one-clip is a pertinent approach to integrate arterial hypertension in animal models of stroke: Serial magnetic resonance imaging studies of brain lesions before and during cerebral ischemia. J Cereb Blood Flow Metab. 2018;38:1769–1780. doi: 10.1177/0271678X17715813

9. Ergul A, Hafez S, Fouda A, Fagan SC. Impact of comorbidities on acute injury and recovery in preclinical stroke research: focus on hypertension and diabetes. Transl Stroke Res. 2016;7:248–260. doi: 10.1007/s12975-016-0464-8

10. Wang F, Tang H, Zhu J, Zhang JH. Transplanting mesenchymal stem cells for treatment of ischemic stroke. Cell Transplant. 2018;27:1825–1834. doi: 10.1177/0963689718795424

11. Diederich K, Schmidt A, Beuker C, Strecker JK, Wagner DC, Boltze J, et al. Granulocyte colony-stimulating factor (G-CSF) treatment in combination with transplantation of bone marrow cells is not superior to G-CSF treatment alone after cortical stroke in spontaneously hypertensive rats. Front Cell Neurosci. 2014;8:411. doi: 10.3389/fncel.2014.00411

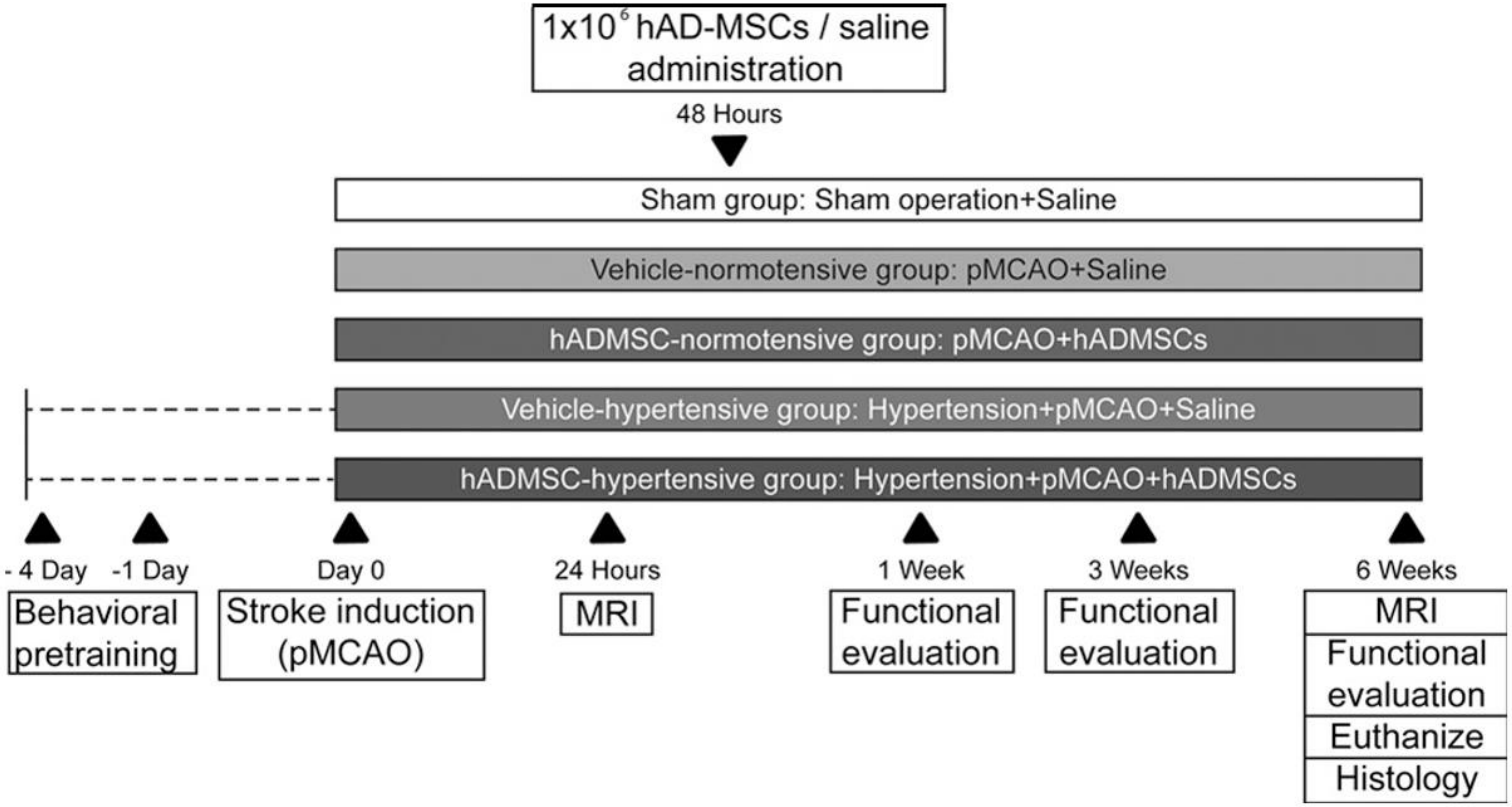


**Figure 1.** Study design. Ischemic stroke was induced by permanent middle cerebral artery occlusion (pMCAO). Human adipose tissue-derived mesenchymal stem cell (hADMSC) or vehicle was administered intravenously 48 h after surgery. Behavioral performance and magnetic resonance imaging (MRI) were evaluated throughout the follow-up. Animals were euthanized after 6 wk.

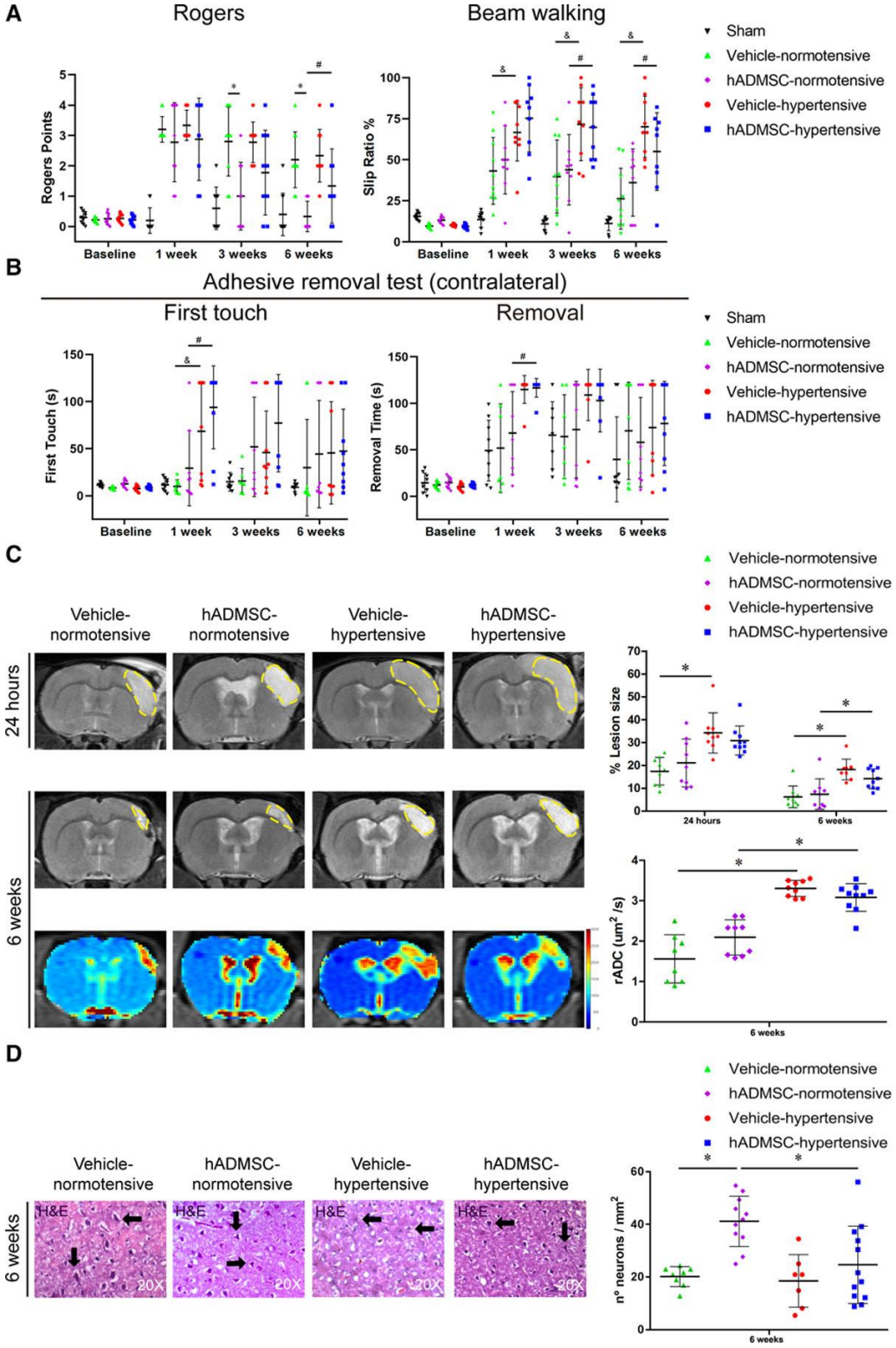


**Figure 2.** Behavioral tests and lesion analysis. **A** and **B**, Functional outcome. Assessment of behavioral outcome in Rogers test (**left**), walking beam (**right**) (**A**), and adhesive-removal test (**B**) at baseline and follow-up. &: vehicle-normotensive vs vehicle-hypertensive; *: vehicle-normotensive vs hADMSC-normotensive; #: hADMSC-normotensive vs hADMSC-hypertensive (n=10 rats per group). **C**, Magnetic resonance imaging (MRI) analysis. Lesion size analysis and relative apparent diffusion coefficient (rADC) measurement at 24 h and 6 wk after treatment (n=10 rats per group). **D**, Histopathologic analysis. Representative images and quantification of the motor neurons in the cortex at 6 wk after treatment (3 rats per group, 4 sections in each rat per group). Data are shown as mean ± SD.*$P$<0.05.

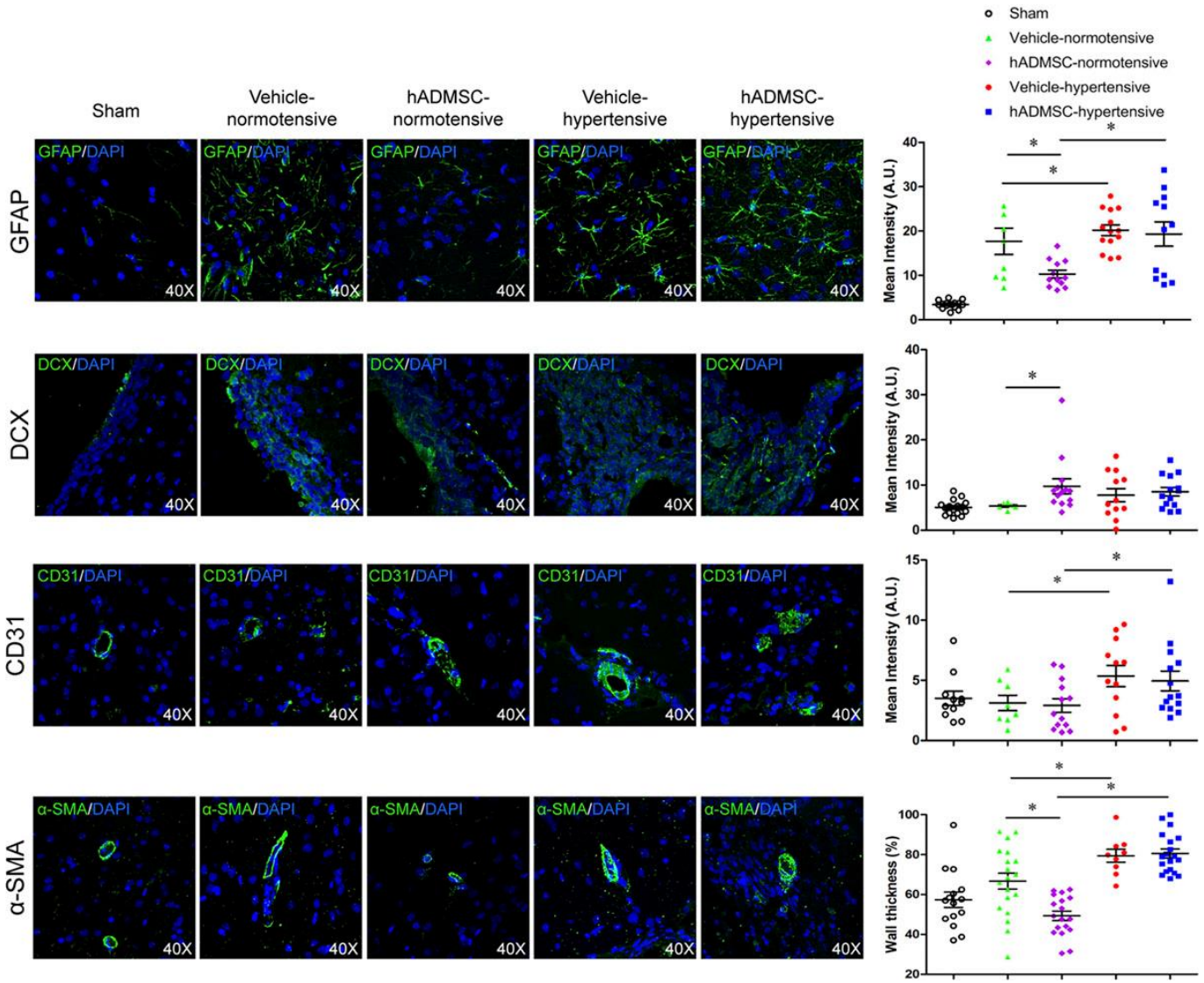


**Figure 3**. Representative images and quantification of GFAP (glial fibrillary acidic protein), doublecortin, CD-31, and α-smooth muscle actin (α-SMA) markers (3 rats per group, 4 sections in each rat per group). Data are shown as mean±SD.*$P$<0.05.

**SUPPLEMENTAL MATERIAL**

**Mesenchymal stem cells from adipose tissue do not improve functional recovery after ischemic stroke in hypertensive rats**

## METHODS

### Cell culture protocol and hADMSC isolation

The hADMSC obtained from the French Blood Establishment (EFS, Grenoble, France) and the Biomedical Research Institute of Malaga (IBIMA, Malaga, Spain) were cultured. The cells were thawed, expanded on tissue culture flasks (Fisher Scientific) with a seeding density of 2.5 x $10^4$ cells/$cm^2$ and maintained in Minimum Essential Media-alpha (1X) (MEM-Alpha, Gibco), supplemented with 5% PLTMax Human Platelet Lysate (Merck) and 1% penicillin/streptomycin with 5% $CO_2$ at 37 ºC. The phenotype pattern of the cells was studied using flow cytometry, the positive expression of CD90, CD73, CD105 and CD44 (≥ 90%), and the lack of expression of CD11b, CD19, CD34, CD45 and HLA-DR (≤ 5%) were detected (Figure I).

Cell viability was studied using 0.4% trypan blue (Trypan Blue solution, Sigma) and a Nikon Inverted Microscope Diaphot-TMD (Japan) with 10x objective lens and a Nikon Phase Contrast-2 ELWD 0.3 Condenser (Japan). When the cells reached > 90% confluence, the hADMSCs were trypsinized (trypsin 0.25%- ethylenediaminetetraacetic acid in Hanks' Balanced Salt Solution [Biowest]) and centrifuged for 10 minutes at 1250 rpm at room temperature. One million cells were re-suspended in 1 ml of saline solution for intravenous administration

### Animals, hypertension and surgery

The experiments were conducted on adult (9-10 weeks old) spontaneously-hypertensive male rats (SHR) and normotensive rats weighing 200–250 g from the Autonomous University of Madrid. The systolic (SBP) and diastolic (DBP) blood pressures of rats from each group were measured on the $7_{th}$ day and then 6 weeks after permanent middle cerebral artery occlusion (pMCAO) using the tail-cuff method.

To induce a cortical ischemic stroke, rats were previously anesthetized intraperitoneally with an injection of a solution of ketamine (25 mg/kg) and diazepam (2 mg/kg) at a dose of 2.5 ml/kg. Analgesia was induced by subcutaneous injection of meloxicam (2 mg/kg). To induce the pMCAO, a small craniotomy was performed; the right MCA was permanently ligated just

before its bifurcation and both common carotid arteries were then occluded for 60 minutes as previously described.[1]

**Functional assessment scales**

Functional assessments were performed on all rats by a blinded observer before surgery and at 1 week, 3 weeks and 6 weeks post-stroke induction. Motor and sensory performance were evaluated using the Rogers, beam-walking and adhesive-removal tests.

A variant of Rogers' functional scale was used to assign scores as follows: 0 = no functional deficit; 1 = failure to fully extend the forepaw; 2 = decreased grip of forelimb while the tail is gently pulled; 3 = spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4 = circling; 5 = walking only when stimulated; 6 = unresponsive to stimulation with a depressed level of consciousness; and 7 = dead.[2]

The beam-walking test evaluated the rats' ability to traverse a wooden beam. We calculated the left hind limb slip ratio as follows: (total slips + 0.5 x half-slips) / total steps x 100%.[3]

For the adhesive-removal test, a sticker was placed on the palm of both of the rat's forelimbs, and contact and removal times were recorded.[4]

***In vivo* magnetic resonance imaging**

Lesion size was analyzed using T2-weighted magnetic resonance imaging (MRI) at 24 hours and at 6 weeks post-stroke using a 7-Tesla horizontal bore magnet (Bruker Pharmascan, Ettlingen, Germany) and a T2-weighted spin echo image. Images were acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial orientations and the following parameters: Number of echo images 2 (TE: 29.54 msec and 88.61 msec), TR = 3000 msec, RARE factor = 4, Av = 3, FOV = 3.5 cm, acquisition matrix = 256 × 256 corresponding to an in-plane resolution of 137 × 137 μm2, slice thickness = 1.00 mm without gap and number of slices = 16. The lesion area was expressed as a percentage of the contralateral hemisphere, after correcting for brain edema. To correct for the brain edema effect, lesion volume was determined by an indirect method: (infarct area) = (area of the intact contralateral hemisphere) - (area of the intact ipsilateral hemisphere) as previously described.[5] Then lesion volume was expressed as a percentage of the intact contralateral hemispheric volume.

Diffusion-weighted imaging (DWI), including apparent diffusion coefficient (ADC) maps, were used at 6 weeks. Images were obtained in three different planes defined by the read, phase, and slice encoding gradients using a multi shot spin-echo echo planar imaging (EPI) sequence.

Acquisition conditions were diffusion gradient duration: 3 msec; diffusion gradient separation: 18 msec; TR: 3000 msec; TE: 50 msec, FOV: 3.8 cm; axial slices (1.5 mm thickness) and 3 b values: 100, 400 and 1000 sec/mm2; acquisition matrix = 128 × 128. To normalize the ADC values, the ROI of the lesion and the same ROI in the contralateral side were divided by the value in the contralateral normal hemisphere and expressed as a relative ADC (rADC) of the region.[6]

**Hematoxylin and Eosin (H&E) staining**

Histopathological changes in the cortex were studied by H&E staining. Slices were immersed for 10 seconds in hematoxylin and then for 1 minute in eosin. They were dehydrated and coverslipped with DePex. The multipolar motor neurons were observed using a 20x objective lens, and processed by image analysis software (Image-Pro Plus 4.1, Media Cybernetics) (3 rats for each group, 4 sections in each rat per group). Cell counts were expressed as individual values and as the mean number of viable neurons/$mm^2$ as previously described.[7]

**Immunofluorescence**

The perilesional area was studied using immunofluorescence for astrocytes with GFAP (1:500, Millipore); neurons with doublecortin (1:250, Santa Cruz); endothelium with platelet endothelial cell adhesion molecule-1 (CD-31) (1:50, Abcam) and alpha-smooth muscle actin (α-SMA) (1:200, Abcam), followed by goat anti-mouse and anti-rabbit Alexa Fluor 488 (1:750, Invitrogen). Immunofluorescence images were acquired as a confocal maximum projection using a Leica TCS-SPE confocal microscope (Leica Microsystems, Heidelberg, Germany), using a 40× objective lens, and analyzed using LAS AF software (Leica). Mean fluorescence intensity was measured by the NIS-Element AR (Nikon) 4.5 Program. The experiments, images and quantification of the samples were performed by blinded observers using the same microscope configurations to eliminate bias due to background normalization.

**Statistical analysis**

The results of the tests were expressed as mean ± standard deviation (SD) and the data were compared using ANOVA adjusted to Bonferroni´s method for normally-distributed data, or the Kruskal-Wallis test followed by the Mann-Whitney U test for data with a non-normal distribution. Values of $p < 0.05$ were considered significant at a 95% confidence interval; the data were calculated using the IBM SPSS 22 statistical program and GraphPad Prism 7 software. The power analysis showed that with non-parametric testing for infarct size and behavioral tests, at least 10 rats needed to be randomly assigned to each group for a significance

level of 5% (alpha) and a power of 80% (1 - beta). Moreover, a post-hoc power analysis was conducted using the software package Pass 11. A sample size of 10 was used for the statistical power analysis. An alpha level of $p < 0.05$, two-tailed was used in this analysis.

The post hoc analyses revealed the statistical power for this study was > 0.8 for all detected effect sizes, showing more than adequate power for all the tests used. The rats removed from the study were replaced by new subjects that were randomly assigned to the experimental groups until a total number of 10 rats per group was reached.

## RESULTS

### Mortality

A total of 81 male rats were used for the study. Thirty one rats were excluded from the study: twenty eight died after surgical induction of pMCAO (nineteen from the vehicle- hypertensive group and nine from the vehicle- normotensive group), one rat died during treatment administration and two were excluded because they did not show lesions on the MRI.

### Effects of hADMSC administration on blood pressure

The SBP and DBP of the hADMSC- hypertensive and vehicle- hypertensive groups were significantly higher than for the vehicle- normotensive group at 7 days and 6 weeks after pMCAO. At 7 days, the SBP/DBP in the vehicle- hypertensive group was 142.5 ± 3.53/110 ± 1.41 mmHg; in the hADMSC-hypertensive group it was 140 ± 2.83/105.5 ± 0.71 mmHg; in the vehicle- normotensive group it was 99 ± 1.41/79.5 ± 0.71 mmHg (hADMSC-hypertensive or vehicle-hypertensive vs. the vehicle- normotensive, $p < 0.01$). At 6 weeks, the SBP/DBP in the vehicle-hypertensive group was 166 ± 2.12/126 ± 1.41 mmHg; in the hADMSC hypertensive group it was 162 ± 1.41/123 ± 1.41 mmHg; in the vehicle- normotensive group it was 102 ± 1.42/80 ± 1.41 mmHg (hADMSC-hypertensive or vehicle- hypertensive vs. the vehicle-normotensive, $p < 0.01$).

## REFERENCES

1. Gutiérrez-Fernández M, Rodríguez-Frutos B, Alvarez-Grech J, Vallejo-Cremades MT, Expósito-Alcaide M, Merino J, et al. Functional recovery after hematic administration of allogenic mesenchymal stem cells in acute ischemic stroke in rats. Neuroscience. 2011;175:394–405.

2. Rogers DA, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. *Stroke*. 1997;28:2060–2066.

3. Schallert T, Woodlee MT, Fleming SM, Schallert T, Woodlee MT FS. Disentangling multiple types of recovery from brain injury recovery of function. *Pharmacol. Cereb. Ischemia.* 2002;201–216.

4. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology.* 2000;39:777–787.

5. Gutiérrez-Fernández M, Rodríguez-Frutos B, Fuentes B, Vallejo-Cremades MT, Alvarez-Grech J, Expósito-Alcaide M, Díez-Tejedor E. CDP-choline treatment induces brain plasticity markers expression in experimental animal stroke. *Neurochem. Int.* 2012;60:310-7.

6. Henriques IL, Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Otero-Ortega L, Navarro Hernanz T, et al. Intralesional patterns of MRI ADC maps predict outcome in experimental stroke. *Cerebrovasc. Dis.* 2015;39:293–301.

7. Gómez-de Frutos MC, Laso-García F, Diekhorst L, Otero-Ortega L, Fuentes B, Jolkkonen J, et al; RESSTORE consortium. Intravenous delivery of adipose tissue-derived mesenchymal stem cells improves brain repair in hyperglycemic stroke rats. *Stem Cell Res. Ther.* 2019;10:212.

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**Figure I:**

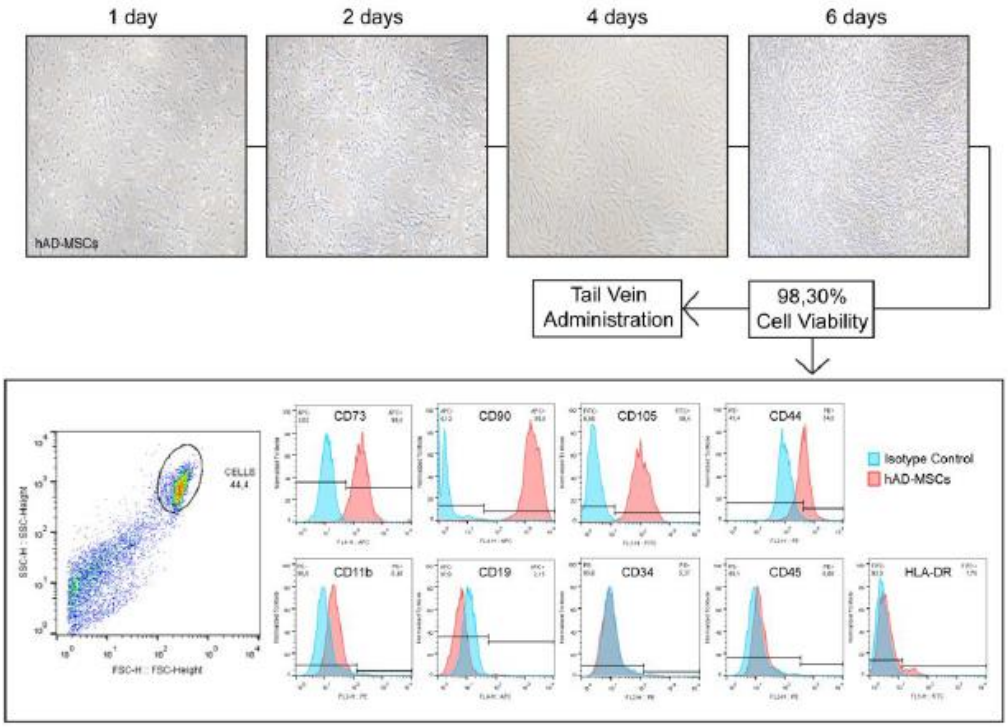


**Figure I. Schematic illustration of the cell culture protocol.** hADMSC were cultured on tissue culture flasks for 6 days and prepared for intravenous administration after a cell viability study. The phenotype pattern of the cells was studied using flow cytometry, the positive expression of CD90, CD73, CD105 and CD44 (≥ 90%), and the lack of expression of CD11b, CD19, CD34, CD45 and HLA-DR (≤ 5%) were detected**.**