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Doctoral Thesis

Allogenic adipose mesenchymal stem cell therapy in ischemic stroke with hypertension and hyperglycemia. Experimental animal models

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Allogenic adipose mesenchymal stem cell therapy in ischemic stroke with hypertension and hyperglycemia. Experimental animal models

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CERTIFICAN:

Que el presente trabajo titulado "**THERAPEUTIC EFFICACY OF ALLOGENIC ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS FOR ENHANCING FUNCTIONAL OUTCOME IN ISCHEMIC STROKE IN AN EXPERIMENTAL ANIMAL MODEL OF HYPERTENSION AND HYPERGLYCEMIA**" ha sido realizado por Don Lucas Alexander Maria Diekhorst bajo su dirección y se encuentra en condiciones de ser leído y defendido como Tesis para alcanzar el grado de Doctor ante el Tribunal correspondiente en la Universidad Autónoma de Madrid.

Madrid,

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Abbreviations

ACA	anterior cerebral artery
ATP	adenosine triphosphate
A.U.	arbitrary units
AD-MSC	adipose-derived mesenchymal stem cell
APC	allophycocyanin
α-SMA	alpha smooth muscle actin
ADC	apparent diffusion coefficient
BBB	blood-brain barrier
BM-MNC	bone marrow-derived mononuclear stem cell
BM-MSC	bone marrow-derived mesenchymal stem cell
BDNF	brain-derived neurotrophic factor
CBF	cerebral blood flow
CD31	cluster of differentiation 31
COL-IV	Collagen-IV
CSD	cortical spreading depression
DAPI	4′6-diamidino-2-phenylindole
DPB	diastolic blood pressure
DWI	diffusion-weighted imaging
DCX	double cortin
EPI	echo-planar imaging

EPC	endothelial progenitor cell
EPO	erythropoietin
EU	European union
FITC	fluorescein isothiocyanate
FOV	field of view
GFAP	glial fibrillary acidic protein
HSC	hematopoietic stem cell
hADMSC	human adipose-derived mesenchymal stem cell
HLA-DR	human leukocyte antigen – DR isotype
IA	intra-arterial
IV	intravenous
IVW	inner vessel wall
IBA-1	ionized calcium binding adaptor molecule 1
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
MAP-2	microtubule-associated protein 2
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
NSPC	neural stem/progenitor cell
NF	neurofilament
NSC	neuronal stem cell
pMCAO	permanent middle cerebral artery occlusion

PBS	phosphate-buffered saline
PE	phycoerythrin
RARE	rapid acquisition with relaxation enhancement
rtPA	recombinant tissue plasminogen activator
ROI	region of interest
rADC	relative apparent diffusion coefficient
SHR	spontaneously hypertensive rat
SD	standard deviation
SC	stem cell
SYP	synaptophysin
SBP	systolic blood pressure
T2-W	T2-weighted
TE	echo time
tMCAO	transient middle cerebral artery
TR	repetition time
TIA	transient ischemic attack
OVW	outer vessel wall
VEGF	vascular endothelial growth factor
VSEL	very small embryonic-like stem cell
WHO	world health organization

Abstract

Introduction

Stroke is one of the leading causes of death and disability worldwide. At present, with the exception of reperfusion therapies with recombinant tissue plasminogen activator (rtPA) or mechanical thrombectomy, there are no other therapeutic interventions to reduce ischemic brain injury and neurological deficits after stroke. However, a large number of patients never receive reperfusion therapies because they are outside the therapeutic window. In this regard, new treatments using stem cells show great promise in preclinical ischemic stroke models and could significantly extend the treatment window. However, successful translation to the clinic is still far away. This is because preclinic stroke models do not reflect clinical reality by using young and healthy animals. In the clinic, the situation is very different, since most patients with cerebral infarction present comorbidity on admission to hospital. Hence, the importance of incorporating comorbidities into experimental animal models of stroke.

Hypothesis

We hypothesize that comorbidities such as hypertension and hyperglycemia will influence ischemic stroke outcome after cerebral infarction, as well as the efficacy of stem cell efficacy. Knowledge of the influence of these comorbidities on stem cell treatment in ischemic stroke could increase chances of successful translation to the clinic and influence future clinical trial design.

Objectives

In an experimental rat model of cerebral infarction by permanent middle cerebral artery occlusion associated with comorbidities, we investigate:

1- The impact of hyperglycemia or hypertension on the functional recovery, lesion size and brain repair processes.
2- The effect of intravenously delivered human adipose-derived mesenchymal stem cell (hADMSCs) in ischemic stroke animals with hyperglycemia or hypertension on functional recovery, lesion size and tissue integrity.
3- Explore the damage/repair markers underlying the therapeutic efficacy of hADMSCs in stroke animals with hyperglycemia or hypertension.

Materials and methods

Hyperglycemia was induced in male rats through an intraperitoneal injection of nicotinamide followed by an injection 15 minutes later of streptozotocin. Nicotinamide was used to protect against the total β-cell destruction produced by streptozotocin. Animals with glucose concentrations above 250 mg/dl were considered hyperglycemic. To mimic hypertension, spontaneous hypertensive rats were used. The rats with comorbidity and their respective controls were then subjected to stroke by permanent middle cerebral artery occlusion. At 48 hours post-stroke, 1.0 × 10^6 hADMSCs or saline were intravenously administered via the tail vein. The behavioral outcome through the walking beam, Roger′s test, and the adhesive dot removal test were evaluated 1 day pre stroke, 1 week, 3 weeks, and 6 weeks after

stroke. Furthermore, the infarct size was measured by magnetic resonance imaging (MRI) 24 hours and 6 weeks after stroke. To assess motor neuron density, an H&E stain was used 6 weeks after stroke. Damage/repair markers involved in gliogenesis [glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule 1 (IBA-1)], neurogenesis [double cortin (DCX)], synaptogenesis [synaptophysin (SYP)] and vascularization [CD31, collagenase (COL-IV) and alpha smooth muscle actin (α-SMA)] were assessed using immunohistochemistry 6 weeks after stroke.

Results

More severe neurological deficits were seen in hypertensive and hyperglycemic rats than their respective normotensive and normoglycemic controls. Motor function was negatively affected by both comorbidities in the beam walking test at 1-, 3-, and 6-weeks post-stroke. However, sensory perception measured by the adhesive dot removal test and hemiplegia by Roger′s test was not significantly affected by both comorbidities. Both comorbidities increased lesion size and diffusion coefficient after stroke compared to the controls. Furthermore, GFAP, IBA-1 and α-SMA were elevated in hyperglycemic rats compared to non-hyperglycemic rats. While in hypertensive rats, GFAP, CD-31 and α-SMA were elevated compared to non-hypertensive rats.

Treatment with hADMSCs in hyperglycemic animals resulted in increased motor function in the walking beam test but not in hypertensive animals at 1, 3, and 6 weeks after stroke. Sensory perception is not influenced in the adhesive removal test in hyperglycemic or hypertensive animals. However, hADMSC treatment does significantly improve Roger's test results of hyperglycemic animals at 3 and 6 weeks

post-stroke but not in hypertensive animals. No significant differences between treated and nontreated hyperglycemic and hypertensive animals were seen regarding lesion size. However, rADC values in treated hyperglycemic animals improved. Treated hyperglycemic animals also had a significantly higher density of motor neurons compared to their control. No difference was seen between treated and untreated hypertensive animals regarding motor neuron density. Furthermore, GFAP, IBA-1 and α-SMA levels were significantly lower in treated hyperglycemic rats compared to nontreated hyperglycemic rats. While between the treated and nontreated hypertensive animals, no significant differences were detected in the tested damage/repair markers (GFAP, DCX, CD31 and α-SMA).

Conclusions

In an experimental rat model of cerebral infarction by permanent middle cerebral artery occlusion associated with comorbidities, we observed that:

1- Hyperglycemia and hypertension contribute to impaired functional recovery, an increased lesion size, and impaired brain repair processes after ischemic stroke.

2- hADMSC therapy:

- in hyperglycemic rats, improves functional recovery, does not reduce lesion volume but improves anatomical tissue preservation.
- in hypertensive rats, does not reduce functional deficits and does not reduce lesion volume or improves anatomical tissue preservation.

3- hADMSC therapy:

- in hyperglycemic rats, reduces the expression of markers implicated in the inflammatory response (GFAP, Iba-1). Markers of neurogenesis (DCX) and synaptogenesis (SYP) were not affected. Furthermore, vascular markers (CD31, COL-IV) did not improve. However, vessel wall thickness significantly reduced (α-SMA).
- in hypertensive rats, had no effect on inflammatory markers (GFAP); nor on neurogenesis (DCX), nor on vascular markers (CD31 and α-SMA).

Resumen

Introducción

El ictus es una de las principales causas de muerte y discapacidad en todo el mundo. En la actualidad, con la excepción de las terapias de reperfusión con activador de plasminógeno tisular recombinante (rtPA) o trombectomia mecánica, no existen otras intervenciones terapéuticas para reducir la lesión cerebral isquémica y los déficit neurológicos tras infarto cerebral. Sin embargo, un gran número de pacientes nunca reciben terapias de reperfusión al encontrarse fuera de la ventana terapéutica. En este sentido, los nuevos tratamientos con células madre parecen muy prometedores en modelos preclínicos de ictus isquémico y podrían ampliar s la ventana terapéutica. Sin embargo, el éxito de la traslación a la clínica aún está lejos. Esto es debido, a que los modelos preclínicos de ictus no reflejan la realidad clínica al usar animales jóvenes y sanos. En la clínica, la situación es muy diferente, ya que la mayoría de los pacientes con infarto cerebral presentan comorbilidad al ingreso en el hospital. De ahí, la importancia de incorporar las comorbilidades a los modelos animales experimentales de ictus.

Hipótesis

Nuestra hipótesis es que las comorbilidades, como la hipertensión y la hiperglucemia, influyen en el resultado funcional tras infarto cerebral, así como en la eficacia del tratamiento con células madre. Conocer la influencia de estas comorbilidades en el tratamiento con células madre en el ictus isquémico podría

aumentar las posibilidades de éxito en la traslación a la clínica e influir en el diseño de futuros ensayos clínicos.

Objetivos

En un modelo animal experimental en rata por oclusión permanente de la arteria cerebral media asociado a comorbilidades, investigamos:

1- El impacto de la hiperglucemia o la hipertensión en la recuperación funcional, tamaño de lesión y procesos de reparación cerebral.

2- El efecto de la administración intravenosa de células madre mesenquimales humanas derivadas de tejido adiposo (hADMSCs) en animales con infarto cerebral e hiperglucemia o hipertensión sobre la recuperación funcional, tamaño de lesión y la integridad del tejido.

3- Explorar los marcadores de daño/reparación subyacentes a la eficacia terapéutica de las hADMSCs en animales con infarto cerebral e hiperglucemia o hipertensión.

Materiales y métodos

La hiperglucemia fue inducida en ratas macho a través de una inyección intraperitoneal de nicotinamida seguida de una inyección, 15 minutos más tarde de estreptozotocina. Se utilizó nicotinamida para proteger contra la destrucción total de células β producida por la estreptozotocina. Los animales con concentraciones de glucosa superiores a 250 mg/dl se consideraron hiperglucémicos. Para simular la hipertensión, se utilizaron ratas macho espontáneamentehipertensas.. Las ratas con

comorbilidad y sus respectivos controles fueron sometidas a un infarto cerebral mediante la oclusión permanente de la arteria cerebral media. 48 horas tras el ictus, se administraron por vía intravenosa 1.0 × 10^6 hADMSCs o suero salino a través de la vena de la cola. El comportamiento funcional se evaluó mediante el test de la barra, el test de Roger's y el test de retirada del adhesivo, 1 día antes del ictus, 1 semana, 3 semanas y 6 semanas tras el infarto cerebral. Además, se midió el tamaño de lesión por Resonancia Magnética (RM) a las 24 horas y 6 semanas tras el infarto cerebral. Para evaluar la densidad de las neuronas motoras, se utilizó la tinción de H&E a las 6 semanas después del infarto cerebral. Los marcadores de daño/reparación implicados en la gliogénesis [proteína ácida fibrilar glial (GFAP), molécula adaptadora de unión de calcio ionizado 1 (IBA-1)], neurogénesis [doblecortina (DCX)], sinaptogénesis [sinaptofisina (SYP)] y vascularización [CD31, colagenasa (COL-IV) y alfa actina de músculo liso (α-SMA)] fueron evaluadas mediante inmunohistoquímica 6 semanas después del infarto cerebral.

Resultados

Se observaron déficits neurológicos más graves en las ratas hipertensas e hiperglucémicas que en sus respectivos controles normotersos y normoglucémicos. La función motora se vio afectada negativamente por ambas comorbilidades, en el test de la barra en la semana 1, 3 y 6 tras el infarto cerebral. Sin embargo, la percepción sensorial medida por el test de retirada del adhesivo y la hemiparesia por el test de Roger's, no fueron significativamente afectadas por las comorbilidades. Ambas comorbilidades incrementaron el tamaño de lesión y el coeficiente de difusión

después del infarto cerebral comparado a los controles. Además, los niveles de expresión de GFAP, IBA-1 y α-SMA estaban elevados en las ratas hiperglucémicas con respecto a las no hiperglucémicas. Mientras que, en las ratas hipertensas, los niveles de expresión de GFAP, CD-31 y α-SMA estaban elevados con respecto a las ratas no hipertensas.

El tratamiento con hADMSCs en los animales hiperglucémicos mostró mejoría en la función motora en el test de la barra, pero no en los animales hipertensos tras 1, 3 y 6 semanas del ictus. La percepción sensorial no estaba afectada en el test de retirada del adhesivo en los animales hiperglucémicos o hipertensos. Sin embargo, el tratamiento con hADMSC mejoró significativamente los resultados en el test de Roger's de los animales hiperglucémicos a las 3 y 6 semanas tras el infarto cerebral, pero no en los animales hipertensos. No se encontraron diferencias significativas entre los animales hiperglucémicos e hipertensos tratados y no tratados con respecto al tamaño de lesión. Pero si se observó una mejoría, en los valores rADC en los animales hiperglucémicos tratados. Los animales hiperglucémicos tratados también tenían una densidad de motoneuronas significativamente mayor que sus controles. No se encontraron diferencias entre los animales hipertensos tratados y no tratados con respecto a la densidad de las neuronas motoras. Además, los niveles de expresión de GFAP, IBA-1 y α-SMA fueron significativamente menores en las ratas hiperglucémicas tratadas comparado con las ratas hiperglucémicas no tratadas. Mientras que entre los animales hipertensos tratados y no tratados, no se detectaron diferencias significativas en los marcadores de daño/reparación estudiados (GFAP, DCX, CD31 y α-SMA).

Conclusiones

En un modelo animal experimental en rata por oclusión permanente de la arteria cerebral media asociado a comorbilidades, observamos que:

1- La hiperglucemia y la hipertensión contribuyen a una peor recuperación funcional, un aumento del tamaño de lesión y a un deterioro de los procesos de reparación cerebral tras un ictus isquémico.

2- La terapia con hADMSC:

- en ratas hiperglucémicas, mejora la recuperación funcional, no reduce el tamaño de lesión, sin embargo,mejora la preservación del tejido.
- en ratas hipertensas, no reduce los deficit funcionales, el tamaño de lesión, ni mejora la preservación del tejido.

3- La terapia con hADMSC:

- en ratas hiperglucémicas, reduce la expresión de marcadores implicados en la respuesta inflamatoria (GFAP, Iba-1). Los marcadores de neurogenesis (DCX) y sinaptogénesis (SYP) no se vieron afectados. Además, los marcadores vasculares (CD31, COL-IV) no mejoraron. Sin embargo, se redujo significativamente el grosor de la pared de los vasos (α-SMA).

- en ratas hipertensas, no tuvo efecto sobre los marcadores inflamatorios (GFAP); ni de neurogénesis (DCX), ni sobre los marcadores vasculares (CD31 y α-SMA).

Introduction

Stroke is one of the leading causes of death and disability in Europe. Cerebrovascular stroke is responsible for over 1 million deaths per year in Europe, and up to one-third of patients lose their independence. (1) Currently, there are no treatment options available after the first few hours post-stroke. (2,3) Stroke occurs when one or more cerebral blood vessels are affected by a pathological process where an area of the brain is permanently or temporarily altered by ischemia or hemorrhage. (4,5) However, ischemic or hemorrhagic stroke does not occur with the same incidence in stroke patients. Ischemic stroke entails 85% of the cases, while hemorrhage only accounts for 15%. (6,7) Ischemic stroke can have several causes that all lead to the same effect. The cerebral blood flow (CBF) is reduced because of the blockage of one or more cerebral blood vessels. (8) Depending on the obstruction´s severity, this could lead to ischemia in the tissues surrounding the blocked vessel(s). (9) As a consequence, neurological deficits and necrosis start to occur if the blood flow is not restored. (10) The longer the blood flow is blocked, the more severe the deficits are and could eventually lead to permanent damage. When the blood flow spontaneously restores, this is called a transient ischemic attack (TIA). (11) The severity of the stroke will depend on the duration of the interruption of blood flow, the affected artery, and the area of the affected tissue.

Epidemiology

Stroke incidence and prevalence are high and account for up to 10% of total deaths globally. They are among the main causes of disability. It is the second cause of death in Europe, with an estimated incidence of 95 to 290 per 100.000 inhabitants per year. (12) In over 90% of cases, patients have a disability; in 26% of the cases, they cannot carry out their usual activities and become dependent. (13) One-month fatalities are between 9% and 19% in Europe for ischemic stroke. (12) However, with the rise of European citizens´ average age, the expectation is that stroke patients will consume a larger part of the overall health budget. Over 3 to 4% of total health care costs in western countries are needed for the treatment of stroke. Resulting in a cost in Europe combined of €26.6 billion in 2010. (13) The majority of the costs are made in the first year post-stroke by inpatient hospital costs and almost double in severe strokes compared to mild strokes. (13) With projections from 1.1 million stroke cases in Europe in 2000 to 1.5 million stroke cases each year in 2025. (12) Furthermore, draining many resources from hospitals and the health system in general.

Risk factors

There is a long list of risk factors that increase the likelihood of suffering from a stroke. These risk factors can be classified into modifiable and non-modifiable risk factors. For example, modifiable risk factors, such as smoking or an unhealthy diet, increase the risk of stroke; however, these risk factors can be influenced. Research indicates that changing these risk factors with help from the health system is much

cheaper than taking care of stroke patients. Non-modifiable risk factors like age, however, cannot be changed. Although there is an extensive list of risk factors that increases the chance of stroke, only 10 risk factors cause over 90% of stroke cases. Narrowing this down shows that only 5 risk factors are responsible for over 80% of stroke cases. Namely, hypertension, current smoking, abdominal obesity, diet, and physical activity. (14)

Stroke interventions

After occlusion of a vessel, brain cells only have a limited time until oxygen deprivation causes irreversible damage. Therefore, time is of the essence in any therapy applied. Acute reperfusion therapy is focused on restoring blood flow. This can be done by the administration of thrombolytic drugs and the use of mechanical thrombectomy.

Thrombolytic drugs

Intravenous (IV) recombinant tissue plasminogen activator (rtPA) is widely used to dissolve clots after ischemic stroke. IV rtPA can only be used in the first 4.5 hours after stroke onset. Originally the recommended time was 3 hours, but recent studies showed that patients still benefited from treatment until 4.5 hours after stroke onset. (15)

However, several contraindications exclude patients from receiving rtPA. (16,17) Due to the extensive list of exclusion criteria, only around 20%, that arrive within the timeframe, of stroke patients receive rtPA after stroke. (16) rtPA is currently the only

available approved anti thrombolytic drug on the market. However, other possibly more potent drugs are being developed.

Mechanical thrombectomy

Several devices on the market are approved for mechanical thrombectomy. They all aim to successfully recanalize proximal arterial occlusions with minimal complications. (18) Moreover, the patients still benefit from treatment until 6–24 hours. (15) Mechanical thrombectomy can be used after the administration of rtPA and improves functional recovery. (19,20) The revascularization rate after mechanical thrombectomy is between 70 to 80% in eligible patients. (21) A considerable advantage of mechanical thrombectomy is that it can still be used in patients with comorbidities that preclude treatment with IV thrombolysis. (22)

Pathogenesis of cerebral infarction

The brain has a vast vascular network and has several unique structures regulating blood flow and providing redundancy, such as the circle of Willis, which can reverse the blood flow in case of a blockage. Furthermore, the cerebral surface is covered by a pial network of vessels, which supplies around 2/3 of the brain. This allows the healthy brain to actively adapt the blood supply both spatially and temporarily to synaptic activity. (23) Ischemic stroke occurs when a blood vessel is blocked either by a thrombotic or embolic clot. (24) After a stroke, the regulation of the blood flow is compromised. Risk factors such as hypertension and diabetes most likely contribute to inadequate perfusion of surrounding areas of the core infarct. Leading to an increase in lesion size because of insufficient collateral blood flow to support neighboring tissues. (23)

Ischemic stroke occurs after CBF drops below a certain point, which leaves the brain cells without enough oxygen to function and, after a while, induces apoptosis and necrosis. After ischemia, an infarct can be divided into three zones: the infarct core, the penumbra, and the benign oligemia (figure 1). (25,26) The ischemic core is the most affected and has no direct sources of oxygen and glucose. Irreversible damage will occur after only minutes. In contrast to the core infarct, the penumbra is the tissue surrounding the core infarct, which is potentially salvable. A major limiting factor is time. The longer it takes to restore blood flow, the more penumbra turns into an ischemic core. Benign oligemia usually surrounds the penumbra and its tissue receives

sufficient oxygen and glucose from peripheral blood vessels leading to spontaneous recovery. The absolute values of blood flow thresholds for humans are difficult to determine. An estimation of blood flow lower than 10mg/100g/min of tissue will result in ischemic core, and between 10 and 17 ml/100g/min is associated with the penumbra. Benign oligemia has a blood flow of higher than 17 ml/100g/min. However, this data is primarily based on animal experiments. (26,27) A systematic review estimated a cerebral blood flow in patients between 14.1 and 35 ml/100g/min in the penumbra and from 4.8 to 8.4 ml/100g/min in the ischemic core. (26)

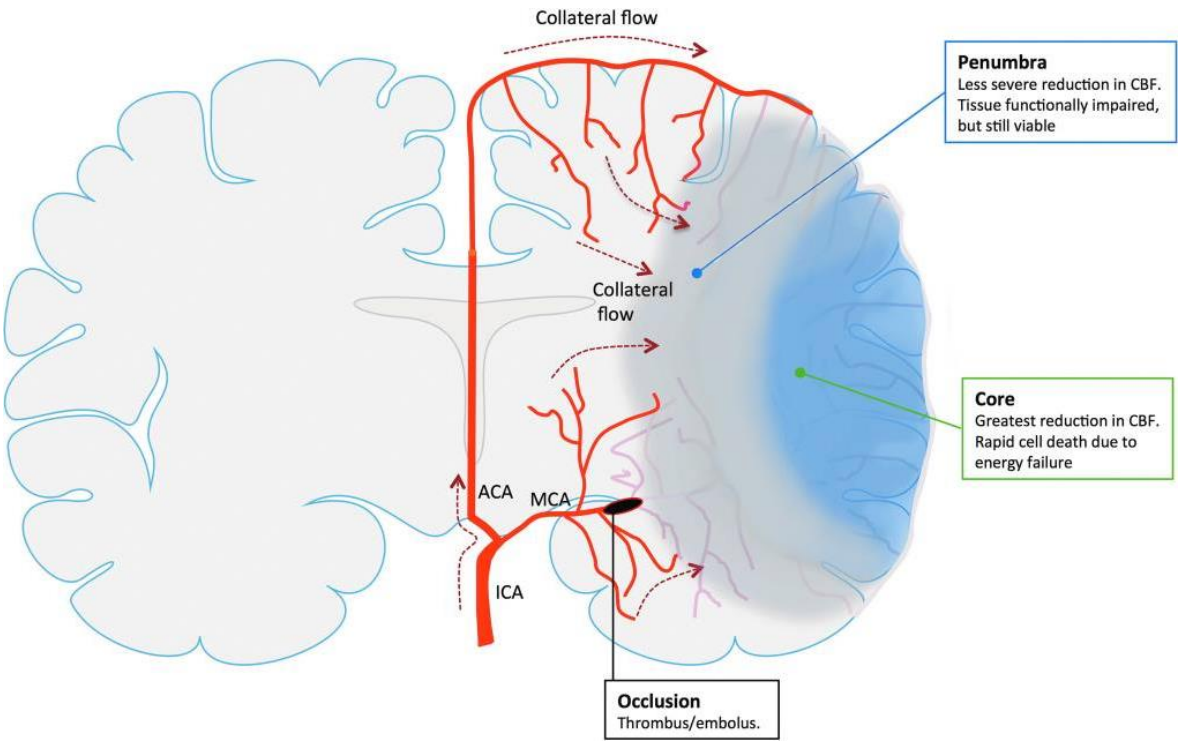


Figure 1, Obstruction of the middle cerebral artery (MCA) will block the cerebral blood flow and deprive brain tissue of oxygen and glucose. Irreversible damage will take place after minutes. The penumbra is surrounding the core tissue and receives limited oxygen and glucose from the collateral flow. For example, as illustrated here, the anterior cerebral artery (ACA) can supply collateral flow to the penumbra. This tissue is salvageable and is currently a target of interest for stem cell therapy. Modified figure from K. Jackman and C. Ladecola, 2015. (23)

If the time of impaired CBF lasts for too long, a cascade of pathophysiological changes occurs in the affected tissue (Figure 2). Ischemia affects all cells, and adenosine triphosphate (ATP) levels are depleted because of insufficient synthesis. This leads to neurotransmitter release and losing the capacity for reuptake. (28) Glutamate (a main excitotoxic neurotransmitter) is especially secreted, leading to a major influx of calcium into the cell. (29) This calcium influx leads to the degradation of essential proteins and membranes inside the neuron. A second consequence is that the glutamate receptors stimulate sodium influx, which leads to cell swelling, shrinking of extracellular space, and edema. Because of the cell´s ionic disruption by calcium influx, another physiological process is activated: cortical spreading depression (CSD). Because of massive calcium and sodium fluxes, neurons start to depolarize, inhibiting normal function. Also, CSD will arise in the peri-infarct zone and spread throughout the penumbra, adding another significant metabolic demand, increasing the infarction volume. (28) Another major factor in acute cerebral ischemia is the immune response. The inflammation response starts moments after vessel occlusion. The vascular endothelium is put under stress by stagnant blood flow, setting in motion a cascade that results in leukocytes´ recruitment. This intravascular inflammation leads eventually to the blood-brain barrier (BBB) breakdown and invasion of the ischemic tissue by leucocytes. (30) Under physiological conditions, the BBB functions as a gatekeeper to supply essential nutrients to the brain and eliminate waste materials. By carefully controlling the cerebral spinal fluid´s ion concentrations, an optimum medium for brain cells is created.

Above mentioned processes occur irreversibly in the ischemic core and will lead to necrotic and apoptotic cell death. The ischemic penumbra is possibly recoverable since, although blood supply is impaired, it still receives some oxygen and nutrients from peripheral vascularization. However, restoration of the blood flow is critical and, therefore, the target of many therapeutical interventions. (31) Furthermore, brain protection and brain repair play vital roles in protecting and recovering affected brain tissue. Currently, no therapies exist that addresses these issues in the clinic. Preclinical and clinical studies have identified a possible candidate for addressing this issue. Stem cells can stimulate endogenous mechanisms in the brain to promote brain plasticity. (32)

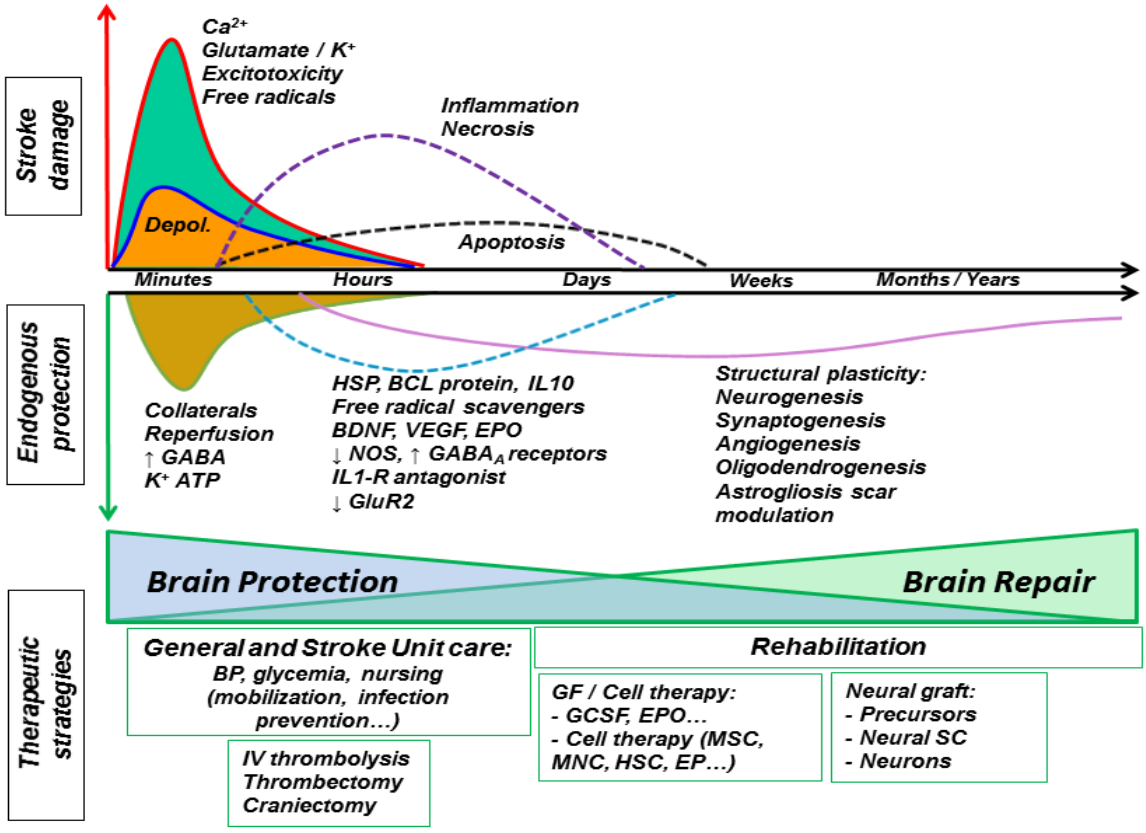


Figure 2, Schematic representation of the pathogenesis of ischemic stroke. The release of damage factors after the stroke sets in motion the activation of pathogenic mechanisms such as inflammation and apoptosis, which in turn activate protection and repair mechanisms (anti-excitation, anti-inflammatory, anti-apoptotic) from the first minutes to weeks after cerebral infarction to prevent the progression of the damage and repair the injury. Modified figure from M. Gutiérrez et al. 2009 (33) and O. Detante et al. 2014 (34)

Brain protection & recovery

For a long time, it has been known that stem cells can differentiate into functional astrocytes, oligodendrocytes, and neurons. (35–39) Two different modes of action have been proposed in how stem cell therapy benefits ischemic stroke recovery. (40) It was once thought that the administered stem cells would home in on the injured site and replace the dead tissue. (41,42) However, recent evidence has shown that it is more likely that the administered stem cells actually work indirectly by section of

trophic factors that activate endogenous repair mechanisms. (40,43) These trophic factors might enhance endogenous repair mechanisms and brain protection. Effects on angiogenesis, neurogenesis, synaptogenesis, white matter remodeling, and immunomodulation have been demonstrated.

- Angiogenesis

Not only should the neuronal circuitry be restored, but supporting tissues such as the vascular network are vital for recovery. Natural-occurring angiogenesis increases after stroke, increasing the vascular volume from 3% to 6%, 90 days after stroke. (44) Angiogenesis is even further promoted after the transplantation of stem cells into the penumbra. Most likely, this is caused by the secretion of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). (45)

- Neurogenesis

Intravenously administrated stem cells can stimulate endogenous neurogenesis. Mesenchymal stem cells (MSCs) after transplantation stimulate neurogenesis by BDNF secretion. (46) Higher levels of endogenous cell proliferation in the subventricular zone were observed, and increased migration of neuronal progenitor cells to the penumbra. Migrated neuronal progenitor cells differentiated into mature neurons and decreased apoptosis. (47)

- Synaptogenesis and white matter remodeling

After a stroke, new connections between neurons will need to be formed to compensate and restore neurological function. For this to happen, new synapses will

need to be formed, and remodeling of the white matter has to take place. Experimental animal models have demonstrated that after permanent middle cerebral artery occlusion (pMCAO) and adipose-derived mesenchymal stem cell (AD-MSC) administration, oligodendrogenesis is stimulated, and substantial reorganization of the fiber tracts with newly formed synapses. (48,49)

- Immunomodulation

The inflammatory response after stroke can result in considerable brain damage and inhibition of brain repair. There are several indications that stem cell transplantation can help in abrogating the immune response. Cytokines play a crucial role in leukocyte recruitment, activation and apoptosis. Stem cell therapy is associated with an increase of anti-inflammatory cytokines and a decrease of pro-inflammatory cytokines. Also, the ratio of M1/M2 microglia changes in favor of the more beneficial M2 phenotype. (50)

Trophic factors

Stem cells are an important source for trophic factors that aid tissue regeneration and brain plasticity after injury. Self-repair processes such as neurogenesis, gliogenesis, synaptogenesis, oligodendrogenesis, and angiogenesis are activated following ischemic stroke. (51) Stem cells naturally express factors such as VEGF and BDNF that are involved in self-repair processes. However, endogenous secreted trophic factors alone are not enough to replace or restore damaged tissue. Transplanted stem cells

can release BDNF and VEGF. Furthermore, MSCs can reduce anti-inflammatory cytokines and, as a consequence, change the immune response. (52)

VEGF has been linked closely to several beneficial effects after stroke. Part of tissue regeneration is the formation of new vasculature. VEGF is a critical player for the activation of angiogenesis. VEGF secreted by MSCs can induce differentiation of endothelial progenitor cells into endothelial cells by paracrine mechanisms. (53) Although the exact processes behind angiogenesis are not yet well understood, decreased VEGF expression in rats inhibits the growth of collateral blood vessels, while increased VEGF expression led to smaller cerebral infarcts. (54) Furthermore, astrocytes and blood vessels nearby transplanted stem cells have increased levels of VEGF. (55) Besides stimulating angiogenesis in the acute phase of stroke, VEGF exerts brain-protective effects by preventing cell death by inhibiting pro-apoptotic genes and reducing brain inflammation. (56,57) Furthermore, VEGF can promote neurogenesis and endogenous migration from the subventricular zone cells in culture. (58)

BNDF is secreted naturally after stroke by brain cells, and levels of circulating BDNF are associated with stroke outcome during the acute phase of stroke. (59) Intravenously administrated MSCs can secrete BDNF. This is of vital importance for the reduction of neurological deficits and reduced brain water content in rats. (60) BDNF plays a vital role in brain protection by inhibiting neurons from going into apoptosis and stimulating endogenous neurogenesis in the dentate gyrus. (61,62) Furthermore, BDNF plays an essential role in synaptic and axonal plasticity. (63)

Cell therapy

The discovery of neuronal stem cells and neurogenesis in the '60s opened up the field for stem cell therapy on neurological disorders. (64) For stem cells to be used after stroke to induce recovery, various steps need to be taken to determine the treatment´s efficacy and safety. This includes a complete characterization of the cells used, several animal models of stroke should be tested, including animals closely related to the stroke demography (age, hypertension, hyperglycemia, diabetes and other comorbidities). Also, safety should be checked in the form of tumor formation, behavioral abnormalities, and adverse physiological alterations. (65)

Stem cell administration: type, route, window and source

With stem cell administration, various factors need to be taken into consideration. The optimal type of cell, administration route, time-window, and the source still have to be determined. (66)

Cellular type

Several types of stem cells have proven efficacy in promoting stroke recovery. However, the use of some types of stem cells raises severe ethical issues. In contrast with embryonic stem cells, adult tissue-derived stem cells do not suffer from these ethical issues and offer other advantages.

Stem cell therapy has proven efficacy in enhancing brain repair after stroke in animal models such as mice and rats. (67) Different types of stem cells can strengthen

brain repair mechanisms. Originally, neuronal stem cells (NSC) were used. Auspicious results have been made using NSCs. NSC grafts in animals induce differentiation into neuroblasts or adult neurons and reduce activated microglia and macrophages. Also, functional recovery improved overtime. (30) However, NSCs have a severe drawback; besides technical difficulties in culturing NSCs, there are also ethical concerns. Since NSCs are mainly harvested from embryonic or fetal tissue. (68)

Other adult-tissue stem cells, such as very small embryonic-like stem cells (VSEL) and endothelial progenitor cells (EPC), have proven efficacy in animal models. Due to their pluripotent capacity, VSELs can differentiate into microglia, oligodendrocytes, and neurons. Their capability to differentiate into key brain cells would make them ideal candidates for stroke therapy. However, there is a limited amount of studies available due to current harvesting protocols resulting in extremely low yields suitable for study. (69,70)

Mesenchymal and hematopoietic stem cells (HSC) are involved in many essential human body regeneration processes. They both form a unique niche in the bone marrow. (71) It was demonstrated that in adults, HSCs could stimulate angiogenesis. (65) When the central nervous system releases chemokines after cerebrovascular insult, HSCs are recruited towards the ischemic injury. (65) Several studies of patient cohorts have demonstrated that HSCs are recruited after ischemic stroke. However, available preclinical research on HSCs with regards to ischemic stroke is minimal. A possible reason for this might be the difficulty of isolation. Furthermore, the efficacy and safety of HSCs are still under investigation.

On the other hand, MSC has a long track record regarding safety and efficacy. They were first described in the late '60s by Friedenstein et al. (30) At first, MSCs were only harvested from bone marrow; however, MSCs can be harvested from various sources such as adipose tissue, the umbilical cord, umbilical cord blood, and dental pulp. (72) In an experimental animal model, it was demonstrated that MSCs derived from bone marrow and adipose tissue increased VEGF levels, synaptophysin (SYP), and neurofilament (NF) after stroke. Also, recovery of the levels of oligodendrocytes was observed. (72) The use of MSCs derived from adipose tissue circumvents several ethical issues since they can be derived from adult tissue generally discarded after a surgical intervention such as liposuction. (73) Another study demonstrated that MSCs derived from adipose tissue outperformed bone marrow MSCs in the secretion of trophic factors, concluding that adipose tissue-derived mesenchymal stem cells (AD-MSC) would be the preferable source of MSCs. (74) Besides, the IV administration of MSCs from human adipose tissue (xenogeneic administration) was shown to be as safe as those from rat adipose tissue (allogeneic administration) in an experimental stroke model, producing no acute adverse effects or evidence of tumor formation during the three-month follow-up. (73)

Administration

After a stroke, the administration route of stem cells is still a heavily debated topic, with no clear consensus. There are currently five methods used in preclinical and clinical research for the administration of stem cells. Each method has distinct advantages and disadvantages. (66)

Initially, intracranial administration was seen as the most promising route. Intracranial administration can be done in several ways, intracerebral, interventricular, or subarachnoid. The original goal of cell therapy was to introduce exogenous stem cells into the ischemic lesion to reconstruct the damaged tissue after stroke. (75) However, only a small percentage of stem cells are functionally incorporated after grafting; this is most likely due to the ischemic zone´s inhospitable milieu. (76) Inflammation, cell death, and glial scarring present a most inhospitable environment for stem cells to survive and differentiate in. Around one-third of surviving stem cells migrate to the ischemic zone. Less invasive grafting methods, such as interventricular or subarachnoid administration, also show the survival of stem cells and migration to the damaged areas. However, a lower percentage of cells arrive at these areas in comparison with intracerebral administration. Another drawback of intracranial grafting of stem cells is the necessity of a craniotomy. This, in turn, increases the risk of complications such as tension pneumocephalus, soft tissue infection, extradural abscesses, and subdural empyema. (45) No direct comparison between different administration methods has been made, so further research is needed to warrant this invasive method of administration.

Intra-arterial administration has demonstrated promising results in experimental animal models. It has several benefits over the intracranial route. It is less invasive, and targeted administration is still possible. Several different types of stem cells have been successfully used using this administration route. (66) Intra-arterial administration of stem cells has significantly higher engraftment levels than

when using intravenous administration. (77) However, several disadvantages have been associated with this method. In some instances, microemboli have been reported, although no direct adverse effects were detected. (66) Embolisms were observed more frequently with an increase in cell dose. Infusion velocity also seems to affect the rate of micro embolisms. (78)

Intravenous administration of stem cells has been widely used in experimental stroke models, and many different sources of stem cells have shown some form of efficacy using this method. (79–81) Intravenous administration of stem cells after stroke is currently the most used clinical trial method (table 1). Although almost no migration of intravenously administered stem cells to the lesion site is detected, the same functional recovery is observed as in other administration routes.(66,82) Therefore, it is not necessary to directly implant stem cells in the injury site since the mode of action is most likely by paracrine mechanisms. Furthermore, of all administration routes, intravenous administration does not have any of the disadvantages intra-arterial administration has. (83)

Alternative methods of administration are possible. However, this has not been sufficiently investigated to be considered a candidate for translational research. Via nasal administration, stem cells can cross from the nose into the brain. (84) Nasal administration of human umbilical cord mesenchymal stem cells 24 hours after ischemic stroke enhanced BBB integrity and improved functional outcome. (85) The application of intraperitoneal administration of stem cells, although possible, has not been actively researched in the area of stroke. It has been hypothesized that

intraperitoneal administration could reduce the risk of embolization. (86) A safety study has been performed in cats, where intraperitoneal injection of autologous MSCs appeared to be a safe treatment approach. (87) Intraperitoneal injection of stem cells is primarily used in neo-natal rats and mice. Moreover, it is the preferred method since the difficulties of IV injections in these young animals. (88) Stem cells can migrate from the abdominal cavity to the ischemic lesion in the brain. (89) However, the number of cells arriving in the brain is significantly lower than when injected IV. (88)

Time-window

The optimal therapeutic window is yet to be determined. Although it seems that stem cell therapy is beneficial to stroke recovery even far into the chronic phase of stroke. (90) It seems that stem cell treatment would give the most benefits when administered in the acute phase of stroke since stem cells would be able to exert protective effects. Stem cell administration has been reported to be effective within the first week after stroke in a stroke animal model. (91) However, stem cell treatment might be effective as early as one hour after stroke. (92)

Source

Not only can the type of stem cell influence stroke recovery, but the source is also important. Allogeneic stem cell administration carries the risk of invoking a strong immune response. (93) From a practical point of view, this would severely limit the use of stem cells in stroke therapy. Stem cells take several weeks to expand after isolation and will not be ready in time for patients to profit from their brain-protective

properties. This is not so in the case of MSCs, which can be derived from a wealth of sources and have low immunogenicity. (93) Several studies have been conducted to determine the effect of allogeneic or even xenogenic MSCs in stroke therapy. The source of AD-MSC does not influence results. Both allogeneic and xenogeneic AD-MSCs have proven to improve several brain repair factors and functional recovery in an experimental animal model. (94,95) This opens up the road for using allogeneic stem cells in human clinical trials. A recent clinical trial evaluated the safety of allogeneic bone marrow-derived mesenchymal stem cells (BM-MSCs) in a single-arm clinical trial. They reported no adverse reactions from the use of allogeneic BM-MSCs after stroke and even suggested behavioral gains. (96)

Table 1, preclinical studies of MSC therapy for ischemic stroke.

Author	**Strain**	**Stroke type**	**Cell type**	**Nº Cells**	**Administration route**	**recovery**
Vahidinia(97)	Wistar	tMCAO	BM-MSC	1 x 10^6	IV	Improved
MU(98)	Sprague-Dawley	pMCAO	AD-MSC	2 x 10^6	IV	Improved
Liu(99)	Sprague-Dawley	tMCAO	BM-MSC	5 x 10^5	IA	Improved
Kho(100)	Sprague-Dawley	Intraluminal suture	Xenogenic placental derived MSCs	1 x 10^6	IV	Not evaluated
Abiko(101)	Sprague-Dawley	tMCAO	Cranial derived MSC and BM-MSC	1 x 10^6	IV	Improved

Otero-Ortega(48)	Sprague-Dawley	pMCAO	AD-MSC	2 x 10^6	IV	Improved
Gutiérrez-Fernández(73)	Sprague-Dawley	pMCAO	AD-MSC	2 x 10^6	IV	Improved
He(102)	Sprague-Dawley	pMCAO	Bone marrow stromal cells	3 x 10^6	IV	Not evaluated
Gutiérrez-Fernández(72)	Sprague-Dawley	pMCAO	BM-MSC or AD-MSC	2 x 10^6	IV	Improved
Moisan(103)	Sprague-Dawley	tMCAO	Human BM-MSCs	3 x 10^6	IV	Improved
Nam(92)	Sprague-Dawley	pMCAO	Human BM-MSCs	2 x 10^6	IV	Improved
Poutheydar(104)	Wistar	Transient CCA suture	BM-MSC	1 x 10^6	IV	Improved
Gutiérrez-Fernández(82)	Sprague-Dawley	pMCAO	BM-MSC	2 x 10^6	IV or IA	Improved
Koh(105)	Sprague-Dawley	tMCAO	Human umbilical cord-derived MSC	6 x 10^5	IV	Improved

tMCAO transient middle cerebral artery occlusion, pMCAO permanent middle cerebral artery occlusion, MSC mesenchymal stem cells, BM-MSC bone marrow-MSC, AD-MSC, adipose tissue derived-MSC, IV intravenous, IA intra-arterial.

Comorbidities

Comorbidities are present in the large majority of stroke patients, yet preclinical research into ischemic stroke does not address this issue (figure 3). Still, the vast majority of ischemic stroke studies are done in healthy animals. To avoid future translational failure of stem cell therapy, STAIR and STEPS guidelines suggest

that further studies should be performed on animals with comorbid conditions to improve the quality of preclinical studies of proposed stroke therapies. (18,106)

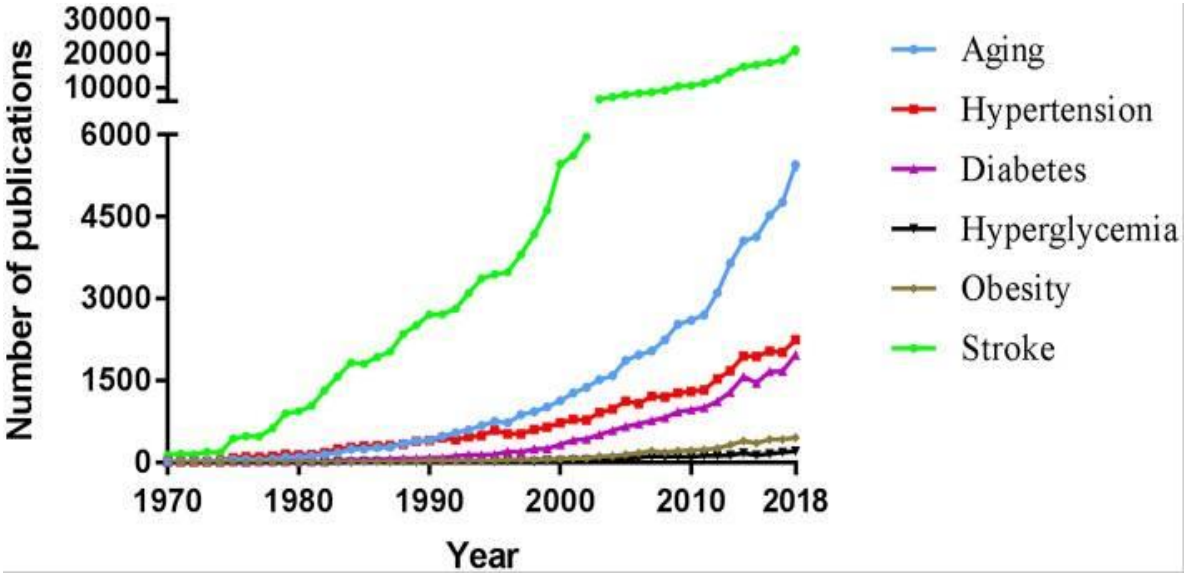


Figure 3 Number of published papers on their respective comorbidity in relationship with stem cell therapy in ischemic stroke. Comorbidities are only mentioned in a fraction of total stroke papers using stem cells for treatment. This figure has been used with permission from the authors Laso et al. (107)

Hypertension

Hypertension is one of the most common vascular risk factors for ischemic stroke and is closely related to stroke severity. Hypertension affects post-stroke edema, BBB integrity, and increases white matter damage. (108) BBB integrity is already compromised during the development of hypertension. In spontaneously hypertensive rats (SHR), BBB impairment is observed after 5 months in the cerebral cortex and deep gray matter if left untreated. Not only does BBB disruption occur in SHRs but also in acute hypertensive models. BBB dysfunction is multifaceted and most likely causes alterations in endothelial cell junctions, dysregulation of ion, and fluid

transport caused by inflammation and oxidative stress. (108) After ischemic stroke, the infarct volume, white matter injury, brain edema, and cognitive deficits are increased with hypertension. The most critical element playing a role is the degradation of the BBB by hypertension. Another major factor contributing to the poor prognosis of stroke with hypertension is the inflammatory response. Leukocyte recruitment results in infiltration into the brain and BBB after stroke. (109)

To test the effect of hypertension on ischemic stroke, several animal models have been developed. Although, in the past, other animals have been used currently, the rat is the commonly used animal in the research of hypertension. There are several methods to induce hypertension in rats, such as stroke-prone spontaneously hypertensive rats, endocrine hypertension by deoxycorticosterone acetate administration, angiotensin II administration, and hypertension induced by stress. However, one of the more popular models is the SHR.

Regarding stroke therapy, MSCs have been rarely used in experimental animal models of hypertension after stroke; mononuclear cells are more often used (table 2). As seen in table 2, bone marrow-derived mononuclear stem cells (BM-MNC) are among the most used stem cells in animal models of ischemic stroke. Intracerebral transplantation of BM-MSCs after cerebral ischemia has demonstrated efficacy by decreasing apoptotic neurons in the neocortex and decreasing overall cell death. Furthermore, it increased the number of microvessels and reactivity to collagen IV. Also, BM-MSC has antioxidant potential and a protective effect in SHR rats after stroke. The transplantation of MSCs increases the density of glial fibrillary acidic

protein (GFAP) positive cells in the penumbra, which might support the regeneration and survival of astrocytes. Which, in turn, leads to a decreased infarct volume 60 days post-stroke and improved functional recovery. (110,111) However, several studies did not detect any effect on stroke outcome after intravenous administration of mononuclear cells. Functional outcome, inflammatory response and infarct volume did not improve independent of the source of the mononuclear cells. (112–115)

Table 2 preclinical studies of MSC therapy for stroke in combination with hypertension

Author	Strain	Stroke type	Comorbidity	Cell type	№ Cells	Administration route	Recovery
Kranz et al. (110)	SHR	MCAO	Hypertension	MSC from placenta	1×10^6	Intravenous	Improved
Minnerup et al.(112)	SHR	MCAO	Hypertension	BMMNC	$1/5/20 \times 10^6$	Intravenous	Did not improve
Weise et al. (113)	SHR	MCAO	Hypertension	HUCBMNC	8×10^6/kg	Intravenous	Did not improve
Pösel et al. (114)	SHR	MCAO	Hypertension	G-CSF + BMMNC	50 µg/kg + 1.5×10^7/kg	Intravenous	Did not improve
Taguchi et al.(111)	SHR	Focal cortical ischemia	Hypertension	BMMNC	5×10^5	Intravenous and intraosseous	Improved
Wagner et al.(115)	SHR	MCAO	Hypertension	BMMNC	8×10^6/kg	Intravenous	Did not improve

SHR spontaneous hypertensive rat, MCAO middle cerebral artery occlusion, MSC mesenchymal stem cells, BMMNC bone marrow-derived mononuclear cells, HUCBMNC human umbilical cord blood mononuclear cells, G-CSF granulocyte colony-stimulating factor.

Hyperglycemia

Hyperglycemia is an important comorbidity in stroke. It has been demonstrated that hyperglycemia can negatively influence BBB function, which results in poor stroke outcomes and increased mortality after stroke. (116–118) Between

8–63% of non-diabetic patients present hyperglycemia at hospital admission, and in diabetic patients, this is between 39 and 83%. (119) The result is increased glucose levels in over 50% of stroke patients. (120) This stresses the importance of hyperglycemic animal models in stroke research. No previous studies have been published that explore the role of this comorbidity and if it affects stem cell treatment after stroke.

Most stroke patients present at the hospital with at least one comorbidity. It is, therefore, clear that the translation from preclinical research to the clinic should include this factor. (121)

Clinical trials

Various sources of stem cells have been used in clinical trials to treat ischemic stroke with various degrees of success (table 3). A meta-analysis of clinical trials of stem cell treatment of ischemic stroke in Asia reported promising results. MSC therapy after ischemic stroke showed improvement in the NIHSS scale at least until 6 months after stroke. Also, no serious adverse events were reported. (122) However, no analysis has been made regarding any differences in MSC treatment effect regarding comorbidities. The vast majority of the included studies used BM-MSCs or BM-MNCs (table 3). Several clinical trials underway assess the safety and efficacy of AD-MSC in ischemic stroke; however, results still have to be published (NCT01678534, NCT04280003). (123,124)

Table 3 Clinical studies using MSC therapy for stroke.

Author	Cases (SC/ control)	Time onset to SC infusion	Cell type	Cells dose	Route of administration	Follow-up
Bang(125)	5/25	32 to 61 days	Autologous BM-MSCs	5×10^7, twice	IV	12 months
Bhatia (126)	10/10	Mean 10 days	Autologous BM-MNCs	Mean 6.1×10^8	IA ipsilateral MCA	6 months
Chen(127)	15/15	6 months to 5 years	Autologous PBSCs	$3–8 \times 10^6$	Stereotaxic implantation	12 months
Díez-Tejedor (123)	10/10	< 14 days	Allogeneic ADMSCs	1×10^6/kg	IV	2 years
Fang(128)	10/6	Mean 33.5 days	Autologous EPSs (50%), autologous BM-MSCs (50%	2.5×10^6/kg, twice	IV	4 years
Hess(129)	67/62	24 to 48 h	Allogeneic multipotent adult progenitor cells	1.2×10^9	IV	12 months
Jin(130)	10/10	3 weeks to 5 months	Autologous BM-MNCs	1×10^7	Subarachnoid infusion	7 years
Lee(131)	16/36	4 to 9 weeks	Autologous BM-MSCs	5×10^7, Twice	IV	5 years
Prasad (132)	60/60	Mean 18.5 days	Autologous BM-MNCs	Mean 2.8×10^8	IV	12 months
Savitz (133)	29/19	13 to 19 days	Autologous bone marrow-derived ALDHbr cells	Mean 3.08×10^6	IA. ipsilateral ICA	12 months
Bhasin (134)	12/12	3 months to 2 years	Autologous BM-MNCs	Mean 5.46×10^7	IV	24 weeks
Bhasin (135)	20/20	3 months to 2 years	Autologous BM-MSCs (30%), autologous BM-MNCs (70%)	Mean 5.54×10^7	IV	24 weeks
Bhasin (136)	10/10	3 months to 1.5 years	Autologous BM-MNCs	Mean 6.28×10^7	IV	8 weeks
Bhasin (137)	6/6	3 months to 2 years	Autologous BM-MSCs	Mean $5–6 \times 10^7$	IV	4 years
Ghali(138)	21/18	12 to 32 days	Autologous BM-MNCs	1×10^6	IA ipsilateral ICA	12 months
Meng(139)	60/60	Mean 21 days	Autologous BM-MSCs	Mean 2.97×10^9	IV	6 months
Moniche (140)	10/10	5 to 9 days	Autologous BM-MNCs	Mean 3.38×10^6	IA ipsilateral MCA	6 months

SC stem cell, MSC mesenchymal stem cells, MNC mononuclear cells, BM-MSC bone marrow derived-MSC, BM-MNC bone marrow-derived-MNC, PBSC peripheral blood stem cell, EPS extended pluripotent stem cell, ALDHbr aldehyde dehydrogenase bright, ADMSC adipose tissue derived-MSC IV intravenous, IA intra-arterial, MCA middle cerebral artery, ICA internal carotid artery.

Hypothesis

Ischemic stroke coincides in most cases with various comorbidities, often also risk factors for cerebral infarction. After a stroke, repair processes are initiated as part of the normal physiological response to brain damage. Trophic and reparative factors may mediate these processes. If and how comorbidities influence these factors is yet unknown. The comorbidities could affect stem cell treatment after stroke. We hypothesize that comorbidities such as hypertension and hyperglycemia will influence ischemic stroke outcome after cerebral infarction, as well as the efficacy of stem cell efficacy. Knowledge of the influence of these comorbidities on stem cell treatment in ischemic stroke could increase chances of successful translation to the clinic and influence future clinical trial design.

Objectives

In an experimental rat model of cerebral infarction by permanent middle cerebral artery occlusion associated with comorbidities, we investigate: the impacts of hyperglycemia and hypertension on the ischemic stroke recovery after intravenously administrated hADMSCs. More specifically, the following factors will be investigated:

1- The impact of hyperglycemia or hypertension on the functional recovery, lesion size and brain repair processes.

2- The effect of intravenously delivered human adipose-derived mesenchymal stem cell (hADMSCs) in ischemic stroke animals with hyperglycemia or hypertension on functional recovery, lesion size and tissue integrity.

3- Explore the damage/repair markers underlying the therapeutic efficacy of hADMSCs in stroke animals with hyperglycemia or hypertension.

Materials and methods

Ethics statement

The procedure was carried out at our Neurological Sciences and Cerebrovascular Research Laboratory, La Paz University Hospital, IdiPAZ Research Institute, Madrid, Spain. All experiments were designed to minimize animal suffering of animals in compliance and approved by our medical school's Ethical Committee for the Care and Use of Animals in Research (Ref. PROEX 249/15) according to the Spanish (RD 1201/2005 and RD53/2013) and European Union (EU) (86/609/CEE, 2003/65/CE, 2010/63/EU) rules. Experiments were conducted according to the ARRIVE guidelines for reporting animal research in terms of randomization, blinding, and statistical power (https://www.nc3rs.org.uk/arrive-guidelines).

Equipment and materials used

All procedures and analyses were done using the following infrastructure and material resources.

The following equipment was used in the **cell culture laboratory**:

- Faster Bio 48 laminar flow hoods (Cultek S.L.U., Madrid, Spain), centrifuge (Heraeus Instruments, Hanau, Germany), Galaxy CO_2 incubators (New Brunswick Scientific, Enfield, CT, USA), thermostatic bath (Raypa, Barcelona, Spain), pipettors (Accu-jet Brand, Wertheim, Germany), suction pumps.
- Inverted optical microscope (Nikon Corporation, Tokyo, Japan). It was used to obtain, isolate and grow AD-MSCs.

The following equipment was used in the **flow cytometry laboratory**:

- FACScalibur Flow Cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and its program CELLquest, for the characterization of MSCs.

The following equipment was used in the **experimental surgery laboratory**:

- Animal facilities: animal rooms, surgery rooms, and storage rooms.
- Leica M300 Surgical Microscope (Leica Microsystems, Heidelberg, Germany).
- Stainless steel macro and microsurgical instruments (Lanbeck, Talexco, Madrid, Spain).
- Thermal blanket (Gaymar, Orchard Park, NY, USA).
- Lifescan mod. one touch II blood glucose monitor with test strips (Johnson & Johnson, New Brunswick, NJ, USA).
- Hero 5 (GoPro, San Mateo, CA, USA).
- Custom made tapered walking beam following instruction from Schallert et al. (141)
- Acrylic cylinder for adhesive dot removal test
- Precision scale model SVA 120 (Gram, Barcelona, Spain).
- 1mm acrylic matrix for rat brain (Stoelting Europe, Dublin, Ireland) and razor blades to cut the fresh brain of the animals.

The following equipment was used in the **immunohistochemistry and molecular biology laboratory**:

- -80ºC freezer Nuaire (Nirco, Madrid, Spain) for sample storage.
- LEICA CM1950 Cryostat (Leica Microsystems Heidelberg, Germany), to cut the frozen brains included in OCT (Tissue-Teck, Sakura Finetek, CA, USA) to a thickness of 10µm.
- Olympus Optical Microscope, BX41 (Olympus Corporation, Tokyo, Japan). It allowed us to observe the histological slice samples.
- Confocal microscope LEICA TCS SPE (Leica Microsystems Heidelberg, Germany) and analysis program LEICA software LAS AF, version 2.0.1 Build 2043, for the immunofluorescence study of brain slices.

Computer equipment and material:

- Software Image J 1.52a (NIH software, Bethesda, MD, USA) and Image-Pro Plus 4.1 (Media Cybernetics, Rockville, MD, USA). It allowed us to obtain an automated measurement of the size of the lesion.
- The software IBM SPSS 22 and Pass 11 were used to record data points and determine statistical connections between groups.

Collaborations

- Finally, the collaboration of the High Field Magnetic Resonance Imaging and Spectroscopy Service (SIERMAC), directed by Dr. Sebastián Cerdán, where the Magnetic Resonance Imaging (MRI) studies were carried out (Bruker Pharmascan, Ettlingen, Germany 7 Tesla horizontal bore magnets) for obtaining the T2 maps and diffusion-weighted imaging.

Cell culture protocol, characterization and hADMSC isolation

The French Blood Establishment (EFS, Grenoble, France) and the Biomedical Research Institute of Málaga (IBIMA, Málaga, Spain) have as part of the RESSTORE consortium supplied the hADMSC that were used in this study. The hADMSC were rapidly thawed and were divided over tissue culture flasks to reach a seeding density of approximately 2.5 10^4 hADMSC/cm^2. The cells were cultured in Minimum Essential Media-alpha (1x) containing 5% PLTMax Human Platelet Lysate and 1% penicillin/streptomycin. The cell cultures were kept constant at 37ºC with an air mixture of 5% CO_2. The hADMSCs phenotype was studied using flow cytometry. The cells were checked for their positive expression of CD44, CD73, CD90 and CD105 (≥ 90%) and negative expression of CD11b, CD19, CD34, CD45 and human leukocyte antigen – DR isotype (HLA-DR) (≤ 5%) to confirm hADMSC status.

Antibodies	**Fluorophore**	**Brand**	**Ref/Cat**
CD11b	FITC	Beckman Coulter	IM0530
CD19	APC	ImmunoStep	19A1-100T

CD34	PE	BD Biosciences	555822
CD44	FITC	Immunotools	21270443X2
CD45	PE	BD Biosciences	555483
CD73	APC	BD Biosciences	560847
CD90	APC	Invitrogen	17-0909-42
CD105	PE	BD Biosciences	560839

To ensure optimal results, cell viability was assessed using 0.4% trypan blue and a Nikon Inverted Microscope using a 10x objective lens and a Phase contrast condenser. The hADMSC were trypsinized when the cells reached > 90% confluence as rated by the observer. After trypsinization, cells were centrifuged for 10 minutes at 1250 rpm at room temperature. Then, 1 million of hADMCs were resuspended in 1 ml of saline for intravenous administration.

Animals

The experiments were conducted using 57 healthy male Sprague-Dawley rats, 42 healthy Wistar rats, and 39 spontaneously hypertensive rats (SHR). All rats weighed between 200 g and 250 g at the start of the experiments and were approximately 9-10 weeks old. During the experiments, the rats were kept in a climate-controlled environment where the temperature was kept constant at 20°C ± 3°C. Rats had ad libitum access to standard rat chow and water. They were kept in groups of 2-4 in transparent cages covered with a metal grid and a separator for the food and water. Cages were enriched using a carton cover large enough for the rats to hide beneath and play with. For the administration of stem cell therapy, 1 million hADMSCs were resuspended in 1 ml of saline. Depending on the group, either 1 ml of the hADMSC suspension was injected by the tail vein or 1 ml of saline as a vehicle.

Rats were divided into a total of 9 groups (Table 1):

1. Sham group: Sprague-Dawley rats were subjected to surgery without inducing a cerebral infarct (n=10).
2. Vehicle normoglycemic group: Sprague-Dawley rats were subjected to pMCAO and were administered with a vehicle (n = 10).
3. Vehicle hyperglycemic group: Sprague-Dawley hyperglycemic rats were subjected to pMCAO and were administered with a vehicle (n = 10).
4. hADMSC-hyperglycemic group: Sprague-Dawley hyperglycemic rats were subjected to pMCAO and hADMSC administration (n = 11).
5. Sham group: Wistar rats were subjected to surgery without inducing a cerebral infarct (n=10).
6. Vehicle-normotensive group: Wistar rats were subjected to a pMCAO and were administered with a vehicle (n= 10).
7. Vehicle-hypertensive group: SHR were subjected to pMCAO and were administered with a vehicle (n=10).
8. hADMSC-normotensive group: Wistar rats were subjected to pMCAO and hADMSC administration (n=10).
9. hADMSC-hypertensive group: SHR were subjected to pMCAO and hADMSC administration (n=10).

Group	**pMCAO**	**treatment**	**comorbidity**	**Strain**
Sham	-	-	-	Sprague -Dawley
vehicle- *normoglycemic*	+	-	-	Sprague -Dawley
vehicle hyperglycemic	+	-	HYPERGLYCEMIA	Sprague -Dawley
hADMSC-hyperglycemic	+	+	HYPERGLYCEMIA	Sprague -Dawley
Sham	-	-	-	Wistar
vehicle-normotensive	+	-	-	Wistar

vehicle-hypertensive	+	-	HYPERTENSION	SHR
hADMSC-normotensive	+	+	-	Wistar
hADMSC-hypertensive	+	+	HYPERTENSION	SHR

Inducing hyperglycemia in rats

When rats reached a weight of 200 - 250 g, they were fixated by hand, and an intraperitoneal injection of nicotinamide (210 mg/kg) (EMD Millipore, Germany) was administered. This was followed, 15 minutes later, by another intraperitoneal injection of streptozotocin (60 mg/kg) (EMD Millipore, Germany). The nicotinamide solution was prepared on the same day as the injection would take place. The nicotinamide was carefully weighed appropriately to the individual animal's body weight and dissolved in saline, after which the solution was vortexed until no discernible particles could be detected in the liquid. The streptozotocin was prepared less than 15 minutes before administration, similar to the nicotinamide; however, 0.5M citrate buffer pH 4.5 was used as a solvent. Glucose levels were determined after 72 hours and again after 6 weeks using a glucose meter (ACCU-CHEK, Performa, Germany). Rats were considered hyperglycemic if they had a blood glucose concentration higher than 250 mg/dl 72 hours after administering the nicotinamide and streptozotocin (figure 4).

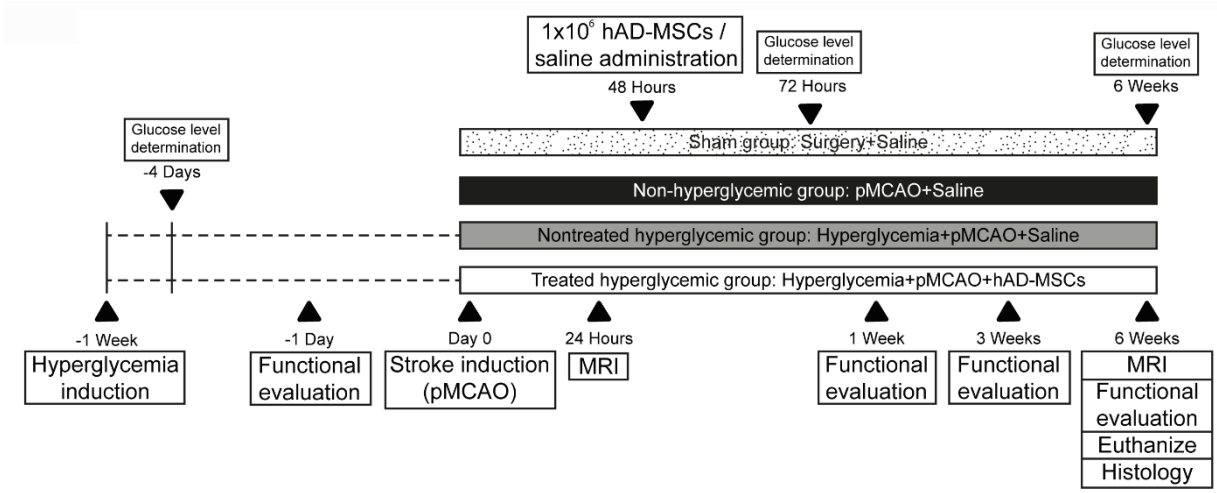


Figure 4 Schematic representation of the study design. 20 rats were injected with nicotinamide and streptozotocin 1 week before stroke induction. All groups received training for the behavioral tasks for 3 days. One day before surgery, the baseline for the behavioral tasks was established. Behavioral tests were repeated at fixed times until the rats were euthanized. Furthermore, MRI scans were obtained 24 hours and 6 weeks post-stroke. At 6 weeks post-stroke, the rats were euthanized, and the brain was processed for histological analyses. Abbreviations: hADMSCs human adipose tissue-derived mesenchymal stem cells, pMCAO permanent middle cerebral artery, MRI magnetic resonance imaging.

Hypertensive rats

The experiments were conducted on SHR and normotensive rats from the Autonomous University of Madrid according to the schedule in figure 5.

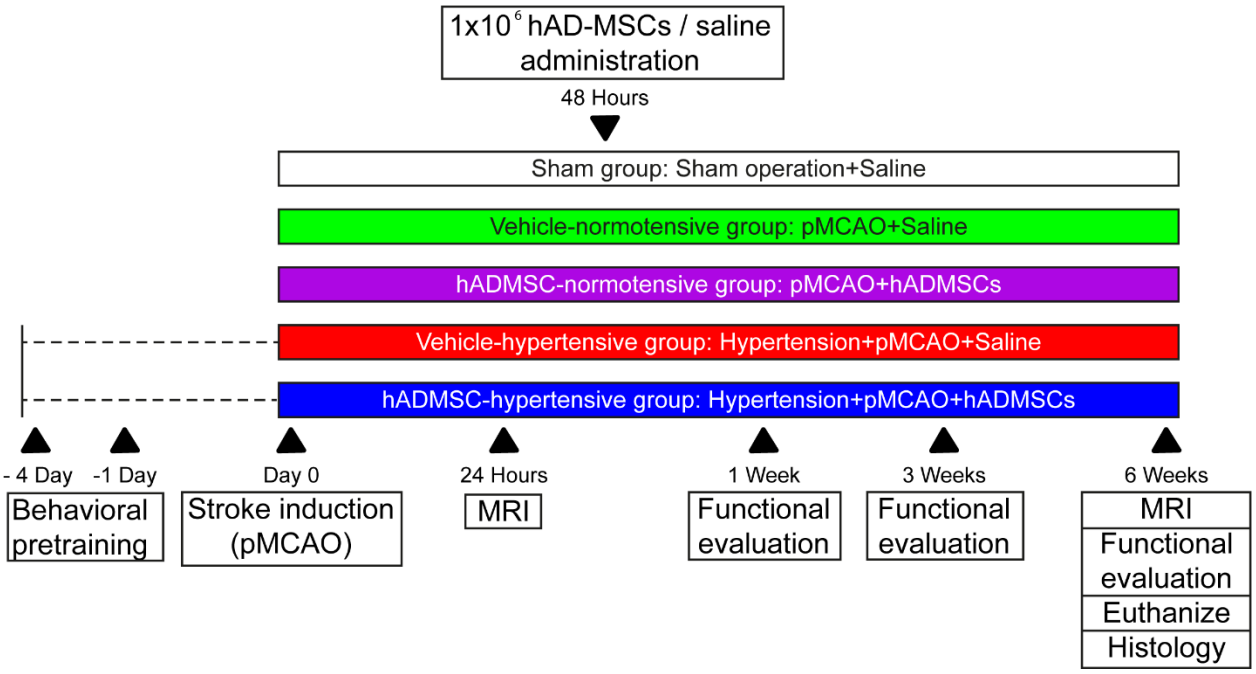


Figure 5 Schematic representation of the study design. All groups received training for the behavioral tasks for 3 days. One day before surgery, the baseline for the behavioral tasks was established. Behavioral tests were repeated at fixed times until the rats were euthanized. Furthermore, MRI scans were obtained 24 hours and 6 weeks post-stroke. At 6 weeks post-stroke, the rats were euthanized, and the brain was processed for histological analyses. Abbreviations: hADMSCs human adipose tissue-derived mesenchymal stem cells, pMCAO permanent middle cerebral artery, MRI magnetic resonance imaging.

Experimental animal model of permanent middle cerebral artery occlusion

Rats were previously anesthetized using an intraperitoneal injection of a solution of ketamine (25 mg/kg) and diazepam (2 mg/kg) at a dose of 2.5 ml/kg. A 1.5 cm scalp incision was made at the midpoint between the right eye and the right ear. The temporalis muscle was separated and lifted to expose the underlying bone. Using microsurgical techniques, a small craniotomy of about 2 mm was made using a drill over the area of the middle cerebral artery (MCA). During the drilling, care was taken to avoid damaging the cortex by either physical or thermal injury. A surgical microscope was used to identify the dura mater and the MCA. The dura mater was carefully pierced and removed. The now exposed MCA was isolated and ligated using

a 10-0 suture just before the bifurcation between the frontal and the parietal branch to achieve a cortical lesion. The temporalis muscle and overlying skin were placed back in their original position and were sutured separately. The common carotid arteries were isolated via a ventral midline cervical incision, and both the common carotid arteries were isolated. A transitory occlusion (60 minutes) of the two common carotid arteries was then performed to eliminate the effect of collateral circulation and reduce variability in infarct size.

Sacrifice of animals and processing of brain

The animals were sacrificed 6 weeks after pMCAO surgery. For this purpose, the animals were anesthetized using sevoflurane (Sevorane®, Abbott Laboratories, Illinois, USA) at 6%, and the heart was stopped by injecting 0.5 ml of KCL directly into the heart. The death of the animal was confirmed by cutting the spinal cord. The skull was then removed using Kelly forceps. The fresh brain was then removed from the skull and placed in an acrylic matrix, and cuts were made at 2 mm anterior of the optic chiasm and the pons. Both posterior and anterior parts were removed, and the remainder was left overnight at 4ºC in 4% paraformaldehyde for fixation of the tissue. After fixation, the brain tissue was left for 72 hours in 30% sucrose before being embedded in OCT (Tissue-Tek, Sakura Finetek, CA, USA) and frozen at -80ºC.

To cut the tissue, the samples were adhered to the cryostat support using OCT and were placed inside the cryostat at -22ºC for 10 minutes until they were completely frozen. The brains were then sectioned into 10 µm thick cuts to be used in various histological studies. The brain sections were placed on glass slides coated with gelatine to facilitate adhesion.

Histological studies

The slides with the samples were put in a metal rack for transport between liquids to perform a Hematoxylin and Eosin stain. The slides were introduced for 5 minutes in a glass cuvette filled with dH_2O. The slides were then transferred into hematoxylin for 30-60 seconds and then quickly washed twice with dH_2O and final dH_2O wash for 2 minutes. The slides were then transferred into the eosin for 4/5 minutes. After this, the slides were washed again with dH_2O and put in 70% ethanol for 5 minutes, followed by 5 minutes in 96% ethanol and 5 minutes in 100% ethanol. As a final step before covering the slides using DPX, the slides stayed a minimum of 5 minutes in xylene.

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/mm2. (142)

Study variables

To evaluate the effectiveness of intravenous administration of hADMSCs in the animal model of cerebral infarction associated with comorbidities (hyperglycemia and hypertension), the following study variables were analyzed: functional recovery, size of the lesion, and expression of markers of brain damage/repair.

Functional evaluation

Different tests were used to evaluate the motor deficit as well as the motor coordination in the animals. The modified Roger´s test was used to assess hemiplegia. The tapered walking beam was used to study hind limb function. And the adhesive removal test was used to test sensorimotor deficits.

The modified Roger´s test

The modified Roger´s test was performed on a flat surface and for certain parts on the metal grid of the rat cages. The rats were left a minimum of 15 minutes before starting the evaluation. A variant of Roger´s functional scale was used to assign scores as follows:

0, no functional deficit.

1, failure to extend forepaw fully.

2, decreased grip of forelimb while tail 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.

7, dead.

Tapered walking beam

Rats were pre-trained for 3 days a week before surgery, and additional training would be performed until the rat performed consistently. During the pre-training, the rat would be placed at increasing distances from the dark-box and left after entering the dark-box alone for 2 minutes before resetting the set-up. One day before stroke and 7 days, 3 weeks, and 6 weeks after stroke, the rats were filmed while traveling the beam's distance towards the dark-box from the contralateral side. The rat would perform 3 trials per evaluation.

In a later stage, the videos were analyzed for contralateral hind paw placement. Depending on the placement, the following scores would be assigned. The total times that the hind paw fully slipped were counted as well as a half-slip (when at

least 3 toes were overhanging but not a full slip). Furthermore, the total amount of steps of the hind paw was counted. The final score was calculated as follows:

$$slip\ ratio = \frac{\left(\frac{1}{2}\text{half slip}\right) + \text{full slip}}{\text{Total steps}} * 100$$

The adhesive dot removal test

Two equal-sized adhesive circular pieces of tape were attached to the dorsal side of the front paws. A camera was set-up, recording through the bottom of the platform to record the time of the first touch of the paw contralateral to the stroke lesion and the removal of the piece of tape. Rats were left on the platform for a maximum of 120 seconds. If they did not remove the piece of tape by that time, the pieces of tape were removed by hand, and the rat was given the maximum score. Scores were recorded in seconds.

Lesions size assessment by magnetic resonance imaging

Lesion size was analyzed 24 hours and 6 weeks post-stroke by magnetic resonance imaging (MRI) (Bruker Pharmascan, Ettlingen, Germany). Rats were anesthetized using isoflurane, and heart rate was monitored throughout the MRI scan. 7-T horizontal bore magnets using T2-weighted (T2-W) spin-echo anatomical images acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial orientations and the following parameters: two echo images (echo time (TE), 29.54 ms and 88.61 ms); repetition time (TR) = 3000 ms; Rapid Imaging with Refocused Echoes (RARE) factor = 4; Av = 3; field-of-view (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.

We used diffusion-weighted imaging (DWI), including apparent diffusion coefficient (ADC) maps. Images were obtained with three different directions 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 ms; diffusion gradient separation, 18 ms; TR, 3000 ms; TE, 50 ms; FOV, 3.8 cm; axial slices (1.5 mm thickness) and 3 b values 100, 400, and 1000s/mm2; acquisition matrix = 128 × 128. To normalize the ADC values, the region of interest (ROI) of the lesion and the same ROI in the contralateral side was divided by the value in the contralateral normal hemisphere and expressed as a relative ADC (rADC) of the region [23].

Immunofluorescence studies

The individual sections were washed with phosphate-buffered saline (PBS) to remove the Tissuetek, and then a hydrophobic barrier was made surrounding the tissue using a PAP pen. A 100 µl of blocking solution was added to each section and left to incubate for 30 minutes at room temperature. After 30 minutes, the blocking solution was removed, and 50 µl of the primary antibody was added to each section and left at 4ºC overnight. The next day, the primary antibody was removed, and the slide was washed 3 times for 5 minutes with PBS. Then 50 µl of the corresponding secondary antibody was added and left to incubate for 45 minutes. Three washes for 5 minutes with PBS were used to remove any residual secondary antibody. After the washes, excess liquid was removed from the slide and mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (vectashield, Vector, USA) staining was applied to each section before cover slipping.

The perilesional area around the lesion core was defined on brain sections at 6 weeks post-stroke by microtubule-associated protein 2 (MAP-2) staining (1:1000, Millipore) and glial fibrillary acidic protein (GFAP) staining (1:500, Millipore). The samples were sectioned at 10 µm thickness using a Leica CM1950 cryostat (Leica). Immunohistochemistry images were obtained using a ×20 and ×40 objective lens and processed by image analysis software (Image-Pro Plus 4.1, Media Cybernetics).

Expression of markers of brain damage/repair by immunofluorescence

GFAP, Doublecortin, CD-31, MAP-2, Synaptophysin, Collagen-IV, and IBA-1 images were analysed using the NIS-element AR 4.5 software package. Images were quantified, averaging out the mean intensity from 4 individual pictures per rat and expressed in arbitrary units (A.U.) The relative vessel wall thickness was assessed by measuring the surface area of the vessel wall as a fraction of the total vessel area using α-smooth muscle actin (α-SMA).

$$relative\ vessel\ wall\ thickness = \frac{Area_{ovw} - Area_{ivw}}{\text{Area}_{ovw}}$$

Area_{ovw}: outer vessel wall

Area_{ivw}: inner vessel wall

Primary antibodies	**Dilution**	**Company**	**Reference number**
GFAP	1:500	Millipore	MAB360
Doublecortin	1:250	Santa Cruz	Sc-271390
CD-31	1:50	Abcam	ab64543
α-SMA	1:200	Abcam	AB7817
MAP-2	1:1000	Millipore	AB5622
Synaptophysin	1:200	Sigma-Aldrich	S5768
Collagen-IV	1:400	Abcam	AB19808
IBA-1	1:1000	Millipore	MABN92
Secondary antibodies	**Dilution**	**Company**	**Reference number**

Anti-mouse Alexa Fluor 488	1:750	Invitrogen	A11001
Anti-rabbit Alexa Fluor 488	1:750	Invitrogen	A11008

Statistical analysis

All results of depicted experiments were expressed as mean ± standard deviation (SD). All statistical tests were performed using the IBM SPSS 22 statistical software. To determine which statistical test would be used, a Shapiro-Wilk Test of Normality was performed. In normal distributed data, an ANOVA analysis was used, and thereafter post-hoc analysis was performed using the Bonferroni´s method. In the case of data with non-normal distribution, the Mann-Whitney U test was performed. A 95% confidence interval was used with *p* values smaller than 0.05 were considered significant. A priori power analysis was performed using non-parametric testing on expected effect size for infarct size and behavioral tests. To obtain a power of at least 80% (1 – beta) and a significance level of 5% (alpha), each group will have to include 10 rats randomly assigned. A posteriori power analysis was conducted using the software package Pass 11. Using a sample size of 10 and an alpha level of P is smaller than 0.05, two-tailed. Statistical power of greater than 0.8 was obtained for all detected effect sizes.

Cell culture protocol, characterization and hADMSC isolation

Several flow cytometry experiments were performed to test whether the cells used for stroke therapy were truly hADMSCs and not yet differentiated (Figure 6). Over 95% of cells tested positive for MSC markers CD73, CD90, CD105, and CD44. Less than 2% of the cells tested positive for hematopoietic markers, CD11b, CD19, CD34, and CD45. Another essential property of MSCs is that they HLA-DR negative, as demonstrated in figure 6. Based on their expression pattern, they conform to MSC expression. Size and complexity also conform to MSCs.

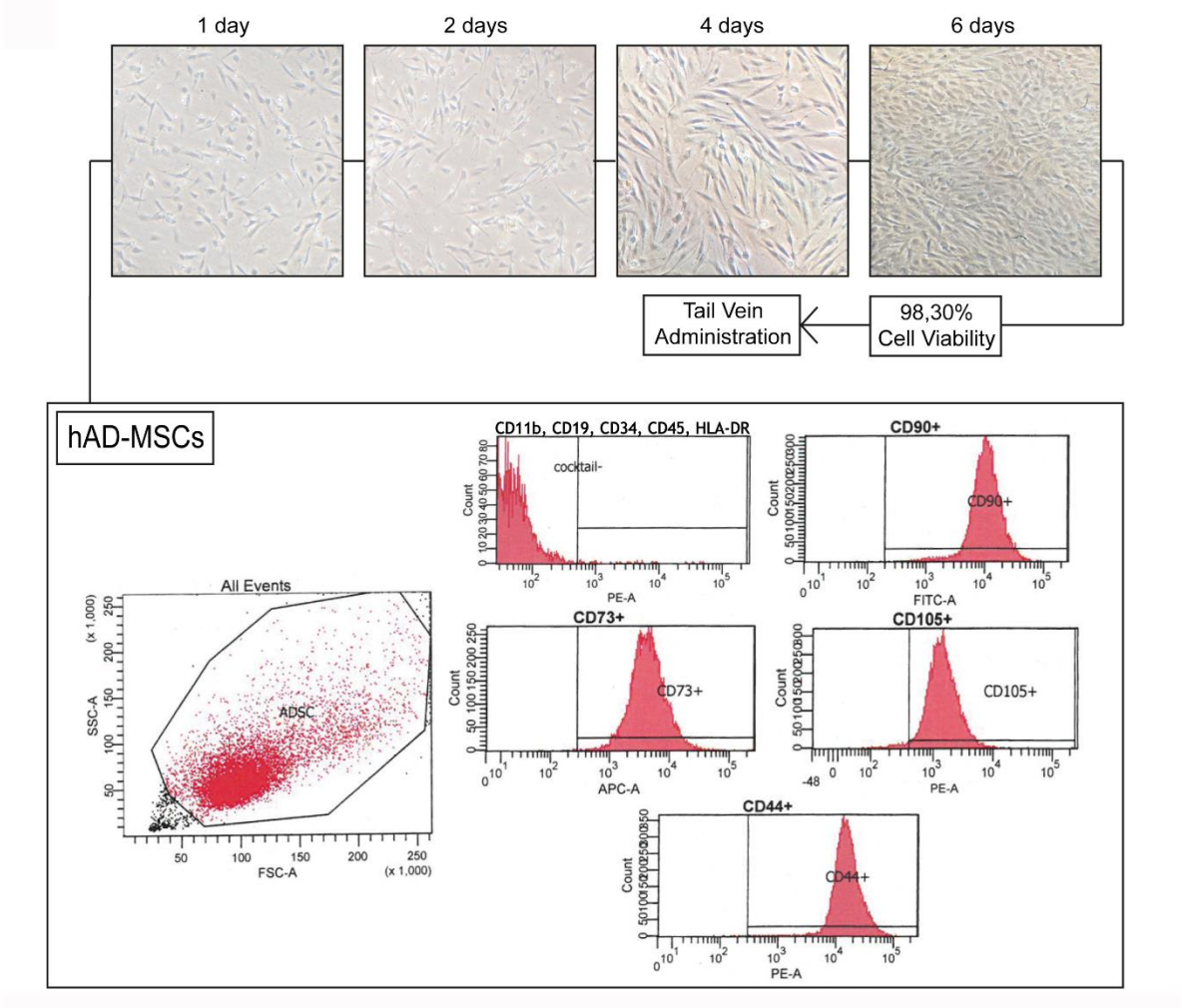


Figure 6 hADMSC culture validation. hADMSCs were thawed and cultured in flasks for 6 days. After 6 days, the cell viability was determined, and the phenotypic pattern was determined. Using flow cytometry, the positive expression of CD44, CD73, CD90 and CD105 (≥90%) and the negative expression of CD11b, CD19, CD34, CD45

and HLA-DR were detected (≤5%). After cell phenotype validation, the cells were prepared for intravenous administration.

The location of the periinfarct tissue was determined using MAP-2 and GFAP staining. Upon visual inspection, a clear distinction could be made between ischemic core tissue and periinfarct tissue by using either MAP-2 or GFAP (figure 7).

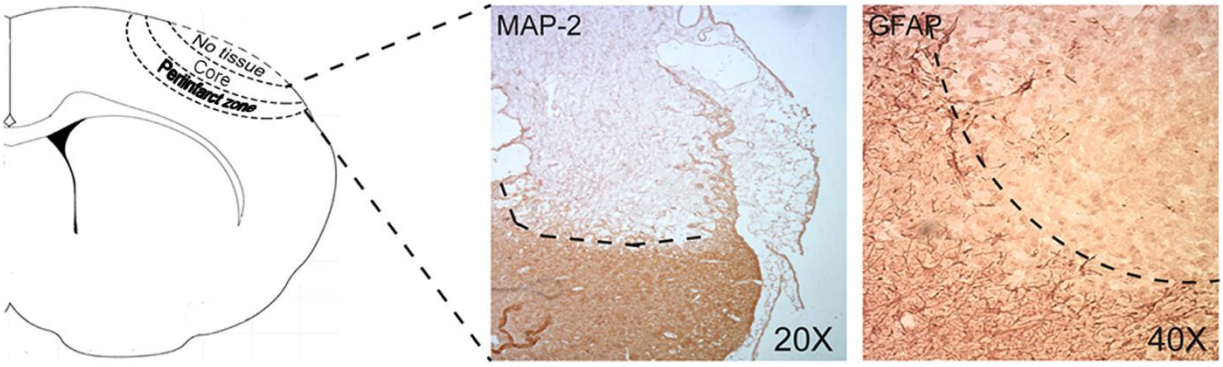


Figure 7 Determination of the periinfarct zone using MAP-2 and GFAP as markers of neurons and astrocytes. The dotted line deliminates the perinfarct zone from the ischemic core tissue.

Study variables in the animal model of cerebral infarction associated with hyperglycemia

Study variables in the animal model of cerebral infarction associated with hyperglycemia

Mortality

For this study, a total of 57 male Sprague-Dawley rats were used. Several animals were excluded from the study due to variations in pMCAO. Sixteen rats were excluded from the study, 13 died after surgical induction of permanent middle cerebral artery occlusion (pMCAO) (10 hyperglycemic animals and 3 nonhyperglycemic animals), and 3 rats were excluded because of a lack of lesions.

Blood glucose levels

To exclude the possibility that the observed recovery of treated hyperglycemic rats was due to decreasing glucose levels, all groups were monitored before MCAO surgery and before euthanasia. No significant difference in blood glucose levels was detected between non-treated hyperglycemic rats and treated hyperglycemic rats ($p>0.05$). Therefore, it is unlikely that recovery is due to any improvement of blood glucose levels (figure 8).

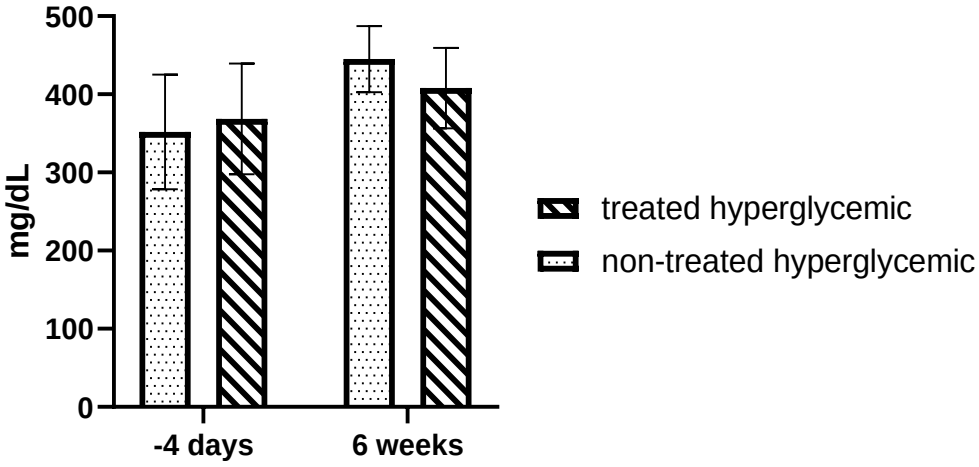


Figure 8 Blood glucose levels 4 days before surgery and 6 weeks after surgery. Glucose levels were in rats above 250 mg/dl were considered hyperglycemic. At the end of the experimental period, glucose levels were not significantly different from the first measurement ($p > 0.05$).

Hyperglycemia-associated stroke-induced impairment of motor function but hADMSC treatment improved neurological outcome in hyperglycemic rats

Animals were evaluated before stroke, 1-, 3- and 6-weeks post-stroke. In Roger's test, beam walking test and the adhesive removal test, all rats scored near perfect at the baseline (figure 9).

The non-hyperglycemic group did not show a significant difference with the nontreated-hyperglycemic group at 1, 3 or 6 weeks in Roger´s test. Therefore, no difference in hemiplegia was detected ($p>0.05$) (figure 9A). However, treatment positively affected recovery since the treated-hyperglycemic group recovered better after 3 to 6 weeks post-stroke than the nontreated-hyperglycemic group (figure 9B). The beam walking test indicated a worse neurological state of nontreated-hyperglycemic animals than non-hyperglycemic animals ($p<0.05$) (figure 9A). Treated hyperglycemic animals recovered better after stroke than hyperglycemic animals without treatment at 1-, 3- and 6-weeks post-stroke ($p<0.05$) (figure 9B). Treated hyperglycemic animals showed comparable results in the beam walking test with the non-hyperglycemic animals ($p>0.05$). For the adhesive removal test, two parameters were recorded: the time it took for an animal to touch for the first time a sticker on the contralateral paw from the lesion (sensitivity) and the time to remove said sticker. No significant difference in sensitivity between groups was detected at all-time points (figure 9C and 9D). No differences between non-hyperglycemic and nontreated hyperglycemic animals were observed ($p>0.05$) (figure 9C). As well as no differences between nontreated hyperglycemic animals and treated hyperglycemic animals ($p>0.05$) (figure 9D). Similar results were observed with the time of removal. There was no significant difference in removal time between groups ($p>0.05$) (figure 9C and 9D). No differences in removal time between non-hyperglycemic and nontreated-hyperglycemic animals were observed ($p>0.05$) (figure 9C). Also, there were no

significant differences between nontreated-hyperglycemic animals and treated hyperglycemic animals regarding removal time (p>0.05) (figure 9D).

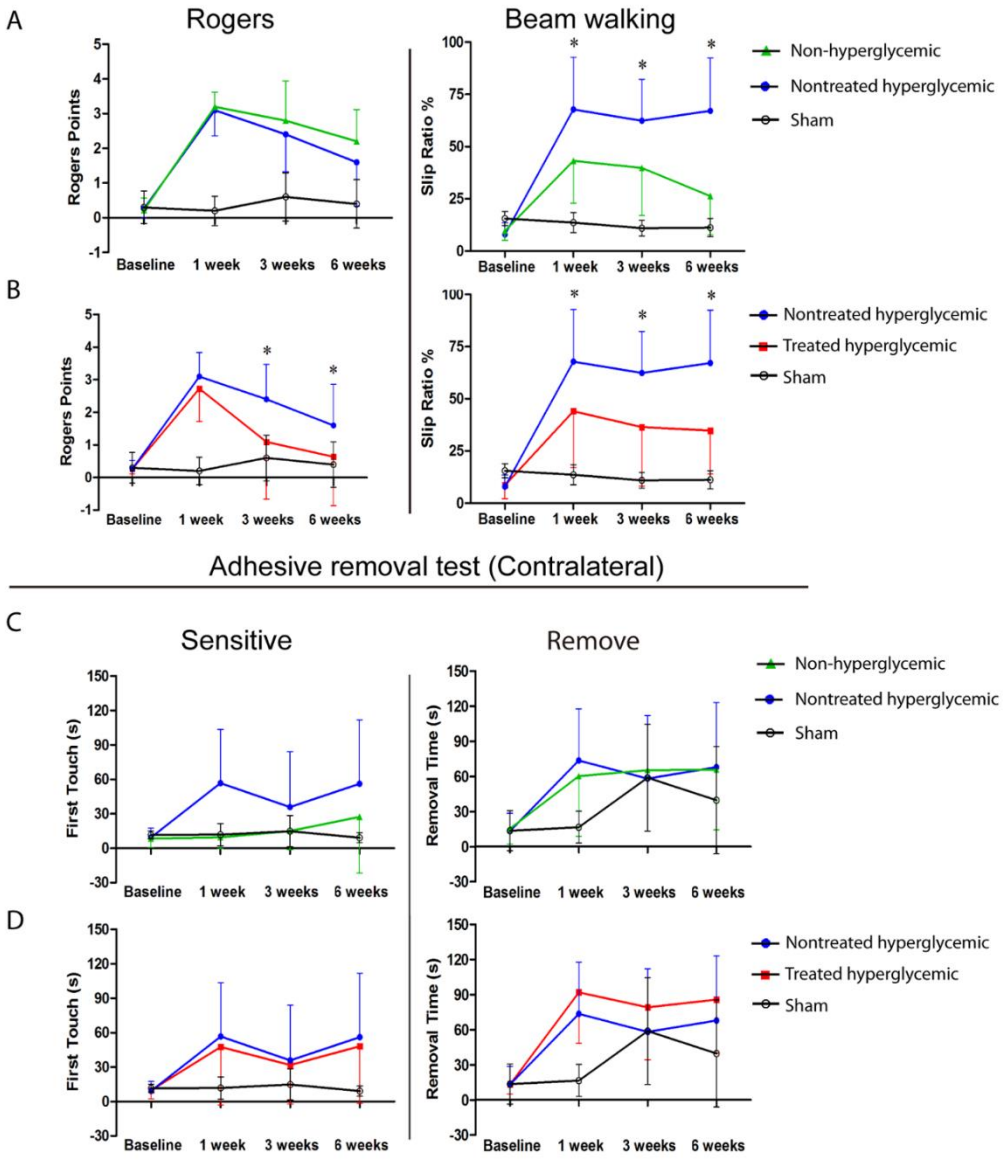


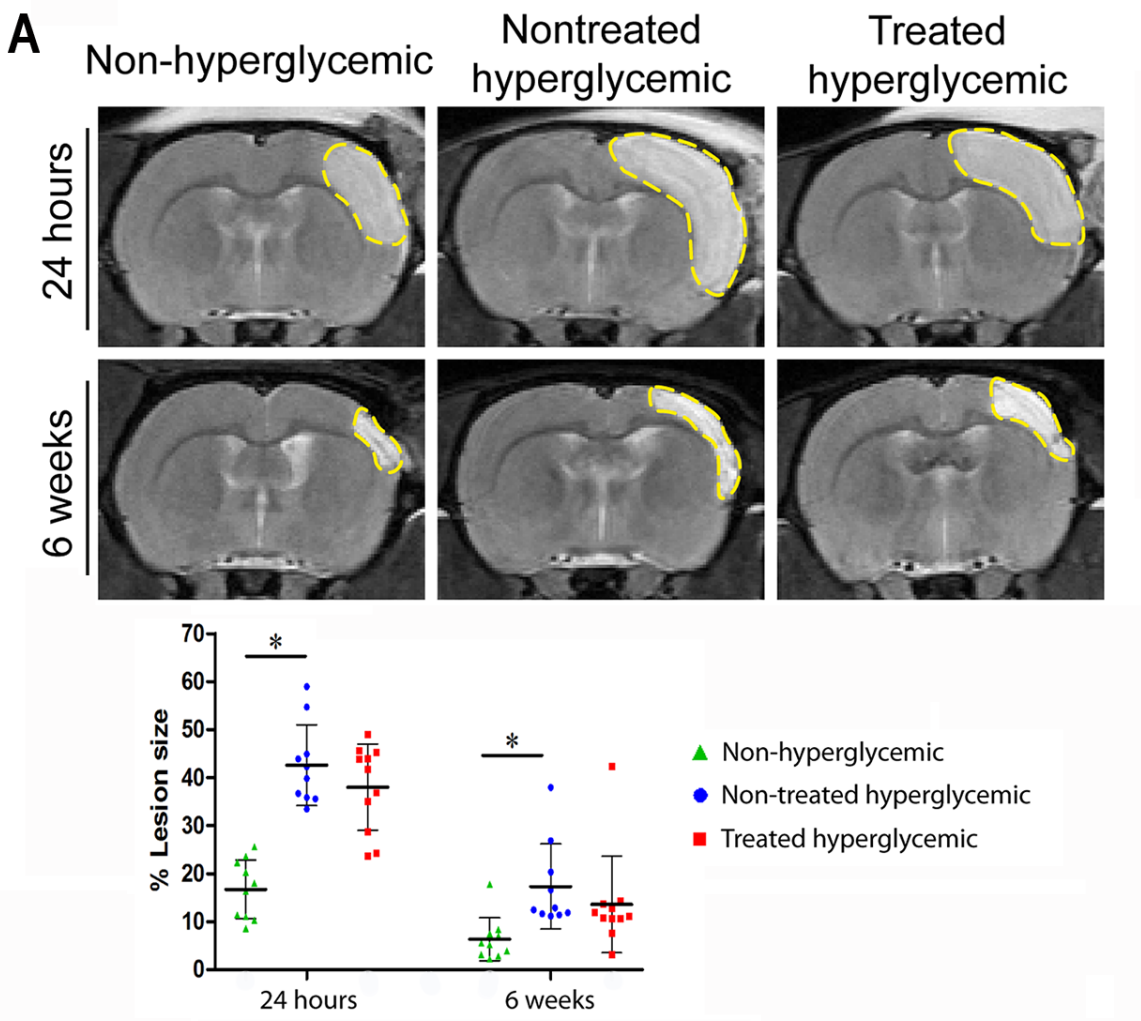
Figure 9 Functional evaluation using a battery of tests (Roger´s test, beam walking test and adhesive removal test). The functional evaluation was conducted 1 day before MCAO surgery (baseline), 1 week after, 3 weeks after and 6 weeks after. A) Comparison of non-hyperglycemic rats with nontreated hyperglycemic rats using the Roger´s and beam walking test. Only in the beam walking test, significant differences in favor of the non-hyperglycemic rats were observed after stroke. B) Comparison of nontreated hyperglycemic rats with treated hyperglycemic rats in the Roger´s and beam walking test. Significant differences were observed in favor of

treated hyperglycemic rats. C) Comparison between non-hyperglycemic rats and nontreated hyperglycemic rats in the adhesive removal test. No significant differences between the two groups were detected. D) Comparison between the nontreated hyperglycemic rats and the treated hyperglycemic rats in the adhesive removal test. No significant differences between groups were detected. The adhesive removal score is expressed in seconds, with a maximum of 120 seconds to complete the task. (n=10 rats per group). Data are presented as mean ± standard deviation. *p<0.05.

Hyperglycemia significantly increased lesion size and diffusion coefficients after stroke in rats

Following MCAO surgery, a unilateral cortical ischemic zone will form within 24 hours. MRI T2 scans were taken 24 hours after stroke and again after 6 weeks to quantify the lesion size and recovery using a coronal section at the height of the ventricles (figure 10a). rADC scans were taken 6 weeks after stroke to quantify anatomical tissue preservation (figure 10b).

In rats that suffered from hyperglycemia without treatment, lesion size was significantly bigger than that of non-hyperglycemic animals after 24 h ($p<0.001$), this difference is still seen after 6 weeks ($p<0.05$); however, the difference is smaller (figure 10a). Treated-hyperglycemic animals did not have a smaller lesion volume than non-treated hyperglycemic animals after 24 h and 6 weeks. Significantly higher diffusion coefficients were observed by rADC analysis in hyperglycemic animals compared to non-hyperglycemic animals after 6 weeks ($p<0.05$) (figure 10b). Treated-hyperglycemic animals have lower diffusion coefficients than nontreated-hyperglycemic animals ($p<0.05$). No significant difference in diffusion coefficients between non-hyperglycemic animals and treated hyperglycemic animals was detected (figure 10b).


A
Non-hyperglycemic
Nontreated hyperglycemic
Treated hyperglycemic
24 hours
6 weeks
% Lesion size
70
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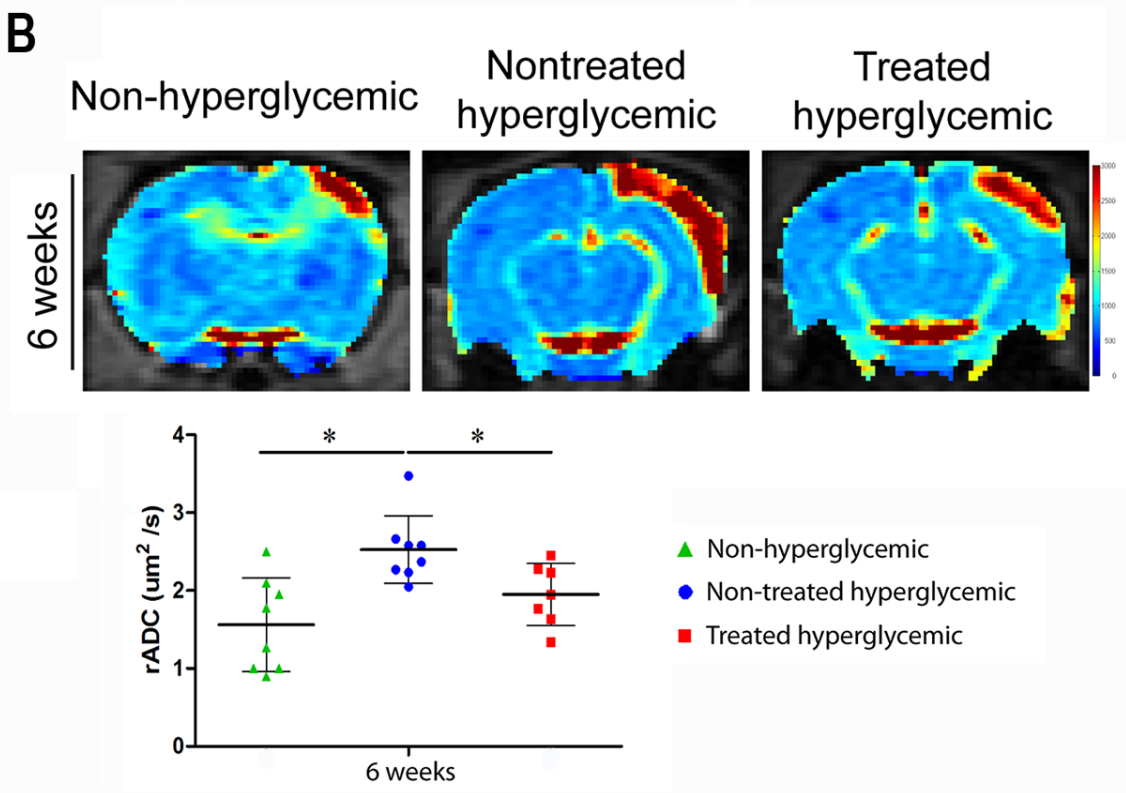
B
Non-hyperglycemic
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2
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0
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*
6 weeks
Non-hyperglycemic
Non-treated hyperglycemic
Treated hyperglycemic

Figure 10 Lesion size and density analysis using MRI. Representative MRI T2 images at 24 hours and 6 weeks post-stroke and DWI images at 6 weeks post-stroke. Quantification of the lesion size and tissue density is represented as their respective percentage and rADC. Lesion size is presented as the ratio of the size of the lesion to the size of the contralateral hemisphere. A) T2 images were obtained 24 hours and 6 weeks after stroke. Lesion size was significantly bigger in non-treated hyperglycemic rats compared to non-hyperglycemic rats. B) DWI images were obtained 6 weeks after stroke, and non-treated hyperglycemic rats have a significantly higher density in the stroke area compared to non-hyperglycemic rats and treated hyperglycemic rats. *p<0.05, (n=10 rats per group) data are shown as mean ± standard deviation.

hADMSC treatment increased the number of surviving neurons post-stroke in hyperglycemic rats

No significant differences between the number of neurons in non-hyperglycemic animals and nontreated hyperglycemic animals were detected. However, treated hyperglycemic animals had a significantly higher level of neurons than non-treated hyperglycemic animals (p<0.05) (figure 11).

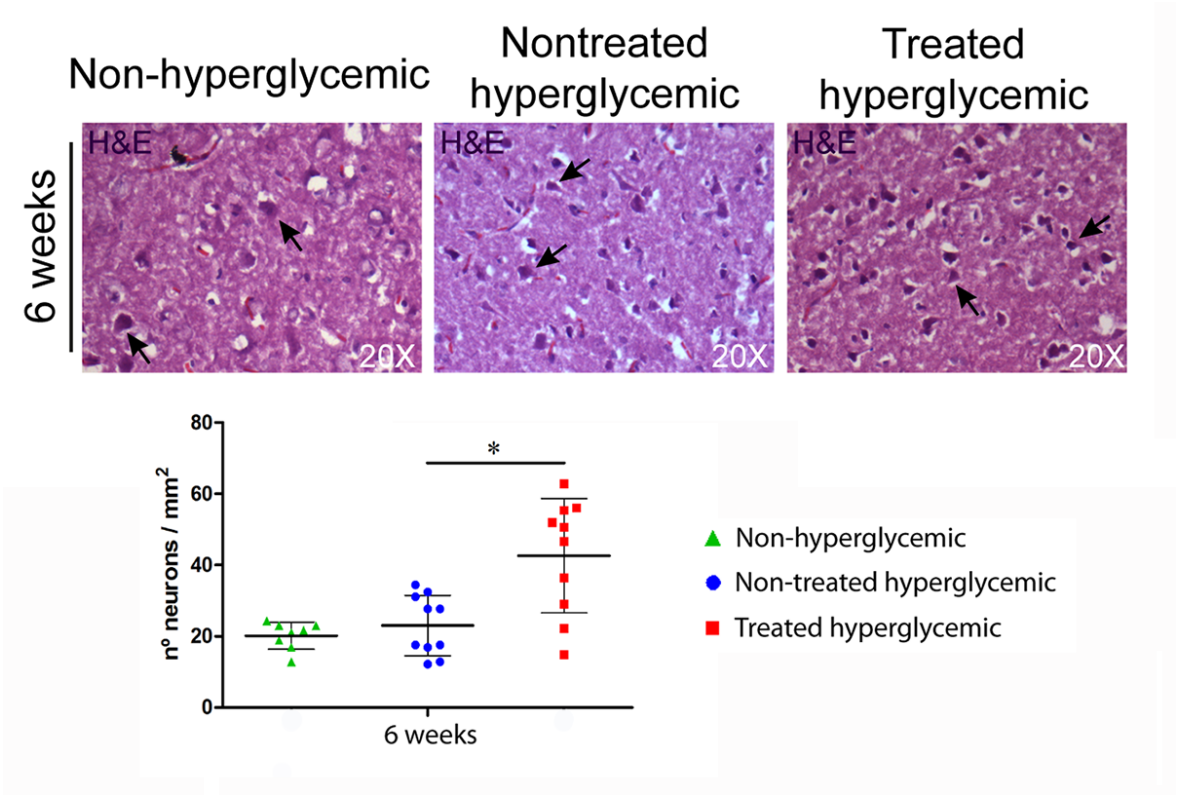

Figure 11 Histopathological analysis of motor neurons using a Hematoxylin and Eosin stain. Representative images of each group and quantification of motor neurons 6 weeks post-stroke. No significant differences in motor neuron density were observed between non-hyperglycemic and non-treated hyperglycemic rats. Treated hyperglycemic rats had a significantly higher motor neuron density than non-treated hyperglycemic

rats. Motor neurons are indicated by arrows. Data are presented as mean ± standard deviation. (3 animals per group, 4 sections per animal) *p<0.05

hADMSC treatment decreased astrocytes and microglia post-stroke in hyperglycemic rats

Levels of astrocytes and microglia were measured using GFAP and Iba-1, respectively. After stroke, GFAP levels vary depending on the study group. GFAP levels were much higher in non-treated hyperglycemic animals than in non-hyperglycemic animals ($p<0.05$). GFAP levels were significantly lower in treated hyperglycemic animals than nontreated hyperglycemic animals ($p<0.001$). However, treated hyperglycemic animals did not have significantly different GFAP levels from non-hyperglycemic animals (figure 12a).

A similar pattern was observed in Iba-1 expression as in GFAP expression. Hyperglycemia and treatment influence Iba-1 levels. The highest levels of Iba-1 were seen in nontreated hyperglycemic animals and are significantly higher compared to non-hyperglycemic animals $p<0.05$). Treated hyperglycemic animals had significantly lower Iba-1 levels than nontreated hyperglycemic animals ($p<0.05$). No significant difference in Iba-1 levels between treated hyperglycemic animals and non-hyperglycemic animals was observed (figure 12b).

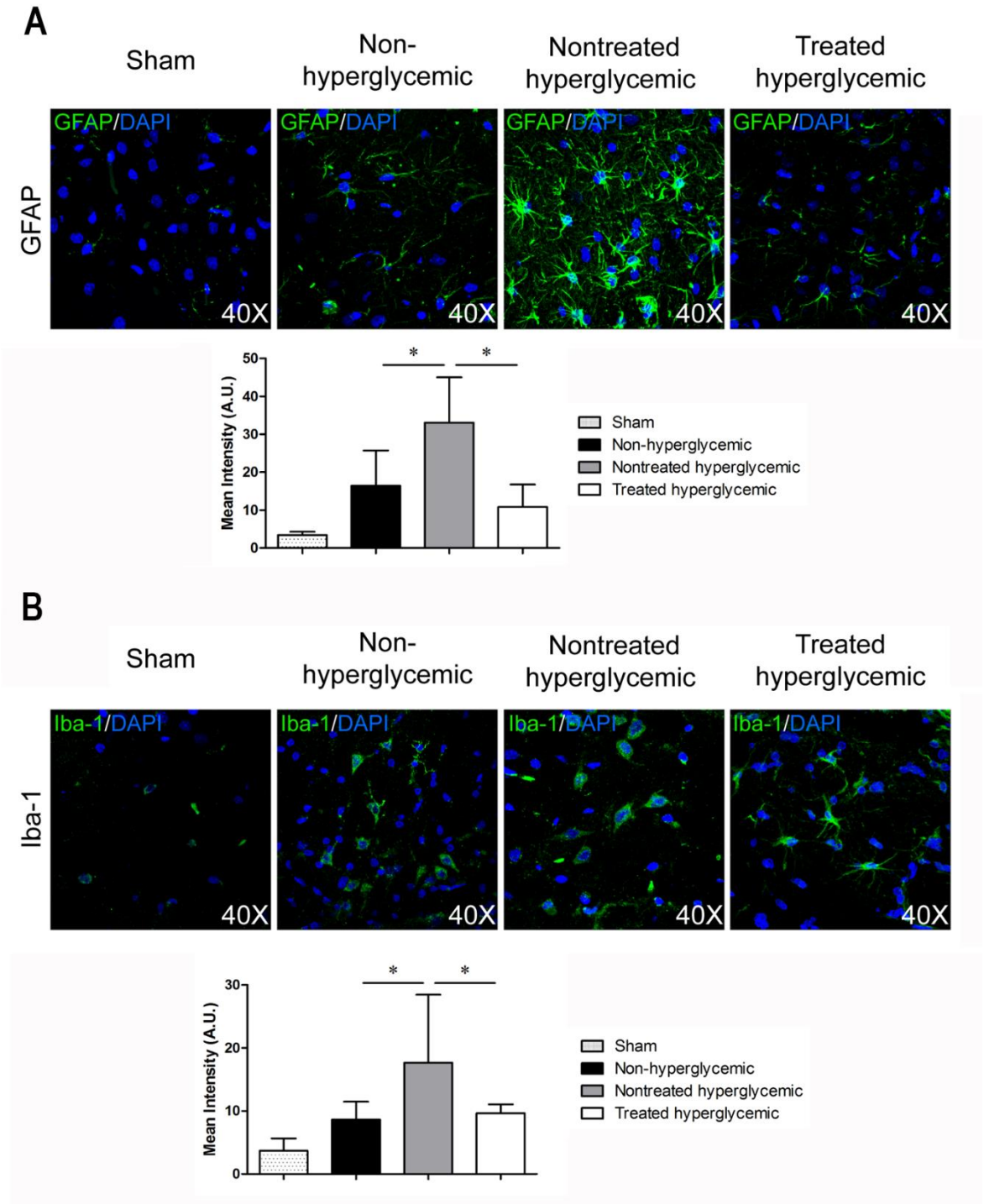

Figure 12 Representative images of astrocyte and microglial markers (GFAP, Iba-1) in the periinfarct of each group. A) GFAP is quantified for each group. Nontreated hyperglycemic rats have significantly higher GFAP expression in the periinfarct than non-hyperglycemic rats and treated hyperglycemic rats. B) Iba-1 expression is significantly higher in nontreated hyperglycemic rats than non-hyperglycemic rats and treated hyperglycemic rats. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *p<0.05.

hADMSC treatment did not promote synaptogenesis and neurogenesis post-stroke in hyperglycemic rats

At 6 weeks, post-stroke synaptophysin levels do not show any significant differences between groups. Although both hyperglycemic groups have the lowest average value for synaptophysin of all groups, no significant difference was detected (figure 13a).

As with synaptophysin, the levels of DCX, a marker for neurogenesis, did not seem to be different between groups. Although non-treated hyperglycemic rats have the lowest average levels of DCX, there were no significant increases or decreases of measured DCX levels in-between any of the groups (figure 13b).

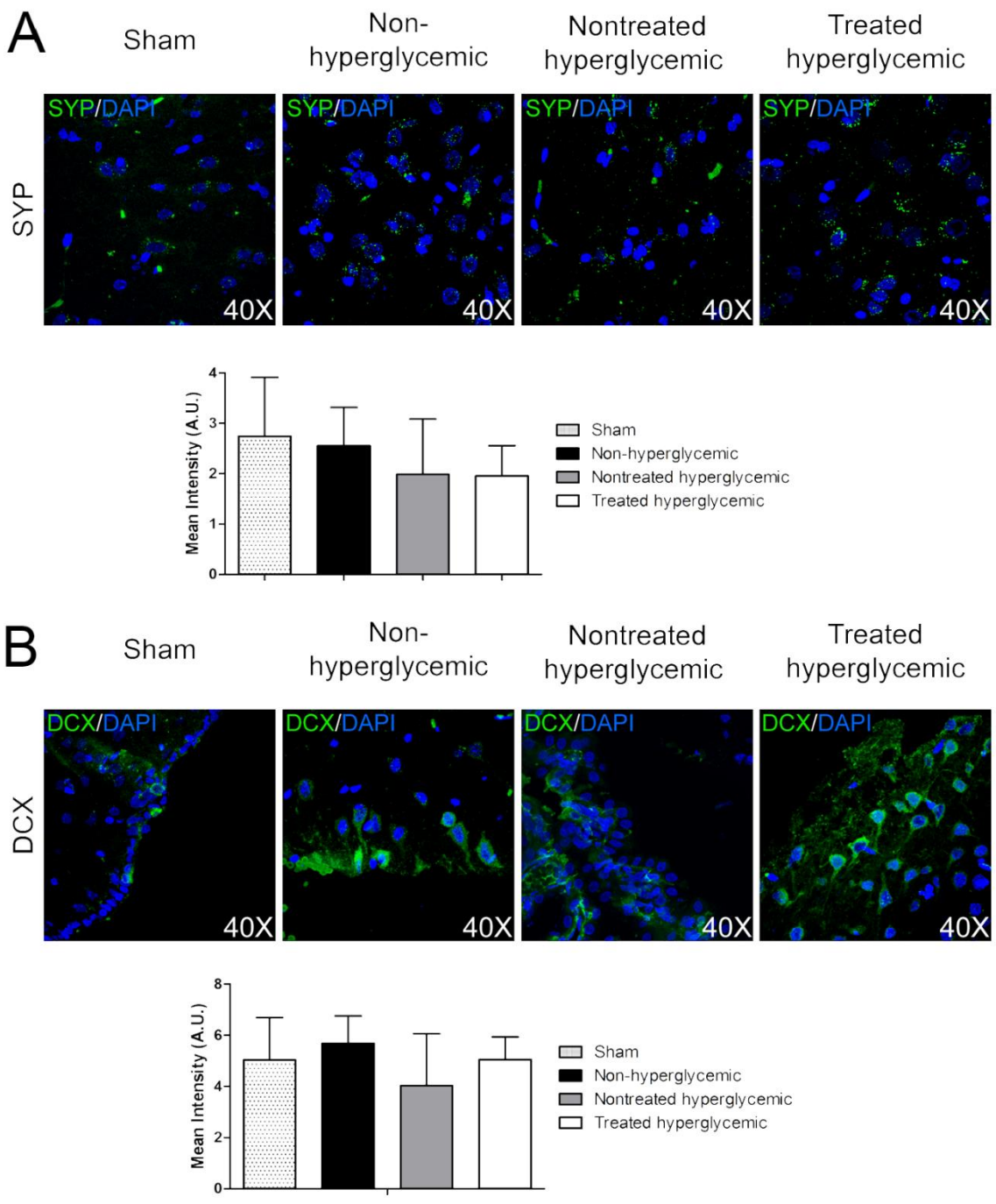

Figure 13 Representative images of synaptogenesis and neurogenesis (SYP, DCX) in the periinfarct of each group. A) No significant difference in expression of SYP was observed between any of the groups. B) No significant difference in expression of DCX was observed between any of the groups. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *p<0.05.

Hyperglycemia affects α-SMA, but not vascular markers CD31 and collagen-IV

No significant differences in the expression of CD31 were observed in any study group (figure 14a). The sham, non-hyperglycemic, and nontreated hyperglycemic were remarkably similar. The treated-hyperglycemic group had a more considerable variability in CD31 levels.

No significant differences in the COL-IV marker were measured between the study groups (figure 14b).

There were, however, significant differences in levels of α-SMA. We found that nontreated hyperglycemia had significantly higher levels of α-SMA compared with the non-hyperglycemic group ($p<0.01$). Furthermore, the treated hyperglycemic group has significantly lower α-SMA levels than the nontreated hyperglycemic group ($p<0.05$). α-SMA levels are not significantly different between the non-hyperglycemic and treated hyperglycemic groups (figure 14c).

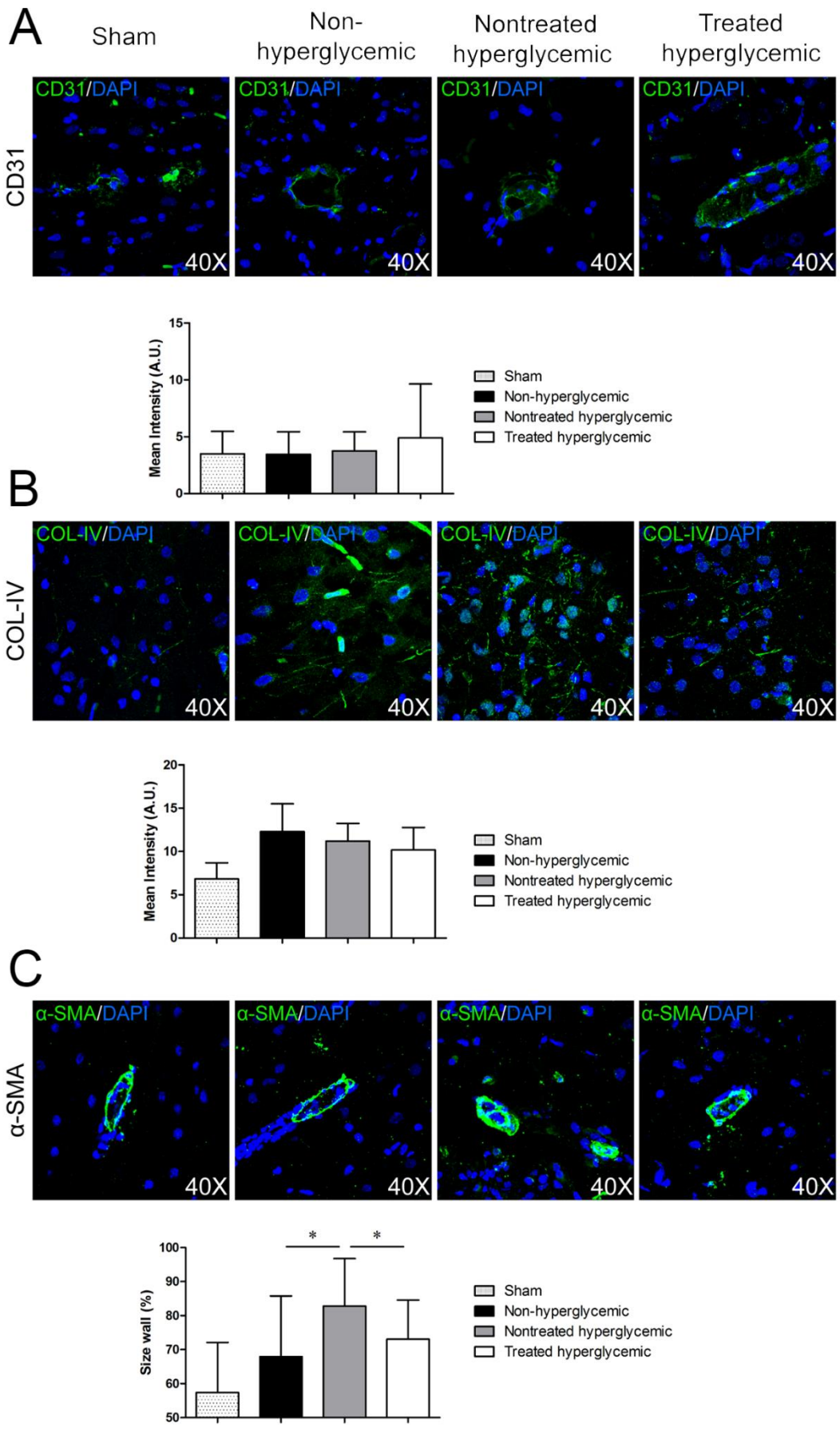
A
Sham
Non-hyperglycemic
Nontreated hyperglycemic
Treated hyperglycemic
CD31
CD31/DAPI
40X
Mean Intensity (A.U.)
15
10
5
0
Sham
Non-hyperglycemic
Nontreated hyperglycemic
Treated hyperglycemic
B
COL-IV
COL-IV/DAPI
40X
Mean Intensity (A.U.)
20
15
10
5
0
Sham
Non-hyperglycemic
Nontreated hyperglycemic
Treated hyperglycemic
C
α-SMA
α-SMA/DAPI
40X
Size wall (%)
100
90
80
70
60
50
*
*
Sham
Non-hyperglycemic
Nontreated hyperglycemic
Treated hyperglycemic

Figure 14 Representative images of vascular markers (CD31, COL-IV and α-SMA) in the periinfarct of each group. A) No significant differences in the expression of CD31 were observed between any of the groups. B) No significant differences in expression of COL-IV were observed between any of the groups C) α-SMA expression was significantly higher in the nontreated hyperglycemic rats compared to the non-hyperglycemic rats and the treated hyperglycemic rats. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *p<0.05.

Study variables in the animal model of cerebral infarction associated with hypertension

Mortality

For this study, a total of 81 male SHR and Wistar rats were used. A total of 31 rats were excluded from the study. Of these 31 rats, 28 died after the pMCAO surgery, 19 were from the vehicle-hypertensive group, and 9 from the vehicle-normotensive group. Two rats were excluded from the study because no signs of a lesion appeared on the MRI T2 scans, and one rat died during treatment administration.

hADMSC administration does not alter blood pressure

To confirm that blood pressure was not affected by stem cell treatment, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured 7 days after pMCAO surgery and 6 weeks after (figure 15). Animals of the hADMSC-hypertensive and vehicle-hypertensive group had significantly higher SBP and DBP than the vehicle-normotensive group at 7 days and 6 weeks post-stroke. Seven days post-stroke, SBP and DBP in the vehicle-hypertensive group and the hADMSC-hypertensive group were significantly higher than the vehicle-normotensive group ($p<0.01$). After 6 weeks, the same result was observed between groups ($p<0.01$) (figure 15).

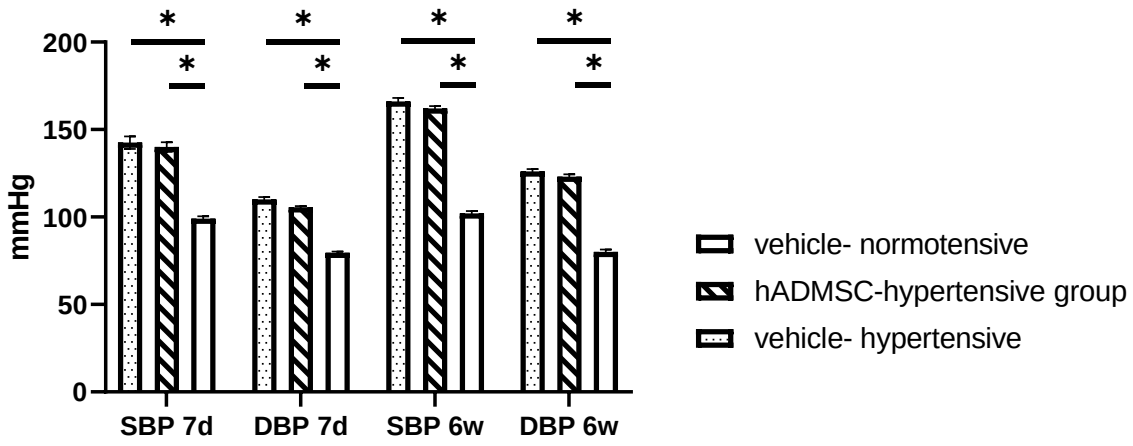

Figure 15 Effects of treatment on blood pressure. Systolic blood pressure (SBP) and diastolic blood pressure (DSP) were determined 7 days and 6 weeks after stroke. No significant differences between vehicle hypertensive and hADMSC-hypertensive groups were observed. The vehicle-normotensive group had at all times a significantly lower blood pressure (SBP and DBP) than the vehicle-hypertensive and hADMSC-hypertensive groups. *p<0.05

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

The rats were subjected to various functional evaluations (Roger´s test, beam walking test and adhesive removal test).

The baseline of Roger´s test showed an almost perfect score of 0 as a baseline with a minimum variation inside and between groups (figure 16a). One week after stroke induction, Roger´s test score increased significantly from baseline in all groups except for the sham group. However, no differences were detected between groups (figure 16a). Three weeks post-stroke, the first effects of stem cell treatment are observed; the hADMSC-normotensive group has a significantly lower score than the vehicle-normotensive group (p<0.05) (figure 16a). Six weeks after stroke, this difference is still observed (p<0.001) and also a significant difference between hADMSC-normotensive and hADMSC-hypertensive (p<0.05) is observed in favor of

the normotensive group (figure 16a). During the 6 weeks after stroke, there was no significant difference detected between the vehicle-hypertensive group or the hADMSC-hypertensive group (figure 16a).

During the beam walking test, rats showed a consistent and low slip ratio at baseline, as well did the sham group during the entire duration of the experiment (figure 16a). After the first week post-stroke, significant differences were detected between vehicle-normotensive and vehicle-hypertensive groups ($p<0.05$) (figure 16a). The same was observed after 3 ($p<0.05$) and 6 weeks ($p<0.01$) (figure 16a). Also, a significant difference between hADMSC-normotensive and hADMSC-hypertensive groups was observed after 3 ($p<0.01$) and 6 ($p<0.05$) weeks (figure 16a). No significant differences between treated and non-treated hypertensive animals were detected at any of the time-points (figure 16a).

Two different parameters of the adhesive removal test were analyzed. The moment the rat touched the contralateral sticker and when it removed the sticker from the contralateral paw. At baseline, all rats displayed a swift and consistent response and removal time (figure 16b). All sham rats performed consistently on their first touch time throughout the experiment. After 7 days, significant differences between the vehicle-normotensive and vehicle-hypertensive groups were detected ($p<0.05$) (figure 16b). Also, between hADMSC-normotensive and hADMSC-hypertensive significant differences occurred (figure 16b). However, no significant difference was detected between any treated and nontreated groups (figure 16b). After 3 and 6 weeks, all groups were recovering from the stroke, and no significant differences between groups were detected (figure 16b).

During the adhesive dot removal test, the second parameter recorded was how much time it took for the rat to remove the dot stuck to their paw. At baseline, all groups performed equally with no significant difference between them (figure 16b). After 1 week post-stroke, all groups took more time to remove the dot, including the sham group (figure 16b). Only a significant difference between the groups was seen

between hADMSC-normotensive and hADMSC-hypertensive groups (figure 16b). No significant differences were detected between vehicle-normotensive and vehicle-hypertensive groups (figure 16b). After 3 and 6 weeks, no significant difference between any of the groups was detected (figure 16b).

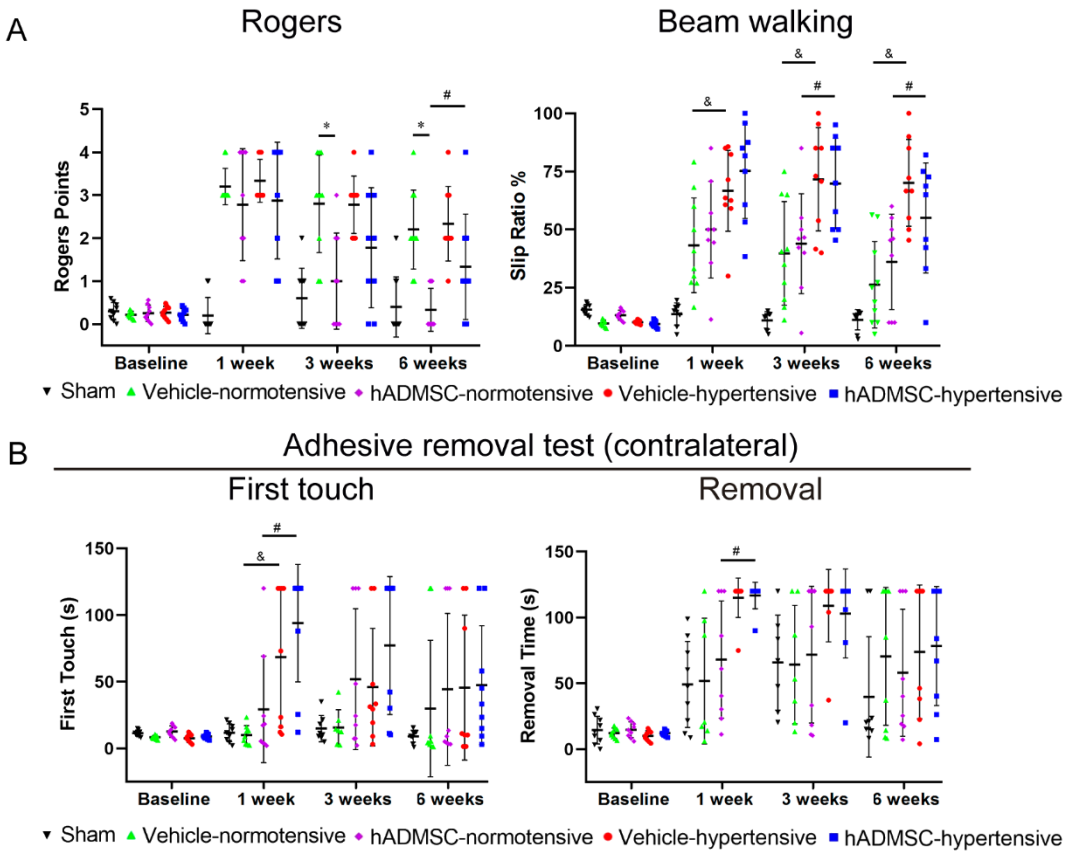

Figure 16 Functional evaluation using a battery of tests. A) Assessment of behavioral outcome using Roger´s test (left) and the Beam walking test (right). After 1 week, the vehicle-normotensive rats have a lower score than vehicle-hypertensive rats, which continues until the 6th week. In the 3rd and 6th week after stroke hADMSC-normotensive rats perform better than vehicle-normotensive rats in Roger´s test, while no significant differences were seen in the beam walking test. Furthermore, at the 3rd and 6th week, the hADMSC-normotensive group outperforms the hADMSC-hypertensive group in the beam walking test and at the 6th week in Roger´s test. B) Assessment of behavioral outcome using the adhesive removal test measuring the time to first touch (left) and removal time (right). Significance (p<0.05) is noted as follows: *: vehicle-normotensive vs. hADMSC-normotensive; &: vehicle-normotensive vs. vehicle-hypertensive; #: hADMSC-normotensive vs. hADMSC-hypertensive (n=10 rats per group). Data are shown as mean ± standard deviation.

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

To quantify the lesion size after stroke, a coronal cross-section was used at the height of the lateral ventricles. Quantification of the size was done after 24 hours and 6 weeks (figure 17). The vehicle-normotensive group had a significantly smaller infarct size than the vehicle-hypertensive group after 24 hours ($p<0.001$). After six weeks, all groups showed partial recovery of the lesion. All hypertensive animals had an increased lesion size compared to their normotensive counterpart after six weeks ($p<0.001$). hADMSC treatment did not decrease lesion size in hypertensive rats when compared to the hypertensive vehicle group ($p>0.05$) or in the normotensive rats compared to the normotensive vehicle group ($p>0.05$) after 6 weeks. hADMSC-hypertensive rats had significantly larger lesions compared to hADMSC-normotensive rats ($p>0.05$ (figure 17a).

At 6 weeks, post-stroke also the relative apparent diffusion coefficient (rADC) was determined. A similar pattern as of the lesion size was detected. rADC was significantly elevated in the vehicle-hypertensive groups compared to the vehicle-normotensive group ($p<0.001$). When comparing the hADMSC- normotensive group and the hADMSC-hypertensive group with their respective vehicle groups, no significant differences in rADC values were seen ($p>0.05$ and $p>0.05$, respectively). When the hADMSC-hypertensive group was compared with the hADMSC-normotensive group, a significantly larger lesion was detected ($p<0.001$) (figure 17b).

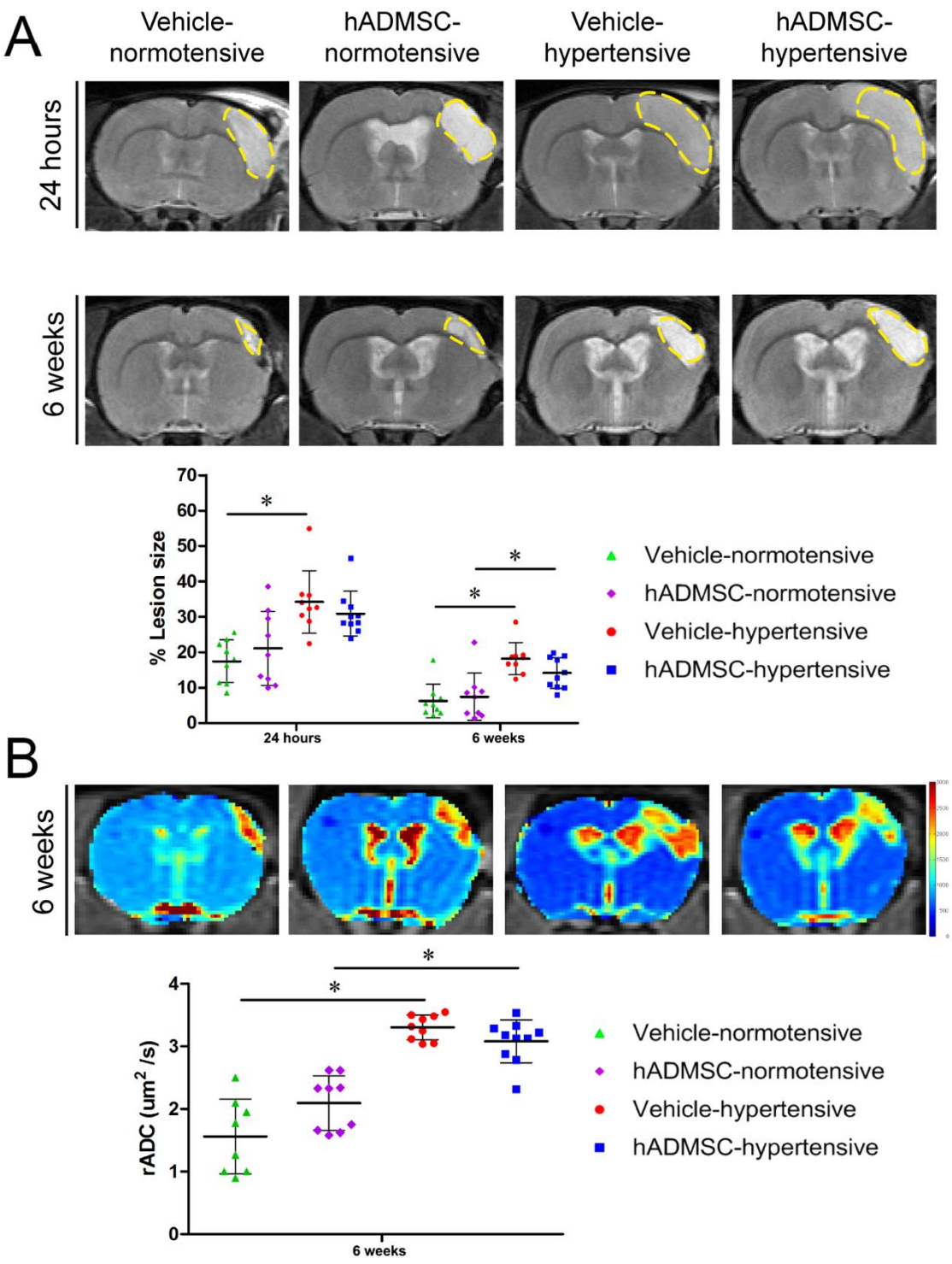

Figure 17 Lesion size and density analysis using MRI. Representative MRI T2 images at 24 hours and 6 weeks post-stroke and DWI images at 6 weeks post-stroke. Vehicle-hypertensive animals have a significantly bigger lesion size than vehicle-normotensive animals at 24 hours and 6 weeks post-stroke. 6 weeks after stroke, hAMDSC-hypertensive animals have a significantly bigger lesion than hADMSC-normotensive animals. rADC values are significantly higher in vehicle-hypertensive rats than in vehicle-normotensive rats. hADMSC-

hypertensive rats have a significantly higher rADC value than hADMSC-normotensive rats. *: $p<0.05$, (n=10 rats per group) data are shown as mean ± standard deviation.

Hypertension did not influence total cortical motor neurons, however, hADMSC treatment increased total cortical motor neurons in hypertensive rats

After 6 weeks, vehicle-hypertensive rats showed no significant differences in the number of cortical motor neurons than vehicle-normotensive rats ($p>0.05$). hADMSC treatment increased the number of motor neurons in the normotensive group ($p<0.001$) but not in the hypertensive group ($p>0.05$) 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 ($p<0.01$) (figure 18).

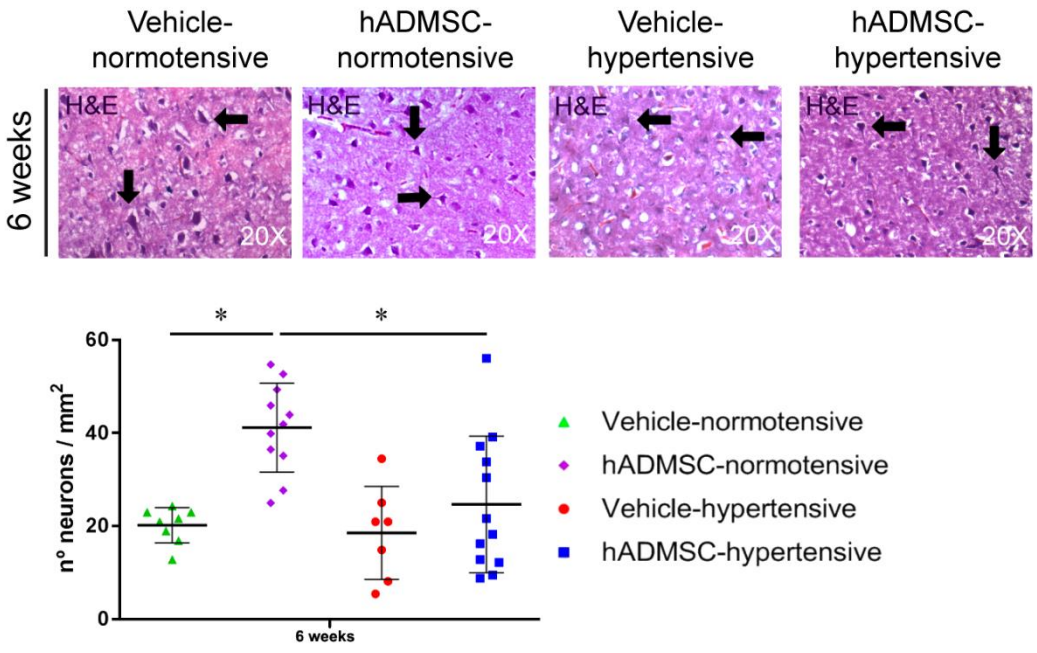

Figure 18 Histopathological analysis of motor neurons. Representative images of each group and quantification of motor neurons 6 weeks post-stroke. hADMSC-normotensive rats have a significantly higher density of motor neurons than hADMSC-hypertensive and vehicle-normotensive rats. Data are presented as mean ± standard deviation. (3 rats per group, 4 sections per animal) *$p<0.05$

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

GFAP, as a marker of astrocytes, was analyzed in the perilesional area of the stroke infarct. Six weeks after stroke, GFAP levels were significantly increased in the vehicle hypertensive group when compared to the vehicle-normotensive group (p<0.05) (figure 19). The treatment effect of hADMSC was investigated in both normotensive and hypertensive animals. hADMSC treatment effectively lowered GFAP levels in normotensive animals compared to the vehicle-normotensive group (p<0.05). However, no changes were observed between the vehicle-hypertensive group and the hADMSC-hypertensive group (p>0.05) (figure 19). When comparing treatment groups of hypertensive versus normotensive animals, significantly higher GFAP levels were detected in the hADMSC-hypertensive group when compare to the hADMSC-normotensive group (p<0.01) (figure 19).

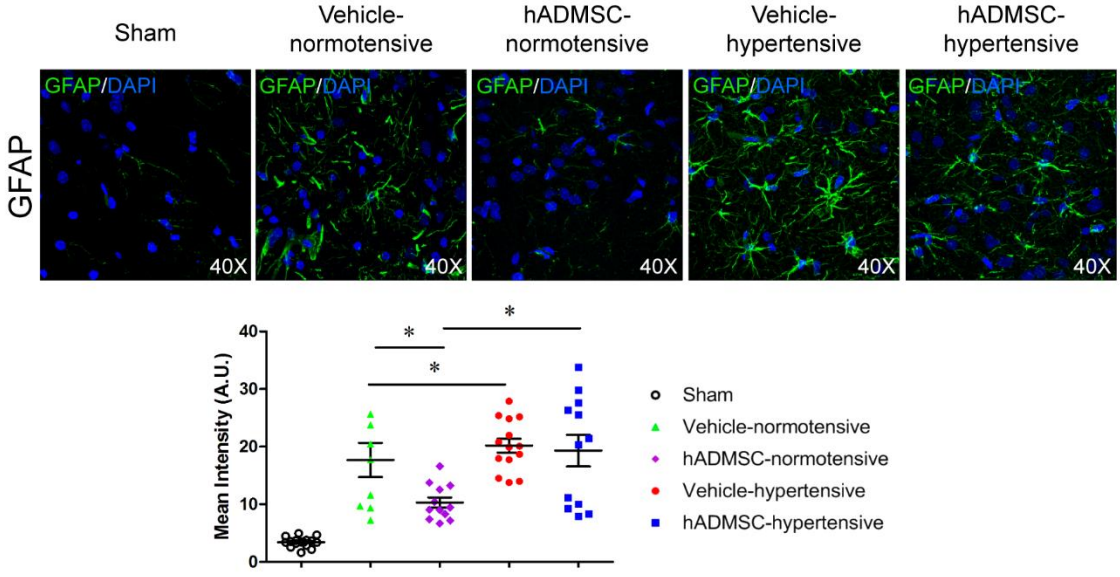

Figure 19 Representative images of glial fibrillary acidic protein (GFAP) in the perilesional area of each group. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *p<0.05.

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

The role of hypertension in stroke and its influence on neurogenesis 6 weeks after stroke was investigated using the marker doublecortin (DCX) (figure 20). No difference in DCX levels was detected between the vehicle-hypertensive and vehicle-normotensive groups ($p>0.05$). Furthermore, no differences in DCX levels were detected between treated hypertensive and normotensive animals ($p>0.05$) and between vehicle-normotensive animals and hADMSC-hypertensive animals ($p>0.05$) (figure 20). Only in the hADMSC-normotensive animals, compared to vehicle-normotensive animals, the treatment had a significant effect ($p<0.01$).

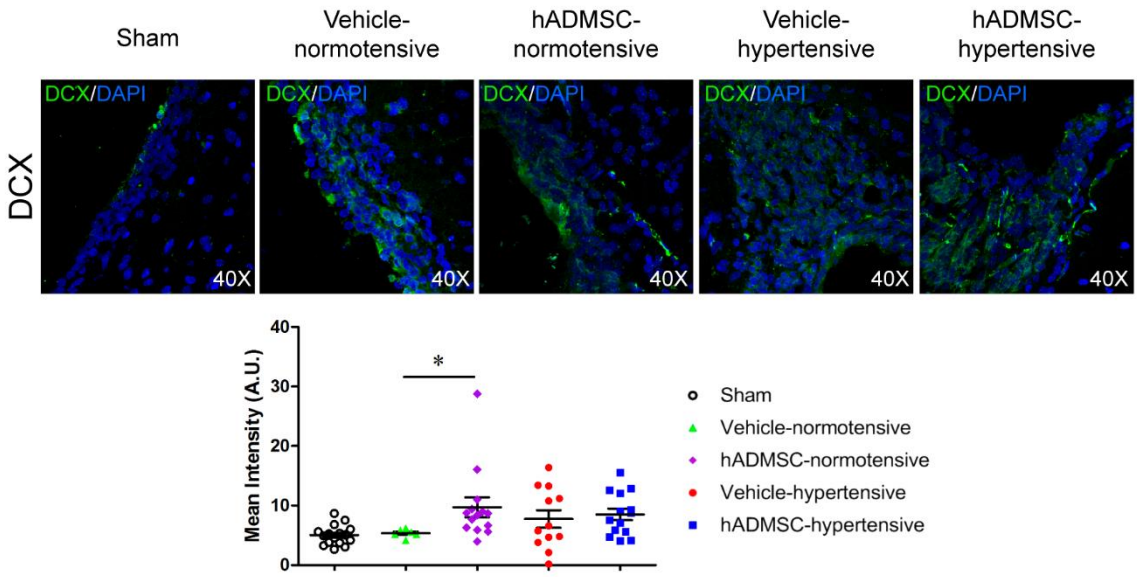

Figure 20 Representative images of doublecortin (DCX) in the penumbra of each group. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *$p<0.05$.

Hypertension Increased CD-31 and α-SMA Signals and hADMSC Had No Effect on These Vascular Proteins

Influences of hypertension on stroke were seen in the endothelial lining and smooth mussels of vascularization of the perilesional tissue. The endothelial lining, as seen by CD-31, was increased in the vehicle-hypertensive group compared with the vehicle-normotensive group ($p>0.001$) (figure 21a). The hADMSC-hypertensive group had significantly higher CD-31 values than the hADMSC-normotensive groups ($p<0.05$). No significant differences in CD-31 values between the hADMSC-hypertensive group and the vehicle-hypertensive group were observed ($p>0.05$). The same effect was absent from the hADMSC-normotensive group and the vehicle-normotensive group ($p>0.05$) (figure 21a).

Smooth mussels were observed with the marker α-SMA to measure vessel wall thickness. Increased wall thickness was observed in vehicle-hypertensive animals when compared with vehicle-normotensive animals ($p<0.001$) (figure 21b). Blood vessel walls did not differ significantly in thickness between the hADMSC-hypertensive group and the vehicle-hypertensive group ($p>0.05$). Treatment had a significant effect on normotensive animals when comparing hADMSC-normotensive with vehicle-normotensive animals ($p<0.05$) (figure 21b). Also, vessel walls were significantly thinner in hADMSC-normotensive animals than in hADMSC-hypertensive animals.

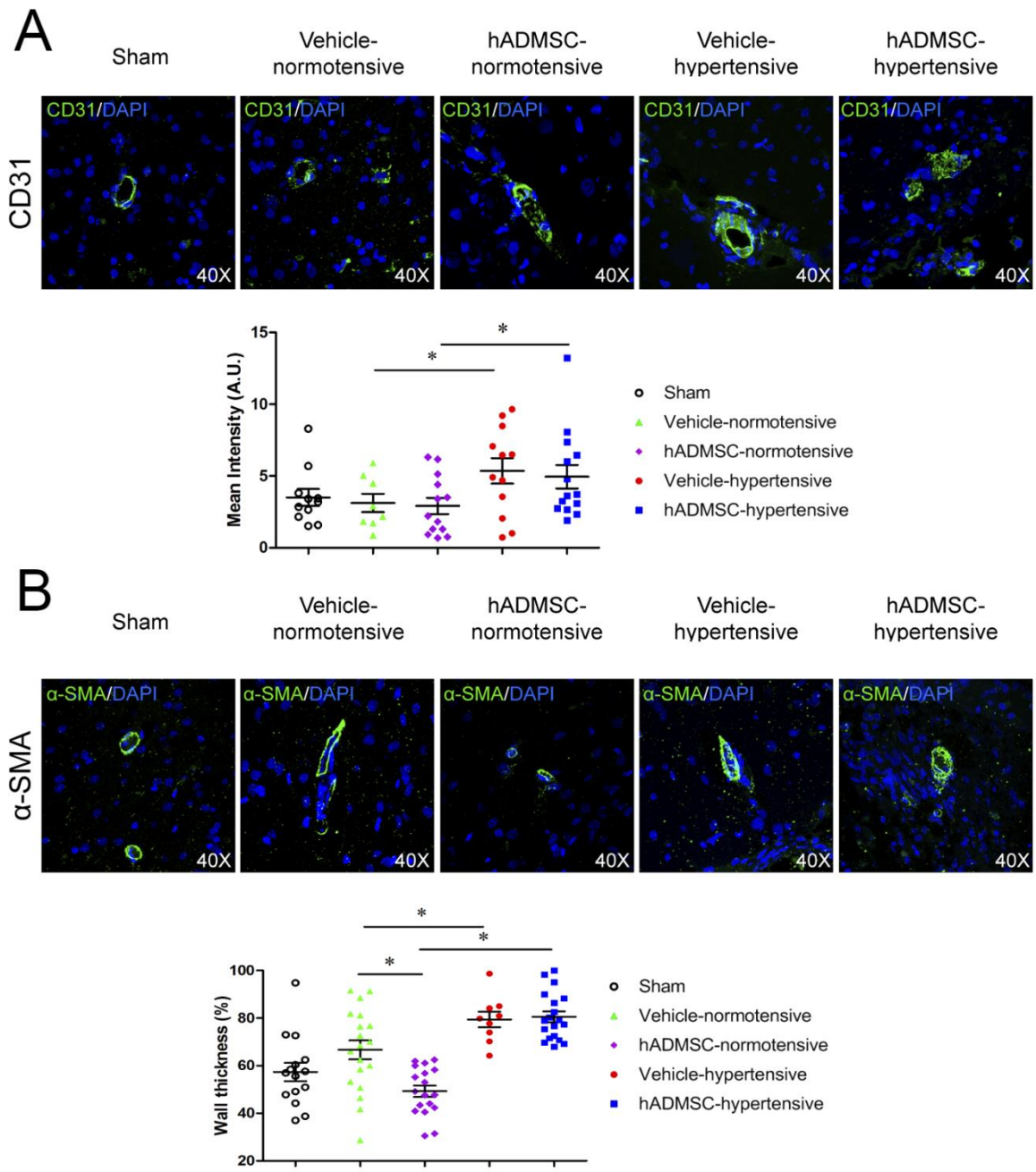

Figure 21 Representative images of vascular markers (CD31, α-SMA) in the periinfarct area of each group. For each group, 3 rats were used, of which 4 sections each were quantified. Data are presented as mean ± standard deviation. *p<0.05.

Discussion

Stem cells are a promising therapeutic option for stroke patients. (143,144) Current treatments exclude the vast majority of patients due to time limits, and even the patients arriving in time for treatment do not always receive it. (145) Stem cell treatment promises extended treatment windows, removing some of the pressure on the health system and the patient receiving treatment. (146) However, certain stem cell types of interest for ischemic stroke therapy have not been well investigated with respect to how two of the most common comorbidities (hyperglycemia and hypertension) influence their efficacy. (107)

The source of MSCs is of importance. Although there is no clear consensus on the difference in efficacy of ADMSCs and BMMSCs, studies have shown that AD-MSCs are at least as good or even better than BM-MSCs.(72,74) Furthermore, from a translational point of view, AD-MSCs can be harvested in greater quantities than BM-MSCs from cosmetic liposuctions. (147) Furthermore, xenogeneic or allogeneic administration of AD-MSC had similar efficacy in treating ischemic stroke in rats. (73) For many years the BM-MSCs were the golden standard in preclinical research as a treatment for ischemic stroke. However, AD-MSCs have some specific advantages over BM-MSCs that make them a better candidate from a translational point of view.

To determine how AD-MSC therapy is influenced by comorbidities, it is vital to incorporate it into experimental animal models of stroke in order to test therapeutic strategies as a previous step to translation to the patient, as recommended by STAIR and STEPS. (18,106)

Animal model of cerebral infarction associated with hyperglycemia

Acute stroke patients suffer from hyperglycemia in over 40-50% of the cases, associated with a poor prognosis and outcome. (148,149) Taking this into account, it

is important to introduce this comorbidity in an experimental animal model of stroke. To induce hyperglycemia in our study, we have used the nicotinamide- streptotozin model. Pancreatic β-cells, the only source of insulin in the body, can be specifically targeted using streptozotocin, which is toxic for those cells. However, high concentrations are needed for introducing hyperglycemia with only a small margin for error, either resulting in killing all pancreatic β-cells or not killing enough to induce hyperglycemia. The cytoprotective substance nicotinamide protects pancreatic β-cells and allows for a more reliable method to produce hyperglycemia. (150)

In animal models, hyperglycemia exacerbates stroke prognosis and outcome. (151) It has been demonstrated that hyperglycemia enhances lactic acid production after ischemic stroke, which increases the damage of the affected zone. During ischemia, the pyruvate oxidation is blocked or slowed, leading to the reduction of pyruvate to lactate. Due to the high glucose concentrations due to hyperglycemia and the lack of oxygen, large quantities of lactate and hydrogen molecules are produced. This, in turn, will lower the pH and promote edema formation. (152) Our study confirmed that ischemic rats who suffer from hyperglycemia have an increased lesion size, impairment of brain repair processes and increased inflammatory response. Furthermore, vessel wall thickness is increased in non-treated hyperglycemic animals. An increase in thickness could be the result of extra stress on the vessel caused by hyperglycemia, impairing the ability of the vessel to relax. (153) This study´s results are consistent with previously published reports; animals who suffer from hyperglycemia have less neurological recovery and larger infarct size than normoglycemic animals. (151,154–157) Furthermore, this study observed enhanced diffusion coefficients in hyperglycemic animals. At 30 days post-stroke, increased rADC values are associated with apoptosis, liquefactive necrosis, shrinkage of the brain, and cell membrane integrity loss. (142) All in all, this suggests that high rADC values less anatomical tissue preservation, although this study did not observe any effect on the total number of surviving neurons in the penumbra.

Regarding the treatment, the impact of hyperglycemia in combination with AD-MSC treatment on stroke recovery has not previously been studied in rats. This study demonstrated that hADMSC treatment promotes functional recovery, although lesion size was not reduced, compared to hyperglycemic animals without treatment. These results align with previously published studies that report stem cell therapy in diabetes type II rats post-stroke. (158–160) Furthermore, rADC values were significantly lower in hyperglycemic animals with hADMSC treatment than without. Lower rADC values are related to the increased repair of axon and myelin and reduced nerve cell damage after 30 days. (142) That rADC values are correlated with increased tissue preservation is supported by the fact that hADMSC treated rats had a much smaller reduction of multipolar motor neurons in the cortex than non-treated hyperglycemic rats. This supports the hypothesis that hADMSC treatment has protective effects on neuronal cells and increases tissue preservation after stroke.

It has been well established that the ischemic stroke cascade activates astrocytes, which has a detrimental effect on the brain in the acute phase of stroke while having a protective role in the chronic phase. (161,162) Diabetic rats have been shown to have inhibited astrocyte activation in the acute phase of stroke compared to non-diabetic animals. (163) Furthermore, this present study observed that GFAP levels were increased in hyperglycemic stroke rats in the chronic phase of stroke. However, hADMSC treatment reduced GFAP levels after administration in accordance with previous studies. (161,164)

Inflammation plays a crucial role in post-stroke recovery. Mild to moderate inflammatory response can benefit brain repair. However, severe inflammation can inhibit recovery by creating an inhospitable environment for brain repair. (157) In this present study, hyperglycemia was associated with an increase of microglia markers after stroke, confirming previously published results. (165) Furthermore, AD-MSC treatment attenuates the microglial response and decreases it compared to non-treated hyperglycemic animals.

To study neurogenesis and synaptogenesis in hyperglycemic animals post-stroke, doublecortin and synaptophysin expression were measured in the periinfarct zone. In the past, it was demonstrated that neural stem/progenitor cells (NSPCs) proliferation is suppressed in the hippocampus by long-term hyperglycemia. (166–168) However, several studies report enhanced proliferation of NSPCs, although their survival is impaired. (169,170) Furthermore, the severity of hyperglycemia is positively correlated with ischemic injury and inhibits subventricular neurogenesis. (171) Also, loss of synaptic structures in the injured area was observed in hyperglycemic animals after stroke compared to controls. (172) However, this study could not detect any effects on synaptogenesis or neurogenesis caused by hyperglycemia. This could be explained by the location and length of the study. Furthermore, differences in used techniques for inducing stroke and hyperglycemia might explain any discrepancies with other studies. Stem cell treatment efficacy in diabetic rats has been shown to increase doublecortin levels after stroke. (159) However, our results do not show increased doublecortin levels 6 weeks after stroke and are similar for synaptophysin, where hyperglycemic animals, with or without stem cell treatment, have similar post-stroke levels. (173)

It is well established that diabetes, or more specifically hyperglycemia, plays a significant role in vascular complications. Therefore, the expression of three vascular markers was evaluated (α-SMA, CD31 and Collagen-IV) 6 weeks after stroke. Several studies have demonstrated that in transient stroke, blood-brain barrier disruption and neurovascular damage are increased by reperfusion. (174–177) Following these studies, our results show that hyperglycemic rats have significantly increased arterial wall thickness compared to non-hyperglycemic rats. Even so, other markers such as CD31 and Collagen-IV were unaffected by hyperglycemia. This discrepancy might be explained by the fact that vascular smooth muscle cells lose the ability to relax in hyperglycemic conditions. (153) This would explain an increase in vessel wall thickness because contraction of the vascular smooth muscle cells would decrease the circumference of the vessel. Furthermore, a difference in methodology might cause

discrepancies between this data and previously published literature. The MCAO surgery used in these experiments was permanent to mimic the largest patient group who do not recanalize, while previous literature used transient MCAO. This might increase neurovascular injury by reperfusion. Comparing treated against nontreated hyperglycemic rats, no differences between CD31 or Collagen-IV were observed. However, vessel wall thickness was significantly decreased.

Animal model of cerebral infarction associated with hypertension

To carry out this part of the study, we used the spontaneous hypertensive rat, a well-established animal model and widely used in hypertensive research. (178) Furthermore, the SHR develops at the same pace as its normotensive control. Also, blood pressure gradually increases with age. (179)

Various preclinical studies have proven the efficacy of ADMSC by improving functional recovery and enhancing endogenous repair mechanisms in normotensive stroke animals. (72,95,180) and their safety [202]. However, this is concerning healthy and relatively young animals, while most stroke patients suffer from comorbidities as hypertension. (181) Despite the STEPS (18,182) and STAIR (183) guidelines, few articles study the effect of hypertension on stroke. (107)

In our study, lesion volume and rADC values were aggravated in hypertensive animals after stroke and also functional recovery was impaired compared to normotensive animals. An increase of rADC values is possibly correlated with an increase in water content in the lesion, previously reported in SHR. (184) Higher water content might indicate higher structural loss and non-salvageable tissue, which might explain why hADMSC did not improve stroke outcomes in hypertensive rats. This study indicates that hADMSC is not able to provide beneficial effects for hypertensive animals after ischemic stroke. Previous studies have shown that hypertension negatively influences stroke therapies by decreasing their effectiveness. (185,186) It has been established that bone marrow-derived mononuclear cells (BMMNC)

administration after transient middle cerebral artery occlusion in hypertensive rats is not effective in improving functional outcome. (112) Consistent with this finding, in our study, hADMSC administration has not exhibited effectiveness in improving functional outcome or reducing the lesion size.

It has been reported that astrocytes are activated in response to central nervous system injury, and the reactive astrocytes play essential roles in neuronal survival and function. (187) Astrogliosis has been related to the cognitive impairment of the SHR model. (121) Our results suggest an increase in GFAP expression in hypertensive rats than normotensive rats, as previously demonstrated by another group. (188) In this case, hypertensive rats with an ischemic stroke display a higher degree of astrocytic activation than the normotensive rats. (189) However, BM-MNC treatment in hypertensive animals does not show significant differences in GFAP-immunoreactivity than the control. (112) This is consistent with our results where hADMSC treatment did not affect GFAP expression.

After stroke, mechanisms of endogenous cerebral protection and repair are activated, such as neurogenesis, synaptogenesis, angiogenesis or remyelination, which contribute to the repair of the neurovascular unit. Previous studies have demonstrated that MSC administration potentiates brain plasticity markers after stroke in normotensive animals. (86) Our results demonstrate that in SHR animals, neurogenesis is not enhanced by cell therapy following stroke. In this sense, the therapeutic modulation of neurogenesis may be limited by hypertension, resulting in reduced effectiveness. Therefore, a potential explanation might be an exhausted neurogenic reserve (190), as suggested for neurodegenerative diseases. (191)

Hypertension participates in the origin and development of stroke, contributing to the proliferation of endothelial cells and smooth muscle cell hypertrophy, aggravating intracerebral vasculopathy. (192) Our findings also suggest an influence of hypertension in the expression of vascular markers (CD31, α-SMA). The changes in the vessels alter blood flow and have been linked to an increase in the

lesion size of SHR animals. (121) In our study, hypertension increased rADC values, which is related to an increase in arterial wall thickness compared to normotensive animals, as previously reported. (121,192) Regarding cell therapy, many studies have used MSC as a treatment after stroke and have shown an increase in angiogenesis. (193) Conversely, our results did not demonstrate any beneficial effects of hADMSC treatment following pMCAO in SHR rats on the vascular markers analyzed.

Hypertension and hyperglycemia

With the results obtained in our study, we observed that rats with hyperglycemic or hypertension ischemic stroke exhibit an increased lesion size and impaired brain repair processes, which likely contribute to the exacerbated behavioral impairments. In hyperglycemic rats, the hADMSC treatment improved anatomical tissue preservation with decreased inflammatory response and the arterial wall thickness, together with an improved behavioral outcome. However, the administration of hADMSC did not reduce lesion volume or functional deficits and did not affect gliosis, neurogenesis, or vascular marker levels in hypertensive rats. The results suggest a negative impact of hypertension on the therapeutic effect of hADMSC after an ischemic stroke.

Stem cell therapy is not effective in restoring glucose or blood pressure levels to normal. The route of action is also the same, so it might be in the difference of damages. Hypertension damages the vessels long term. However, the stroke does not seem to be much stronger than in hyperglycemia. Seeing the relationship between the brain and the spleen in the regulation of blood pressure, (194), hypertension can prime the immune response of the spleen. (195) It would be worth investigating if hypertension causes an increased splenic response after ischemic stroke than hyperglycemic stroke or if splenectomy might make the hypertensive animal model susceptible to stem cell treatment.

To investigate whether I.V. stem cell therapy had any effect on the comorbidity, blood pressure and glucose levels were measured throughout the study. Both glucose levels

and blood pressure remained stable throughout the study. No differences were seen between vehicle and treated hypertensive animals, indicating that any observed effects were solely due to the stem cell treatment and not due to the solvent or injection itself. Stem cell therapy over time does not significantly alter blood pressure or glucose levels. Although not controlled for in the study design, the administration of a liquid might increase blood pressure. However, this seems unlikely to happen from a single injection. This is confirmed by a study that observed blood pressure increases only after the 8th day of daily repeated I.V. injections of saline, but not before. (196)

Future perspectives

The previously discussed results clearly show an essential role for comorbidities in preclinical stroke research. The translational success rate from bench to clinic might be increased by including comorbidities in preclinical stroke research. The animal models used in this research are based on patient conditions, hyperglycemia and hypertension. However, the rats did not receive the same treatment as patients. Most patients with diagnosed high blood pressure are treated for this, so most likely, the BBB will be less affected than in a rat who has been with untreated high blood pressure during its entire life span. (197) The hyperglycemic animal model is only started several days before pMCAO surgery. Therefore, it more accurately represents the clinical reality. (198) Two-thirds of patients with impaired glucose tolerance and previously unrecognized diabetes stroke had persistent hyperglycemia 3 months after ischemic stroke. (199) However, as with hypertensive patients, hyperglycemia will be treated in patients.

An important recommendation from the STEPS guidelines is to use more animals with permanent MCAO, whereas transient MCAO is currently the most used stroke model. (18,200) Most stroke patients do not recanalize. (200,201) Therefore, the permanent MCAO model reflects better the patient population.

As in most research, preclinical research often uses surrogate endpoints. This is partly done from an ethical perspective, not letting the animals suffer unnecessarily, and also from a financial perspective. (202) Using biomarkers as a surrogate endpoint has several advantages and disadvantages. It can be used to determine disease severity, progression and response to therapy. Furthermore, biomarkers could be used for predicting prognosis. However, the use of biomarkers as a surrogate endpoint is often poorly validated. In other words, the evidence that improvement of the surrogate endpoint leads to improvement of the target outcome has not always been demonstrated. (202) Long term outcome of hyperglycemia and hypertension after stroke and the effect of stem cell therapy is not yet known. (203)

Regarding cryopreservation, there is an ongoing debate on whether cell cryopreservation, being mandatory in a clinical scenario, compromises the cell's therapeutic impact. (204) It seems that the cryopreservation of cells might play a role. (113) There are strong indications that cryopreservation alters stem cell viability and subpopulation composition. (205) Although the current hyperglycemia study indicates the efficacy of stem cells, it does not explore the possibility that fresh stem cells might outperform cryopreserved stem cells. Also, while fresh cells might perform better than frozen cells, this might be highly impractical in the clinic since studies indicate that stem cell therapy shows greater efficacy, and faster administration takes place after stroke. Current freezing methods might be further optimized, and more emphasis might be given to the protocol. (206) Therefore, excluding as a parameter of influence on stem cell therapy efficiency.

Investigation of the role of various comorbidities and how they influence stroke outcome is of vital importance. These two studies report widely different outcomes depending on the comorbidity. Currently, many clinical trials do not account for different comorbidities. Also, many clinical trials have not published any results. The mismatch between preclinical and clinical results obtained from stem cell therapy might at least in part lie in differences between comorbidities. Although true, the used

animal models here do not reflect (at least for hypertension) clinical reality since hypertension is often treated. Follow-up studies would need to address this.

Conclusions

In an experimental rat model of cerebral infarction by permanent middle cerebral artery occlusion associated with comorbidities, we observed that:

1- Hyperglycemia and hypertension contribute to impaired functional recovery, an increased lesion size, and impaired brain repair processes after ischemic stroke.

2- hADMSC therapy:

- in hyperglycemic rats, improves functional recovery, does not reduce lesion volume but improves anatomical tissue preservation.
- in hypertensive rats, does not reduce functional deficits and does not reduce lesion volume or improves anatomical tissue preservation.

3- hADMSC therapy:

- in hyperglycemic rats, reduces the expression of markers implicated in the inflammatory response (GFAP, Iba-1). Markers of neurogenesis (DCX) and synaptogenesis (SYP) were not affected. Furthermore, vascular markers (CD31, COL-IV) did not improve. However, vessel wall thickness significantly reduced (α-SMA).
- in hypertensive rats, had no effect on inflammatory markers (GFAP); nor on neurogenesis (DCX), nor on vascular markers (CD31 and α-SMA).

Conclusiones

En un modelo animal experimental en rata por oclusión permanente de la arteria cerebral media asociado a comorbilidades, observamos que:

1- La hiperglucemia y la hipertensión contribuyen a una peor recuperación funcional, un aumento del tamaño de lesión y a un deterioro de los procesos de reparación cerebral tras un ictus isquémico.

2- La terapia con hADMSC:

- en ratas hiperglucémicas, mejora la recuperación funcional, no reduce el tamaño de lesión, sin embargo,mejora la preservación del tejido.
- en ratas hipertensas, no reduce los deficit funcionales, el tamaño de lesión, ni mejora la preservación del tejido.

3- La terapia con hADMSC:

- en ratas hiperglucémicas, reduce la expresión de marcadores implicados en la respuesta inflamatoria (GFAP, Iba-1). Los marcadores de neurogenesis (DCX) y sinaptogénesis (SYP) no se vieron afectados. Además, los marcadores vasculares (CD31, COL-IV) no mejoraron. Sin embargo, se redujo significativamente el grosor de la pared de los vasos (α-SMA).

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Appendix

This thesis has resulted in the following publications:

Laso-García, F., Diekhorst, L., Gómez-de Frutos, M. C., Otero-Ortega, L., Fuentes, B., Ruiz-Ares, G., ... & Gutiérrez-Fernández, M. (2019). Cell-based therapies for stroke: promising solution or dead end? Mesenchymal stem cells and comorbidities in preclinical stroke research. *Frontiers in neurology*, *10*, 332.

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REVIEW
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Cell-Based Therapies for Stroke: Promising Solution or Dead End? Mesenchymal Stem Cells and Comorbidities in Preclinical Stroke Research

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Stroke is a major health problem worldwide. It has been estimated that 90% of the population attributable risk of stroke is due to risk factors such as aging, hypertension, hyperglycemia, diabetes mellitus and obesity, among others. However, most animal models of stroke use predominantly healthy and young animals. These models ignore the main comorbidities associated with cerebrovascular disease, which could be one explanation for the unsuccessful bench-to-bedside translation of protective and regenerative strategies by not taking the patient's situation into account. This lack of success makes it important to incorporate comorbidities into animal models of stroke in order to study the effects of the various therapeutic strategies tested. Regarding cell therapy, the administration of stem cells in the acute and chronic phases has been shown to be safe and effective in experimental animal models of stroke. This review aims to show the results of studies with promising new therapeutic strategies such as mesenchymal stem cells, which are being tested in preclinical models of stroke associated with comorbidities and in elderly animals.

Keywords: aging, hypertension, diabetes, hyperglycemia, obesity, comorbidity, mesenchymal, stroke

INTRODUCTION

Stroke is still the most common cause of permanent disability in adults and the second leading cause of death in the world (1). The pathology of stroke is poorly understood; however, it has been shown that the majority of patients with stroke have at least one comorbidity (2). The contribution of various risk factors to worldwide stroke burden is unknown. The INTERSTROKE study has demonstrated that five risk factors accounted for more than 80% of the global risk for all strokes (either ischemic stroke or intracerebral hemorrhage [ICH]): hypertension, current smoking, abdominal obesity, diet and physical activity (3). Furthermore, stroke incidence rises with increased in age (4). This high prevalence of comorbidities in stroke patients indicates the need for therapies in preclinical studies that take these comorbidities into account (**Figure 1**).

Of 502 experimental therapies for acute focal ischemic stroke, only 10% were tested in animals with hypertension. Hypertensive animals have larger infarct sizes and reduced efficacy with

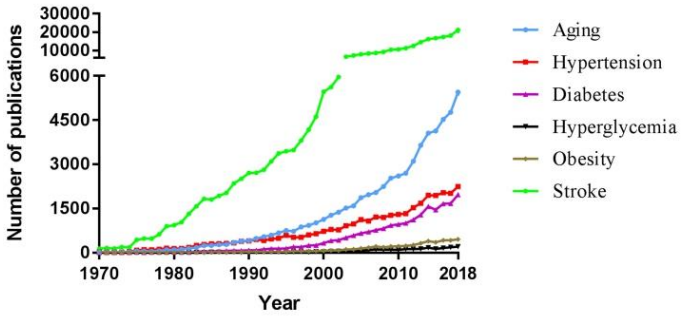

FIGURE 1 | Comparative evolution of the evaluation of the effect of various comorbidities in animal models of stroke as well as in clinical research. An advanced search was performed in PubMed in December 2018 to find, for each year of publication, all articles using the text word stroke with the given term as text word: age or aging; hypertension or hypertensive or high blood pressure; diabetes or diabetes mellitus; hyperglycemia or hyperglycemic or high blood sugar or high glucose levels; or obesity or overweight. Year of final publication (and not advanced online date) of articles in English (and not other languages) was taken into account. Manual elimination of articles describing a non-comorbid stroke association were excluded.

therapeutic intervention (5, 6). Even fewer preclinical studies assess the effects of diabetes or acute hyperglycemia on the response to therapeutic intervention (7). The majority of preclinical studies for novel therapies use young healthy animal models and this may play a role in the fact that of 1,026 treatments tested on animal models, only one has been effective in clinical trials (8).

In particular, stem cell therapy has been proven to be effective mostly in healthy animals. Various types of stem cells have been used in preclinical stroke models: embryonic stem cells, neural stem cells, induced pluripotent stem cells, mesenchymal stem cells (MSCs) and hematopoietic stem cells (9). Cell therapy has been shown to promote functional recovery, participating in processes such as immunomodulation, neurogenesis, synaptogenesis, oligodendrogenesis, axonal connectivity, and myelin formation, improvement in blood brain barrier (BBB) integrity, neovascularization and reduced lesion size, showing efficacy not only in grey matter, but also white matter injury (10–17). However, its mechanisms of action has not yet been clarified. Recent evidence has suggested that it might be related to long-distance cell-to-cell communication by paracrine function through secretory factors in the extracellular environment. Intercellular communication between stem cells and the damaged organ was thought to be regulated via the release of free molecules that transmit the signal by binding to a receptor. These molecules could in part be trophic factors, inflammation modulators and even exosomes. In order to avoid previous translation failure in stem cell therapy, STAIR guidelines suggest that further studies should be performed on animals with comorbid conditions such as hypertension and diabetes in order to improve the quality of preclinical studies of purported stroke therapies (18).

This review is focused on MSC therapies being tested in preclinical models of stroke with the most common comorbidities (hypertension, hyperglycemia, diabetes, obesity), as well as in elderly animals. We intend to provide insight into the viability of this new strategy, which could lead to an improved translation of cell therapy from bench to bedside.

Abbreviations: ICH, intracerebral hemorrhage; MSCs, mesenchymal stem cells; BBB, blood-brain barrier; BMMNCs, bone marrow mononuclear cells; BMSCs, bone marrow stromal cells; G-CSF, granulocyte colony-stimulating factor; BMMSCs, bone marrow mesenchymal stem cells; SHRs, spontaneously hypertensive rats; SHR-SPs, stroke-prone SHRs; HUCBCs, human umbilical cord blood cells; DM-BMSCs, BMSCs derived from type I diabetes rats.

MESENCHYMAL STEM CELLS IN PRECLINICAL STUDIES

Aging in Stroke

Age is the most important risk factor for developing a stroke. Age has been proven to be a predictive factor for recovery after stroke, independent of stroke severity, characteristics and complications (19). Stroke incidence is also strongly but not solely correlated with an increase in age, in addition to the patient's general fitness (4). As previously stated, patient data shows a direct correlation between age and the occurrence of stroke. For these reasons, the impact of age should be considered carefully in preclinical studies given that the mechanisms of stroke and response to drugs can be very different in the developing, juvenile, adult and elderly brain (20). However, despite being the most important risk factor, there are currently few studies (only 7) on aged animals that evaluate the effect of administering MSC after ischemic stroke (**Table 1**). In this regard, systemic administration of bone marrow mononuclear cells (BMMNCs) in the acute phase after stroke reduced neurological deficits (21, 22) and reduced infarct volume, modulating post-ischemic inflammatory cytokines within the brain in older rats (21). Also in the acute phase, intravenous administration of human umbilical tissue-derived cells improved recovery of neurological function in aged rats after stroke and was associated with activation of repair processes (23). This beneficial effect of cell therapy is not only observed in the short

term, but also in the long term. In one study, intra-arterial administration of bone marrow stromal cells (BMSCs) at 1 day after ischemic stroke had long-lasting beneficial effects on recovery of neurological functional (24). One interesting strategy for reducing ischemic damage to the brain would be based on a combination of therapies to act in different steps of the ischemic cascade. In this sense, the combination the granulocyte colony-stimulating factor (G-CSF) with bone marrow mesenchymal stem cells (BMMSCs) increased neurogenesis and improved microvessel recovery and density in the aged brain (25). However, another study demonstrated that the combination of G-CSF and bone marrow mononuclear cells (BMMNCs) did not further improve post-stroke recovery (26).

Currently, all studies with MSCs in elderly animals have been performed on ischemic stroke, none on hemorrhagic stroke. Also, no standardized protocols were used in the above cell therapy studies for observing the various routes of administration and doses used. The number of studies is still very limited and further preclinical research is needed to determine the efficacy of MSCs in aged animal models. In view of the results, however, the aged brain retains the capacity for repair in response to cell therapy.

Hypertension in Stroke

Hypertension is considered one of the most common and important vascular risk factors for stroke (3) and is responsible for approximately 52% of strokes (5); it is also closely correlated with stroke severity. Hypertension has numerous effects, such as reducing BBB integrity and promoting white matter damage and post-stroke edema (5).

In order to test a new therapeutic strategy, a good experimental animal model must first be selected. Several models have been used to induce hypertension in animals. In the past, dogs were used in experiments as a hypertension model. Currently, the rat has become the common model for research as a cost-effective alternative. There are various ways to induce hypertension in rats; for example, spontaneously hypertensive rats (SHRs), stroke-prone SHRs (SHR-SPs), endocrine hypertension by deoxycorticosterone acetate administration, angiotensin II administration and hypertension induced by stress (47–49). Based on consistent reproducibility, the SHR is probably the best model with which to observe hypertension.

Regarding cell therapy, MSCs have been used in hypertensive rats to evaluate the efficacy of functional recovery in animals (**Table 1**).

In cerebral ischemia, intracerebral transplantation of BMMSCs has been shown to decrease apoptotic neurons in the neocortex and ameliorate brain damage by decreasing cell death (27). This has been shown to play a primary role in brain protection. BMMSCs also act on vasculogenesis in SHR rats. Thus, the cells significantly increased the number of microvessels and their reactivity to collagen IV in the neocortex, which indicates protection of the neurovascular unit and improvement of vascular integrity (27). In this regard, BMMSCs also increase of levels of the antiapoptotic B-cell lymphoma 2 (Bcl-2) gene and decrease superoxide, demonstrating that MSC has antioxidant potential and a protective effect in SHR rats with stroke (28). Also, intravenously administered dual transplantation of human maternal or fetal placenta MSCs produces increased density of glial fibrillary acidic protein-positive cells in the area adjacent to the infarct border which may increase survival rates of regenerative astrocytes, leading to a decrease in infarct volume on day 60, triggering functional improvement in SHRs (29). However, not all studies have reported favorable functional outcomes after ischemic stroke with hypertension SHRs. BMMNCs or cryopreserved human umbilical cord blood mononuclear cells given intravenously did not show a beneficial effect on infarct volume, behavioral outcomes or inflammatory response (30, 31). Pösel et al. performed a study to determine a possible synergistic effect of G-CSF and BMMNCs after stroke in SHR rats, in which they found administration of G-CSF improved long-term functional recovery. However, this effect was negated by cotransplantation of BMMNCs as provoked splenic accumulation of granulocytes and transplanted cells, accompanied by a significant rise in circulating granulocytes and infiltration in the ischemic brain, which was detrimental to stroke outcome (32).

In addition, the Framingham Study clearly demonstrated the relevance of age and high blood pressure for lifetime risk of stroke (50), indicating the need to mimic these risk factors in preclinical stroke studies. Along these lines, animals transplanted with intravenous bone marrow cells from young SHR-SPs displayed an increase in microvasculature density in the peri-infarction zone which led to reduced ischemic brain damage and improved neurological function (33). However, in the same study BM cells led to a significant increase in levels of cytokines such as interleukin 1β (IL-1β) and monocyte chemoattractant protein 1(MCP-1) in the brain and a decrease of IL-6 levels in serum. These results suggest that modulation in the expression of inflammatory cytokines (i.e., favoring recovery/decreased inflammatory profile) did not occur and, therefore, is not likely to explain the beneficial effect of the response to cerebral ischemia observed in older SHR-SPs transplanted with BM cells from young SHR-SPs (33). Additionally, further studies reported negative results, such as the study by Wagner et al. which evaluated the therapeutic efficacy of intravenously transplanted young and aged BMMNCs in aged hypertensive rats. The authors concluded that BMMNCs from both juvenile and elderly donors failed to decrease lesion volume and functional recovery was not improved (34).

There are currently two studies in SHRs with ICH. In both studies, intravenously (35) or intracerebrally (36) transplanted BMMSCs improved neurological function and integrity of the BBB by preventing extravasation of blood through the endothelium (35, 36), resulting in improvements such as reduced brain edema and decreased cell apoptosis (36).

Although several different doses and administration routes have been used for treatment in hypertensive animals post-stroke, contradictory results have been found between different research groups using MSCs in hypertensive animals (**Table 1**). More studies should be performed to evaluate whether MSC therapy is effective not only in brain protection, but also in brain repair in the treatment of stroke in hypertensive animals.

TABLE 1 | Original studies evaluating the effect of MSC administration in ischemic stroke models using aged animals, hypertension- and diabetes-induced stroke models.

References	Species	Stroke type	Cell type	N° Cells	Administration Route	Recovery	Comorbidity
Brenneman et al. (21)	Long Evans	MCAO	BMMNC	4×10^6	Intra-arterial	Improved	Aging
Coelho et al. (22)	Wistar	Focal cortical ischemia	BMMNC	3×10^7	Intravenous	Improved	Aging
Zhang et al. (23)	Wistar	MCAO	HUTC	1×10^7/kg	Intravenous	Improved	Aging
Shen et al. (24)	Wistar	MCAO	BMSC	2×10^6	Intra-arterial	Improved	Aging
Balseanu et al. (25)	Sprague-Dawley	MCAO	G-CSF + BMMSC	50 μg/kg + 1×10^6/kg	Intravenous	Improved	Aging
Buga et al. (26)	Sprague-Dawley	MCAO	G-CSF + BMMNC	50μg/kg + 1×10^6/kg	Intravenous	Improved	Aging
Ito et al. (27)	SHR	Stroke prone	BMSC	5×10^5	Intracranial	Not evaluated	Hypertension
Calió et al. (28)	SHR	Stroke prone	BMMSC	1×10^6	Intracranial	Not evaluated	Hypertension
Kranz et al. (29)	SHR	MCAO	MSC from maternal or fetal placenta	1×10^6	Intravenous	Improved	Hypertension
Minnerup et al. (30)	SHR	MCAO	BMMNC	$1/5/20 \times 10^6$	Intravenous	Did not improve	Hypertension
Weise et al. (31)	SHR	MCAO	HUCBMNC	8×10^6/kg	Intravenous	Did not improve	Hypertension
Pösel et al. (32)	SHR	MCAO	G-CSF + BMMNC	50 μg/kg + 1.5×10^7/kg	Intravenous	Did not improve	Hypertension
Taguchi et al. (33)	SHR	Focal cortical ischemia	BMMNC	5×10^5	Intravenous and intraosseous	Improved	Hypertension
Wagner et al. (34)	SHR	MCAO	BMMNC	8×10^6/kg	Intravenous	Did not improve	Hypertension
Wang et al. (35)	SHR	Intracerebral hemorrhage	BMMSC	1×10^6	Intravenous	Improved	Hypertension
Ding et al. (36)	SHR	Intracerebral hemorrhage	BMSC	1×10^6	Intracranial	Improved	Hypertension
Yan et al. (37)	Wistar	MCAO	HUCBC	5×10^6	Intravenous	Improved	Diabetes Type I
Cui et al. (38)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type I
Chen et al. (39)	Wistar	MCAO	BMSC	3×10^6	Intravenous	Did not improve	Diabetes Type I
Yan et al, (40)	Wistar	MCAO	BMSC + Niaspan	5×10^6 + 40 mg/kg	Intravenous	Did not improve	Diabetes Type I
Ye et al. (41)	Wistar	MCAO	BMSC + Niaspan	5×10^6 + 40 mg/kg	Intravenous	Not evaluated	Diabetes Type I
Yan et al. (42)	Wistar	MCAO	HUCBC	5×10^6	Intravenous	Improved	Diabetes Type II
Ding et al. (43)	Wistar	MCAO	BMSC	5×10^7	Intravenous	Improved	Diabetes Type II
Hu et al. (44)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type II
Xiang et al. (45)	Wistar	MCAO	BMSC-CM	10 ml/kg	Intravenous	Improved	Diabetes Type II
Yan et al. (46)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type II

MCAO, middle cerebral artery occlusion; BMMNC, bone marrow mononuclear cell; HUTC, human umbilical tissue-derived cell; BMSC, bone marrow stromal cell; G-CSF, granulocyte colony-stimulating factor; BMMSC, bone marrow mesenchymal stem cell; SHR, spontaneously hypertensive rats; G-CSF, granulocyte colony-stimulating factor; HUCBMNC, human umbilical cord blood mononuclear cell; HUCBC, human umbilical cord blood cell; BMSC-CM, bone marrow stromal cell conditioned medium.

Diabetes in Stroke

Diabetes is divided into two types: type 1, in which the beta-cells of the pancreas are damaged and affected people need external administration of insulin; and type 2, which is peripheral insulin resistance, and is present in 85% of the patients with diabetes (51, 52). Diabetes causes several metabolic and pathological changes that lead to stroke including arterial stiffness, systematic inflammation, endothelial dysfunction and heart failure (53). In addition, stroke in diabetes patients increases hospital mortality (54).

There are many methods to try to mimic type 1 and 2 diabetes in rats (55). One of the most commonly used models for diabetes type 1 are the Biobreeding rats; these rats develop diabetes spontaneously or it is induced by a virus (55). Recently, however, injection of chemicals to destroy beta-cells in the islets of Langerhans has been growing in importance. Alloxan has been used for some time as a good diabetic model but it has problems such as spontaneous recovery from the diabetic condition or renal toxicity. To solve this, another beta-cytotoxic agent, streptozotocin, is used. One advantage of this chemical is that the damage is dose-dependent, which allows researchers to control the severity of that animals' hyperglycemia (56).

To replicate type 2 diabetes, various spontaneous models are used in laboratories such as the spontaneously diabetic tori rat (57), due to gradual beta-cell degeneration, or Goto-Kakizaki rats, which develop peripheral insulin resistance after 56 days (58). Another option is induction by a high fat diet and intraperitoneal streptozotocin administration, in which the rats develop hyperinsulinemia, obesity and a reduction in beta-cells (59). In addition, administration of nicotinamide intraperitoneally prior to administration of a low dose of

streptozotocin protects the cells by attenuating the effect of streptozotocin (60).

Regarding MSC therapy, human umbilical cord blood cells (HUCBCs) and BMSCs have been shown to contribute to an increase in phosphorylated neurofilament marker SMI-31 and synaptophysin expression. These markers are involved in axonal and synaptic plasticity that promotes white matter remodeling in the ischemic brain (37, 38, 41). Vascular remodeling was revealed by an increase in the expression of smooth muscle actin (α-SMA) and Von Willebrand Factor (vWF) (37) in the ischemic brain which led to an improvement in functional outcomes (37, 38). In addition, BMSCs decreased miR-145 expression, which reduces endothelial cell proliferation, contributing to increased functional cells and restorative effects in type 1 diabetic rats (38). However, contradictory results have been reported by other authors. BMSC treatment by tail vein starting 24h after middle cerebral artery occlusion in diabetes type 1 rats resulted in increased brain hemorrhage, BBB leakage and higher expression of angiogenin. This causes accelerated cerebral arteriosclerosis and prevents improvement in functional outcomes (39).

In subsequent studies, however, the harmful effects of BMSC administration were negated when the treatment was administered in combination with Niaspan. Despite the combination, BMSC and Niaspan treatment for stroke did not improve functional outcomes. However, it did decrease BBB leakage and atherosclerotic-like changes (40) and promoted white matter remodeling in type 1 diabetes rats after stroke (41) (**Table 1**).

Regarding type 2 diabetes, all experimental animal studies have shown a beneficial effect of MSCs. Independent of treatment with HUCBCs, BMSCs or bone marrow stromal cell-conditioned medium initiated at 24 h or 3 days after stroke via intravenous administration improved functional recovery, promoted restorative effects and reduced BBB disruption after stroke in type 2 diabetes rats (42–46) (**Table 1**). As in type 1 diabetes, MSC therapy is also associated with white matter remodeling in type 2 diabetes (42, 46), participating in axonal regeneration, sprout and remyelination which led to improved long-term functional outcomes (46). MSC therapy after stroke also contributes to vascular remodeling in type 2 diabetes. Specifically, BMSC-CM treatment enhanced expression of angiopoietin 1 (Ang1), tyrosine-protein kinase receptor Tie-2 (45), α-SMA, and vWF (39, 43), which indicates higher cerebral artery and vascular density (42). Ang1 also seems to be related with a reduction in BBB leakage and promotes vascular stabilization in the ischemic brain. Moreover, it plays a role in white matter remodeling (37, 41), which may improve functional outcome. Regarding the immune system, several authors defend the idea that MSC treatment can also regulate pro-inflammatory factors. This has been shown by a decrease in expression of the receptor for advanced glycation end-products (RAGE) after HUCBC (42) and BMSC (44) treatment in diabetic rats. This indicates a decrease in inflammation, neuronal death, vascular injury and brain damage following ischemia in type 2 diabetic rats. HUCBC and BMSC treatment of type 2 diabetic stroke rats also had an effect on macrophage polarization, promoting decreases inflammation (42, 46), decreased the expression of the proinflammatory protein toll-like receptor 4 (TLR4) (42), increasing brain platelet-derived growth factor (PDGF) expression in the ischemic brain, contributing to restoration (46) and promoting functional improvement after stroke (42).

In summary, with regard to type 1 diabetes, although the experimental studies are homogeneous in terms of the route of administration, cell dose and stroke location, the results are contradictory with no good functional recovery observed in any of them. This reveals the need for further research to increase understanding of the interaction between type 1 diabetes mellitus and cell-based therapy with MSCs. Although all the studies performed thus far reveal that treatment of type 2 diabetes with MSCs in ischemic stroke models can be successful, to our knowledge no studies have been conducted to test the efficacy of MSCs in hemorrhagic stroke. The meager interest aroused could be due to the fact that the prevalence of diabetes is higher in patients with ischemic compared with hemorrhagic stroke, as recently demonstrated by a meta-analysis (61).

Hyperglycemia in Stroke

Hyperglycemia plays an important role in stroke and is associated with poorer functional recovery and an increase in mortality in ischemic and hemorrhagic stroke (62, 63). It has been observed that after stroke, hyperglycemia is present in over 50% of patients (64). Given the high percentage of patients who develop post-stroke hyperglycemia, it is important to conduct research in hyperglycemic animal models. Two options have been used to reproduce hyperglycemic situations in preclinical stroke models, depending on the researchers' requirements: acute hyperglycemia after anesthesia can be mimicked in animals with intravenous infusion of glucose (65); or intravenous administration of streptozotocin and continuous administration of insulin, in which the animals develop hyperglycemia after 1 week (66). To our knowledge, no data have been published on how MSC-based therapy affects stroke associated with hyperglycemia.

Obesity in Stroke

Obesity, especially in the abdominal zone (67), is an important risk factor at all ages. An increase in body mass index (BMI) significantly increases the risk of stroke. Compared with healthy weight individuals, the obese population has a 64% greater probability of experiencing an ischemic stroke (68). The association between BMI and ischemic stroke is linear, without differences between sex or race (69). Other studies also linked obesity with hemorrhage, in which people with a high BMI have a 37% higher incidence (70).

Various animal models have been established to induce obesity in rats (71). Yet despite the high prevalence of obesity in stroke, no research has been performed on how MSC therapy affects the pathology of stroke associated with obesity. In conclusion, studies should be conducted to show the interactions between obesity and MSC treatment after stroke.

Take Home Message

Most of the studies carried out on this subject indeed favor BMMSCs. However, compared to other cell types, adipose tissue-derived mesenchymal stem cells (ADMSCs) have

several advantages in clinical applications for neurological disorders (72). ADMSCs are derived from adipose tissue and thus are abundant, accessible and easy to obtain using lipoaspiration techniques. Moreover, they provide proliferation and differentiation potential (73) without adverse side effects (12, 74, 75) and they can be administered without ethical concerns. All of these advantages mean that ADMSCs present a great opportunity for the treatment of diseases such as comorbidities in stroke. Further studies should take this into consideration.

It should be emphasized that patients present not only one but also several associated comorbidities at a time. Therefore, it is clear that there is a need for multimodelling for successful translation of preclinical research to the clinic (6). In this sense, not only modifiable factors, but also non-modifiable risk factors such as age and sex, are important to include in animal model studies (6). Moreover, animal models with co-morbidities show higher variability in outcome measures and therefore, higher sample sizes should be estimated with the specific disease model in mind (6). Also, aged animals take longer to recover after stroke, but eventually recovered to the same degree as young mice, making clear the importance of implementing long-term studies (76).

Adequate selection of the experimental model for stroke and comorbidity induction is important to reduce mortality, as it is often higher in models with preexisting comorbid conditions. This strategy leads to decreased costs. Besides, outcome measures should be optimized and adequate for these studies as there is variability in outcomes compared to healthy animals (6).

CONCLUSION

The high prevalence of comorbidities in patients with stroke indicates the need for therapies in preclinical studies that take into account these comorbidities in order to avoid failures in translation to the patient. Preclinical studies are beginning to evaluate the efficacy of MSC treatment in stroke associated with comorbidities, especially hypertension, for ischemic and hemorrhagic stroke. Regarding aging and diabetes, only ischemic stroke studies have been performed. For the moment, few studies have been performed and contradictory results are being reported. These contradictory results may be due to the use of different stroke and comorbidity models, and to the use of different protocols for administering cell-based therapies. This situation indicates a further need to promote standardization of cell concentration and administration routes. Obesity and hyperglycemia have been completely ignored, although they are frequently present in patients with stroke. For this reason, the role of comorbidities should have a more prominent place in preclinical stroke studies. This will hopefully improve bench-to-bedside translation and identify viable therapeutic options.

AUTHOR CONTRIBUTIONS

FL-G and LD wrote the first draft of the manuscript. MG-dF and LO-O wrote sections of the manuscript. BF, GR-A, and ED-T contributed to manuscript revision and read and approved the submitted version. MG-F contributed to the conception of the study, wrote and revised the manuscript, and approved the submitted version.

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Intravenous delivery of adipose tissue-derived mesenchymal stem cells improves brain repair in hyperglycemic stroke rats

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Abstract

Background: Over 50% of acute stroke patients have hyperglycemia, which is associated with a poorer prognosis and outcome. Our aim was to investigate the impact of hyperglycemia on behavioral recovery and brain repair of delivered human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) in a rat model of permanent middle cerebral artery occlusion (pMCAO).

Methods: Hyperglycemia was induced in rats by the administration of nicotinamide and streptozotocin. The rats were then subjected to stroke by a pMCAO model. At 48 h post-stroke, 1×10^6 hAD-MSCs or saline were intravenously administered. We evaluated behavioral outcome, infarct size by MRI, and brain plasticity markers by immunohistochemistry (glial fibrillary acidic protein [GFAP], Iba-1, synaptophysin, doublecortin, CD-31, collagen-IV, and α-smooth muscle actin [α-SMA]).

Results: The hyperglycemic group exhibited more severe neurological deficits; lesion size and diffusion coefficient were larger compared with the non-hyperglycemic rats. GFAP, Iba-1, and α-SMA were increased in the hyperglycemic group. The hyperglycemic rats administered hAD-MSCs at 48 h after pMCAO had improved neurological impairment. Although T2-MRI did not show differences in lesion size between groups, the rADC values were lower in the treated group. Finally, the levels of GFAP, Iba-1, and arterial wall thickness were lower in the treated hyperglycemic group than in the nontreated hyperglycemic group at 6 weeks post-stroke.

Conclusions: Our data suggest that rats with hyperglycemic ischemic stroke exhibit increased lesion size and impaired brain repair processes, which lead to impairments in behavioral recovery after pMCAO. More importantly, hAD-MSC administration induced better anatomical tissue preservation, associated with a good behavioral outcome.

Keywords: Adipose tissue, Behavioral outcome, Brain repair, Experimental model, Hyperglycemia, Mesenchymal stem cells

Background

Stroke is a significant public health issue and is the most common cause of death and disability worldwide [1]. To date, only intravenous thrombolysis (tPA) and mechanical thrombectomy have been shown to be effective in the acute phase of ischemic stroke. However, the narrow therapeutic window (< 4.5 h for tPA and < 6–24 h for endovascular treatment [2, 3]) limits its application to a small percentage of patients. Cell therapy is an interesting and promising approach in stroke research. Adipose tissue-derived mesenchymal stem cells (AD-MSCs), among others, have been shown to improve functional recovery, with an increase in markers related to brain repair in experimental animal models of stroke [4, 5].

Hyperglycemia is present in a significant proportion of patients without diabetes [6]. Various studies have demonstrated that more than 50% of acute stroke patients

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present hyperglycemia on admission which predicts higher mortality and morbidity [7–9]. In experimental animal models, hyperglycemia in acute stroke is associated with a poor outcome, exacerbating processes involved in ischemic brain injury [10, 11]. Despite the high percentage of patients with comorbidities, most of the preclinical models are performed on healthy and young animals. Moreover, the effects of comorbidities including hyperglycemia on therapeutic effects are poorly studied [12], although this aspect is emphasized in The Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) recommendations [13, 14].

To our knowledge, this study is the first to investigate whether the MSC treatment could improve functional outcome post-stroke in rats, even in hyperglycemic conditions. Therefore, our aim was to assess the impact of hyperglycemia on infarct size and behavioral recovery in a rat model of permanent middle cerebral artery occlusion (pMCAO). We also sought to evaluate the effect of hyperglycemia on the therapeutic response to intravenous administration of human AD-MSCs (hAD-MSCs) in an experimental model of ischemic stroke.

Materials and methods

Ethics statement

The procedure was carried out at our Cerebrovascular and Neuroscience Research Laboratory, La Paz University Hospital, Madrid, Spain. All experiments were designed to minimize animal suffering of animals in compliance and approved by our medical school's Ethical Committee for the Care and Use of Animals in Research (Ref. PROEX 249/15) according to the Spanish (RD 1201/2005 and RD53/2013) and European Union (EU) (86/609/CEE, 2003/65/CE, 2010/63/EU) rules. Experiments were conducted according to the ARRIVE guidelines for reporting animal research in terms of randomization, blinding, and statistical power (https://www.nc3rs.org.uk/arrive-guidelines).

Cell culture protocol, characterization, and hAD-MSC isolation

The hAD-MSCs obtained from the French Blood Establishment (EFS, La Tronche, France) were cultured. The cells were thawed, expanded (using a seeding density of 2×10^3 viable cells/cm^2) on tissue culture flasks (Fisher Scientific), 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 °C. The phenotypic 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 were detected (Fig. 1a).

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

Animals, hyperglycemia induction, and surgery

Hyperglycemia was induced using nicotinamide-streptozotocin. Streptozotocin has a nonspecific action in the body that causes organ deterioration due to its cytotoxic action [15, 16]. Nicotinamide was used to prevent organ damage [15]. In the present study, hyperglycemia was induced by an intraperitoneal injection with nicotinamide (210 mg/kg) (EMD Millipore, Germany) followed 15 min later with an intraperitoneal injection of streptozotocin (60 mg/kg) (EMD Millipore, Germany). After 72 h and again at 6 weeks, blood glucose concentration was determined using a glucose meter (ACCU-CHEK, Performa, Germany), and the animals with a blood glucose concentration above 250 mg/dl were considered hyperglycemic [17].

A total of 57 male Sprague-Dawley rats (8–9 weeks old, weighing 200–250 g) were used for the study. The rats were anesthetized via intraperitoneal 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 perform the pMCAO, a small craniotomy was performed; the right middle cerebral artery (MCA) was permanently ligated just before its bifurcation and both common carotid arteries were then occluded for 60 min as previously described [18].

The rats were randomly assigned to the following groups: the sham group was subjected to surgery without infarction ($n = 10$); the non-hyperglycemic group was subjected to a pMCAO and received intravenous saline ($n = 10$); the nontreated hyperglycemic group was subjected to a pMCAO and received intravenous saline ($n = 10$); and the treated hyperglycemic group was subjected to a pMCAO and received intravenous hAD-MSCs ($n = 11$). Saline solution or 1×10^6 hAD-MSCs in 1 ml of saline solution was administered via the tail vein at 48 h after surgery. All the animals were euthanized at 6 weeks post-stroke (Fig. 1b).

Functional evaluation scales

Functional evaluations were performed on all the rats by a blind observer before surgery and at 1 week, 3 weeks, and 6 weeks post-stroke induction. Motor and sensory

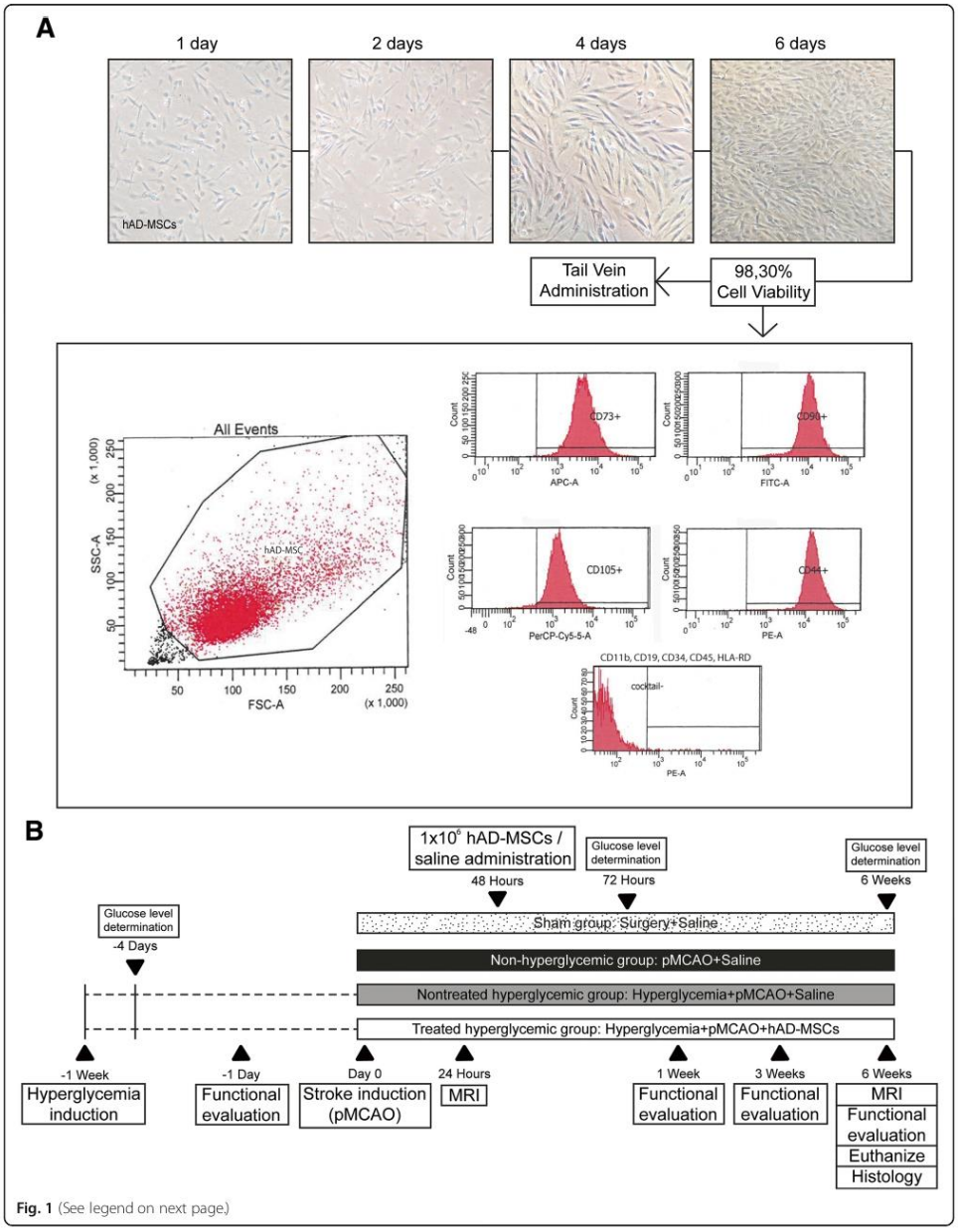

Fig. 1 (See legend on next page.)

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Fig. 1 a Human AD-MSC cell culture protocol. Human AD-MSCs were thawed and cultured for their expansion on tissue culture flasks for 6 days. The phenotypic 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 were detected. Cell viability was studied and the cells were prepared for intravenous administration. **b** Experimental animal protocol. One week before stroke induction, hyperglycemia was induced in the rats with two intraperitoneal injections (nicotinamide and 15 min later streptozotocin). After 72 h and 6 weeks, blood glucose concentrations were determined. Rats were subjected to a cortical stroke by permanent middle cerebral artery occlusion and divided into groups. Forty-eight hours after surgery, 1×10^6 hAD-MSCs or saline solution was administered. Functional evaluation was performed before surgery and at 1 week, 3 weeks, and 6 weeks post-stroke, and MRI was analyzed at 24 h and 6 weeks post-stroke. Six weeks post-stroke, the animals were euthanized and histological analyses were performed. Abbreviations: pMCAO: permanent middle cerebral artery occlusion; MRI: magnetic resonance imaging; hAD-MSCs: human adipose tissue-derived mesenchymal stem cells

performance was 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 extend forepaw fully; 2, decreased grip of forelimb while tail 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 [19]. The beam walking test evaluated hindlimb functions [20] by the capacity of the rats to traverse a wooden beam. We calculated the left hind limb slip ratio as follows: (total slips + 0.5 × half slips)/total steps × 100% [21]. To assess the forelimb sensory asymmetry, the adhesive removal test was performed [20]. For this test, a sticker was placed on the palm of both forelimbs of the rat and contact and removal times were recorded [22].

In vivo magnetic resonance imaging

Lesion size was analyzed at 24 h and 6 weeks post-stroke by magnetic resonance imaging (MRI) (Bruker Pharmascan, Ettlingen, Germany); 7-T horizontal bore magnets using T2-weighted (T2-W) spin-echo anatomical images acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial orientations and the following parameters: two echo images (TE, 29.54 ms and 88.61 ms); TR = 3000 ms; RARE factor = 4; Av = 3; FOV = 3.5 cm; acquisition matrix = 256 × 256 corresponding to an in-plane resolution of 137 × 137 μm^2; slice thickness = 1.00 mm without gap; and number of slices = 16.

We used diffusion-weighted imaging (DWI) including apparent diffusion coefficient (ADC) maps. Images were obtained with three different directions 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 ms; diffusion gradient separation, 18 ms; TR, 3000 ms; TE, 50 ms; FOV, 3.8 cm; axial slices (1.5 mm thickness) and 3 *b* values 100, 400, and 1000s/mm^2; 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 [23].

Hematoxylin and eosin (H&E) staining

The histopathological changes in the cortex were studied by H&E staining. Slices were immersed 10 s in hematoxylin and 1 min in eosin. Finally, they were dehydrated and coverslipped with DePex. The multipolar motor neurons were observed using a × 20 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 [24].

Immunohistochemistry and immunofluorescence

The perilesional area around the lesion core was defined on brain sections at 6 weeks post-stroke by microtubule-associated protein 2 (MAP-2) staining (1:1000, Millipore) and glial fibrillary acidic protein (GFAP) staining (1:500, Millipore) (Additional file 1: Figure S1). The samples were sectioned at 10 μm thickness using a Leica CM1950 cryostat (Leica). Immunohistochemistry images were obtained using a × 20 and × 40 objective lens, and processed by image analysis software (Image-Pro Plus 4.1, Media Cybernetics).

The perilesional area was studied in detail using immunofluorescence for astrocytes with GFAP (1:500, Millipore); microglia with Iba-1 (1:1000, Millipore); synaptic plasticity with synaptophysin (1:200, Sigma); neurons with doublecortin (1:250, Santa Cruz); endothelium with platelet endothelial cell adhesion molecule-1 (CD-31) (1:50, Abcam), collagen-IV (1:400, 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 (3 rats for each group, 4 sections in each rat per group).

Statistical analysis

The results were expressed as mean ± standard deviation (SD), and the data were compared using Kruskal–Wallis test followed by the Mann–Whitney *U* test as the data followed 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 statistical program 22 and GraphPad Prism 7 software. The rats removed from the study were immediately replaced by new subjects that were randomly allocated to the experimental groups until a total number of 10 rats per group was reached. The power analysis showed that with nonparametric testing for infarct size and behavioral tests, at least 10 rats needed to be randomized to each group for a significance level of 5% (alpha) and a power of 80% (1-beta).

Results

Mortality

Sixteen rats were excluded from the study: 13 died after surgical induction of permanent middle cerebral artery occlusion (pMCAO) (ten from the hyperglycemic group and three from the nonhyperglycemic group) and three were excluded because they did not show lesions on MRI analysis.

Hyperglycemia-associated stroke-induced impairment of motor function

The hyperglycemic group exhibited more severe neurological deficits, measured by the beam walking test at 1, 3, and 6 weeks post-stroke, compared with the non-hyperglycemic group ($p < 0.05$). The Rogers and adhesive removal tests showed no significant differences between the non-hyperglycemic and the hyperglycemic groups at all study times ($p > 0.05$) (Fig. 2a, c).

hAD-MSC treatment significantly improved post-stroke neurological outcome in hyperglycemic rats

hAD-MSC treatment after pMCAO in hyperglycemic rats significantly improved neurological recovery compared with the nontreated hyperglycemic group, as indicated by the Rogers (at 3 and 6 weeks) and walking beam (at 1, 3, and 6 weeks) tests ($p < 0.05$).

The adhesive removal test showed no significant differences between the nontreated hyperglycemic and the treated hyperglycemic groups ($p > 0.05$) (Fig. 2b, d).

There were no significant differences in blood glucose level between non-treated hyperglycemic rats (before MCAO 351.75 ± 73.35 mg/dl; before euthanasia 445.0 ± 42.42 mg/dl) and treated hyperglycemic rats (before MCAO 368.5 ± 70.76 mg/dl; before euthanasia 408.0 ± 51.50 mg/dl) $P > 0.05$. It can be inferred that hAD-MSC treatment induces recovery which is not related to changes in blood glucose levels.

Hyperglycemia significantly increased lesion size and diffusion coefficients after stroke in rats

The lesion size of the hyperglycemic group was significantly higher than that of the non-hyperglycemic group at 24 h ($p < 0.001$) and 6 weeks ($p < 0.04$) (Fig. 3a).

The rADC analysis showed significantly higher diffusion coefficients in the hyperglycemic rats compared with the non-hyperglycemic ($p < 0.04$) at 6 weeks post-stroke (Fig. 3a).

Hyperglycemia had no effect on the number of surviving neurons compared to non-hyperglycemic rats ($p > 0.05$) (Fig. 3b).

hAD-MSC treatment did not reduce lesion size but significantly decreased diffusion and increased the number of surviving neurons post-stroke in hyperglycemic rats

At 24 h and 6 weeks, hAD-MSC treatment in the hyperglycemic rats did not decrease lesion size compared with the nontreated hyperglycemic group ($p > 0.05$) (Fig. 3a).

At 6 weeks post-stroke, the rADC values were higher in the nontreated hyperglycemic rats than in the treated hyperglycemic group ($p < 0.03$) (Fig. 3a).

The treated hyperglycemic group showed an increased in the number of surviving multipolar motor neurons compared with the nontreated hyperglycemic group ($p < 0.003$) (Fig. 3b).

Hyperglycemia contributed to increased astrocyte and microglia markers

There were increases in GFAP ($p < 0.03$) and Iba-1 ($p < 0.012$) levels in the hyperglycemic group compared with the non-hyperglycemic group (Fig. 4).

hAD-MSC treatment decreased astrocytes and microglia post-stroke in hyperglycemic rats

To evaluate the effect of hAD-MSC treatment on glial cells, brain tissues were stained for the astrocyte marker GFAP and the microglia marker Iba-1. The expression of GFAP ($p < 0.001$) and Iba-1 ($p < 0.03$) was lower in the treated hyperglycemic group than in the nontreated hyperglycemic group at 6 weeks post-stroke (Fig. 4).

Hyperglycemia induction has no effect on synaptogenesis and neurogenesis

The non-hyperglycemic rats did not show differences in the expression of synaptophysin and doublecortin compared with the hyperglycemic rats ($p > 0.05$) (Fig. 4).

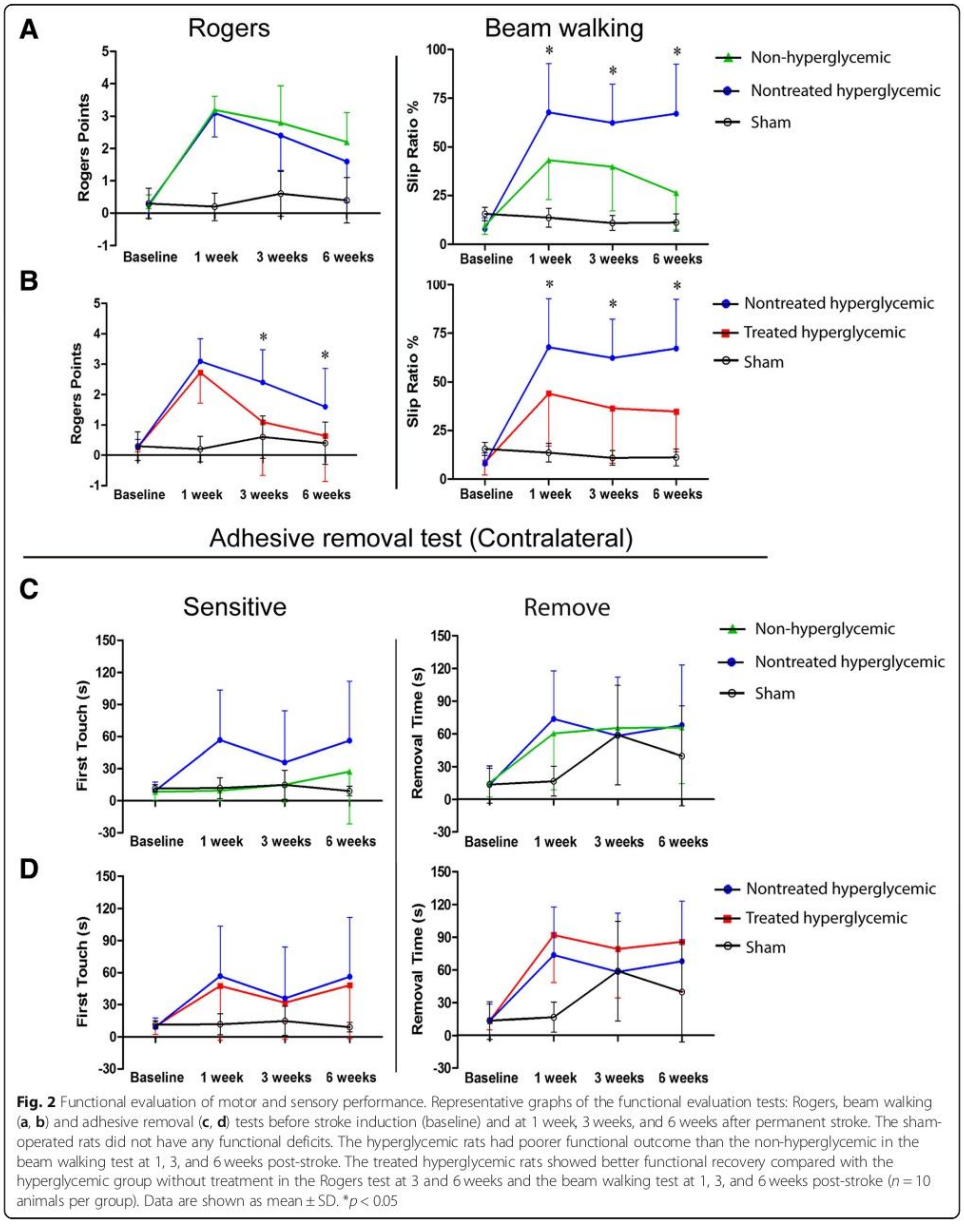

Fig. 2 Functional evaluation of motor and sensory performance. Representative graphs of the functional evaluation tests: Rogers, beam walking (**a**, **b**) and adhesive removal (**c**, **d**) tests before stroke induction (baseline) and at 1 week, 3 weeks, and 6 weeks after permanent stroke. The sham-operated rats did not have any functional deficits. The hyperglycemic rats had poorer functional outcome than the non-hyperglycemic in the beam walking test at 1, 3, and 6 weeks post-stroke. The treated hyperglycemic rats showed better functional recovery compared with the hyperglycemic group without treatment in the Rogers test at 3 and 6 weeks and the beam walking test at 1, 3, and 6 weeks post-stroke ($n = 10$ animals per group). Data are shown as mean ± SD. *$p < 0.05$

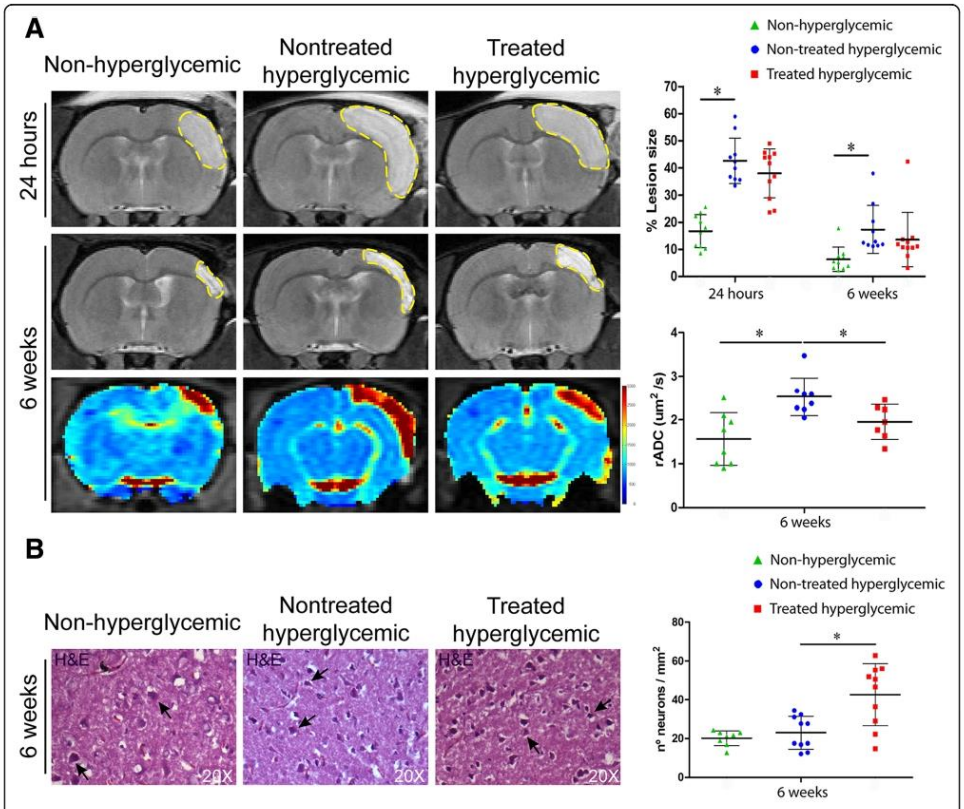

Fig. 3 a MRI analysis. Representative T2-weighted MRI at 24 h and 6 weeks and rADC images at 6 weeks post-stroke. At 24 h and 6 weeks post-stroke, quantitative graphs of T2-weighted MRI showed a significantly higher lesion size in the hyperglycemic group compared with the non-hyperglycemic group. At 6 weeks post-stroke, the non-hyperglycemic and the treated hyperglycemic groups in the rADC images showed lower diffusion coefficients compared with the hyperglycemic rats ($n = 10$ animals per group). **b** Histopathological analysis. Representative H&E staining images in the cortex at 6 weeks. A significant increase in the number of surviving neurons was observed in the hyperglycemic treated group compared to the nontreated hyperglycemic group. (3 animals per group, 4 sections in each animal per group). Data are shown as mean ± SD. *$p < 0.05$. Abbreviations: MRI: magnetic resonance imaging; rADC: relative apparent diffusion coefficient; H&E: hematoxylin and eosin

hAD-MSC treatment did not promote synaptogenesis and neurogenesis post-stroke in hyperglycemic rats

No differences were found between the hyperglycemic and treated rats in relation to synaptophysin and double-cortin staining at 6 weeks post-stroke ($p > 0.05$) (Fig. 4).

Hyperglycemia had an effect on α-SMA, but not on vascular markers CD31 and collagen-IV

We found no differences in the expression of CD31 and collagen-IV markers between the non-hyperglycemic and hyperglycemic rats ($p > 0.05$). However, we found that hyperglycemia significantly increased α-SMA compared with the non-hyperglycemic group ($p < 0.004$) (Fig. 5).

hAD-MSC treatment did not promote the formation of blood vessels but decreased arterial wall thickness

Our results suggest that the treatment did not promote blood vessel reconstruction in terms of CD-31 and collagen-IV markers compared with the hyperglycemic group ($p > 0.05$). However, we observed that α-SMA significantly decreased in the treated hyperglycemic rats compared with the hyperglycemic group ($p < 0.015$) (Fig. 5).

Discussion

To date, the majority of the experimental animal studies of stroke associated with hyperglycemia have been performed using ischemia/reperfusion models.

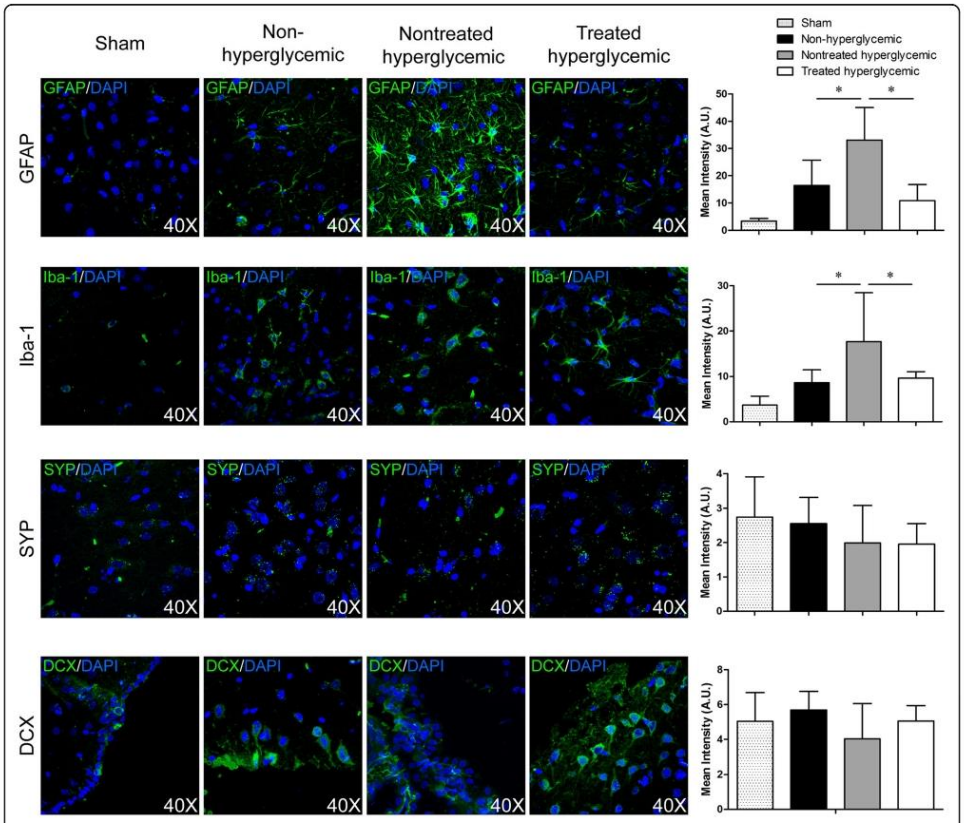

Fig. 4 Brain repair markers related to glia and neurons at 6 weeks post-stroke by immunofluorescence. Representative images and quantification of immunofluorescence of GFAP, Iba-1, synaptophysin, and doublecortin. At 6 weeks post-stroke, the hyperglycemic rats showed significantly higher levels of GFAP and Iba-1 compared with the non-hyperglycemic and treated hyperglycemic groups. 4′,6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining (3 animals per group, 4 sections in each animal per group). Data are shown as mean ± SD. *$p < 0.05$. Abbreviations: GFAP: glial fibrillary acidic protein; SYP: synaptophysin; DCX: doublecortin

However, a high percentage of patients do not recanalize. Thus, it is important to introduce permanent ischemia models in order to understand the exact mechanisms by which hyperglycemia leads to poorer functional outcomes, as well as to continue investigating therapies that have demonstrated efficacy in stroke models without associated comorbidities.

Previous studies from our group have demonstrated that AD-MSC administration improves functional recovery, decreased cell death and increased brain plasticity markers after pMCAO [5, 25]. However, the present study is the first to investigate whether AD-MSC treatment could improve the functional outcome post-stroke in hyperglycemic rats, a comorbidity that remains relatively under explored.

It has been shown that hyperglycemia worsens damage probably by enhancing the production of lactic acid during ischemia. Ischemia blocks or retards the stage of pyruvate oxidation and leads to the reduction of pyruvate to lactate. The net result of the anaerobic metabolism of glucose is the production of lactate- and of H^+. Thus, the enhancement of glucose supply by hyperglycemia may exaggerate the acidosis. The adverse effects of acidosis may promote edema formation by inducing Na^+ and Cl^- accumulation via coupled Na^+/H^+ and $C1^-/HCO3^-$ exchange. Thus, the severity of the acidosis and exacerbated brain damage correlates to the preischemic hyperglycemia. This brain injury implicates damage to neurons, glial cells, and/ or vascular endothelium as well [26]. Our study confirmed

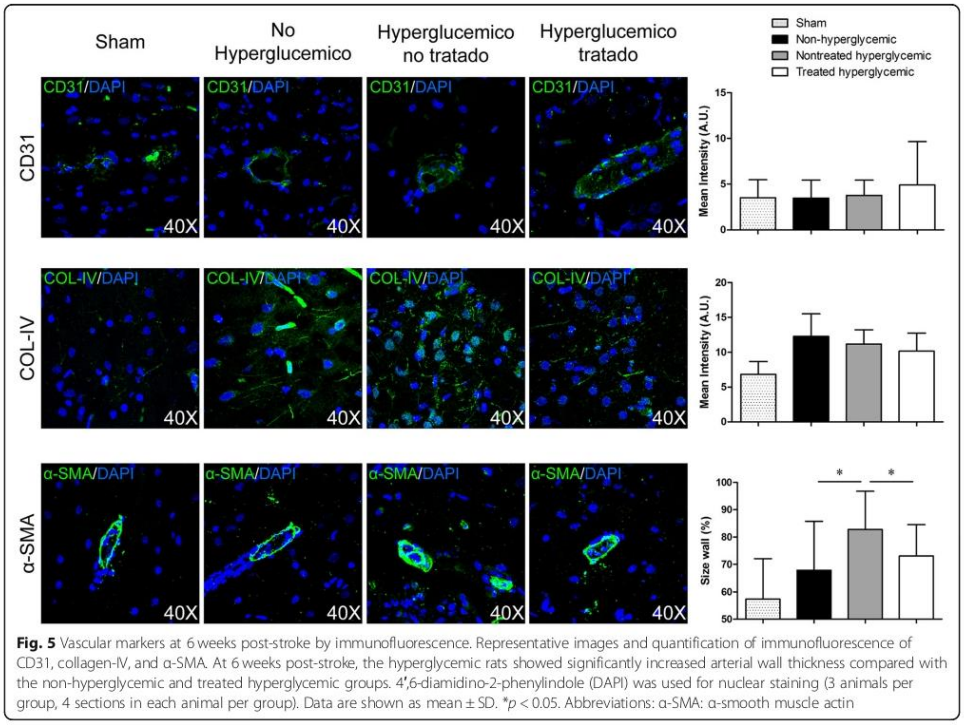

Fig. 5 Vascular markers at 6 weeks post-stroke by immunofluorescence. Representative images and quantification of immunofluorescence of CD31, collagen-IV, and α-SMA. At 6 weeks post-stroke, the hyperglycemic rats showed significantly increased arterial wall thickness compared with the non-hyperglycemic and treated hyperglycemic groups. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining (3 animals per group, 4 sections in each animal per group). Data are shown as mean ± SD. *$p < 0.05$. Abbreviations: α-SMA: α-smooth muscle actin

that hyperglycemic ischemic rats exhibit an increased lesion size and impaired brain repair processes, and demonstrated an increase in the inflammatory response (Iba 1 and GFAP) and in the thickness of the arterial wall (α-SMA), which likely contribute to exacerbated impairments of behavioral recovery.

We also evaluated the repair effect of intravenous delivery of hAD-MSCs in a rat model of pMCAO associated with hyperglycemia. Our results demonstrated that hAD-MSC treatment induces lower diffusion coefficients with a decrease in the inflammatory response and in the thickness of the arterial wall, which is associated with a good behavioral outcome.

Stroke patients are a heterogeneous population that has associated comorbidities, such as hyperglycemia. Over 50% of acute stroke patients suffer from hyperglycemia, which is associated with a poorer prognosis and outcome [7, 9, 11]. Moreover, comorbidities themselves may exert a detrimental impact on treatment efficacy [27]. For this reason, it is important to introduce comorbidities in the experimental animal stroke model to mimic the patient's situation and to test the efficacy of new treatments, avoiding pitfalls in translational research [27].

The exacerbated damage from hyperglycemia is typically observed in animal ischemic stroke models [10]. Our results are consistent with previous experimental data in which, animals with ischemic stroke and hyperglycemia tended to have poorer neurological recovery and increased ischemic infarct size compared to ischemic controls [10, 28–31]. Additionally, in our study, hyperglycemia enhanced diffusion coefficients. High rADC values are associated with loss of cell membrane integrity, apoptosis, shrinkage and tissue liquefaction necrosis at 30 days post-stroke [24], suggesting that higher diffusion coefficients represent poorer anatomical tissue preservation, without affecting the number of surviving neurons in the present study. Preclinical studies have demonstrated the safety and efficacy of AD-MSCs in animal models of ischemic stroke [5, 32–34]. However, whether hyperglycemia has an impact on therapeutic response has not yet been studied. In the present study, we demonstrated that the rats treated with hAD-MSCs achieved a good behavioral recovery without reducing lesion size compared with the

nontreated hyperglycemic group. In line with our results, previous animal studies have reported that post-stroke cell therapy in type 2 diabetic rats promotes neurological functional outcome without affecting infarction volume [35–37]. However, in the present study, the rADC values were significantly lower in the group that received hAD-MSCs at the chronic phase. It has been described that low rADC levels are related to reduced nerve cell damage and promoting the repair of axon and myelin on the 30th day post-stroke [24]. In this sense, we have shown that hAD-MSCs administration significantly increased the number of multipolar motor neurons in the cortex, suggesting that treatment protected neuronal cells, indicating better tissue preservation post-stroke.

Ischemic injury has been related to reactive astrocytes [38, 39]. However, it has been reported that diabetic rats show inhibited astrocyte activation after ischemic stroke [39]. Conversely, in our study, GFAP levels were increased in hyperglycemic stroke rats. This controversy is probably due to differences between the diabetes model (only streptozotocin) and the stroke model used (intraluminal filament and exsanguination) compared with our study. We also detected a reduction in the glial marker GFAP after hAD-MSC administration, in accordance with previous studies [38, 40].

Inflammatory responses play an important role in post-stroke recovery. Although mild to moderate inflammation can be beneficial to brain repair, exacerbated inflammation can impede recovery and create an inhospitable environment for brain repair. In accordance with earlier studies [31, 41], we found an increase in the levels of microglia marker in hyperglycemic pMCAO rats compared with non-hyperglycemic rats. In addition, we observed that the cell treatment decreased the expression of the microglia marker.

We evaluated the expression of doublecortin and synaptophysin in the peri-infarct zone to study the role of hyperglycemia in neurogenesis and synaptogenesis. Previous studies have indicated that long-term hyperglycemia suppresses the proliferation of hippocampus neural stem/progenitor cells (NSPCs) [42–44], whereas other researchers have found an enhanced proliferation of NSPCs [45, 46]. Other authors have demonstrated that severe (20 mM) instead of mild (10 mM) hyperglycemia exacerbates ischemic injury and inhibits stroke-induced subventricular zone neurogenesis [47] and that diabetes leads to greater post-stroke spine loss which could indicate a decrease in synapses [48]. In our study, however, hyperglycemia had no effects on neurogenesis and synaptogenesis. The discrepancy among studies could be attributed to the different models used to induce hyperglycemia and stroke (transient and permanent). The efficacy of stem cell treatment on neurogenesis has been investigated by a number of studies, in which human marrow stromal cell administration increases doublecortin density post-stroke in diabetic rats [36]; however, we found that the administration of hAD-MSCs at 48 h post-stroke had no effect on the levels of doublecortin marker. Instead, our results regarding synaptophysin, a marker related to synaptic plasticity, are consistent with some articles that showed similar levels of synaptophysin between diabetic animals with and without bone marrow stromal cells [49].

Hyperglycemia and diabetes play an important role in the pathogenesis of vascular complications. We evaluated the expression of several vascular markers (CD31, collagen-IV, and α-SMA) in pMCAO model in rats. Experimental animal models in transient stroke have demonstrated increased cerebral ischemia–reperfusion-induced blood–brain barrier (BBB) disruption and neurovascular damage [11, 50–52]. Consistent with these studies, the rats with hyperglycemia showed significantly increased arterial wall thickness compared with the non-hyperglycemic group. However, our results showed that hyperglycemia did not affect the expression of CD31 or collagen-IV, perhaps because we used a permanent model instead of a transient one, contributing to exacerbated neurovascular injury as a consequence of the reperfusion. Finally, when we evaluated the effect of cell therapy, hAD-MSCs only had a significant effect on the decrease in arterial wall thickness, but it had no effect on the expression of the other markers analyzed in the study.

The precise mechanism of the therapeutic action of MSCs remains unclear. Many molecular and cellular mechanisms such as enhanced endogenous neurogenesis, trophic factor secretion, cell replacement, the formation of biobridges, and more have been suggested but not fully documented [53]. In order to elucidate these mechanisms, previous studies from our laboratory analyzed the migration and implantation of MSC when they were administered intravenously. MSC were magnetically labeled using Endorem™ (superparamagnetic iron oxide) and with a lipophilic agent using DiI and their biodistribution was analyzed in vivo by magnetic resonance [18] and after sacrifice by immunofluorescence [18, 54]. MSC were not observed in the brain, but they were found in peripheral organs (liver, lung and spleen). Even from the distance, in both studies MSC showed efficacy in promoting brain repair. These results suggest that MSC does not need to reach the brain to exert recovery after a stroke. These results agrees with other studies where estimated that only 1% of the injected cells were detected in the brain after i.v. administration of MSC [55] and that most of these cells had accumulated in internal organs such as the lungs, liver, and spleen [25, 56]. In this regards, transient early lung trapping was observed after intravenous administration of MSC radiolabeled with 370 MBq of 99mTc the HMPAO and

visualized by Whole-Body Nuclear Imaging. In this study, some MSC seems to be able to migrate to the ischemic brain lesion and are eliminated by kidneys [57]. These data suggest that administered MSC have the potential to enhance brain repair mechanisms from the distance by releasing products such as trophic factors, immunomodulatory molecules, and even extracellular vesicles. Therefore, preclinical data is still needed to understand the mechanism of action of these cells to better interpret their therapeutic effects.

Regarding cryopreservation, there is an ongoing debate whether cell cryopreservation, being mandatory in a clinical scenario, compromises the cell's therapeutic impact [58]. It seems that the cryopreservation of cells might play a role [59]. Future preclinical studies should be conducted with fresh cells to study whether their therapeutic effect could be affected by this cryopreservation.

Thus, the present data on permanent ischemia models highlights novel mechanisms how hyperglycemia leads to a worse behavioral outcome, establishing a basis for further studies investigating therapies effects comorbid conditions.

Conclusions

In conclusion, our data suggest that rats with hyperglycemic ischemic stroke exhibit an increased lesion size and impaired brain repair processes, which likely contribute to exacerbated behavioral impairments. In addition, hAD-MSC treatment showed better anatomical tissue preservation with a decrease in the inflammatory response and in the thickness of the arterial wall, together with the improved behavioral outcome.

Additional file

Additional file 1: Figure S1. Immunohistochemistry study Delimitation of the perilesional tissue with microtubule-associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP) labeling by immunohistochemistry. (TIF 1038 kb)

Abbreviations

ADC: Apparent diffusion coefficient; AD-MSCs: Adipose tissue-derived mesenchymal stem cells; BBB: Blood–brain barrier; DAPI: 4′,6-Diamidino-2-phenylindole; DWI: Diffusion-weighted imaging; GFAP: Glial fibrillary acidic protein; H&E: Hematoxylin and eosin; hAD-MSCs: Human adipose tissue-derived mesenchymal stem cells; MRI: Magnetic resonance imaging; NSPCs: Neural stem/progenitor cells; pMCAO: Permanent middle cerebral artery occlusion; rADC: Relative apparent diffusion coefficient; SD: Standard deviation; STEPS: Stem Cell Therapies as an Emerging Paradigm in Stroke; tPA: tisular Plasminogen Activator; α-SMA: α-Smooth muscle actin

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Authors' contributions

MCGF contributed to the collection and assembly of data, manuscript writing, data analysis, and interpretation. FLG contributed to the collection and assembly of data, manuscript writing, data analysis, and interpretation. LD contributed to the collection and assembly of data, manuscript writing, data analysis, and interpretation. LOO contributed to the collection and assembly of data. JJ, OD, and AM contributed to the data analysis. AMA contributed to the collection and assembly of data. EDT contributed to the conception and design, data analysis, and interpretation. MGF contributed to the conception and design, collection and assembly of data, manuscript writing, data analysis, and interpretation. All authors read and approved the final manuscript.

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Availability of data and materials

The original data are available from the corresponding author on request.

Ethics approval and consent to participate

The procedure was carried out at our Cerebrovascular and Neuroscience Research Laboratory, La Paz University Hospital, Madrid, Spain. All experiments were designed to minimize animal suffering of animals in compliance and approved by our medical school's Ethical Committee for the Care and Use of Animals in Research (Ref. PROEX 249/15) according to the Spanish (RD 1201/2005 and RD53/2013) and European Union (EU) (86/609/CEE, 2003/65/CE, 2010/63/EU) rules.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

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

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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 1×10^6 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.

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

Key Words: brain ■ comorbidity ■ hypertension ■ neurogenesis ■ stem cells

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

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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 1×10^6 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).

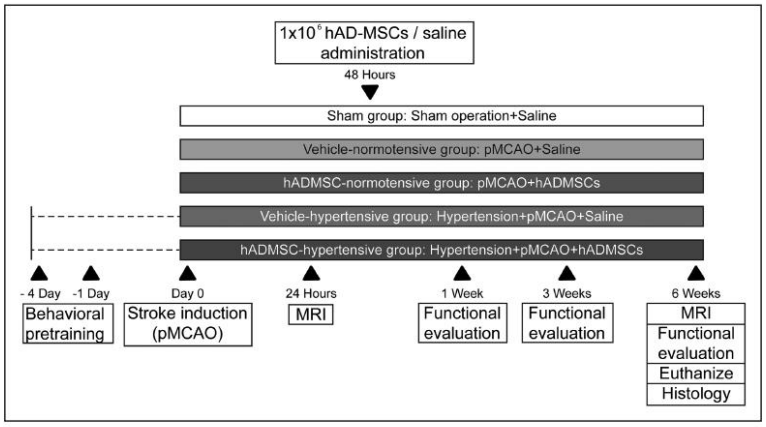

Figure 1. Study design. Ischemic stroke was induced by permanent middle cerebral artery occlusion (pMCAO). Human adipose tissue-derived mesenchymal stem cell (hADMSC) or vehicle was administered intravenously 48 h after surgery. Behavioral performance and magnetic resonance imaging (MRI) were evaluated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

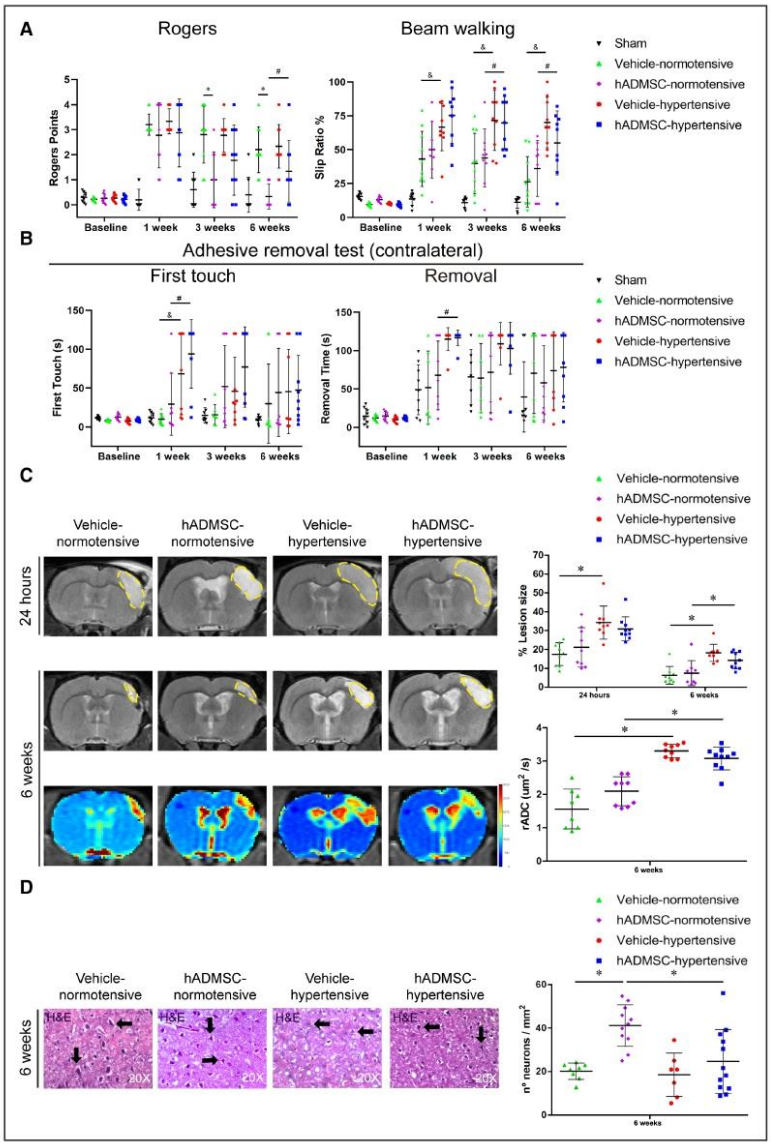

Figure 2. Behavioral tests and lesion analysis. **A** and **B**, Functional outcome. Assessment of behavioral outcome in Rogers test (**left**), walking beam (**right**) (**A**), and adhesive-removal test (**B**) at baseline and follow-up. &: vehicle-normotensive vs vehicle-hypertensive; *: vehicle-normotensive 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.

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

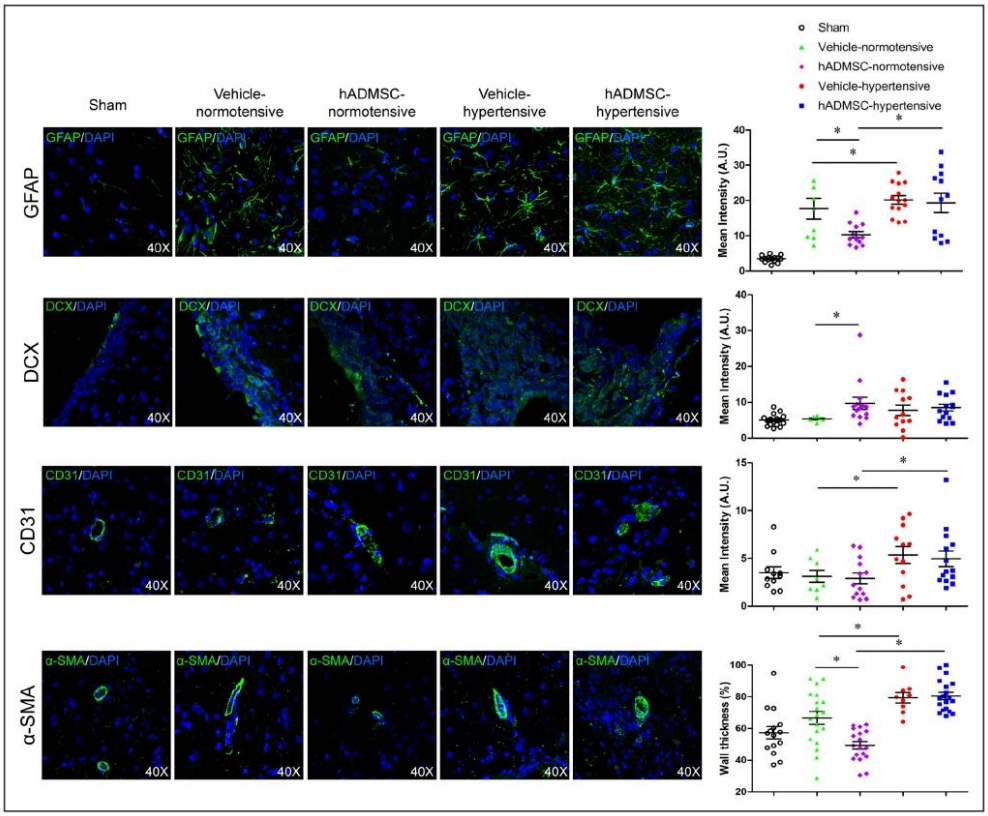

Figure 3. Representative images and quantification of GFAP (glial fibrillary acidic protein), doublecortin, CD-31, and α-smooth muscle actin (α-SMA) markers (3 rats per group, 4 sections in each rat per group). Data are shown as mean±SD.*P<0.05.

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.

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Disclosures

None.

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