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Neurorestorative responses to delayed hMSC treatment of stroke in type 2 diabetic rats

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Abstract

Background and Purpose—Co-morbidity of diabetes mellitus and stroke results in worse functional outcome, poor long term recovery and extensive vascular damage. We investigated the neurorestorative effects and mechanisms of stroke treatment with human bone marrow derived mesenchymal stromal cells (hMSCs) in type two diabetes mellitus (T2DM) rats.

Methods—Adult male Wistar rats were induced with T2DM, subjected to 2 hours of middle cerebral artery occlusion (MCAo) and treated via tail-vein injection with: 1) PBS (n=8); 2) hMSCs (n=10, 5×10^6) at 3 days after MCAo.

Results—In T2DM rats, hMSCs administered at 3 days after MCAo significantly improves neurological function without affecting blood glucose, infarct volume and incidence of brain hemorrhage in comparison to T2DM-MCAo PBS treated rats. Delayed hMSC treatment of T2DM stroke significantly improves blood brain barrier integrity, increases vascular and arterial density and cerebral vascular perfusion, and promotes neuroblast cell migration and white matter remodeling as indicated by increased doublecortin, axon, myelin and neurofilament density, respectively. Delayed hMSC treatment significantly increases platelet-derived growth factor (PDGF) expression in the ischemic brain, decreases pro-inflammatory M1 macrophage and increases anti-inflammatory M2 macrophage compared to PBS treated T2DM-MCAo rats. In vitro data show that hMSCs increase sub-ventricular zone explant cell migration and primary cortical neuron neurite outgrowth while inhibition of PDGF decreases hMSCs induced SVZ cell migration and axonal outgrowth.

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Conclusion—In T2DM stroke rats, delayed hMSC treatment significantly improves neurological functional outcome, and increases neurorestorative effects and M2 macrophage polarization. Increasing brain PDGF expression may contribute to hMSC induced neurorestoration.

Keywords

Stroke; ischemia; type 2 diabetes mellitus; mesenchymal stromal cells; platelet-derived growth factor

Introduction

Diabetes mellitus (DM) is a high risk factor for ischemic stroke and stroke patients with DM battle higher mortality rates and poor long term recovery than non-DM stroke patients[1]. In non-DM rodents, marrow stromal cell (MSC) therapy for stroke facilitates functional recovery by stimulating angiogenesis, vascular stabilization and white matter (WM) remodeling in the injured brain[2, 3]. However, MSCs therapy for stroke in non-DM rats does not successfully translate to DM stroke, and type one DM (T1DM) rats subjected to stroke and treated with MSCs 24 hours later, suffer from significantly increased brain hemorrhage, blood brain barrier (BBB) leakage and treated rats do not show functional improvement compared to T1DM stroke control rats[4]. Since nearly 90% of DM patients suffer from type 2 DM (T2DM), in this study, we investigated the therapeutic effects of delayed (3 days) human MSC (hMSC) treatment in T2DM stroke rats.

MSCs secrete several angiogenic, trophic and growth factors[5]. Platelet-derived growth factor (PDGF) in particular, is a potent neuroprotective molecule, and is secreted abundantly by MSCs[6]. PDGF is highly expressed in WM, and can induce axonal regeneration[7]. PDGF-BB and its receptors are also expressed on endothelial cells, and may play an important role in post stroke angiogenesis[7]. Macrophages are major regulators of vascularization and axonal remodeling[8]. Macrophages can be classified broadly into two types, the pro-inflammatory and anti-angiogenic M1 phenotype, and the anti-inflammatory and pro-angiogenic M2 phenotype[8]. Whether hMSC treatment regulates PDGF expression and M2 macrophage polarization in T2DM-stroke rats has not been investigated.

In this study, we investigate the therapeutic effects and underlying mechanisms of delayed hMSC treatment of stroke in T2DM rats. We hypothesize that delayed hMSC treatment of T2DM stroke significantly improves functional outcome, and induces neurorestorative effects and PDGF and M2 macrophage polarization may partially contribute to hMSC induced neurorestoration.

Materials and methods

All experiments were conducted in accordance with the standards and procedures of the American Council on Animal Care and Institutional Animal Care and Use Committee of Henry Ford Health System.

Diabetes induction

Adult Male Wistar rats (175–200g, Charles River) were induced with T2DM using a combination of high-fat diet (HFD) and low dose of Streptozotocin (STZ)[9, 10]. Body weight, blood glucose, high density lipoprotein (HDL), and total cholesterol were measured before and 10 days after STZ injection. Rats with fasting (8 hours) plasma glucose ≥300 mg/dl were considered diabetic.

MCAo model and experiment groups

T2DM rats were subject to transient (2 hours) middle cerebral artery occlusion (MCAo) by intraluminal suture model[3, 10]. Rats were randomized (n=10/group) and treated 3 days after MCAo via tail vein injection with: 1) phosphate-buffered saline (PBS); 2) 5×10^6 hMSCs (Cognate Bioservices, Inc.). T2DM stroke rats treated with PBS or hMSCs at 3 day after stroke were sacrificed at 4 weeks after MCAo to evaluate long term effects and blood serum and brain tissues were collected for ELISA and immunostaining. Mortality rate was 20% in each group. The sample size, 10 per group, was predefined to detect an effect size of 1.33 with a power of 80%.

Functional tests

To assess neurological functional outcome, a battery of tests including a modified neurological severity score test (mNSS)[11], adhesive removal test[3] and foot-fault test[10] were performed before MCAo and after MCAo on days 1, 7, 14, 21 and 28 by an investigator who was blinded to the experimental groups.

Histological and immunohistochemical assessment

Rats were transcardially perfused with 0.9% saline, brains immediately removed and fixed in 4% paraformaldehyde. A standard paraffin block was obtained from the center of the lesion (bregma −1mm to +1mm). Every 10^{th} coronal section for a total of 5 sections (6μm thick) was used for immunohistochemical staining. Antibody against Von Willebrand Factor (vWF, an endothelial cell marker, 1:400; Dako), α-smooth muscle actin (αSMA, smooth muscle cell marker, mouse monoclonal IgG 1:800, Dako), ED1 (microglia/macrophages marker, 1:30; AbD Serotec), CD163 (M2 macrophage marker, 1:500, Abcam), Doublecortin (DCX, a protein expressed in migrating neurons, 1:200, Santa Cruz), SMI-31 (phosphorylated neurofilament marker, 1:1000, Covance), PDGFRa (platelet derived growth factor receptor, 1:400, Santa Cruz) and PDGFRb (1:100, R&D systems) were employed. Bielschowsky silver (BS) and luxol fast blue (LFB) staining was used to demonstrate axons and myelin respectively. Antibody against albumin (albumin-FITC, polyclonal, 1:500, Abcam) was used to demonstrate BBB leakage. Gomori One-Step Trichrome Stain was used to evaluate arteriosclerosis. For control experiments non-immune serum was substituted for the primary antibody.

Quantification analysis

The ischemic border zone (IBZ) is defined as the area surrounding the lesion. For quantitative measurements, five slides from each brain, with each slide containing 4 fields from striatum bundle of the IBZ (BS and LFB) or 8 fields from striatum and cortex of the

IBZ (ED1, CD163, PDGFRa, PDGFRb, DCX, albumin, αSMA, vWF and SMI) were digitized under a 20× or 40× objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research)[10]. Data were analyzed in a blinded manner and positive areas or positive cell numbers were measured in the IBZ.

Arterial density, wall thickness, and diameter and occluded artery measurement

The number of arteries stained with αSMA were counted and analyzed with regard to small and large vessels (≥10μm diameter). The arterial density in the IBZ, and the 10 largest arterial wall thicknesses and diameter were measured.

Trichrome immunostaining and measurement

Using Gomori One-Step Trichrome Stain (Sigma), brain sections were post fixed in Bouin fixative. Nuclei were stained with Weigert hematoxylin and then stained in Gomori trichrome stain followed by a 0.5% acetic water rinse. Connective tissue and collagen are stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. Artery intimae, media, and artery diameter (minimum diameter) were measured in the internal carotid artery (ICA).

Cerebral blood perfusion measurement

To test cerebral vascular perfusion, an additional set of animals (n=5/group) were prepared and FITC-dextran (FD2000S, Sigma) 50mg/rat in 2ml PBS was injected intravenously 5 minutes before sacrifice[12, 13]. Brain tissues were fixed by 4% paraformaldehyde for 48 hours then were processed to acquire adjacent 100μm thick coronal sections using a vibratome. Five sections from the bregma (−1mm to +1mm) were imaged using a fluorescence microscope (Zeiss Axiophot 2) and FITC-dextran labeled vessels were quantification using ImageJ.

Angiogenesis ELISA array assay

Mouse angiogenesis antibody array kit (R&D Systems), including 55 angiogenesis-associated proteins and cytogeneses, was employed to test angiogenic protein level in ischemic brain tissue according to the manufacturer's instructions.

SVZ cell migration assay

Rats were subjected to 2 hours of MCAo and SVZ explants were isolated from the ipsilateral side at 24 hours after stroke. To further test whether hMSCs affect neuroblast migration, we used a previously described in vitro SVZ explant culture model[14]. The cultured SVZ explants were treated as per the following experimental groups: 1) control; 2) +50% hMSCs conditioned media; 3) +50% hMSCs conditioned media + PDGF inhibitor (20μg). The average linear distance of cell migration from the edge of the SVZ explant was measured using the MCID software.

Primary cortical neuron axonal outgrowth assay

Axonal outgrowth was measured, as previously described[15]. Briefly, primary cortical neurons were harvested from pregnant embryonic (day 18) Wistar rats. A microfluidic chamber (Standard Neuron Device, Xona Microfluidics) was used to separate axons from neuronal soma[16]. The experimental groups included: 1) control no treatment; 2) +50% hMSC conditioned media; 3) +50% hMSC conditioned medium +PDGF inhibitor (20μg).

Statistical analysis

All measurements and analyses were performed by normality of distribution, and the homogeneity of variances was tested including the functional tests, biochemistry and immunostaining. The Global test using GEE were employed to test effects on functional recovery measured from three behavioral tests at each time point. The analysis began with testing hMSC effect on the overall functional recovery, followed by testing the treatment effect on the individual test. One-way analysis of variance (ANOVA) was used for the immunostaining analysis. All data are presented as mean ± standard error (SE).

Results

hMSC treatment of stroke in T2DM rats significantly improves functional outcome but does not affect blood glucose level, lesion volume and brain hemorrhage transformation

Delayed hMSC treatment initiated at 3 days after MCAo significantly improves long term neurological function compared to T2DM MCAo control rats as indicated by mNSS test, foot-fault test and adhesive removal test ($p<0.05$, n=8/group, Figure 1A). Data were evaluated for normality and ranked data were used for the analysis because the data were not normally distributed. The data show that the overall group effect was significant at 14, 21 and 28 days after stroke ($p<0.05$). However, hMSC treatment of stroke in T2DM rats does not significantly decrease lesion volume and brain hemorrhage as well as the blood glucose/lipid levels compared to non-treatment T2DM stroke controls ($p>0.05$) (Supplementary Table 1).

hMSC treatment of stroke in T2DM rats promotes neurovascular remodeling

To test the mechanisms of hMSC treatment induced improvement of functional outcome after stroke in T2DM rats, vascular remodeling and cerebral perfusion were evaluated. We found that hMSC treatment of stroke initiated at 3 days after MCAo significantly decreases BBB leakage (Figure 1B), and increases cerebral vascular and cerebral arterial density (Figures 1C–D) in the IBZ compared to T2DM-MCAo rats after stroke ($p<0.01$). Figure 2A shows that the cerebral vascular perfusion is significantly increased in the T2DM-hMSCs rats compared with control group.

hMSC treatment of stroke in T2DM rats may have potential adverse effects such as arteriosclerosis-like changes

hMSC treatment significantly increases cerebral artery wall thickness, artery intimae thickness and ICA wall thickness; and significantly decreases cerebral artery diameter compared to vehicle control in T2DM rats after stroke (Figure 2B–C). The data are

consistent with our previous study that MSC treatment in T1DM MCAo rats increases atherosclerotic-like vascular changes[4].

hMSC treatment of stroke in T2DM rats promotes neural progenitor cell migration and axonal/WM remodeling in IBZ

To test whether hMSC treatment affects axon/WM remodeling, BS (axon), LFB (myelin), SMI-31 (phosphorylated neurofilament), staining were performed. To test whether hMSC treatment regulates neural progenitor cell migration, DCX immunostaining was performed. Figure 3 show that hMSC significantly increases SMI-31 (A), myelin (B) and axon (C) density in the striatum bundle of the IBZ as well as increases neural progenitor cell migration (D) after T2DM-MCAo.

hMSC treatment of T2DM stroke rats promotes M2 macrophage polarization in the ischemic brain

To investigate underlying mechanisms of hMSC treatment induced neurorestorative effects in T2DM-MCAo rats, M2 macrophage polarization was evaluated. Figure 4 shows that hMSC treatment significantly decreases M1 macrophage ED1 (A) and significantly increases M2 macrophage CD163 (B) expression compared to PBS treated T2DM MCAo rats. The data suggest that hMSC treatment initiated at 3 days after MCAo in T2DM promotes M2 polarization and decreases pro-inflammatory effects post ischemia.

hMSC treatment of stroke in T2DM increases PDGF and its receptor expression in the ischemic brain

To identify potential molecular mechanism underlying hMSC induced dual effects of neurorestorative and atherosclerotic-like vascular damage, an angiogenic antibody array was performed. We found that hMSCs treatment of T2DM-MCAo rat's increases PDGF-AA and PDGF-BB levels compared to PBS treated T2DM-MCAo rats (Figure 5A). Consistent with the ELISA array, immunostaining (Figure 5B–C) show that PDGFRa and PDGFRaa expression are significantly increased in hMSC treated T2DM-MCAo rats compared to PBS treated T2DM-MCAo control.

Inhibition of PDGF attenuates hMSC induced axonal/WM remodeling and decreases SVZ explant cell migration

To test whether PDGF plays an important role in hMSC treatment derived neurorestorative effects, in vitro studies were performed. We found that hMSC treatment increases PCN axonal outgrowth and SVZ explant cell migration (Figure 6). Inhibition of PDGF decreases hMSC induced axonal outgrowth changes and SVZ explant cell migration. These data indicate that increase in PDGF may play a key role in hMSC-induced neurorestorative effects.

Discussion

In this study, we present for the first time that delayed (3 day) treatment for T2DM stroke using hMSCs not only increases angiogenesis, axonal/white matter remodeling, but also increases neuroblast cell migration in the ischemic brain. We also specifically demonstrate

the contribution of PDGF in hMSC-induced axonal/white matter remodeling as well as in SVZ explant migration.

Diabetes triggers a cascade of events leading to vascular endothelial cell dysfunction, increased vascular permeability, vigorous angiogenesis but dysfunctional neovascularization, and poor recovery after ischemic stroke[4, 17]. We have previously shown that MSCs secrete several growth and trophic factors, one of which is VEGF (Vascular endothelial growth factor)[5]. It has been previously demonstrated that acute (1 hr after stroke) administration of VEGF enhances cerebral microvascular perfusion, increases BBB leakage, cerebral hemorrhage, infarction volume after stroke, while delayed (48 hours) administration of VEGF enhances angiogenesis in the ischemic penumbra and significantly improves neurological functional recovery[13]. Since diabetic rats suffer from vascular damage[18] which is aggravated after an ischemic insult[12], the effects of VEGF may be exacerbated in diabetic stroke rats compared to non-diabetic stroke rats. Our data show that VEGF is significantly increased in the ischemic brain of T2DM rats compared to wild type (WT) non-DM rats at 1 day after stroke (Supplementary figure 2). VEGF is significantly decreased by 3 days after stroke in the ischemic brain of T2DM stroke rats (Supplementary figure 3). In T1DM rats, when MSC therapy was initiated at 1 day after stroke, it induced brain hemorrhage and BBB leakage[4, 19]. In the acute phase after stroke, loss of BBB integrity and brain hemorrhage may override the beneficial effects of MSC treatment such as vascular and axonal/WM remodeling[4]. In the current study, we have demonstrated that treatment initiation at a delayed time point i.e. 3 days after stroke, in T2DM rats significantly improves neurological functional outcome, does not increase BBB leakage and brain hemorrhage, and significantly promotes vascular remodeling, as demonstrated by increased vascular and arterial density and vascular perfusion. Hence, for cell therapy for DM subjects, treatment initiation time point is extremely critical and should be considered when treating diabetic stroke. Since the time point of hMSC treatment in the current T2DM stroke study (3 days after stroke) differs from that of the T1DM stroke treatment (I day after stroke), we are unable to compare the apparent divergent outcomes between T1DM and T2DM MSC treatments. However, comparison of response to cell-based therapy performed under the identical treatment protocols in T1DM and T2DM rats with stroke warrants investigation.

Angiogenesis plays an important role in improving post stroke neurological function[20]. Angiogenic events increase blood supply to the ischemic brain tissue, and are also tightly coupled to neurogenesis[21, 22]. In this study, we found that hMSC treatment of stroke increases vascular density and cerebral blood perfusion in T2DM rats. Post ischemia, there is an exuberant expansion of neural progenitor cells in the SVZ, differentiation into mature neurons, astrocytes, and oligodendrocytes and migration to the IBZ[20]. Neural progenitor cell migration is closely associated with blood vessels which serve as scaffolds, and guide the migration of neural progenitor cells from the SVZ towards damaged brain regions[23]. Our results show that hMSC therapy significantly increases SVZ explant cell migration and neural progenitor cell migration identified by increased DCX density in the IBZ. These data suggest that hMSC treatment of stroke in T2DM rats promotes neurovascular remodeling which may partially contribute to the functional outcome after stroke.

Stroke and diabetes cause axonal/WM damage which induces long-term disability due to the brain's limited capacity of axonal regeneration and inhibitory environment for axon regrowth, sprouting and remyelination[24]. Post stroke protection of neurons in the gray matter is not sufficient, as WM damage would still hinder neuronal connectivity and functioning. Therefore, targeting WM remodeling is crucial for improving long term functional outcome after stroke. In this study, we found that hMSC treatment of stroke initiated at 3 days after MCAo in addition to promoting neurovascular remodeling, also increases axonal/WM remodeling in the ischemic brain.

M1 macrophages are neurotoxic, while M2 macrophages promote a regenerative growth response in adult sensory axons[8]. M2 phenotype polarization creates a conducive environment for axonal extension and functional recovery[25]. Post ischemia, the local and infiltrating microglia and macrophages assume an anti-inflammatory and protective M2 phenotype[26]. Extending this M2 phase of these macrophages and microglia and delaying their transition into the pro-inflammatory M1 phenotype is a desirable effect. Macrophage invasion typically starts around 24 hours post stroke and increases by 3 to 7 days after stroke; however, recent studies have indicated that the increased level of macrophage accumulation in the brain persists to at least 28 days after stroke[27]. Our data show that hMSC treatment of stroke in T2DM rats significantly induces M2 macrophage polarization as well as increases vascular integrity and axonal/WM remodeling in the ischemic brain. MSCs secrete several growth and trophic factors which may mediate several pathways and contribute towards neurorestoration after stroke in T2DM rats. Using the experimental design and methods used in this study, it is difficult to dissect whether M2 macrophage polarization has a direct or indirect (by creating a hospitable environment for brain repair) role in hMSC induced neurorestorative effects after stroke in T2DM rats. This is an important question and future studies are warranted.

Accumulating evidence suggests that the neurorestorative effects of cell therapy is primarily derived from the secretion of trophic and growth factors that stimulate endogenous brain repair and remodeling to induce neurological recovery[20]. We acknowledge the similarities observed in treatment derived benefits from our other cell-based treatment strategies such as human umbilical cord blood cells (HUCBCs). Accordingly, we have shown that for T2DM stroke, delayed (3 day) cell therapy with HUCBCs[10, 28] and hMSCs significantly promote long term neurological functional outcome without affecting blood glucose and infarction volume. Similar to HUCBCs[10, 20], hMSC treatment also promotes neurorestorative effects such as vascular and white matter remodeling and induces anti-inflammatory effects such as M2 macrophage polarization (Supplementary figure 2). However, the mechanism of action is different for HUCBC and hMSCs. We have reported that HUCBC treatment in T2DM stroke promotes serum, brain and brain endothelial cell microRNA-126 (miR-126) expression which stimulates vascular and white matter remodeling in the ischemic brain[28]. Compared to HUCBCs, hMSCs secrete significantly less miR-126 in-vitro, and in T2DM stroke rats, treatment with hMSCs stimulate significantly less circulating miR-126 compared to treatment with HUCBCs (Supplementary figure 3). In the present study, we demonstrate that hMSC treatment of T2DM stroke significantly increases PDGF expression, which then plays a key role in promoting neurovascular and WM remodeling.

PDGF signaling mediates vascular smooth muscle cell (SMC) and pericyte function and regulates vessel integrity. PDGF treatment decreases axonal abnormalities and improves remyelination by promoting proliferation of oligodendrocytes and oligodendrocyte progenitor cells[29]. Our data show that hMSC treatment of stroke in T2DM rats significantly increases PDGF expression in the ischemic brain and promotes neurovascular and WM remodeling. Inhibition of PDGF attenuates hMSC induced SVZ cell migration and PCN axonal sprouting. However, PDGF signaling is also a key mediator of SMC proliferation and plays an important role in arteriosclerosis[30]. PDGF triggers the switching of SMC from a quiescent, contractile phenotype to a proliferative, migratory, and synthetic phenotype[31]. PDGF is a major stimulus for the abnormal migration and proliferation of SMCs and contributes critically to vascular disease. Our data show that hMSC therapy in T2DM stroke rats not only increases neurovascular and axonal/WM remodeling but also increases arteriosclerosis-like changes compared to PBS treatment. Therefore, increasing PDGF by hMSC treatment of stroke in T2DM rats not only promote neurovascular and axonal/WM remodeling, but also may induce vascular arteriosclerosis-like changes.

Conclusions

T2DM stroke treatment using hMSCs initiated 3 days post the ischemic insult significantly improves neurological functional recovery by promoting neurovascular and axonal/WM remodeling in the ischemic brain. Our data suggest that M2 macrophage polarization and increase of PDGF may contribute to the mechanisms underlying hMSC treatment derived neurorestorative and atherosclerotic-like effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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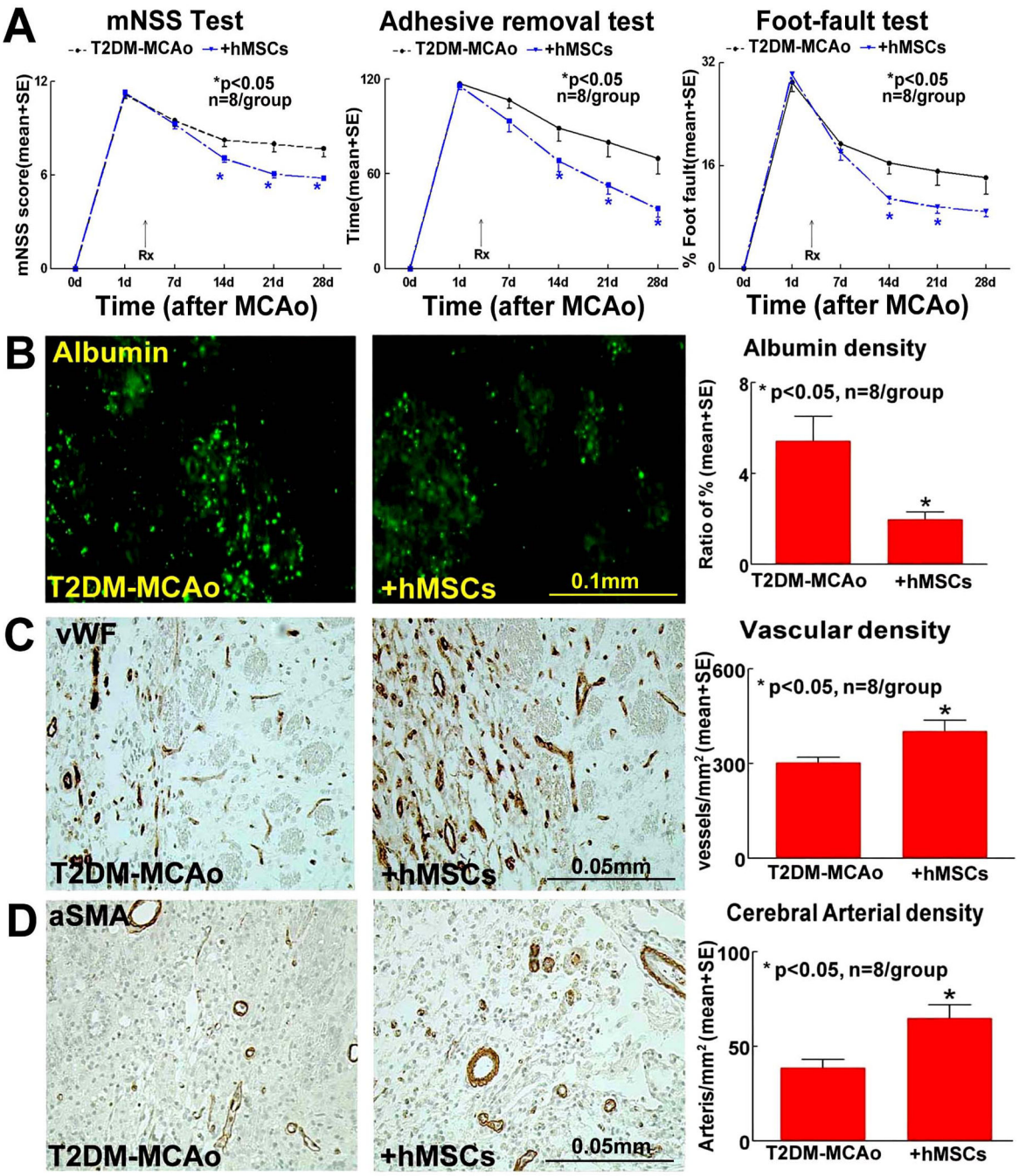

Figure 1.

In T2DM-MCAo rats, compared to PBS treatment, hMSC treatment 3 days post stroke significantly improves functional outcome indicated by A) mNSS Test, Adhesive removal test, Foot-fault test; significantly decreases BBB leakage as indicated by B) FITC-albumin staining, and improves vascular remodeling as indicated by: C) vWF and D) αSMA immunostaining and quantification data in the IBZ.

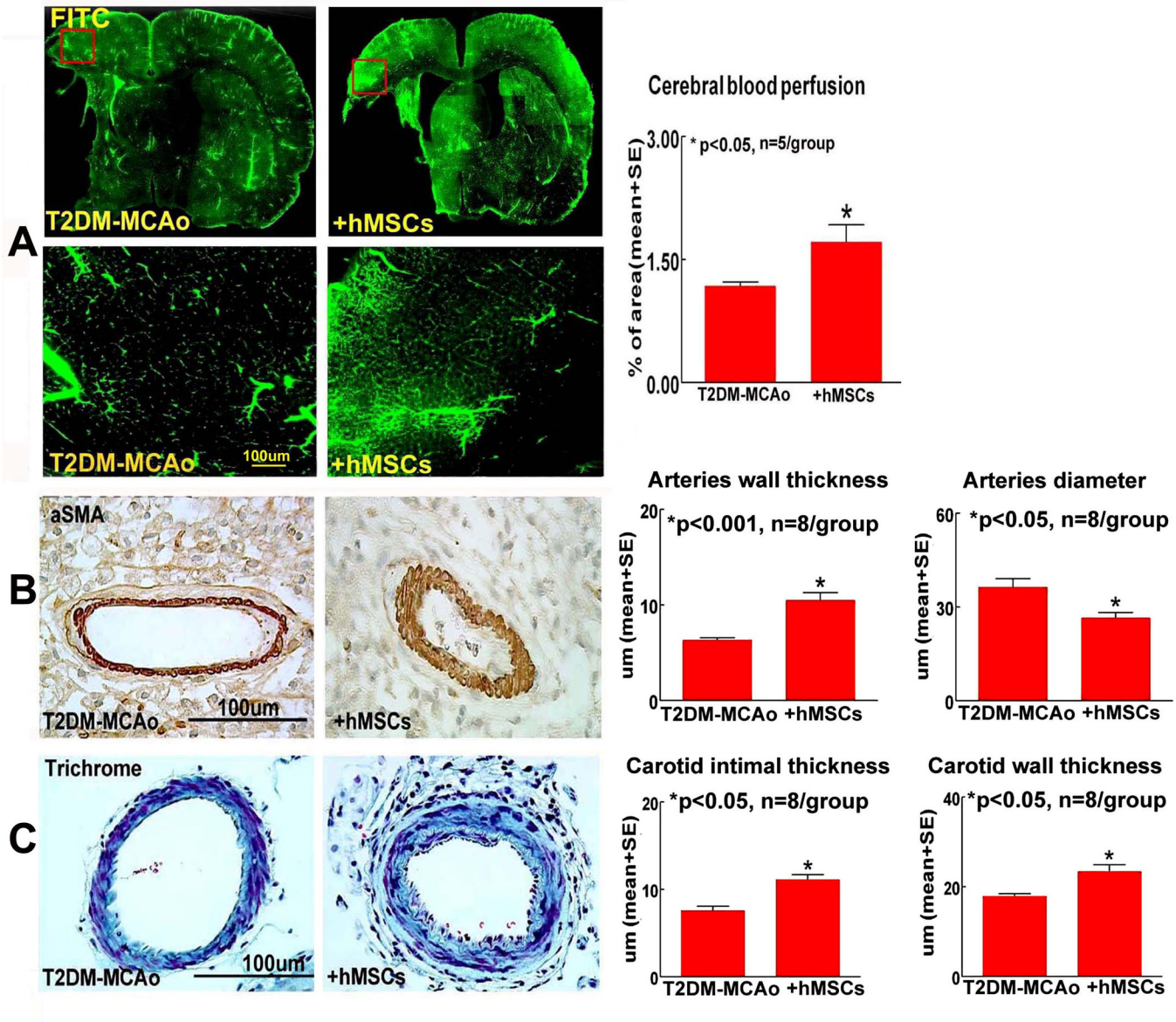

Figure 2.

In T2DM-MCAo rats, delayed hMSC therapy significantly A) improves cerebral perfusion, B) increases cerebral artery wall thickness (αSMA) and decreases artery diameter (αsMA); C) increases intimae thickness and increases internal carotid wall thickness (Trichrome) compared to PBS treated T2DM-MCAo rats.

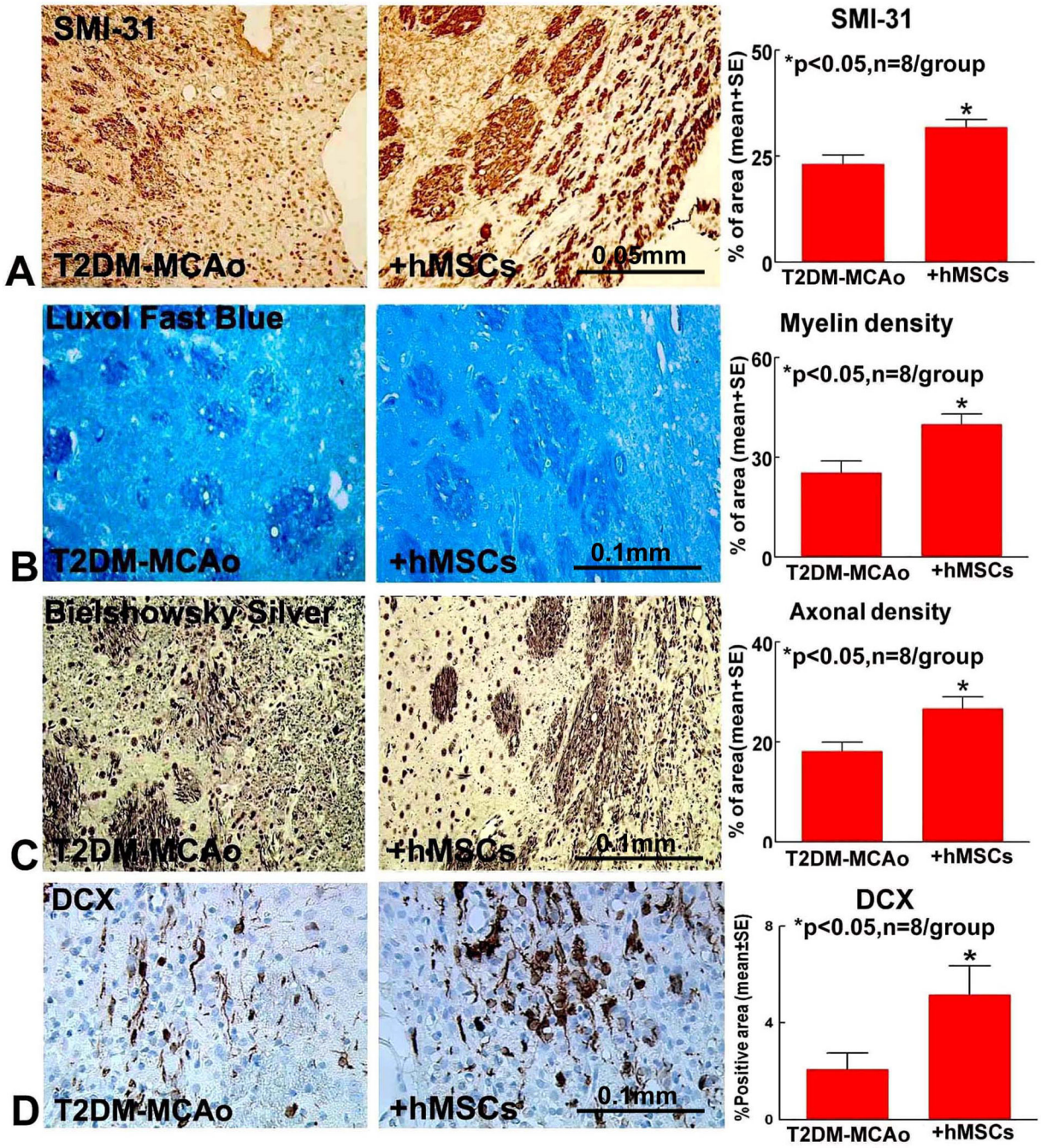

Figure 3.

Compared to PBS treatment, hMSC treatment 3 days post stroke in T2DM MCAo rats significantly promotes WM remodeling indicated by A) SMI-31; B) Luxol fast blue; C) Bielschowsky silver immunostaining and quantification analysis. hMSC therapy also promotes neural progenitor cell migration indicated by D) DCX immunostaining.

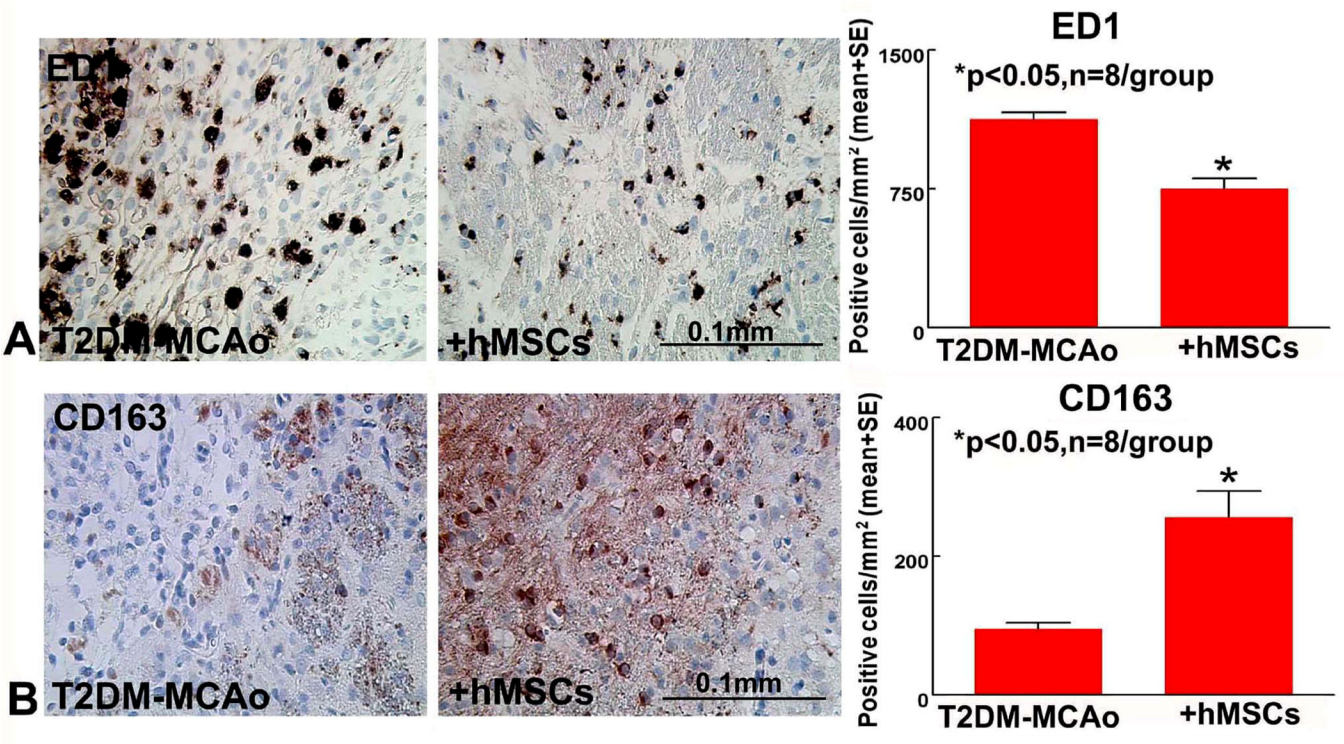

Figure 4.

Compared to PBS treatment, hMSC treatment 3 days post stroke in T2DM MCAo rats significantly promotes macrophage polarization indicated by: A) decreasing M1 macrophage ED1 and B) increasing M2 macrophage CD 163.

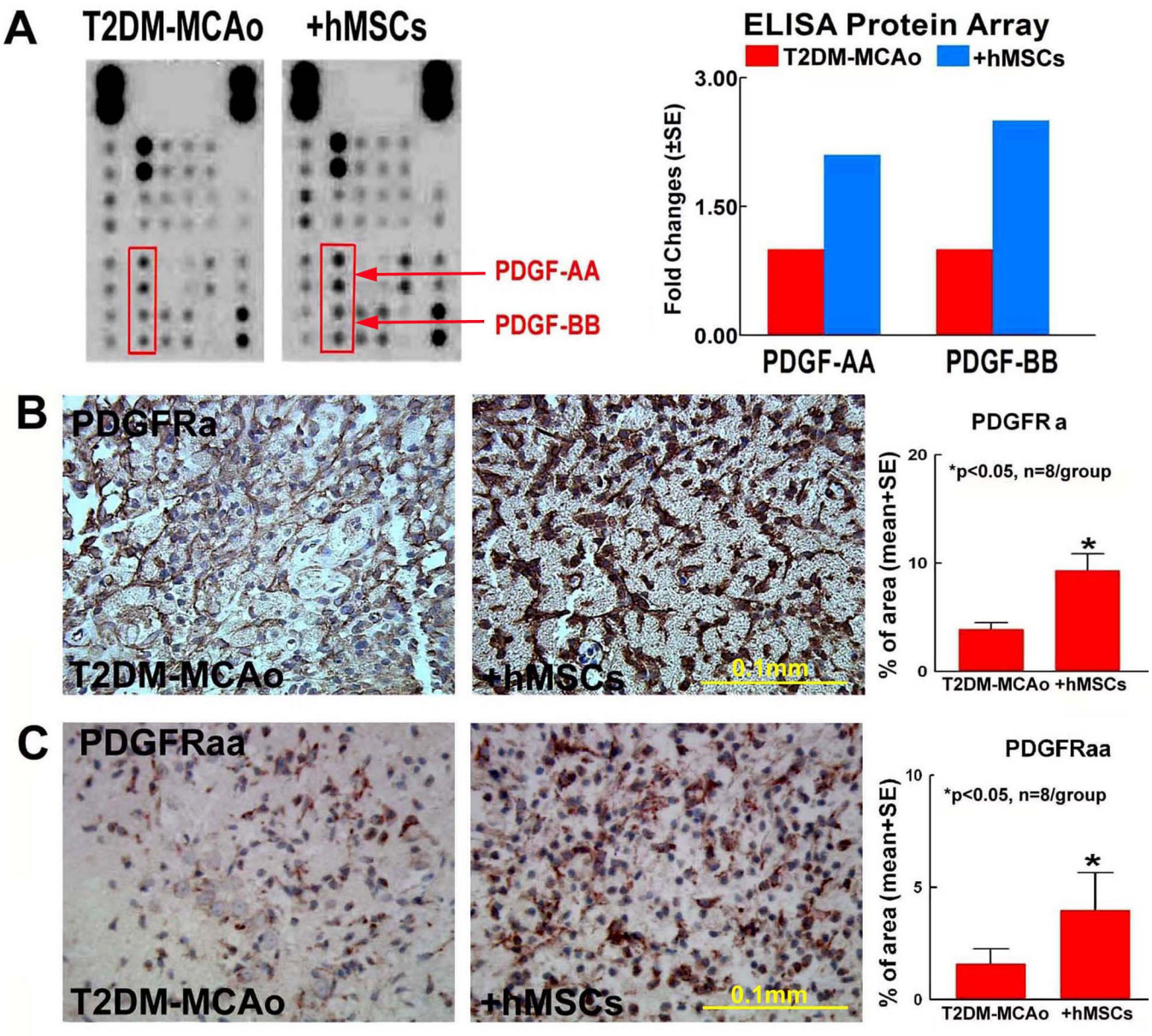

Figure 5.

A) Angiogenesis ELISA protein array shows that hMSCs treatment of T2DM stroke increases PDGF-AA and PDGF-BB, and these results are confirmed by: B) PDGFRa and C) PDGFRaa immunostaining and quantification analysis.

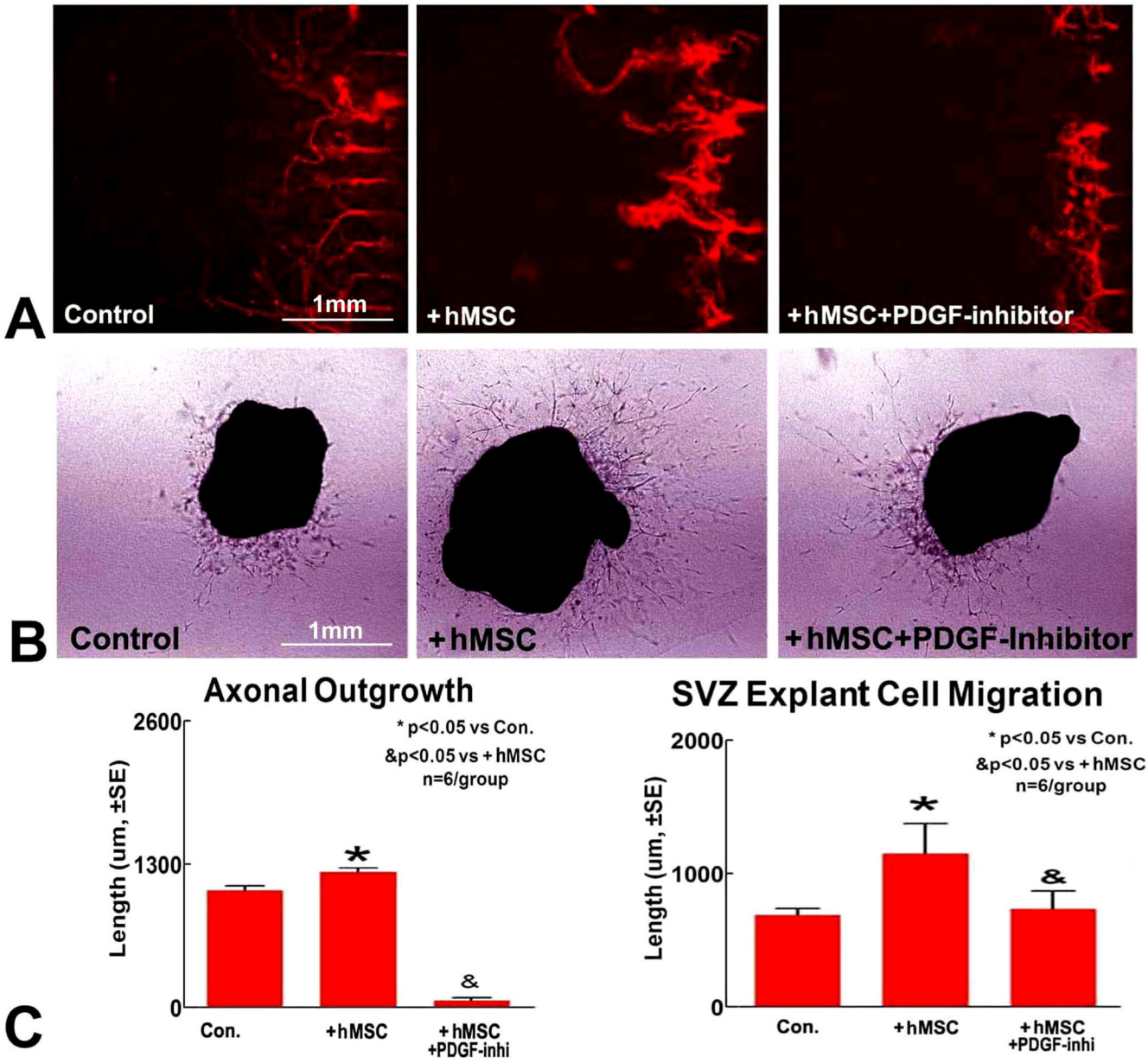

Figure 6.

A) Primary cortical neuron axonal outgrowth assay indicates that hMSC enhances axon outgrowth, and inhibition of PDGF attenuates hMSC induced axon outgrowth. B) SVZ explant cell migration assay shows that hMSC enhances SVZ cell migration, and inhibition of PDGF attenuates hMSC induced axon outgrowth. C) Quantification analysis.