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Neurorestorative Therapy of Stroke in Type two Diabetes Rats Treated with Human Umbilical Cord Blood Cells

Tao Yan, PhD[1,2,*], **Poornima Venkat, BS**[1,3,*], **Michael Chopp, PhD**[1,3], **Alex Zacharek, MS**[1], **Ruizhuo Ning, MD**[1], **Yisheng Cui, BS**[1], **Cynthia Roberts, BS**[1], **Nicole Kuzmin-Nichols, PhD**[4], **Cyndy Davis Sanberg, PhD**[4], and **Jieli Chen, MD**[1,2,#]

[1]Neurology, Henry Ford Hospital, Detroit, MI, USA

[2]Tianjin Neurological Institute, Neurology of Tianjin Medical University General Hospital, China

[3]Physics, Oakland University, Rochester, MI, USA

[4]Saneron CCEL Therapeutics, Inc. Tampa, FL 33612, USA

Abstract

Background and Purpose—Diabetes mellitus is a high risk factor for ischemic stroke. Diabetic stroke patients suffer worse outcomes, poor long term recovery, risk of recurrent strokes and extensive vascular damage. We investigated the neurorestorative effects and the underlying mechanisms of stroke treatment with human umbilical cord blood cells (HUCBCs) in Type two diabetes mellitus (T2DM) rats.

Methods—Adult male T2DM rats were subjected to 2 h of middle cerebral artery occlusion (MCAo). Three days after MCAo, rats were treated via tail-vein injection with: 1) phosphate-buffered-saline (PBS); 2) HUCBCs (5×10^6); n=10/group.

Results—HUCBC stroke treatment initiated 3 days after MCAo in T2DM rats did not significantly decrease blood-brain-barrier (BBB) leakage (p=0.1) and lesion volume (p=0.078), but significantly improved long term functional outcome and decreased brain hemorrhage ($p<0.05$) when compared to the PBS-treated T2DM-MCAo control group. HUCBC treatment significantly promoted white matter (WM) remodeling as indicated by increased expression of Bielschowsky silver (axons marker), Luxol fast blue (myelin marker), SMI-31 (neurofilament) and Synaptophysin in the ischemic border zone (IBZ). HUCBC promoted vascular remodeling, and significantly increased arterial and vascular density. HUCBC treatment of stroke in T2DM rats significantly increased M2 macrophage polarization (increased M2 macrophage CD163, CD 206; decreased M1 macrophage ED1 and iNOS expression) in the ischemic brain compared to PBS-treated T2DM-MCAo controls ($p<0.05$). HUCBC also significantly decreased pro-inflammatory factors i.e., matrix metalloproteinase 9 (MMP9), receptor for advanced glycation end-products (RAGE) and toll like receptor 4 (TLR4) expression in the ischemic brain.

[#]please send all correspondence to: Jieli Chen, MD, Senior Staff Investigator, Henry Ford Hospital, Neurology Research, E&R Building, 3091, Detroit, MI, 48202, Phone: (313) 916-1991; Fax: (313) 916-1318.
[*]These authors contributed equally to this work

Disclosures

JC is a consultant to Saneron CCEL Therapeutics, Inc. Also, CDS & NKN are inventors on cord blood patents/applications. CDS is Sr. VP of R&D, and NKN is the President & COO at Saneron CCEL Therapeutics, Inc.

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Conclusion—HUCBC treatment initiated 3 days after stroke significantly increased WM and vascular remodeling in the ischemic brain as well as decreased neuroinflammatory factor expression in the ischemic brain in T2DM rats and promoted M2 macrophage polarization. HUCBC reduction of neuroinflammation and increased vascular and WM-axonal remodeling may contribute to the HUCBC induced beneficial effects in T2DM stroke rats.

Keywords

HUCBC; neurorestorative therapy; stroke; T2DM; vascular remodeling; white matter remodeling

Introduction

The global concern for stroke, a cardiovascular disease that can lead to long term disability or even death, is rapidly growing. Among the prominent risk factors for ischemic stroke is Diabetes Mellitus (DM). The combination of diabetes and stroke leads to extensive brain damage and worse post stroke outcomes[1]. The T2DM-stroke combination puts the patient at a risk of recurrent strokes[2] and massive vascular damage leading to poor long term functional recovery and even lifelong paralysis. While the only FDA approved drug for ischemic stroke is tPA (tissue plasminogen activator) that can breakdown the blood clot and restore blood flow to the brain, the treatment is challenged by practicality because of its narrow treatment window (3–4.5 hrs after stroke onset). Thus, there is a compelling need to develop and test neurorestorative therapeutic approaches for stroke, with treatment initiated days after stroke[3]. One such therapy employs HUCBCs to induce neurorestorative effects post ischemic stroke.

HUCBCs therapy administered via tail vein injection within 24–72 hrs post an ischemic insult in non diabetic stroke rats has significant functional benefits associated with the regulation of inflammatory and immune responses[4, 5]. In T1DM rats, initiation of HUCBC treatment (5×10^6 cells) via tail vein injection 24 hrs post stroke had several beneficial effects including improved functional outcome, enhanced vascular and WM remodeling[6]. The primary mechanism of action was attributed to increasing angiopoetin-1 (Ang-1) and decreasing inflammatory factor RAGE (receptor for advanced glycation end products) expression in the ischemic border zone (IBZ) in T1DM rats[6]. T2DM mice that underwent stroke suffered from worse functional outcome, increased brain hemorrhage, severe WM damage and increased inflammatory factor MMP9 (matrix mettaloproteinase-9) expression[1]. Mortality rates in diabetic stroke mice were significantly greater than in wild type mice[7]. With T2DM being the most common form of diabetes and the success of HUCBC treatment in non diabetic and T1DM stroke animals, our aim in this study is to evaluate the therapeutic efficiency and underlying mechanism of delayed HUCBC treatment in T2DM rats when treatment is initiated 3 days after stroke.

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.

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Diabetes induction

T2DM was induced using a combination of 2 weeks high fat diet followed by low dose of streptozotocin (STZ, 35 mg/kg, Sigma Chemical Co., St. Louis, MO) intraperitoneal injection in adult Male Wistar rats and a continued high fat diet for another 2 weeks. Two weeks after STZ injection, the fasting blood glucose level was tested using a glucose analyzer (Accu-Chek Compact System; Roche Diagnostics, Indianapolis, IN) and animals with fasting blood glucose >300 mg/dl underwent 2 hrs of transient MCAo.

Middle cerebral artery occlusion (MCAo) model and experiment groups

Twenty four T2DM rats were subjected to transient (2 hrs) right MCAo via intraluminal vascular occlusion, as previously described[8, 9]. The exclusion criteria were rats with modified neurological severity score (mNSS) less than 6 (possibly small to no lesion) or over 13 (poor survival) at 24 hrs after MCAo (prior to treatment). Accordingly, 4 rats were excluded from this study. The rats were randomly assigned to different groups and treated with 1) PBS as vehicle control (n=10); 2) HUCBCs (5×10^6, n=10) (Saneron CCEL Therapeutics) via tail-vein injection starting at 3 days post MCAo. The treatment time point of 72 hrs after stroke in T2DM rats was employed to investigate HUCBC induced neurorestorative effects as well as to accommodate a wide treatment window. Rats were sacrificed 28 days after MCAo for immunostaining quantification analysis.

Neurological functional tests

An investigator was blinded to the experimental groups to perform a battery of functional tests including foot-fault[10], adhesive removal[8] and evaluation of mNSS[8] before MCAo, and after MCAo on days 1, 7, 14, 21 and 28.

Histological and Immunohistochemical assessment

Brains were fixed using transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. Then the brains were embedded in paraffin and a standard block was obtained from the center of the lesion (bregma −2 mm~+2 mm). A series of 6μm thick sections were cut from the block. Hematoxylin and eosin (H&E) stained seven coronal sections of tissue were used for lesion volume calculation and presented as a percentage of lesion compared to the contralateral hemisphere.

Brain coronal tissue sections were prepared and antibody against α-smooth muscle actin (α-SMA, mouse monoclonal IgG, 1:800; Dako); Von Willebrand Factor (vWF, 1:400; Dako); SMI-31 (Neurofilaments, phosphorylated monoclonal, 1:1000, Covance), Synaptophysin (monoclonal; 1:500, Millipore); RAGE (1:400; Dako), TLR4 (goat polyclonal IgG; dilution 1:100; Santa Cruz Biotechnology); MMP9 (1:500, Santa Cruz Biotechnology); CD163 (1:500, Abcam Cambridge, MA); ED1 (a mouse mAb against rat microglia/macrophages, monoclonal, 1:30; AbD Serotec); CD 206 (1:3000, Abcam), iNOS (1:200, Millipore) were employed.

Antibody against albumin (albumin-FITC, polyclonal, 1:500, Abcam) was used to demonstrate BBB leakage and Prussian blue staining used to evaluate hemorrhage. Bielschowsky-silver (BS) immunostaining was used to demonstrate axons; luxol fast blue

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(LFB) staining was used to demonstrate myelin. Control experiments consisted of staining brain coronal tissue sections as outlined above, but non-immune serum was substituted for the primary antibody.

Quantification analysis

All the immunostaining quantification analysis was performed by an investigator who was blinded to the experimental groups. Five slides from each brain, with each slide containing 8 fields from striatum of the IBZ were digitized under a 20x objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research, St. Catharines, Canada). For BS and LFB measurements positive areas of immunoreactive cells were measured in the WM bundles of the striatum in the IBZ[11]. For other immunostaining (albumin-FITC, Prussian blue, Synaptophysin, SMI-31, RAGE, MMP9 and TLR4), positive areas of immunoreactive cells were measured in the IBZ and for CD163, iNOS, ED1 and CD206 positive cell number was measured in the IBZ[12].

Vascular density measurement

To measure the vascular density in the IBZ, 8 fields of view of vWF immunostaining from the IBZ were digitized using a 20×objective via the MCID software[13]. The α-SMA stained arteries were analyzed with regard to small and large vessels (10μm diameter). The arterial density in the IBZ was measured[14].

Statistical Analysis

One-way Analysis of Variance (ANOVA) was used for the evaluation of functional outcome and histology, respectively. "Contract/estimate" statement was used to test the group difference. If an overall treatment group effect was detected at $p<0.05$, pair-wise comparisons were made. All data are presented as mean ± standard error (SE).

Results

HUCBC treatment significantly improved long term functional outcome but did not significantly decrease BBB leakage and lesion volume

Long term functional benefit derived from HUCBC treatment initiated at 3 days after MCAo in T2DM rats was assessed using a battery of behavioral tests. N=10/group was employed and with a mortality rate of 20% (between days 7–14 after MCAo), 8 rats/group survived at the end of experiments in both treatment and control groups. Figure 1A presents mNSS, adhesive removal and Foot fault data for 28 days after stroke that indicate significantly improved functional outcome in T2DM MCAo rats treated with HUCBCs compared to PBS treated control group ($p<0.05$).

HUCBC treatment significantly decreased hemorrhage in the brain identified by Prussian blue staining ($p<0.05$, Fig. 1D) compared to PBS-treated T2DM stroke rats. HUCBC treatment in T2DM rats did not alter body weight, blood glucose level, did not significantly decrease lesion volume ($p=0.078$, Fig. 1C) and BBB leakage as indicated by FITC-albumin immunostaining ($p=0.1$; Fig. 1B), compared to PBS-treated T2DM-MCAo rats.

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HUCBC stroke treatment significantly promoted vascular remodeling

To test the beneficial effects of HUCBC treatment, vascular remodeling was evaluated using α-SMA and vWF immunostaining. Figure 2 indicates that HUCBC significantly enhanced cerebral artery density (α-SMA, Fig. 2A) and vascular density (vWF, Fig. 2B) compared to PBS treated control T2DM stroke rats ($p<0.05$).

HUCBC stroke treatment promoted white matter remodeling and increased axonal and synaptic plasticity

BS and LFB staining were used to evaluate the beneficial effects of HUCBC treatment on WM remodeling. HUCBC treatment significantly increased BS and LFB expression (Fig. 3A–B) in the WM bundles compared to PBS-treated T2DM-MCAo rats ($p<0.05$). HUCBC treatment also regulated axonal and synaptic plasticity, indicated by significantly increased SMI-31 (Fig. 3C) and Synaptophysin (Fig. 3D) expression levels in the IBZ compared to PBS-treated T2DM-MCAo rats ($p<0.05$).

HUCBC stroke treatment significantly decreased neuroinflammatory factor expression and increased M2 macrophage polarization in the IBZ

To understand the mechanisms of HUCBC therapy derived benefits, neuroinflammation was evaluated in the ischemic brain. Expression levels of inflammatory factors TLR4, MMP9 and RAGE were measured in the IBZ. As indicated in Figure 4, HUCBC treatment significantly decreased TLR4, MMP9 and RAGE ($p<0.05$) expression compared to PBS-treated T2DM stroke rats ($p<0.05$).

To test the effect of HUCBC treatment on macrophage polarization, expression levels of M2 macrophage markers CD163, CD206 and M1 macrophage markers ED1, iNOS were measured. Figure 5 shows that HUCBC treatment in T2DM MCAo rats significantly increased M2 and decreased M1 macrophage expression in the ischemic brain in comparison to PBS-treated T2DM stroke rats ($p<0.05$).

Discussion

HUCBCs have received a great deal of attention as a treatment option for hematological disorders and malignancies as they are a rich source of hematopoietic stem cells. The driving advantages in using HUCBCs include easy availability as it is discarded post birth, lack of ethical conflicts and none to low severity effects like GVHD (graft versus host disease) from donor-recipient HLA (human leukocyte antigen) mismatch[15]. This tolerance of HUCBC therapy to donor recipient HLA mismatch aided by cord blood's naïve and immature immune function[15] play a key role in translating this therapy to the clinic. We found that HUCBC therapy in T2DM rats initiated intravenously at 3 days post stroke, did not significantly decrease BBB leakage and lesion volume, but significantly decreased brain hemorrhagic transformation and significantly increased vascular and WM remodeling and improved functional recovery. The underlying mechanisms of HUCBC-induced benefits may be decreased neuroinflammatory effects and promotion of M2 macrophage polarization.

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Several stroke treatments in the diabetic rat population have failed including bone marrow stromal cells[16] and tPA[17]. These failures were associated with increased BBB leakage, brain hemorrhagic transformation and inflammation in T1DM[16, 17] and T2DM [18] rats. In this study, significant functional benefit was derived upon HUCBC therapy, although we found that HUCBC treatment initiated 3 days after stroke in T2DM did not significantly decrease BBB leakage and lesion volume compared to T2DM PBS-treated stroke rats. These data suggest that HUCBC induced therapeutic and functional benefits in T2DM stroke rats may primarily be derived from the regulation of vascular and WM remodeling. While in non diabetic rats, hemorrhagic transformation and BBB leakage occur early after stroke, in diabetic rats these events have an extended time window and are present at 14 days after stroke[12, 16]. Although the 28 day time point is not ideal for studying BBB permeability and hemorrhagic transformation which occur acutely after stroke[19, 20], since we observed HUCBC treatment induced functional benefits starting at 14 days after stroke, we elected not to perform these measurements at an early time point.

A multifaceted mechanism of action has been suggested by several studies employing cell based therapies to promote post stroke long term beneficial effects; minor benefit derived from a small portion of infused cells migrating to the brain and differentiating into neuronal cells, and major benefit derived by promoting various aspects of neurorestoration like WM remodeling, vascular remodeling, synaptogenesis and neurogenesis by: a) enhancing endogenous brain repair mechanisms, and b) secreting trophic and growth factors. Stroke decreases cerebral blood flow and triggers vascular remodeling to improve blood supply via angiogenesis and arteriogenesis[21]. Increasing angiogenesis and arteriogenesis are correlated with neurological functional outcome after stroke[22]. In T2DM mice, poor functional outcome, compared with non diabetic stroke mice was correlated with exacerbated WM and vascular damage[1]. HUCBC treatment increased cerebral arterial and vascular density in the IBZ. These results indicate that HUCBC stroke treatment induces an increase in cerebral vascular remodeling in T2DM rats.

WM of brain is highly susceptible to ischemic stress primarily because of its relatively limited blood supply. Hence, after a stroke, WM recovery is critical for sustained long term functional recovery. HUCBC treatment enhanced the expression of Bielschowsky silver, SMI-31 and Luxol fast blue in the IBZ indicative of enhanced axonal plasticity and myelin regeneration. Several studies point towards axonal remodeling to improve brain repair, attenuate stroke induced neurological deficits as well as contribute to long term benefits in functional improvement[23–25]. At the same time, worse outcomes in DM stroke subjects have been associated with defective or restricted axonal regeneration[26, 27]. The importance of enhanced axonal myelination centers on faster communication and sensory/motor reflexes which helps restore lost neurological function and decreases stroke induced paralytic symptoms. Stroke leads to loss of myelin in the peri infarct area at 48 hrs after onset, but also stimulates the formation of new myelin around the damaged and sprouting axons[28]. This endogenous brain repair process is significantly enhanced by HUCBC treatment to promote the expression of LFB, a myelin marker, in the ischemic brain regions. HUCBC treatment also enhanced synaptic plasticity, indicated by increased Synaptophysin expression in the IBZ. Intercellular communication between neurons and other neurons/cells is facilitated via a synapse in the central nervous system, and improved synaptic plasticity

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indicated by enhanced Synaptophysin expression has been reported to mediate stroke treatment benefits[29]. Enhanced axonal and myelin remodeling, and axonal and synaptic plasticity may contribute to the observed HUCBC treatment induced post stroke recovery in T2DM rats.

Regulation of the inflammatory responses induced by stroke early after onset lasting up to weeks later, is crucial for effectiveness of stroke treatments. While mild inflammation can be favorable for brain repair in a chronic stage[30], in the acute phase, upon uncontrolled inflammation the activated microglia, astrocytes and macrophages can exacerbate damage and/or death to the injured brain by releasing pro-inflammatory factors and by creating an inhospitable environment for neurovascular plasticity[31, 32]. MMP9 has been implicated in enhancing T2DM induced WM and axonal damage[1, 33]. TLR4 and RAGE are both inflammatory factors typically increased in diabetic stroke animals and have been implicated in exacerbating brain damage[34, 35]. HMGB1 (high-mobility group box 1) is an inflammatory mediator secreted upon injury by immune cells or injured cells. HMGB1 release can trigger an inflammatory cascade and binds to its receptors TLR4 and RAGE. It has been reported that in cerebral ischemia HMGB1 triggers MMP9 increase in neurons and astrocytes mainly through TLR4[36]. Hence, treatments that can regulate the HMGB1/TLR4 signaling pathway can potentially decrease tissue damage by controlling post ischemic inflammatory responses. HUCBC treatment significantly decreased the expression levels of these detrimental inflammatory factors (TLR4, RAGE, and MMP9) in the IBZ and induces restorative effects in T2DM stroke rats.

M2 macrophage polarization has been associated with decreased neuroinflammation and enhanced axon growth in injured mouse spinal cord[37]. Our data show that M2 macrophage polarization was significantly increased by HUCBC treatment in the IBZ of T2DM MCAo rats. M2 macrophage polarization, marked by increased M2 macrophage CD163 and decreased M1 macrophage ED1 expression, was evident with HUCBC treatment. The M2 macrophage polarization mechanism can improve functional outcome post stroke[38]. Microglia and macrophages upon ischemic insult can assume an anti inflammatory M2 activation and protect neurons; which is a potential target for neurorestorative therapies[39, 40]. Soon after focal cerebral ischemia, the local and infiltrating macrophages assume M2 phenotype and decrease the expression of inflammatory factors thereby extending a protective effect to the neurons and improving their survival in the ischemic environment[39]. Extending the M2 phase of these macrophages and microglia and delaying their transit into M1 phenotype which is detrimental to the ischemic brain due to increased pro-inflammatory factor production, is a desirable effect. HUCBC treatment promotes M2 macrophage polarization which may contribute to improved neurological outcome. While it is common knowledge that macrophage invasion starts around 24 hrs after stroke and increases by 3 to 7 days after stroke; recent studies have revealed that the increased level of macrophage accumulation in the brain persists to at least 28 days after stroke[41], lasting up to 1 year after stroke[42]. The links between M2 polarization and HUCBC induced regulation of neuroinflammation, WM and vascular remodeling leading to beneficial effects are not clear and further studies are warranted.

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Conclusions

Treatment of stroke initiated 3 days post the ischemic insult via intravenous administration of HUCBCs in T2DM rats significantly improves functional recovery by enhancing WM-axonal and vascular remodeling in the ischemic brain. Our data suggest that decreasing neuroinflammatory factors and increasing M2 macrophage polarization may be contributing mechanisms underlying HUCBC treatment derived beneficial effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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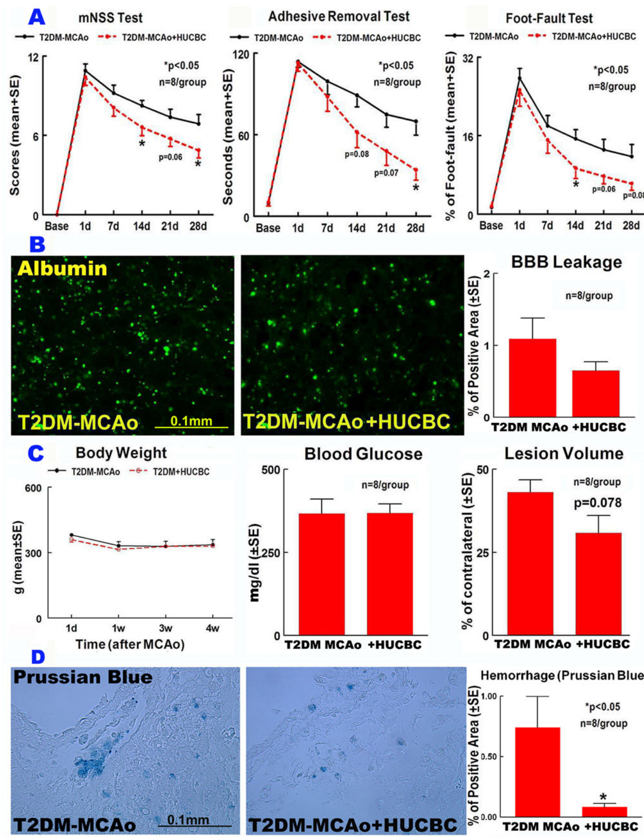

Figure 1.

HUCBC treatment 3d post stroke in T2DM-MCAo rats significantly improved functional outcome **A)** mNSS, Adhesive removal test, Foot-fault; **B)** did not significantly decrease BBB leakage, **C)** body weight, blood glucose or lesion volume but significantly decreased **D)** brain hemorrhage compared to PBS treated T2DM MCAo rats.

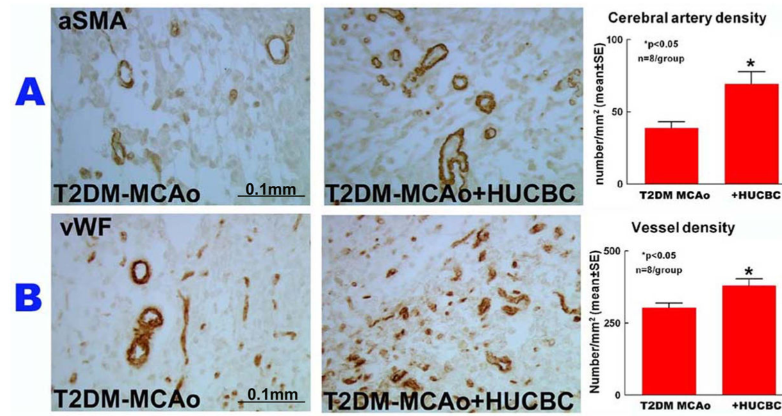

Figure 2.

HUCBC treatment 3d post stroke in T2DM-MCAo rats significantly improved vascular remodeling as indicated by **A)** a-SMA and **B)** vWF immunostaining and quantification data in the IBZ compared to PBS treated T2DM MCAo rats.

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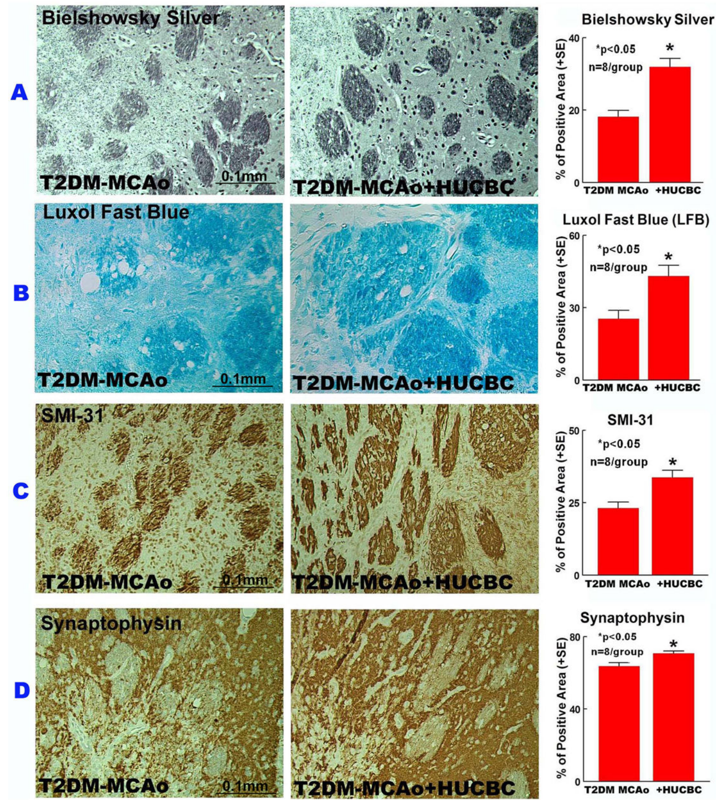

Figure 3.

HUCBC treatment 3d post stroke in T2DM-MCAo rats significantly increased WM remodeling, axon density and synaptic protein expression in the ischemic brain. **A**) Immunostaining with Bielschowsky silver, **B)** Luxol fast blue, **C**) SMI-31, **D)** Synaptophysin and quantification data in the IBZ compared to PBS treated T2DM MCAo rats.

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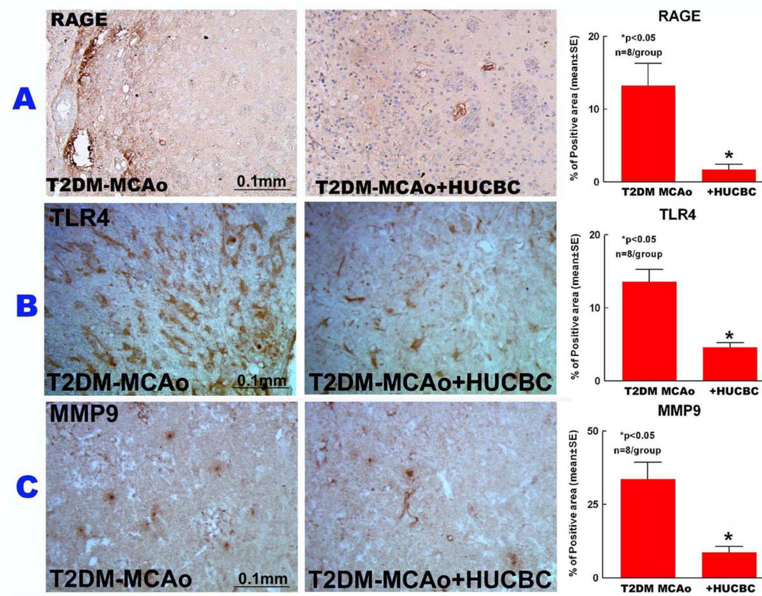

Figure 4.
HUCBC treatment 3d post stroke in T2DM-MCAo rats significantly decreased inflammatory factors expression in the ischemic brain. **A)** Immunostaining with Rage, **B)** TLR4, **C)** MMP9 and quantification data in the IBZ compared to PBS treated T2DM MCAo rats.

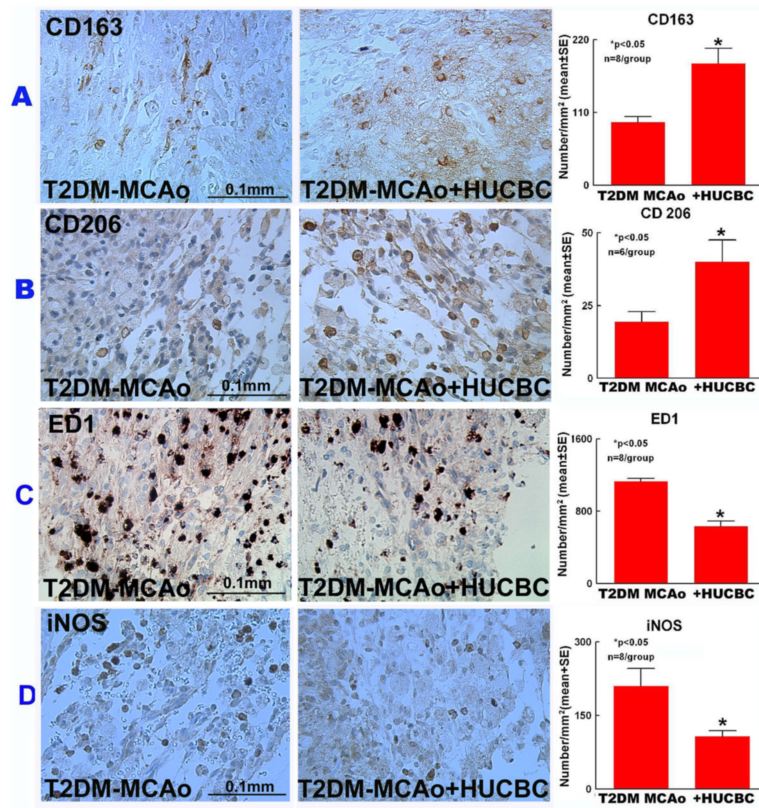

Figure 5.

HUCBC treatment 3d post stroke in T2DM-MCAo rats significantly increased M2 macrophage polarization in the ischemic brain. **A**) Immunostaining with CD 163, **B**) CD 206, **C**) ED1, **D**) iNOS and quantification data in the IBZ compared to PBS treated T2DM MCAo rats.

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