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Research paper

Exosomes derived from bone marrow mesenchymal stem cells harvested from type two diabetes rats promotes neurorestorative effects after stroke in type two diabetes rats

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ARTICLE INFO

Keywords:
Exosomes
Mesenchymal stromal cells
miR-9
Neurorestoration
Stroke
T2DM

ABSTRACT

Background and purpose: Diabetes elevates the risk of stroke, promotes inflammation, and exacerbates vascular and white matter damage post-stroke, thereby hindering long term functional recovery. Here, we investigated the neurorestorative effects and the underlying therapeutic mechanisms of treatment of stroke in type 2 diabetic rats (T2DM) using exosomes harvested from bone marrow stromal cells obtained from T2DM rats (T2DM-MSC-Exo).

Methods: T2DM was induced in adult male Wistar rats using a combination of high fat diet and Streptozotocin. Rats were subjected to transient 2 h middle cerebral artery occlusion (MCAo) and 3 days later randomized to one of the following treatment groups: 1) phosphate-buffered saline (PBS, i.v), 2) T2DM-MSC-Exo, (3 × 10¹¹, i.v), 3) T2DM-MSC-Exo with miR-9 overexpression (miR9+/+-T2DM-MSC-Exo, 3 × 10¹¹, i.v) or 4) MSC-Exo derived from normoglycemic rats (Nor-MSC-Exo) (3 × 10¹¹, i.v). T2DM sham control group is included as reference. Rats were sacrificed 28 days after MCAo.

Results: T2DM-MSC-Exo treatment does not alter blood glucose, lipid levels, or lesion volume, but significantly improves neurological function and attenuates post-stroke weight loss compared to PBS treated as well as Nor-MSC-Exo treated T2DM-stroke rats. Compared to PBS treatment, T2DM-MSC-Exo treatment of T2DM-stroke rats significantly 1) increases tight junction protein ZO-1 and improves blood brain barrier (BBB) integrity; 2) promotes white matter remodeling indicated by increased axon and myelin density, and increases oligodendrocytes and oligodendrocyte progenitor cell numbers in the ischemic border zone as well as increases primary cortical neuronal axonal outgrowth; 3) decreases activated microglia, M1 macrophages, and inflammatory factors MMP-9 (matrix metalloproteinase-9) and MCP-1 (monocyte chemoattractant protein-1) expression in the ischemic brain; and 4) decreases miR-9 expression in serum, and increases miR-9 target ABCA1 (ATP-binding cassette transporter 1) and IGF1 (Insulin-like growth factor 1 receptor) expression in the brain. MiR9+/+-T2DM-MSC-Exo treatment significantly increases serum miR-9 expression compared to PBS treated and T2DM-MSC-Exo treated T2DM stroke rats. Treatment of T2DM stroke with miR9+/+-T2DM-MSC-Exo fails to improve functional outcome and attenuates T2DM-MSC-Exo treatment induced white matter remodeling and anti-inflammatory effects in T2DM stroke rats.

Conclusions: T2DM-MSC-Exo treatment for stroke in T2DM rats promotes neurorestorative effects and improves functional outcome. Down regulation of miR-9 expression and increasing its target ABCA1 pathway may...
1. Introduction

Stroke is predominantly a vascular disease with devastating neurological aftermath, often resulting in long term disability or death. Despite a trend of declining ischemic stroke incidence in the United States as well as globally largely due to control of risk factors such as hypertension and smoking (Vangen-Lonne Anne et al., 2017), there is a dramatic worldwide increase in incidence of type 2 diabetes mellitus (T2DM) (Read et al., 2018). Diabetes increases the risk of stroke by 3 to 4 fold, and approximately 30% of stroke patients suffer from diabetes (Ergul et al., 2016; Mast et al., 1995; Megherbi et al., 2003). Treating diabetic stroke patients is challenging due to the extensive damage they sustain to the cerebral vasculature, exacerbated neurological deficits, enhanced inflammatory responses and their susceptibility to recurrent strokes (Callahan et al., 2011; Chen et al., 2011b; Chen et al., 2011c). Pre-clinical studies have indicated that therapeutic strategies that are successful in non-diabetic stroke may not readily translate to diabetic stroke treatment (Chen et al., 2011d; Ning et al., 2012). Thus, there is a compelling need to develop therapeutic strategies specifically to improve neurological function after stroke in the diabetic population.

Ischemic stroke induced neural damage and death is rapid, thereby challenging neuroprotective strategies with a narrow intervention time frame. Even when T2DM stroke patients receive timely thrombolytic intervention, they still face an increased risk of death, intracerebral hemorrhage and unfavorable 90-day outcome proportional to admission hyperglycemia (Poppe et al., 2009). Treatment of stroke using cell therapy is emerging as a promising treatment option, particularly at delayed time points (Chen et al., 2014; Sarmah et al., 2018). Spontaneous recovery driven by endogenous brain remodeling that occur post stroke may be amplified by exogenously administered neurorestorative agents, such as cell therapy (Chen et al., 2014). Among the cells used for post stroke therapy, bone marrow stromal cells (MSC) have great potential as therapeutic agents in stroke management, as they are easily obtained and can be rapidly expanded ex vivo for transplantation (Chen et al., 2014; Deng et al., 2019; Otero-Ortega et al., 2019).

MSCs interact with and alter brain parenchymal cells via the secretion of trophic and growth factors as well as exosomes to exert therapeutic effects (Otero-Ortega et al., 2019; Xin et al., 2012b). Exosomes are small extracellular vesicles (30–100 nm) that facilitate cell-cell communication. Exosome therapy has several advantages over cell therapy. Exosomes do not elicit immune rejection, do not have a vascular obstructive effect and have low risk of triggering tumors or malignant transformation (Xin et al., 2014). Facilitating clinical translation, a relatively large quantity of exosomes can be derived from a small quantity of cells; exosomes are stable and can be stored until therapeutic need (Codispoti et al., 2018). Thus, systemic administration of exosomes may be a means by which to deliver the active components of cell-based therapy to the CNS (Moon et al., 2019; Xin et al., 2014; Xin et al., 2013a). The release and transfer of exosomal cargo, which consists of proteins, lipids, and RNA molecules including messenger RNA (mRNA) and microRNA (miR), can regulate protein synthesis within recipient cells. MSCs then communicate with parenchymal cells via microvesicles including exosomes which transfer miRs to recipient cells.

MiRs are short sequences of non-coding RNA (ca. 22 nucleotides) and post-transcriptionally regulate gene expression (Chen et al., 2014; Moon et al., 2019; Otero-Ortega et al., 2019). MiRs can regulate many genes, pathways, and biological networks, either acting alone or in concert with other miRs (Chen et al., 2014). MiR-9 is among the highly expressed miRs in the developing and adult vertebrate brain and has been implicated to exert protective as well as adverse effects under various disease conditions (Coolen et al., 2013). In patients with pre-diabetes or T2DM, significantly increased miR-9 expression in peripheral blood has been reported (Al-Muhtares and Al-Kafaji, 2018). Increased serum miR-9 expression has also been associated with poor prognosis in diabetic nephropathy (Xiao et al., 2017). Increased expression of miR-9 in serum exosomes of acute ischemic stroke patients has been identified as a potential disease biomarker, and is associated with stroke severity (Ji et al., 2016). Previous studies have demonstrated that treatment of stroke with exosomes derived from MSCs of non-diabetic animals (MSC-Exo) improves neurological function in non-diabetic stroke animals (Xin et al., 2012a; Xin et al., 2013a; Xin et al., 2013b). We have also demonstrated that MSCs derived from diabetic rats promotes neurorestorative effects after stroke in T1DM rats (Cui et al., 2016). However, whether exosomes derived from bone marrow cells of T2DM rats (T2DM-MSC-Exo) can be employed to treat stroke in T2DM rats has not been investigated. In this study, we are the first to investigate the therapeutic efficacy and underlying mechanisms of stroke treatment in T2DM rats with T2DM-MSC-Exo.

2. Material 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. This manuscript has been prepared in accordance with ARRIVE guidelines.

2.1. Diabetes induction

To induce T2DM in male adult Wistar rats (175-200 g, Charles River), a combination of high fat diet (HFD, D12492, Research Diets) for 2 weeks followed by single intraperitoneal injection of low dose Streptozotocin (STZ, 35 mg/kg, Enzo) with continued HFD for another 2 weeks was employed. This widely accepted model of T2DM induces metabolic characteristics of T2DM, such as high glucose, peripheral insulin resistance and reduced β-cell mass (Islam and Loots du, 2009). Two weeks after STZ injection, fasting blood glucose level was tested using a glucose analyzer (AgaMatrix Advanced blood glucose monitoring system) and animals with fasting blood glucose > 300 mg/dl were subjected to stroke. Fasting blood glucose was measured again before sacrifice of animals to test the effect of treatment on glucose modulation. Blood lipids and triglycerides were measured using CardioChek Plus analyzer (Fischer Scientific).

2.2. Middle cerebral artery occlusion model

T2DM rats were subjected to transient (2 h) right middle cerebral artery occlusion (MCAo) via intraluminal vascular occlusion, as previously described (Chen et al., 1992). Briefly, rats were anesthetized with 2% isoflurane and maintained with 1.5 isoflurane in 70% N₂O and 30% O₂ by a face mask and regulated with a modified FLUOTEC 3 Vaporizer (Fraser Harlake). Rectal temperature was maintained at 37 °C throughout the surgical procedure by means of a feedback-regulated water heating system. A 4–0 nylon suture with its tip rounded by heating near a flame was inserted into the external carotid artery (ECA). The length of nylon suture, determined according to the animal's weight, was gently advanced from the ECA into the lumen of the internal carotid artery until the suture blocked the origin of the MCA. After 2 h of MCAo, animals were re-anesthetized, and the filament was...
gently withdrawn to restore blood flow.

2.3. MSC culture

Four normoglycemic rats and four rats with induced T2DM, as described above (fasting glucose > 300 mg/dl), were sacrificed and bone marrow was obtained from the long bones (femurs and tibias). Bone marrow cells were incubated at 37 °C with MSC culture medium (Chen et al., 2001a). Non-adherent cells were removed at 72 h and fresh medium was added. Day 7–10, adherent cells were recovered by trypsinization and cells were re-plated at a density of 5 × 10^6 cells per dish. MSC cultures were maintained by re-plating cells when they reach 80–90% confluence.

2.4. MiR-9 transfection

A mixture of 100 μl Ingenio Electroporation Solution (Mirus) and 5 μl of rat rno-miR-9a-5p mimic (Dharmacon) was prepared. T2DM-MSCs were harvested as described above, and 1–2 × 10^6 cells were re-suspended in the electroporation solution. The cell solution was then loaded into Ingenio cuvettes with a 0.2 cm gap (Mirus). The program run for the electroporation depended on the cell type being transfected (rat MSC used U023). After electroporation, cells were removed from the cuvettes and put into culture and allowed to grow for 24 h at which point the media was changed to media containing exosome depleted FBS and cells were cultured for 48 h. Following miR-9 transfection in DM-MSCs, we measured the expression of miR-9 using real time PCR and found that miR9+/+ DM-MSCs have significantly elevated miR-9 expression compared to DM-MSCs (Supplementary Fig. 1).

2.5. Exosome isolation

To isolate exosomes, culture media was filtered using a 0.22 μM syringe filter (Millipore) to remove any particulate matter and then Exoquick TC (System Biosciences) was added at the ratio of 2 ml Exoquick/10 ml media. The media was stored overnight at 4 °C and then centrifuged at 1500 g for 30 min. Supernatant was removed and the pellet re-suspended in PBS. The protein concentration was determined using BCA Protein Assay Kit (Pierce) and particle size and the number were analyzed using a qNano nanopore-based exosome detection system following the manufacturer’s instructions (Izon).

2.6. MiR9 measurement using real time PCR

Total RNA was isolated from serum, MSC and MSC-Exo with TRIzol (Invitrogen). Then, 2 μg total RNA was used to make cDNA using M-MLV (Invitrogen), following standard protocol. Of this cDNA, 2 μl was used to run a quantitative PCR using the SYBR Green real time PCR method. Quantitative PCR was performed on a ViiA 7 PCR instrument (Applied Biosystems) using 3-stage program parameters provided by the manufacturer, as follows; 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was tested in triplicate, and analysis of relative gene expression data using the 2-ΔΔCT method.

2.7. Experimental groups

Three days after MCAo, T2DM rats were randomized and assigned to one of the following treatments administered via tail-vein injection: 1) PBS-vehicle control (n = 10); 2) T2DM-MSC-Exo (n = 10, 100 μg protein concentration ~3 × 10^{11} exosomes); 3) miR9+/+T2DM-MSC-Exo (n = 6, 100 μg protein concentration ~3 × 10^{11} exosomes); 4) Nor-MSC-Exo (n = 6, 100 μg protein concentration ~3 × 10^{11} exosomes). T2DM-sham control group (n = 5) was included as reference. All treatments were administered once at 3 days after stroke. Since the infarct volume is fully evolved, stroke treatment initiated at this time point does not target decreasing lesion volume, but enhancing functional outcome via neurorestorative effects (Saver, 2010). Also, a majority of stroke patients can be treated at 3 days after stroke. Rats were sacrificed 28 days after MCAo for immunostaining quantification analysis.

2.8. Neurological function tests and exclusion criteria

An investigator was blinded to the experimental groups to perform a battery of functional tests including adhesive removal test (Chen et al., 2001b) and modified neurological severity score (mNSS) evaluation (Chen et al., 2001b) before MCAo, and on days 1, 7, 14, 21 and 28 after MCAo. Animals with mNSS scores < 6 (possibly small to no lesion) or > 13 (poor survival) at 24 h after MCAo were excluded. Body weight was recorded weekly.

2.9. Immunohistochemical assessment

Brains were fixed using transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. The brains were then embedded in paraffin and a standard block was obtained from the center of the lesion (bregma −2 mm ~ +2 mm). A series of 6 μm thick sections cut from the block were then prepared and antibody against specific protein of interest in this project used. To measure ischemic lesion volume, Hematoxylin-eosin (H&E) staining was employed and 7 brain sections were traced with the use of MCID image analysis system (Imaging Research). The indirect lesion area, in which the intact area of the ipsilateral hemisphere is subtracted from the area of the contralateral hemisphere was calculated (Chen et al., 2001a). Infarction volume is presented as a percentage of the lesion compared with the contralateral hemisphere. Lesion volume measurement was performed by an investigator blinded to the experimental groups.

Brain coronal tissue sections were prepared and antibody against APC (adenomatous polyposis coli, oligodendrocyte (OL) marker, Genway, 1:20), NG2 (neural/glial antigen 2, oligodendrocyte progenitor cell (OPC) marker, Chemicon (EMD Millipore), 1:400), ZO-1 (Zona occludens-1, Invitrogen, 1:500), ABCA1 (adenosine triphosphate-binding cassette transporter 1, Novus, 1:200), NG2 (neural/glial antigen 2, oligodendrocyte (OL) marker, Serotec), MMP-9 (matrix metalloproteinase-9, 1:500, Santa Cruz), CNPase (2′,3′-Cyclic-nucleotide 3′-phosphodiesterase, a prenylated myelin protein, Millipore, 1200), DAPI (4′,6-diamidino-2-phenylindole, used as a nuclear counterstaining, Vector Laboratories), GFAP (gliarial fibrillary acidic protein, astrocytes marker, Aves, 1:500), NeuN (neuronal nuclear antigen, marker for neurons, Chemicon, 1:200), vWF (von Willebrand factor, endothelial cell marker, 1:400; Dako), IBA1 (ionizing calcium-Binding Adaptor molecule 1, activated microglia marker, dilution 1:1000, Wako), ED1 (microglia/macrophages marker, 1:30; AbD Serotec), MMP-9 (matrix metalloproteinase-9, 1:500, Santa Cruz Biotechnology), and MCP-1 (monocyte chemoattractant protein 1) were employed. Antibody against albumin (albumin-FITC, polyclonal, 1:500, Abcam) was used to demonstrate blood brain barrier (BBB) leakage and Prussian blue staining used to evaluate hemorrhage. Bielschowsky-silver staining was used to demonstrate axons and luxol fast blue staining was used to demonstrate myelin. Control experiments consisted of similar procedures without addition of primary antibody.

In situ hybridization (ISH) was performed using the miRCURY LNA miRNA ISH Optimization Kit for formalin-fixed paraffin embedded tissue samples (Qiagen) following manufacturer’s protocol. Locked nucleic acid (LNA) probes against miR-9, U6-positive, and scrambled-negative probes (Exiqon) were used for ISH to detect mature miR. All probes were digoxigenin-labeled LNA probes at 25 nM in hybridization buffer.
2.10. Quantification analysis

All the immunostaining quantification analysis was performed by an investigator who was blinded to the experimental groups. Six-eight fields of view of the ischemic boundary zone (IBZ) were digitized under a 20× objective (Olympus BX40) using a 3-CCD colour video camera (Sony DYC-970MD) interfaced with an MCID image analysis system (Imaging Research). For each field of view, positive cell numbers (APC, NG2, ED1) were counted or the positive stained areas (BS, LFB, albumin-FITC, Prussian blue, ZO-1, ABCA1, IGF1, IBA1, MMP-9, MCP-1) were measured using a built-in densitometry function (MCID image analysis system) with a uniform threshold set above unstained for all groups.

2.11. Primary cortical neurons (PCN) axon outgrowth assay

PCNs were obtained from pregnant (day 18) embryonic Wistar rats (Charles River) and cultured with Neural basal-A medium (GIBCO) containing 2% B27 medium-supplement in vitro (Chen et al., 2011b; Ning et al., 2017). To separate axons from neuronal soma, a microfluidic chamber (Xona Microfluidics) was used (Zhang et al., 2013). PCNs were diluted to a concentration of 15–20 × 10⁶ cells/ml, and then 10 μl was placed in each microfluidic chamber and allowed to adhere overnight. The following day, cells were subject to oxygen-glucose deprivation (OGD) in a hypoxia chamber (Forma Anaerobic System, Thermo Scientific) with 37 °C incubator for 2 h. Then, cells were cultured in high glucose media (37.5 mmol/l glucose) for 3 days and treated with 1) Control; 2) + T2DM-MSC-Exo (20 ng); and 3) + miR9+/+-T2DM-MSC-Exo (20 ng); n = 2 chambers/group. pNFH (mature axon marker) positive axons were photographed at 20× magnification to obtain 11 fields of view using a video camera interfaced with MCID image analysis system. The average length of axonal outgrowth of pNFH positive cells was measured using Image J.

3. Statistical analysis

For behavioral tests, two-way repeated Analysis of Variance (ANOVA) followed by Bonferroni post tests were performed for multiple comparisons. A value of p < .05 was considered significant. One-way ANOVA was used for the evaluation of histology. “Contrast/estimate” statement was used to test the group difference. If an overall treatment group effect was detected at p < .05, pair-wise comparisons were made. All data are presented as mean ± standard error (SE).

4. Results

4.1. Treatment with T2DM-MSC-Exo significantly improves functional outcome after stroke in T2DM rats

To test the therapeutic efficacy of T2DM-MSC-Exo, a battery of function tests including mNSS evaluation and adhesive removal test were performed. T2DM-MSC-Exo treatment in T2DM stroke rats...
significantly improves neurological functional indicated by lower mNSS score and improved sensorimotor function indicated by adhesive removal test compared to Nor-MSC-Exo treated and control T2DM stroke rats \( (p < .05, \text{Fig. 1a–b}) \). T2DM-MSC-Exo treated rats had significantly lower weight loss after stroke compared to Nor-MSC-Exo treated and control T2DM stroke rats \( (p < .05, \text{Fig. 1c}) \), which is typically associated with better stroke outcome \( \text{(Jönsson et al., 2008)} \). No significant differences in infarct volume \( (p > .05, \text{Fig. 1d}) \), blood glucose or lipid levels were observed \( (p > .05, \text{Fig. 1e}) \).

4.2. Treatment of T2DM stroke rats with T2DM-MSC-Exo treatment significantly decreases BBB disruption and hemorrhage after stroke

DM stroke is associated with increased BBB leakage and hemorrhage \( \text{(Chen et al., 2011c; Cui et al., 2011)} \). Since an intact BBB prevents albumin from entering the brain, we employed FITC-albumin staining to evaluate the integrity of BBB. The expression of tight junction protein ZO-1 was evaluated around vessels in the IBZ and hemorrhage was measured using Prussian blue staining. Stroke in T2DM rats significantly \( (p < .05) \) increases BBB leakage \( \text{(Fig. 2a)} \) and hemorrhage \( \text{(Fig. 2b)} \) and decreases tight junction protein ZO-1 expression \( \text{(Fig. 2c)} \), compared to T2DM sham control rats. T2DM-MSC-Exo treatment significantly decreases BBB leakage \( \text{(Fig. 2a)} \) and hemorrhage \( \text{(Fig. 2b)} \) while increasing tight junction protein ZO-1 \( \text{(Fig. 2c)} \) post stroke in T2DM rats \( (p < .05) \).

4.3. Treatment of T2DM stroke rats with T2DM-MSC-Exo significantly improves white matter remodeling after stroke

T2DM impairs oligodendrogenesis and white matter remodeling after cerebral ischemia which affects long term functional recovery \( \text{(Ma et al., 2018)} \). Stroke in T2DM rats significantly decreases axon density \( \text{(Bielschowsky silver, axon marker, Fig. 3a)} \) and myelin density \( \text{(luxol fast blue, myelin marker, Fig. 3b)} \) compared to T2DM sham control rats. T2DM-MSC-Exo treatment significantly improves axon density \( \text{(Fig. 3a)} \), myelin density \( \text{(Fig. 3b)} \), OL number \( \text{(Fig. 3c)} \) and OPC number \( \text{(Fig. 3d)} \) in the IBZ of T2DM stroke rats compared to control T2DM stroke rats \( (p < .05) \).

4.4. T2DM-MSC-Exo treatment significantly decreases inflammatory factor expression in ischemic brain of T2DM stroke rats

Consistent with previous findings \( \text{(Chen et al., 2011c; Ma et al., 2018; Tureyen et al., 2011)} \), Fig. 4 indicates that T2DM stroke is associated with aggravated inflammatory responses and proinflammatory microglial/macrophage phenotype. T2DM-MSC-Exo treatment in T2DM stroke rats significantly decreases activated microglia \( \text{(IBA1, Fig. 4a)} \), M1 macrophage \( \text{(ED1, Fig. 4b)} \), and inflammatory factors MMP-9 \( \text{(Fig. 4c)} \) and MCP-1 \( \text{(Fig. 4d)} \) expression in the ischemic brain, compared to control T2DM stroke rats \( (p < .05) \).

4.5. Treatment of T2DM stroke rats with T2DM-MSC-Exo significantly decreases miR-9 expression

To test mechanisms of T2DM-MSC-Exo derived therapeutic benefits

![Fig. 2. T2DM-MSC-Exo treatment significantly improves blood brain barrier function and integrity while decreasing brain hemorrhage after stroke in T2DM rats. Compared to sham T2DM rats, T2DM stroke rats exhibit significantly a) increased BBB leakage identified by FITC-Albumin immunostaining, b) increased brain hemorrhage identified by Prussian blue immunostaining and c) decreased tight junction protein ZO-1 expression in the ischemic brain. Compared to PBS treated T2DM-stroke rats, T2DM-MSC-Exo treated T2DM stroke rats exhibit significantly a) decreased BBB leakage, b) decreased brain hemorrhage and c) increased tight junction protein ZO-1 expression in the ischemic boundary zone. Over expression of miR9 (miR9 +/+ -T2DM-MSC-Exo) does not significantly inhibit T2DM-MSC-Exo induced BBB protection or alter brain hemorrhage in T2DM stroke rats. Scale bar: 0.05 mm. Sample size: T2DM sham: n = 5; T2DM-MCAo: n = 10; T2DM-MCAo + T2DM-MSC-Exo: n = 10; T2DM-MCAo + miR9 +/+ -T2DM-MSC-Exo: n = 6. *p < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
in T2DM stroke, we tested the expression of several miRs that are associated with T2DM and stroke in serum of T2DM stroke rats treated with PBS and T2DM-MSC-Exo. As shown in Fig. 5a–b, we found that miR-9 expression was significantly decreased in T2DM-MSCs and T2DM-MSC-Exo compared to MSCs and MSC-Exo, respectively. Fig. 5c shows that stroke in T2DM rats significantly increases serum miR-9 expression compared to T2DM sham rats. Our data also show that compared to PBS treated T2DM stroke rats, miR-9 expression is significantly decreased in serum of T2DM-MSC-Exo treated T2DM stroke rats and significantly increased in serum of T2DM stroke rats treated with miR9+/+-T2DM-MSC-Exo. ISH using LNA probes specific for miR-9 in brain sections from T2DM-stroke rats indicates that miR-9 is expressed in the nucleus and cytoplasm of brain cells, while U6 was positive in the nucleus and scramble probe did not detect any signal (Fig. 5d). Stroke in T2DM rats increases miR-9 expression in the brain and treatment with T2DM-MSC-Exo decreases miR-9 expression in the brain. Treatment with miR9+/+-T2DM-MSC-Exo increases miR-9 expression. To identify the neurovascular locations of cerebral miR-9 expression, after ISH, immunofluorescent staining was performed with antibody against GFAP (astrocyte marker), vWF (endothelial cell marker) and NeuN (neuron marker). We observe co-localization of miR-9 with neurons, endothelial cells and astrocytes (Supplementary Fig. 3).

4.6. MiR-9 may partially mediate T2DM-MSC-Exo induced neurorestorative effects in T2DM stroke rats

To test the role of miR-9 in mediating the therapeutic effects of T2DM-MSC-Exo in T2DM stroke rats, we evaluated the effects of miR9+/+-T2DM-MSC-Exo treatment. Our data indicate that miR9+/+T2DM-MSC-Exo treatment fails to improve neurological function (Fig. 1a-b) and does not improve BBB integrity (Fig. 2a) or tight junction protein expression (Fig. 2c) compared to control T2DM stroke rats. However, T2DM stroke rats treated with miR9+/+T2DM-MSC-Exo exhibit significantly decreased hemorrhage (Fig. 2b) and improved myelin density (Fig. 3b) and oligodendrogenesis (Fig. 3c-d) compared to control T2DM stroke rats; and miR9+/+T2DM-MSC-Exo treatment improves primary cortical neuron (PCN) axon outgrowth compared to control group (Fig. 5e). Overexpression of miR-9 significantly
attenuates T2DM-MSC-Exo treatment induced improvement in axon density, myelin density, OL and OPC number (Fig. 3a–d), and PCN axon outgrowth (Fig. 5e) compared to the T2DM-MSC-Exo treated group. Overexpression of miR-9 significantly attenuates T2DM-MSC-Exo treatment induced anti-inflammatory effects and increases IBA1 and MMP9 expression compared to T2DM-MSC-Exo treated group (Fig. 4a,c). Therefore, it is likely that miR-9 partially contributes to T2DM-MSC-Exo treatment induced neurorestorative effects and other signaling pathways are also involved.

4.7. Treatment of T2DM stroke rats with T2DM-MSC-Exo significantly increases ABCA1 and IGF1R expression after stroke

ABCA1 and IGF1 are targets of miR-9 (D’Amore et al., 2018). T2DM is associated with reduced ABCA1 gene expression, protein and function (Patel et al., 2011; Tang et al., 2020). Data in Fig. 6a–b show that stroke in T2DM rats significantly decreases ABCA1 expression in the brain compared to T2DM sham rats and T2DM-MSC-Exo treatment in T2DM stroke rats significantly increases ABCA1 and IGF1 expression compared to T2DM-MCAo control. We also found that ABCA1 expression co-localizes mostly with neurons (NeuN) compared to oligodendrocytes (CNPase) or astrocytes (GFAP) in the IBZ (Supplementary Fig. 2). However, miR9 +/+ T2DM-MSC-Exo treatment fails to increase ABCA1 and IGF1R expression (Fig. 6a,b) compared to T2DM-MSC-Exo treated T2DM stroke rats.

5. Discussion

In this study, we have demonstrated for the first time that treatment of T2DM stroke with exosomes derived from MSCs of T2DM rats exerts therapeutic effects resulting in neurological functional recovery compared to Nor-MSC-Exo treated or PBS treated control T2DM stroke rats. Our data indicate that T2DM-MSC-Exo treatment attenuates BBB leakage, hemorrhage, weight loss, and inflammatory responses, while increasing white matter remodeling. T2DM-MSC-Exo induced therapeutic effects maybe partially mediated by decreasing miR-9 and up-regulating ABCA1-IGF1R pathway.

Neurorestorative therapies aim to improve neurological function by promoting brain remodeling and plasticity after stroke. In non-diabetic rats subjected to stroke, both MSCs and MSC-Exo treatment improves neurological outcome by promoting neurogenesis, angiogenesis, neurite remodeling and neurovascular remodeling (Chen et al., 2003; Xin et al.,...
Our data indicate that T2DM-MSC-Exo but not Nor-MSC-Exo improves neurological outcome in T2DM rats subjected to stroke. This is consistent with our previous study in which treatment of T1DM stroke rats with DM-MSCs improved neurological functional outcome, BBB integrity, vascular remodeling and white matter remodeling, while treatment of T1DM stroke with Nor-MSCs did not improve neurological outcome or promote neurorestorative effects (Chen et al., 2011; Cui et al., 2016). Significant improvement in neurological outcome after treatment of T2DM stroke with human MSCs, rat-non-DM MSCs and rat-non-DM MSC-conditioned medium has been previously reported (Hu et al., 2016; Xiang et al., 2017; Yan et al., 2016). In humans as well as rats, MSCs derived from normal or diabetic subjects exhibit similar

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**Fig. 5.** Treatment of T2DM stroke rats with T2DM-MSC-Exo significantly decreases serum miR-9 expression. MiR-9 may mediate T2DM-MSC-Exo induced axonal outgrowth.

MiR-9 expression is significantly decreased in a) T2DM-MSCs compared to non-DM MSCs and b) exosomes derived from T2DM-MSCs compared to exosomes derived from non-DM MSCs. c) Compared to T2DM sham rats, T2DM stroke rats exhibit significantly increased serum miR-9 expression. Compared to PBS treated T2DM stroke rats, miR-9 expression is significantly decreased in serum of T2DM-MSC-Exo treated T2DM stroke rats and significantly increased in serum of T2DM stroke rats treated with miR9+/+T2DM-MSC-Exo. d) In situ hybridization using LNA probes specific for miR-9 in brain sections from T2DM-stroke rats indicates that miR-9 is expressed in the nucleus and cytoplasm of brain cells, while U6 was positive in the nucleus and scramble probe did not detect any signal. Stroke in T2DM rats increases miR-9 expression in the brain and treatment with T2DM-MSC-Exo decreases miR-9 expression in the brain. Treatment with miR9+/+T2DM-MSC-Exo increases miR-9 expression. e) In PCNs subject to ischemia and high glucose conditions, T2DM-MSC-Exo and miR9+/+T2DM-MSC-Exo treatment significantly improve axonal outgrowth compared to control group. However, miR9+/+T2DM-MSC-Exo significantly decreases PCN axon outgrowth compared to T2DM-MSC-Exo treated group. Sample size: T2DM sham: n = 5; T2DM-MCAo: n = 10; T2DM-MCAo + T2DM-MSC-Exo: n = 10; T2DM-MCAo + miR9+/+T2DM-MSC-Exo: n = 6. *p < .05.
morphology, surface marker expression, growth kinetics and differentiation potential in vitro (Gabr et al., 2013; José et al., 2017; Yaochite et al., 2016). However, the secretome of MSCs differs between normoglycemic and hyperglycemic conditions (Morris et al., 2018; Ribot et al., 2017). By employing T2DM Zucker diabetic fatty rats and Zucker LEAN littermates as non-DM control, it has been demonstrated that short-term diabetes alters MSC secretome composition and promotes angiogenic capabilities (Ribot et al., 2017). Priming or pre-conditioning MSCs with hypoxia, growth factors, high glucose, oxidative stress, LPS stimulation etc. improves the therapeutic effects of MSCs in various diseases (Noronha et al., 2019). DM-MSCs secrete soluble, bioactive, angiogenic and chemotactic mediators which promote endothelial cell tube-like formation and migration (Ribot et al., 2017). Proteomic analysis of non-DM-MSCs and DM-MSCs conditioned media also showed differences in proteins related to extracellular matrix remodeling and glucose metabolism (Ribot et al., 2017). Among these factors, vascular endothelial growth factor (VEGF) expression levels are significantly reduced in DM-MSCs compared to Non-DM-MSCs (Khan et al., 2013; Ribot et al., 2017; Zacharek et al., 2007). VEGF is a key mediator of angiogenesis and vascular permeability (Keck et al., 1989).

While increasing VEGF in the recovery phase of non-DM stroke is associated with improved angiogenesis and neurological function, in diabetic stroke, elevated VEGF expression is associated with increased BBB permeability and brain swelling, resulting in worse outcome and larger infarct size (Kim et al., 2018; Zhang et al., 2000). However, it is likely that increased BBB leakage and/or brain hemorrhage override potential beneficial effects of Nor-MSC/Nor-MSC-Exo in DM stroke. Thus, variations in secreted factors that mediate vascular remodeling and stabilization, and BBB integrity may contribute to therapeutic differences between nor-MSC-Exo and DM-MSC-Exo.

Poor neurological functional outcome in diabetic stroke has been attributed in part to microvascular dysfunction, increased BBB permeability and aggravated inflammatory responses (Chen et al., 2011b; Ding et al., 2015; Field et al., 2015; Husseini et al., 2017; Uzu et al., 2010). In addition, T2DM results in greater structural and functional disruption of white matter after stroke which is associated with worse long term functional outcome (Ma et al., 2018). Tight junction proteins such as ZO-1, claudin-5 and occludin play critical roles in maintaining an intact and functioning BBB (Ueno, 2007). Similarly, in this study we found that stroke in T2DM rats significantly increases BBB leakage, decreases tight junction protein expression, decreases axon and myelin density, reduces the number of OLs and OPCs and increases inflammation in the brain compared to T2DM sham rats. These data are consistent with prior reports that T2DM-stroke increases pathological vascular remodeling, increases hemorrhagic transformation, aggravates ischemic injury and exacerbates white matter damage and inflammation in the brain (Chen et al., 2011a; Ergul et al., 2007). We also found that T2DM stroke rats treated with T2DM-MSC-Exo exhibit significantly increased tight junction protein expression and improved BBB function compared to control T2DM-stroke rats. Improved BBB function is a key factor in improving neurological outcome and promoting neurorestorative effects in diabetic stroke animals and has been reported in MSC and human umbilical cord blood cells treatments of diabetic stroke (Cui et al., 2016; Venkat et al., 2018; Yan et al., 2015; Yan et al., 2016). Compared to non-diabetic stroke mice, T2DM stroke mice exhibit significantly greater demyelination, impaired oligodendrogenesis and worse functional outcome at 35 days after ischemic stroke (Ma et al., 2018). Therefore, improving white matter remodeling is essential to improve long term neurological functional outcome. Our data show that T2DM-MSC-Exo treatment of stroke in T2DM rats significantly improves axon and myelin density and improves oligodendrogenesis compared to control T2DM stroke rats.

A leaky BBB aggravates inflammatory response by facilitating invasion of peripheral immune cells, inflammatory factors and macrophages thereby creating an inhospitable environment for brain repair (Jiang et al., 2018). Inflammatory factor MMP-9 is elevated after stroke and has been implicated in aggravating BBB disruption, neuronal death, myelin degradation and white matter injury (Clark et al., 1997; Ji et al., 2017). Particularly in T2DM stroke mice, increased MMP-9 exacerbates white matter injury (Chen et al., 2011a). Inflammatory factor MCP-1 is elevated in serum of diabetic patients as well as from stroke patients (Arakelyan et al., 2005; Vinagre et al., 2014). Compared to wild type control, MCP-1 deficient mice (MCP-1−/−), and rats with non-functional MCP-1 develop significantly smaller infarct volume after stroke and have significantly lower accumulation of phagocytic M1 macrophages in the IBZ (Bose and Cho, 2013; Hughes et al., 2002). In response to ischemia, microglia become reactive and release of cytotoxic factors and oxidative metabolites which contribute to neuronal injury and
elevated inflammatory status in the ischemic brain (Kim and Cho, 2016). Following ischemic stroke, hyperglycemia favors pro-inflammatory state of macrophages and inhibits macrophage polarization to a non-inflammatory state (Khan et al., 2016; Ma et al., 2018). Hyperglycemia shifts microglia to an inflammatory M1 phenotype which impairs OPC differentiation and maturation (Ma et al., 2018). In T2DM stroke mice, proinflammatory microglia/macrophage responses impairs oligodendrogenesis and inhibits white matter repair (Ma et al., 2018). Ablation of peripheral monocytes inhibits the adverse effects of hyperglycemia indicating that monocytes play an important role in the pathological cascade following stroke in hyperglycemic mice (Khan et al., 2016). Our data show that T2DM stroke rats treated with T2DM-MSC-Exo exhibit significantly decreased IBA1, ED1, MMP-9 and MCP-1 expression in the ischemic brain while increased tight junction protein and improved BBB integrity.

MiRs are emerging as key players in the pathogenesis of T2DM and stroke and subsequent vascular and white matter damage (Shantikumar et al., 2012; Zampetaki et al., 2010; Zampetaki and Mayr, 2012). It has been previously demonstrated that MSC-Exo and their cargo miRs promote neurite remodeling and functional recovery after stroke in rats (Xin et al., 2012a; Xin et al., 2013b). In a cohort of newly diagnosed T2DM patients, 7 serum miR's including miR-9 were upregulated (Kong et al., 2011). MiR-9 is one of the most abundant miRs in the developing and adult vertebrate brain (Coolen et al., 2013). Under normal conditions, miR-9 expression in blood is very low (Ogata et al., 2015). Given the specificity of miR-9 to adult CNS, release of miR-9 into the circulation is considered an indicator of neural damage and neurotoxicity (Hachisuka et al., 2014; Ji et al., 2016; Ogata et al., 2015; Xue et al., 2018a). In acute ischemic stroke patients, serum and serum exosomal miR-9 expression were significantly increased compared to healthy control patients (Ji et al., 2016; Xue et al., 2018b). Increased serum miR-9 and serum exosomal miR-9 expression were also found to positively correlate with the increased infarct volume, poor neurological outcome and increased expression of inflammatory factors in serum compared to the healthy control group (Ji et al., 2016; Xue et al., 2018a). In these patients, it is likely that the increased miR-9 in peripheral blood is derived from the injured brain tissue (Ji et al., 2016; Xue et al., 2018b). Likewise, our data indicate that stroke in T2DM rats significantly increases serum and brain miR-9 expression. In addition, DM-MSCs and DM-MSC-Exo exhibit reduced miR-9 expression compared to MSCs and MSC-Exo, respectively, and T2DM-MSC-Exo treatment significantly decreases serum and brain miR-9 expression.

Decreasing miR-9 increases ABCA1 expression (D’Amore et al., 2018). ABCA1 is expressed in cells that constitute the neurovascular unit and thereby plays an important role in maintenance of the BBB and stability of the neurovascular unit (Kim et al., 2006). ABCA1 is highly expressed in neurons, microglia, astrocytes and OLA (Kim et al., 2006; Koldamova et al., 2003) and is required for myelination, dendritic outgrowth and synaptic activity (Karaiskina et al., 2009). We have previously demonstrated that deficiency of ABCA1 in brain induces worse neurological functional deficits after stroke, increases BBB leakage and aggravates demyelination, OL axonal injury and neurofilament loss (Cui et al., 2015; Cui et al., 2017; Wang et al., 2018). Our data show that treatment with T2DM-MSC-Exo significantly increases ABCA1 and IGF1R expression compared to T2DM stroke rats. We also found that, in T2DM stroke rats treated with T2DM-MSC-Exo, ABCA1 expression co-localizes mostly with neurons (NeuN) compared to oligodendrocytes (CNPase) or astrocytes (GFAP) in the ischemic boundary zone. In a previous study, transgenic mice lacking brain ABCA1 (ABCA1<sup>−/−</sup>) as well as mice selectively lacking ABCA1 in neurons (ABCA1<sup>N−/−</sup>) or astrocytes (ABCA1<sup>A/-/−</sup>) were examined for neuroinflammation in the brain (Karaiskina et al., 2013). ABCA1<sup>−/−</sup> mice exhibited cortical astrogliosis and elevated inflammatory gene expression profile, ABCA1<sup>N−/−</sup> mice exhibited cortical astrogliosis without changes in inflammatory genes while ABCA1<sup>A/-/−</sup> mice did not develop astrogliosis or elevated expression of inflammatory markers (Karaiskina et al., 2013). This indicates that loss of neuronal ABCA1 alone can lead to increased presence of reactive astrocytes without microglial activation and coordinated ABCA1 activity across neurons and glial cells affects neuroinflammation and neurodegeneration in the brain (Karaiskina et al., 2013). IGF1 mediates brain growth and development, as well as neurorestorative effects such as myelination, neurogenesis and oligodendrogenesis. IGF1 also exerts neuroprotective effects and improves BBB integrity post stroke. IGF1 decreases cholesterol efflux via ABCA1 and scavenger receptor class B type I expression. Hence the ABCA1 and IGF1 pathway may play a key role in neurorestorative post stroke.

To test the role of miR-9 in mediating T2DM-MSC-Exo derived therapeutic effects after T2DM stroke in rats, we treated a group of T2DM stroke rats with miR9<sup>+/+/+</sup>-T2DM-MSC-Exo. Our data indicate that miR9<sup>+/+/+</sup>-T2DM-MSC-Exo treatment fails to improve neurological function or BBB integrity and significantly attenuates T2DM-MSC-Exo induced WM remodeling and anti-inflammatory effects. In response to ischemic injury to the brain, microglia become activated, undergo morphological change, express surface markers similar to macrophages and circulating monocytes, release proinflammatory factors, and function as phagocytes (Taylor and Sondas, 2013; Yenari et al., 2010). While the activation of microglia evolves over the hours and days following ischemic injury, their phagocytic function facilitates debris removal and neurorepair in the ischemic brain, yet, their generation of proinflammatory cytokines, cytotoxic factors and oxidative metabolites contribute to neuronal injury and secondary brain damage (Kim and Cho, 2016; Taylor and Sondas, 2013; Yenari et al., 2010). Microglia under normal conditions do not express miR-9; however, in response to activation such as LPS stimulation, Iba-1 positive microglia express mature miR-9 (Yao et al., 2014). MiR-9 mediated activation of rat primary microglial cells shift these cells to M1 phenotype and increases the expression of proinflammatory mediators such as interleukin (IL)-1β, IL-6, tumor-necrosis factor-α (TNF-α), MCP-1 and nitric oxide (NO) (Yao et al., 2014). Our data indicate that T2DM stroke is associated with increased miR9 and aggravated inflammatory responses, and increased proinflammatory microglial/macrophage phenotype. However, treatment with T2DM-MSC-Exo significantly decreases miR9 and thereby decreases activated microglia and M1 macrophage and inflammatory factors MMP-9 and MCP-1 expression in the ischemic brain, compared to control T2DM stroke rats. Conversely, miR9<sup>+/+/+</sup>-T2DM-MSC-Exo treatment exacerbates inflammatory responses with increased IBA1, and MMP-9 expression compared to T2DM-MSC-Exo treated T2DM stroke rats. Thus, decreasing miR9 may reduce inflammatory responses and thereby reduce secondary brain damage and contribute to the T2DM-MSC-Exo induced improvement in stroke outcome in T2DM rats. In neonatal rat neuronal cells, transfection of neuronal cells with a miR-9 mimic increased OGD induced apoptosis while transfection with a miR-9 inhibitor decreased OGD induced neuronal apoptosis (Xue et al., 2018a). We found that in PCN’s subject to OGD under high glucose conditions, miR-9 overexpression attenuates T2DM-MSC-Exo induced attenuates axon outgrowth. However, the effects on BBB integrity between T2DM-MSC-Exo and miR9<sup>+/+/+</sup>-T2DM-MSC-Exo treatments are not significantly different, and miR9<sup>+/+/+</sup>-T2DM-MSC-Exo treatment improves myelination in T2DM-MCAo rats and improves PCN axonal outgrowth compared to ischemic control group. Therefore, it is likely that miR-9 and its targets such as ABCA1 only partially contribute to T2DM-MSC-Exo treatment induced neurorestorative effects, and it is likely that other signaling pathways are also involved. The miR-145/ABCA1 pathway was found to play a major role in DM-MSCs treatment induced neurorestorative effects after stroke in T1DM rats (Cui et al., 2016). Both miR-145 and miR-9 can regulate ABCA1 expression, and it is likely that in addition to miR-9; other miR’s such as miR-145 may contribute to therapeutic effects of T2DM-MSC-Exo treatment in T2DM stroke. Further studies are warranted to investigate mechanisms of T2DM-MSC-Exo derived therapeutic effects in T2DM stroke.
Mobilization of stem cells from bone marrow to peripheral blood followed by migration to injured brain tissue contributes to early endogenous repair mechanisms in the ischemic brain (Borlongan, 2011; Courties et al., 2015; Rochefort et al., 2006; Wang et al., 2014; Zhang et al., 2002). In patients with acute ischemic stroke, there was a significant increase in non-apoptotic, MSC-derived microparticles in the circulation indicating that stroke induces mobilization of bone marrow derived MSCs and MSC derived microparticles (Kim et al., 2012). Induction of transient MCAo in mice rapidly activates bone marrow myeloid cells, releases CXCR2-positive granulocytes from the bone marrow and increases bone marrow leukocyte response (Denes et al., 2011). Stroke also alters the sub-population of bone marrow derived mononuclear cells and their release of bioactive factors and cytokines which exert anti-apoptotic, pro-angiogenic and pro-neurogenic effects as well as modulate pro-inflammatory and anti-inflammatory responses (Yang et al., 2012). Exogenous stem cell therapy also activates and facilitates migration of endogenous stem cells from neurogenic niches to injured brain tissue to promote repair and recovery (Crowley and Tajiri, 2017; Tajiri et al., 2013; Yoo et al., 2008). Therefore, in addition to injured brain tissue to promote repair and recovery (Crowley and Tajiri, 2017; Tajiri et al., 2013; Yoo et al., 2008). Therefore, in addition to the therapeutic effects of exogously administered T2DM-MSC-Exo, it is likely that the endogenous stem cells and their exosomes also contribute to improvement of neurological outcome and neurorestorative effects in T2DM rats.

There are a few limitations to this study. The source of MSCs is known to influence the biological function of MSCs (Bonab et al., 2006). We have previously reported that non-diabetic MSCs administered at 1 day after stroke have adverse effects in T1DM stroke rats (Chen et al., 2011d), while human MSCs administered at 3 days after stroke significantly improves neurological function and promotes neurorestorative effects after stroke in T2DM rats (Van et al., 2016). Here we show that T2DM-MSC-Exo but not Nor-MSC-Exo, improves neurological function after stroke in T2DM rats. It is important to investigate the mechanisms by which treatment with MSCs and MSC-Exo from different sources affect stroke outcome.

The changes in BBB integrity, white matter remodeling and inflammatory responses after treatment with T2DM-MSC-Exo in T2DM stroke rats have been evaluated using immunohistochemical staining and analysis, and further biochemical analyses are warranted. We have not investigated sex effects on T2DM-MSC-Exo treatment for T2DM-stroke in rats. We are aware that the optimized treatment protocol and neurovascular responses for males may not be the same for females. Therefore, investigations of the effect of sex differences of T2DM-MSC-Exo treatment for T2DM stroke are warranted.

6. Conclusions

T2DM rats subject to stroke and treated with T2DM-MSC-Exo exhibit significant neurological functional recovery compared to Nor-MSC-Exo and PBS treated T2DM stroke rats. MiR-9/ABC1 pathway may play an important role in T2DM-MSC-Exo induced white matter remodelling and attenuation of inflammatory responses.

Author contributions

All authors have read and agreed to the published version of the manuscript.

Funding

N/A.

Declaration of Competing Interest

The authors declare no conflict of interest.


