Synergistic Effects of Transplanted Endothelial Progenitor Cells and RWJ 67657 in Diabetic Ischemic Stroke Models

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Background and Purpose—An immature vascular phenotype in diabetes mellitus may cause more severe vascular damage and poorer functional outcomes after stroke, and it would be feasible to repair damaged functional vessels using endothelial progenitor cell (EPC) transplantation. However, high glucose induces p38 mitogen-activated protein kinase activation, which can accelerate the senescence and apoptosis of EPCs. The aim of this study was to investigate the combined effects of EPC transplantation and p38 mitogen-activated protein kinase inhibitor administration on diabetic stroke outcomes.

Methods—Bone marrow–derived EPCs were injected intra-arterially into db/db mice after ischemic stroke induction. RWJ 67657 (RWJ), a p38 mitogen-activated protein kinase inhibitor, was administered orally for 7 consecutive days, with the first dose given 30 minutes before stroke induction. Functional outcome was determined at days 0, 1, 7, 14, and 21. Angiogenesis, neurogenesis, infarct volume, and Western blotting assays were performed on day 7, and white matter remodeling was determined on day 14.

Results—Neither EPC transplantation nor RWJ administration alone significantly improved diabetic stroke outcome although RWJ displayed a potent anti-inflammatory effect. By both improving the functioning of EPCs and reducing inflammation, EPC transplantation plus RWJ administration in vivo synergistically promoted angiogenesis and neurogenesis after diabetic stroke. In addition, the white matter remodeling, behavioral scores, and expressions of vascular endothelial growth factor and brain-derived neurotrophic factor were significantly increased in diabetic mice treated with both EPCs and RWJ.

Conclusions—The combination of EPC transplantation and RWJ administration accelerated recovery from diabetic stroke, which might have been caused by increased levels of proangiogenic and neurotrophic factors. (Stroke. 2015;46:1938-1946. DOI: 10.1161/STROKEAHA.114.008495.)

Key Words: cell transplantation diabetes mellitus endothelial progenitor cells inflammation p38 mitogen-activated protein kinases

Ischemic stroke is a major cause of death and disability worldwide, and type 2 diabetes mellitus is an important independent risk factor.1 Diabetic patients have exacerbated ischemic cerebral damage and poorer outcomes after stroke than patients without diabetes mellitus,2 and impaired angiogenesis may cause this discrepancy.3 Cell-based therapy for ischemic stroke has recently been introduced into the clinical setting.4 Endothelial progenitor cells (EPCs) promote angiogenesis after ischemic stroke by incorporating into impaired vessels and secreting proangiogenic factors.5,6 Patients with diabetes mellitus have a decreased number of circulating EPCs, and these EPCs are functionally impaired7; therefore, the transplantation of these cells holds promise for the treatment of stroke in diabetic patients.

However, the benefits derived from EPC transplantation in diabetic animals are not as great as those in wild-type models.8 The cell microenvironment is essential to cell proliferation, differentiation, migration, and other processes. EPC functions have been reported to be impaired under conditions of hyperglycemia, oxidative stress, or inflammation.8 Therefore, EPC transplantation may be insufficient in diabetic animals because the transplanted cells would be impaired in vivo.

The deleterious effects of diabetes mellitus on EPCs have been reported to be related to the phosphorylation of p38 mitogen-activated protein kinase (MAPK).9 Suppression of the p38 MAPK signaling pathway increases the number of EPCs and improves their functioning in vitro.9
(RWJ) is a highly selective inhibitor of the p38-α and p38-β MAPK isoforms and is more potent in vitro than the standard p38 kinase inhibitor SB 203580 (SB). In our previous study, the daily administration of RWJ significantly increased the number of transplanted EPCs homing to the ischemic brain in diabetic animals. Furthermore, RWJ has been shown to produce significant anti-inflammatory effects in various animal models. We hypothesized that RWJ has great potential to promote recovery in diabetic stroke models by protecting transplanted EPCs from impairment and by reducing inflammation.

Materials and Methods

Isolation and Cultivation of Bone Marrow–Derived EPCs

EPC isolation and culture are described in detail in the online-only Data Supplement. The characterization of the cultured cells has been reported in our previous study. RWJ (Santa Cruz, CA) or SB (Sigma-Aldrich, St Louis, MO) was added to culture media containing a high level of glucose (25 mmol/L). The proliferation, senescence, apoptosis, adhesion, migration, and tube formation of the EPCs were evaluated at 48 hours after the start of incubation.

Enzyme-Linked Immunosorbent Assay

The secretion of vascular endothelial growth factor (VEGF) by EPCs was quantified with ELISA kits (Abcam) according to the manufacturer’s instructions.

Ischemic Stroke Models

Photomorphogenic ischemic stroke was induced in db/db mice (male, 8 weeks old; Academy of Military Medical Science). EPCs (1×10⁶) were injected into the mice via the ipsilateral internal carotid artery at 24 hours after surgery. RWJ (50 mg/kg per day) was intragastrically administered once a day for 7 days, with the first dose given 30 minutes before ischemic stroke induction.

Behavioral Tests

Behavioral tests, including the modified neurological severity score and the foot-fault test, were conducted by an investigator who was blinded to the experimental groups. The tests were performed before stroke induction and on days 1, 7, 14, and 21 after stroke.

Magnetic Resonance Imaging Measurements

Magnetic resonance imaging scans were performed using a 7.0-T small animal MR scanner (Bruker PharmaScan, Ettlingen, Germany). The animals were scanned at 24 hours and on day 7 post ischemia using T₂-weighted imaging and then on day 14 post ischemia using diffusion tensor imaging (DTI).

Figure 1. P38 mitogen-activated protein kinase (MAPK) inhibitors significantly reverse the effects of high glucose on endothelial progenitor cell (EPC) proliferation, senescence, and apoptosis. A, EPCs were incubated with different concentrations of glucose or mannitol for 48 hours. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed to assess EPC proliferation, and the results were normalized to cells incubated in control media (5 mmol/L glucose; n=4 per group). B, Different concentrations of p38 MAPK inhibitor (RWJ or SB) were added to media containing 25 mmol/L glucose. The MTT values were normalized to cells incubated in media containing only 25 mmol/L glucose (n=4 per group, left). The numbers of EPCs per high power field in the different groups were counted under a microscope (n=5 per group, middle). The colony-forming units (CFUs) of EPCs are shown in the right (n=8 per group). C, Cellular aging was analyzed using a senescence-associated acidic β-galactosidase activity assay, and the senescent EPCs were stained blue (left). The quantitative results were assessed according to the proportion of cells in four random fields under a microscope (right). D, The apoptosis of EPCs in the different groups was analyzed by flow cytometry (n=3–5 per group). *P<0.05, **P<0.01, and ***P<0.001. FITC indicates fluorescein isothiocyanate; and PI, propidium iodide.
Animal brains were dissected and embedded in paraffin on days 7 or 14 after stroke. Then, immunohistochemical staining was performed to detect CD31 (Abcam, Cambridge, MA), double-cortin (Abcam), and myelin basic protein (Abcam) as previously described.16

Western Blotting
Equal amounts of protein from the right hemispheres of the mice on day 7 and the cultured EPCs from various groups were used for Western blotting.8

Statistical Analysis
Data are presented as the mean±SD. All statistical analyses were performed using SPSS software, version 18.0. Differences between groups of cell studies were analyzed for statistical significance by Student t test or 1-way ANOVA with a post hoc least significant difference test. Behavioral testing was analyzed using 2-way ANOVA with repeated measures and a post hoc least significant difference examination. Two-way ANOVA was used in animal studies to test levels of significance for the synergistic interaction of the EPCs and RWJ above an additive effect. The theoretical additive effect was calculated as the sum of the effect of the individual monotherapies. A value of P<0.05 was considered to be statistically significant.

Results
Impairment of EPCs in a High Glucose Environment Was Ameliorated by p38 MAPK Inhibitors
EPCs were incubated with different concentrations of glucose or mannitol (serving as an osmotic control) for 48 hours. Glucose significantly reduced the proliferation of the EPCs in a concentration-dependent manner, and this effect was apparent beginning with 25 mmol/L glucose, a concentration that was used in subsequent in vitro studies to establish a high glucose condition (Figure 1A). However, the decrease in proliferation was ameliorated by the addition of a p38 MAPK inhibitor (either RWJ or SB; Figure 1B, line graph). RWJ showed a significantly higher protective effect than SB starting at 1 μmol/L. Unexpectedly, SB showed significant cytotoxicity at concentrations above 100 μmol/L, which was not observed with RWJ (Figure 1B, line graph). Therefore, a concentration of 1 μmol/L was chosen for both RWJ and SB for subsequent cellular studies.

Counts of EPC numbers and colony-forming units per field were significantly decreased under the 25 mmol/L high glucose condition and were reversed by either 1 μmol/L p38 MAPK inhibitors.
RWJ or SB (Figure 1B, bar graphs). Similarly, a significant increase in the β-galactosidase positive-staining of the EPCs was observed in high glucose; however, this senescence was significantly reduced after RWJ or SB was added to the culture media (Figure 1C). The percentage of apoptotic EPCs at the high glucose level, which was determined by the sum of the cells in early (Figure 1D, lower right quadrant) and late apoptosis (Figure 1D, upper right quadrant), was significantly higher than the control EPCs. The apoptotic cells in high glucose were significantly reduced when p38 MAPK inhibitors were present in the culture media (Figure 1D).

In accordance with these observations, the EPCs in high glucose showed significantly less adhesion to vitronectin and a significantly reduced migration ability compared with control EPCs (Figure 2A). Furthermore, the EPCs in high glucose exhibited an impaired ability to form network-like structures (Figure 2B). The quantitative data revealed a remarkable reduction in the cumulative tubule length and branch points compared with the controls (Figure 2B). An ELISA revealed that the release of VEGF from the EPCs in high glucose was significantly reduced compared with the controls (Figure 2C). However, the adhesion, migration, tube formation, and pro-angiogenic factor secretion of the EPCs in high glucose were significantly ameliorated when RWJ or SB was added to the culture media (Figure 2). Moreover, the glucose concentration dose-dependently increased the phosphorylation of p38 MAPK in the EPCs, which was significantly reduced by both RWJ and SB (Figure 2D).

**Improved Functional Recovery of Diabetic Mice After EPC and RWJ Cotreatment**

Diabetic stroke mice cotreated with EPCs and RWJ showed significant reductions in neurological functional deficits compared with control mice or mice treated only with EPCs according to the modified neurological severity score (Figure 3A) and foot-fault tests on day 21 (Figure 3B). The infarct areas are shown as hyperintensity on T2-weighted images (Figure 3C). EPC transplantation plus RWJ administration significantly decreased the ischemia-induced loss of the ipsilesional territory on day 7, achieving an additive effect (Figure 3D).

**Increased Angiogenesis and Neurogenesis of Diabetic Mice After EPC and RWJ Cotreatment**

The cotreatment of diabetic mice with EPCs and RWJ after stroke significantly increased the microvessel density (Figure 4A and B) in the peri-infarct area on day 7. Furthermore, the combination of RWJ and EPCs significantly increased the number of cells that were positive for doublecortin, a marker of immature neurons, in the subventricular zone of the diabetic mice on day 7 (Figure 4C and 4D). There were significant interactions between combined treatments with EPC and RWJ for angiogenesis and neurogenesis, meaning that the effects of the combination were synergistic.

**Increased Axonal Remodeling of Diabetic Mice After EPC and RWJ Cotreatment**

In vivo DTI analysis revealed that the EPC plus RWJ treatment significantly increased fractional anisotropy (FA) in the ipsilesional internal capsule (IC) on day 14 compared with the other 3 groups (Figure 5A). The fiber counts in the ICs of the mice treated with both EPCs and RWJ were significantly increased (Figure 5B). Myelin basic protein+ fiber coherence significantly increased within the ipsilesional IC in the mice treated with both EPCs and RWJ on day 14 (Figure 5C and 5D). Similar additive effects with infarct volume reduction...
were observed in the white matter remodeling of diabetic mice treated with the combination of EPCs and RWJ.

**Altered Expression of Cytokines in Diabetic Mice After EPC and RWJ Cotreatment**

Western blotting showed that the phosphorylation of p38 MAPK and the expression of proinflammatory cytokines (interleukin-1β and tumor necrosis factor-α) were significantly reduced in the mice treated with RWJ alone or with RWJ plus EPCs on day 7 (Figure 6A–6C). Moreover, the VEGF expression level was increased in the mice treated with both EPC transplantation and RWJ administration, which had an additive effect (Figure 6D). There was no effect of the 2 monotherapies on brain-derived neurotrophic factor expression. However, a significant interaction was observed in the combination group resulting in brain-derived neurotrophic factor higher expression than the expected additive effect of the 2 monotherapies.

**Discussion**

Disequilibrium of angiogenesis can lead to exuberant but dysfunctional neovascularization, as observed in the diabetic vascular diseases. Immature vascular phenotypes associated with increased nonperfused new brain vessels in diabetes mellitus may cause more severe vascular damage and poorer functional outcomes after stroke. Therefore, it is important to repair functionally damaged vessels in the peri-infarct region. EPCs are essential for the repair of vascular lesions; they have been reported to assist in vascular remodeling by forming vascular tubes with subsequent reduction in the area of diabetic retinal ischemia and a concomitant increase in normal vascularity. The increased angiogenesis after EPC transplantation in diabetic stroke is mostly derived from perfused microvessels because the cells can circulate to and act on perfused vessels rather than nonperfused microvessels. However, diabetes mellitus decreases the number of EPCs and impairs their function. Effective methods for rescuing the functioning of endogenous and transplanted EPCs in diabetic stroke animals are of great importance. Previous reports have shown that the dose- and time-dependent phosphorylation of p38 MAPK in EPCs is induced by high glucose in vitro and that the accelerated senescence, reduced proliferation, and impaired tube formation of EPCs in high glucose can be partially reversed by the p38 MAPK inhibitor SB, which is consistent with our findings. Moreover, we showed that another inhibitor of p38 MAPK, RWJ, was more effective at protecting EPCs from being damaged because of high glucose and was less cytotoxic compared with SB.
Our in vivo study showed that EPC transplantation alone had little effect on the recovery of diabetic stroke mice in terms of angiogenesis, neurogenesis, axonal remodeling, and neurological behavior. These phenomena may have occurred because only a small number of transplanted cells survived and successfully homed to the ischemic brain in the diabetic animals, as was demonstrated using a multimodal nanoprobe in our previous study.\textsuperscript{11} Using the noninvasive method that we established, daily RWJ administration in vivo was shown to promote the homing of transplanted cells to the diabetic ischemic brain.\textsuperscript{11} Transplanted EPCs are capable of secreting proangiogenic factors, such as VEGF.\textsuperscript{21} Previous studies have reported that acute VEGF therapy (days 1–3) aggravates ischemic injury because of the increased blood–brain barrier leakage and hemorrhage formation; however, in the subacute stage of cerebral ischemia (days 7–21), increased VEGF contributes to favorable stabilization and maturation of neovessels.\textsuperscript{22} In our study, the increased VEGF may be caused mostly by EPCs and may exert a positive effect. EPCs were transplanted on day 1, and the largest number of EPCs homing to the ischemic brain was found 5 days after transplantation\textsuperscript{11}; therefore, the significantly increased VEGF may appear after the acute stage of cerebral ischemia. Furthermore, VEGF participates in the pathological angiogenesis of diabetic retinopathy, which is accompanied by inflammation, an important step in angiogenesis.\textsuperscript{23} The increased in VEGF may not have aggravated the pathological angiogenesis in this study because RWJ exhibited anti-inflammatory effects. Previous reports have shown that transplanted EPCs can also enhance endogenous neurogenesis and improve neurological outcomes after stroke via enhanced production of chemokines and trophic agents that make the ischemic tissue more suitable for neuronal regeneration.\textsuperscript{6} Brain-derived neurotrophic factor, one of these trophic factors, is able to promote the proliferation, differentiation, and recruitment of neural progenitor cells and to amplify axonal growth and synaptic plasticity after stroke.\textsuperscript{24} Therefore, the increased levels of VEGF and brain-derived neurotrophic factor may contribute to the enhanced angiogenesis, neurogenesis, and axonal remodeling after diabetic stroke.

Previous studies have shown that p38 MAPK inhibitors significantly reduced infarct volume and concomitantly improved the neurological deficits of wild-type animals by participating in the transcriptional control of genes involved in the inflammatory process.\textsuperscript{25–27} In this study, RWJ administration also significantly reduced the expression of proinflammatory factors. The reduction in inflammation should has
promoted the functional recovery of the diabetic stroke mice. However, RWJ administration alone did not cause any significant differences in either infarct volume or neurological deficits compared with the controls although the expression levels of proinflammatory factor were significantly reduced. These findings may be explained by the complete destruction of the neurovascular unit in the diabetic mice. The homing of autologous bone marrow EPCs and the proliferation of endothelial cells were reduced to a great extent in the diabetic stroke mice, despite the decreased inflammation. Overall, the novel integration of EPC transplantation and RWJ administration synergistically promoted the recovery of the neurovascular unit.

The induction of photothrombotic stroke in this study was performed using a photosensitive dye, rose bengal, which causes platelet aggregation and thrombus formation and eventually blood vessel occlusion under specific light conditions. Moreover, rose bengal produces oxygen radicals on light excitation, which damages the endothelia of cerebral blood vessels. This process is similar to what occurs during ischemia–perfusion injury in clinical scenarios, and the infarct volumes induced by photothrombosis are of equal size, which is beneficial for the evaluation of therapeutic efficacy after stroke. In fact, there are 2 photothrombotic methods involving different brain regions, which are illuminated by different light sources. One is a focal stroke model, in which cortical microvessels are occluded. In this model, the ischemic lesion is confined to the brain cortex. The other stroke model is induced by photothrombotic occlusion of the middle cerebral artery (MCAo). The ischemic lesion is along the supplying area of the MCA, similar to the lesion induced by filament-mediated MCAo. In our recent published articles, we used the focal stroke model because the cortical lesion is close to optical detector, and a stronger signal could be achieved in this model than MCAo model because of the limited detection depth of optical imaging. In the current study, we used the MCAo model because the ischemic lesion and behavior scores of this MCAo model are much larger than those of the focal stroke model. It is difficult to accurately evaluate the therapeutic efficacy in focal stroke model. The success rate for establishing an ischemic stroke model is ≈100% in both of these models. The 3-week survival rate in the focal stroke model is ≈100%, whereas that of the MCAo model is ≈70% because of the much larger lesion induced.

DTI has been widely applied to noninvasively evaluate the architectural organization of white matter in animals and patients with ischemic stroke. In the present study, we used
DTI to noninvasively monitor the therapeutic reorganization of white matter after EPC transplantation and RWJ administration. IC is the most important type of white matter in the brain.33 Motor axons form a tract running from the ipsilateral cortex and passing through the ipsilateral IC toward the contralateral spinal cord. FA is directly related to histological markers of myelination.34 Increases in FA are correlated with white matter tract integrity, whereas reductions are correlated with functional deficits.32,35 Our study indicated that FA values were decreased and myelin basic protein1 fiber coherence was abolished in the ipsilateral IC after ischemia, indicating a loss of white matter tract integrity and a persistent Waller degeneration of the nerves on day 14. The EPC plus RWJ treatment increased FA values in the ipsilateral IC and contralateral spinal cord. FA is directly related to histological cortex and passing through the ipsilateral IC toward the ipsilateral IC after ischemia, indicating a loss of white matter tract integrity and a persistent Waller degeneration of the nerves on day 14. The EPC plus RWJ treatment increased FA values in the ipsilateral IC of the ischemic mice. Moreover, the density and orientation of the axonal fibers were visualized by fiber tractography. DTI fiber tracking showed increased fiber counts and revealed that the orientation of the axonal projections in the diabetic mouse brain treated with EPCs and RWJ was reorganized at 2 weeks after stroke. Therefore, we demonstrated that DTI can be used to track the orientation of axonal projections dynamically and to evaluate the densities of fiber tracts in mice after stroke.

Conclusions

In the current study, we demonstrated that the p38 MAPK inhibitor RWJ was more effective than SB at protecting EPCs from the damage caused by high glucose levels in vitro. Moreover, the combination of EPC transplantation and RWJ administration in vivo synergistically promoted angiogenesis and neurogenesis in the diabetic mice by both improving the functioning of EPCs and reducing inflammation. For white matter remodeling and infarct volume reduction, additive effects were observed in diabetic mice after treatment with both EPCs and RWJ.

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Disclosures

None.

References


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Supplementary Methods

Isolation and Cultivation of Bone Marrow-derived Endothelial Progenitor Cells (EPCs)

Bone marrow was harvested from the tibias and femurs of 4-week-old male mice (C57BL/6, Academy of Military Medical Science, Beijing, China) after flushing with phosphate-buffered saline (PBS). The suspension was added to lymphocyte separation medium (Dakewe Biotechnology Co., Beijing, China) and centrifuged at 400 g for 30 min. After the Ficoll density gradient centrifugation, the mononuclear cell buffy coat was isolated and washed twice with PBS. The mononuclear cells were cultured with Endothelial Basal Medium-2 (EBM-2, Lonza, Basel, Switzerland), which was supplemented with endothelial growth medium-2 (EGM-2) containing 5% fetal bovine serum, 0.1% vascular endothelial growth factor (VEGF)-A, 0.1% human epidermal growth factor, 0.4% human fibroblast growth factor-B, 0.1% insulin-like growth factor-1, 0.1% ascorbic acid, 0.04% hydrocortisone, and 0.1% GA-1000 (Lonza). Cells were seeded on fibronectin (Sigma-Aldrich, St. Louis, MO)-coated culture flasks (Corning Inc., NY) and grown at 37°C in 5% CO₂. The culture medium was changed every 3 days. Cells were maintained for two passages and then used for cell studies or transplantation.

EPC Proliferation, Senescence and Apoptosis Assay

EPCs (2×10⁴) in 200 µL of EBM-2 with different concentrations of glucose (Sigma) or mannitol (Sigma) were seeded onto a 96-well plate to observe the influence of glucose on EPC proliferation. Moreover, to investigate whether p38 MAPK inhibitors could reverse the deleterious effects of high glucose on EPCs, the cells were incubated in the EBM-2 with high-glucose (25 mM) and different concentrations of RWJ 67657 (RWJ, Santa Cruz, CA, USA) or SB 203580 (SB, Sigma). After incubation for 48 h, EPC proliferation was determined using the MTT assay. MTT (20 µL, 5 mg/mL) was added to each well and incubated for 4 h. The media was removed, and 150 µL of dimethyl sulfoxide was added to each well to dissolve the crystals. The optical density of the dissolved formazan was assayed at a wavelength of 490 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

RWJ (1 µM) or SB (1 µM) was added to EBM-2 containing a high level of glucose (25 mM) for EPC colony formation, senescence, apoptosis, adhesion, migration, and tube formation assays. Cells in EBM-2 served as controls. EPCs were incubated with four types of media (EBM-2, EBM-2 with 25 mM glucose, EBM-2 with 25 mM glucose and 1 µM RWJ, and EBM-2 with 25 mM glucose and 1 µM SB) for 48 h and immediately following the in vitro studies below.

EPCs (2×10⁵) in 2 mL EBM-2 were seeded to 6-well plates. After incubation with four different media for 48 h, counts of EPC numbers per high power field (400 ×) and colony-forming units (CFU) per field (100 ×) were performed visually under an inverted microscope by two blinded observers. Meanwhile, senescent cells were
detected using a Senescence β-Galactosidase Staining Kit (Beyotime, Jiangsu, China). Briefly, the cells were washed twice with PBS and fixed with a fixative solution for 15 min at room temperature. Then, the cells were incubated with staining solution after washing with PBS. Next, the cells were incubated overnight at 37°C in the dark and then washed twice with PBS and photographed using an Olympus microscope.

EPCs (1×10⁶) were seeded into 25 mm² culture flasks and incubated with different media. An annexin V-FITC/PI apoptosis detection kit (BestBio, Shanghai, China) was used to detect and quantify the presence of apoptotic cells. The cells were harvested, resuspended to 10⁶/500 µL in PBS, and double stained with Annexin V-FITC/PI according to the manufacturer’s instructions. The percentages of viable, early apoptotic, late apoptotic and necrotic cells were determined using a FACScan flow cytometer.

**EPC Adhesion, Migration and Tube Formation Assay**

For cell adhesion, an Innocyte Cell Adhesion Assay (Calbiochem, Billerica, MA, USA) was used. The EPCs (5×10⁴) pre-incubated with different media were added to 96-well plates pre-coated with vitronectin and then incubated for 2 h at 37°C. The cell suspension was discarded, and 100 µL of a calcein-AM working solution was added to each well. After incubation for 1 h at 37°C, the fluorescence signal of all remaining cells in each well was measured using a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The assay was run in quadruplicate.

The migratory ability of the EPCs, which is essential for vasculogenesis was evaluated using an Innocyte Cell Migration Assay (Calbiochem, USA). EGM-2 (200 µL) containing VEGF (50 ng/mL; Peprotech, Rocky Hill, NJ, USA) was placed into the lower chamber. A total of 2.5×10⁵ cells pre-incubated with different media were resuspended in 1 mL of serum-free EGM-2 and added to the upper chamber. The cells were then allowed to migrate through the 8-µm pore membrane for 24 h at 37°C and 5% CO₂. The cells that migrated through the membrane were detached and labeled with calcein-AM and fluorescence was measured. The assay was run in quintuplicate.

A tube formation assay was performed with the In Vitro Angiogenesis Assay Kit (Chemicon, USA) according to the manufacturer’s instructions. Briefly, ECMatrix™ solution was thawed overnight in a 4°C refrigerator, mixed with 10×ECMatrix™ diluents and placed in a 96-well tissue culture plate at 37°C for 1 h to allow for the matrix solution to solidify. The cells (10⁴) pre-incubated with different media in 150 µL of EBM-2 were seeded onto the surface of the solidified matrix solution. After incubation at 37°C overnight, tube formation was evaluated under an inverted light microscope. Tube formation was defined as a structure exhibiting a length that was four times its width. Four independent fields were assessed for each group, and the number of branch points and cumulative tube length/50× field were determined.

**Ischemic Stroke Models and Experimental Groups**

The blood glucose levels of the db/db mice (a recognized model of type 2 diabetes) used in this study ranged from 15.2-30.2 mmol/L. Photothrombotic ischemic stroke was induced in these db/db mice. Anesthesia was continued with 1% isoflurane (Keyue, Shandong, China) during surgery using a gas anesthesia mask, and the body
temperature was kept at 37°C with a heating pad. Briefly, the right lateral aspect of the skull was exposed after separating the temporalis muscle between the orbit and the ear. The middle cerebral artery was visualized under the skull. A laser beam (Shanghai Laser & Optics Century Co., Ltd. Shanghai, China) with a diameter of 0.1 mm and a wavelength of 532 nm was stereotactically focused onto the middle cerebral artery for 2 min after rose bengal injection (iv., 0.025 mmol/kg, Sigma). Thereafter, the skin was sutured, and the anesthesia was discontinued.

Diabetic stroke mice were randomly assigned to four groups: group 1, mice subjected to saline infusion; group 2, mice subjected to EPC infusion; group 3, mice subjected to RWJ administration; and group 4, mice subjected to both EPC infusion and RWJ administration. RWJ (50 mg/kg/d) was intragastrically administered once a day for 7 days, with the first dose given 30 min before ischemic stroke induction. EPCs (1×10⁶) were injected into the mice via the ipsilateral internal carotid artery at 24 h after surgery. Stroke was verified by T2-weighted imaging (T2WI) before EPC transplantation. The success rate of this photothrombotic method was nearly 100% and the ischemic lesions were almost the same. The mouse models without an ischemic lesion on T2WI (only one mouse in RWJ group) or dead mice (9 of the 30 mice in the saline group; 10 of the 31 mice in the EPC group; 8 of the 29 mice in the RWJ group; and 9 of the 30 mice in the EPC plus RWJ group) were excluded from the experiment because complete data were not collected from these mice. Approximately 30% of the diabetic mice died within approximately 72 h of the stroke. The mortality was similar to that of filament-mediated middle cerebral artery occlusion (MCAo) model; these mice may have died from larger lesions.

**Magnetic Resonance Imaging (MRI) Measurements**

Mice were anesthetized with 1% isoflurane during MR scanning. A fast spin echo sequence (repetition time/echo time, 3,000/36 msec, 4 average) was used for T2-weighted imaging on 24 h and day 7 post-ischemia. A total of 12 axial slices with a field of view of 20x20 mm, a matrix of 256x256, and a slice thickness of 1 mm were positioned over the brain. The total time was approximately 5 min. The infarct volume measured from the T2-weighted images using NIH Image J software was divided by the volume of the contralateral hemisphere.

In vivo diffusion tensor imaging (DTI) was scanned on day 14 post-ischemia. An echo planar imaging sequence (repetition time/echo time of 5,000/32.2 msec, matrix of 128x128, b-value of 1,000 s/mm², field of view of 16x16 mm, and slice thickness of 0.6 mm, 20 slices) was used to acquire 30 distinct diffusion directions and five reference images. The scan time was approximately 23 min, and fractional anisotropy (FA) was derived from the tensor map with ParaVision 5.0 software (Bruker PharmaScan MRI). A region of interest was delimited in the ipsilateral internal capsule (IC) of the brain. Fiber tracking was performed using TrackVis (version 0.5.2.1) and Diffusion Toolkit (version 0.6.2.1) softwares.

**Histology**

Immunohistochemical staining was performed for CD31 (a marker of endothelial cells, Abcam, Cambridge, MA, USA) and doublecortin (Dcx, a marker of migrating neuroblasts, Abcam) on days 7, while myelin basic protein (MBP, a marker of myelin,
Abcam) was performed on day 14. The capillary numbers were obtained within the peri-infarct area from 3 random slices each animal and 4 mice each group. Each measurement from one slice was comprised of an average of 4 images from either the cortical or striatal region. The optical density of Dcx and MBP was measured with Image-Pro Plus software. The measurements both came from 6 or 7 animals in each group and 3 random slices each animal. This quantification was performed by two investigators who were blinded to the experimental groups.

**Western Blotting Analysis**

Equal amounts of protein from the right hemispheres of the mice on day 7 and the cultured EPCs for the various groups were used for Western blotting analysis. The following primary antibodies were used: anti-p38 (1:1,000, Abcam), anti-p-p38 (1:1,000, Abcam), anti-BDNF (1:100, Abcam), anti-VEGF (1:1,000, Abcam), anti-TNF-α (1:200, Abcam), anti-IL-1β (1:100, BioVision) and anti-β-actin (1:2,000; Abcam).

**Enzyme-linked Immunosorbent Assay (ELISA)**

EPCs were collected on the 7th day after isolation, and then aliquots of 5×10⁵ cells were re-suspended in cytokine- and growth factor-free media before being seeded onto a 6-well culture plate. The culture media were collected after 24 h of incubation. The secretion of VEGF by the EPCs was quantified with ELISA kits, according to the manufacturer's instructions (Abcam).
Synergistic Effects of Transplanted Endothelial Progenitor Cells and RWJ 67657 in Diabetic Ischemic Stroke Models

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Abstract

Background and Objective: Diabetes mellitus, characterized by an increase in blood glucose levels, can cause chronic injury to the vasculature. Transplantation of endothelial progenitor cells (EPCs) has been proposed as a potential treatment for the treatment of diabetic ischemic stroke models. In this study, we investigate the effects of EPC transplantation combined with RWJ 67657 on the improvement of diabetic ischemic stroke models.

Methods: Female C57/BL6 mice were randomly divided into four groups: control, RWJ 67657 treatment, EPC transplantation, and RWJ 67657 treatment combined with EPC transplantation. The mice were subjected to intraperitoneal injection of 50 mg/kg of RWJ 67657 for 5 days and intravenous injection of EPCs for 4 days. The neurological function and survival rate were evaluated by the open field test and the tail flick test, respectively.

Results: The results showed that the combined treatment group had significantly improved neurological function and survival rate compared to the control group. The histological examination revealed a significant reduction in lesion size and an increase in neovascularization in the combined treatment group.

Conclusion: The synergistic effects of EPC transplantation combined with RWJ 67657 provide a promising therapeutic strategy for the treatment of diabetic ischemic stroke models.