SUPPLEMENTARY MATERIAL

An eye opener in stroke: Mitochondrial dysfunction and stem cell repair in MCAO-induced retinal ischemia

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Running head: Retinal mitochondrial deficits after stroke

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Supplemental Methods

Middle Cerebral Artery Occlusion

Adult male Sprague-Dawley rats (approximately 250g) were anesthetized by a mixture of 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. Body temperature was maintained at 37 ± 0.3 °C during the surgical procedures. The midline skin incision was made in the neck with subsequent exploration of the right common carotid artery (CCA), the external carotid artery, and internal carotid artery. A 4-0 monofilament nylon suture (27.0–28.0 mm) was advanced from the CCA bifurcation until it blocked the origin of the middle cerebral artery (MCA). The contralateral CCA was temporally ligated for 15 minutes to ensure a consistent blood flow occlusion (including collaterals) to the brain. Animals were allowed to recover from anesthesia during MCAO. After 60 minutes of transient MCAO, animals were re-anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask and reperfused by withdrawal of the nylon thread. Animals receiving the sham operation were anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. A midline incision was made in the neck and the right CCA was isolated. The animals were then closed and allowed to recover from anesthesia.

Laser Doppler Blood Flow Measurement

Brain and eye retinal blood flow measurements were obtained using a laser doppler (Perimed, Periflux System 5000). The animal was under deep anesthesia during the measurement and the animal’s head was shaved for brain measurement. For brain perfusion, the laser doppler probe was placed over the right frontoparietal cortical area supplied by the MCA. For eye perfusion, the laser doppler probe was placed over the retina of the right eye. Measurements were made at baseline, during MCAO, and 5-minutes after reperfusion. Ophthalmic ointment was applied and the animals were allowed to recover from anesthesia.

MSCs Transplantation

On the day of transplantation, MSCs were detached using TrypLE (Gibco 12604-021). Complete media was used to rinse the flask for maximizing cell yield. The MSCs were centrifuged at 300x g for 10 minutes. After the supernatant was aspirated, the cells were re-suspended in normal media. A small volume of cells were set aside for cell count. The cells were then centrifuged at 300x g for 5 minutes. Once the supernatant was aspirated, the cells were re-suspended in final concentration of 4x10^6 cells/500 μL of sterile phosphate-buffered saline (PBS). The animals were anesthetized and transplanted intravenously via jugular vein with MSCs or with PBS only.

Optic Nerve Measurement & Immunohistochemistry

On the day of tissue collection, the animals were euthanized with CO₂ and perfused with 0.9% saline. The animals’ eyes were quickly harvested and the retina were isolated in ice cold PBS, pH 7.4. The optic nerves and the retina were fixed with 4% paraformaldehyde (PFA; 158127; Sigma) in PB for 2 hours at 4°C. The tissues were washed with ice cold PBS for several times and kept in this solution at 4°C.
The retina were embedded in 4% agarose and sectioned with a vibratome at 30 μm thickness in ice cold PBS. The tissues were incubated in blocking buffer (5% normal goat serum and 0.1% Tween 20 in PBS) for 3 hours at 4°C. The tissues were then incubated with NeuN (1:500; ab104225, Abcam) in blocking buffer at 4°C overnight. Then the tissues were washed three times with PBS followed by incubated with Alexa 488 secondary antibody (1:500) for 4 hours at 4°C. The tissues were washed three times with PBS and mounted with anti-fade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories)

**RPE Cells and MSC Culture**

Retinal pigmented epithelium (RPE, CRL-4000; ATCC) cells were cultured in Dulbecco’s Modified Eagle Media/F-12 (DMEM/F-12, 11320033; Gibco) containing 10% fetal bovine serum (FBS; FBS001; Neuromics) and 0.01 mg/ml hygromycin B (10687010; Gibco) in incubator (37°C humidified, with 5% CO2, 95% air).

MSCs were maintained with α-MEM (12561056; Gibco) supplemented with 20% FBS (FBS001; Neuromics), 1% penicillin/streptomycin (15140122; Gibco), 1% non-essential amino acids (11140050; Gibco), 1% GlutaMax-I (35050061; Gibco) in incubator (37°C humidified, with 5% CO2, 95% air).

**Oxygen Glucose Deprivation and Co-culture**

A day prior to the experiment, RPE cells were seeded at 1.5x10^5 cells/well in 6-well plate. On the day of experiment, the media of the RPE cells was changed to Dulbecco’s phosphate-buffered saline (DPBS; 14040133; Gibco). The cells were placed in a sealed hypoxia incubator chamber (27310; StemCell Technologies) containing 95% N2 and 5% CO2) for 3 hours, mimicking the ischemic stroke. We chose 3 hours because based on our experience with RPE cells, 3 hours OGD provided 50-60% cell death. After the 3 hours period, fresh media was reintroduced and the cells were incubated in normoxia condition (37°C humidified atmosphere containing 5% CO2) for 24 hours, which simulated the reperfusion in clinical setting.

MSCs were seeded at 0.5 x 10^5 cells/insert and separately cultured in cell culture inserts (353493, Falcon). After exposing RPE cells to OGD, the RPE cells were allowed to co-culture in a non-contact manner with MSCs by placing the inserts into the wells for 24 hours. Prior to any assay, the inserts that contained the MSCs were discarded. Supplemental Figure IV illustrates the experimental timeline and design.

**Mitochondrial Respiration Assay**

On the day of experiments, RPE cells were detached from cell culture plates and seeded to a Seahorse 96-well plate (101085-004; Agilent) coated with Poly-D-lysine (100μg/ml; P7886; Sigma) at 5.0 x 10^4 cells/well. The cells were immobilized by centrifugation method. Briefly, the Seahorse 96-well plate was centrifuged in swing bucket rotator with slow acceleration (4 on a scale of 9) to a max speed of 450 rpm with 0 brake. Then, the plate orientation was reversed and centrifuged again to max speed of 650 rpm with 0 brake. To determine cellular oxygen consumption rate (OCR), the Seahorse extracellular flux analyzer XFe96 (102416; Agilent) was
used in combination with sequential injection of various compounds (1 µmol/L oligomycin, 1 µmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 µmol/L Rotenone and Antimycin A). OCR measurements were performed by the manufacturer’s protocol.

**Mitochondrial Network Analysis**

The RPE cells were stained with MitoTracker (500 µmol/L; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Then, the cells were carefully washed few times with DPBS, pH 7.4 to remove any excess dye residue. The cells were fixed with 4% PFA at room temp for 30 minutes. Finally, the cells were washed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories). Images were captured using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope and analyzed using ImageJ (NIH) with mitochondrial network analysis (MiNA) plugin.

**Mitochondria Live Cell Imaging**

The mitochondria of RPE cells were incubated with either mitochondrial membrane potential probe JC-1 (2 µg/mL; T3168; Invitrogen) or with MitoTracker (500 µmol/L; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO₂, 95% air). Then, the cells were carefully washed few times with PBS, pH 7.4 to remove any excess dye residue. Finally the media was changed to DMEM without phenol red (21063029, Gibco). Live images were captured at 5-minute interval over 25 minutes using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope.

**Immunocytochemistry**

The RPE cells were rinsed few times with DPBS and then fixed with 4% PFA for 20 minutes at room temperature. The cells were rinsed again for few times and permeabilized with 0.3% Triton-X (X100; Sigma) for 5 minutes at room temperature. The cells were rinsed few times and incubated with blocking buffer containing 5% goat serum (50062Z; Invitrogen) at room temperature for 60 minutes. The cells were incubated with primary antibodies either rabbit anti Ki67 (1µg/ml; NCL-Ki67P; LeicaBiosystems), DRP1 (70278; Life Technologies), or MFN2 (711803; eBioscience) at 4°C overnight. After rising several times with DPBS, the cells were incubated with Alexa Fluor 488 goat anti-mouse (Life Technologies) or Alexa Fluor 488 goat anti-rabbit for (Life Technologies) 60 minutes at room temperature. The cells were then rinsed with DPBS and covered with anti-fade mounting medium containing DAPI (H-1500; Vector Laboratories).
Supp. Figure I: MCAO caused a significant decrease in optic nerve width at day 3 and day 14 compared to sham. (A) Representative images and quantification of optic nerve width. (B) Quantification of optic nerve width at day 3 and day 14. Contra: Contralateral; Ipsi: Ipsilateral. ANOVA with Bonferroni’s post-hoc test *p<0.05; **p<0.01; ***p<0.001. Scale bar 500 µm.
Supplemental Figure II: MSCs’ mitochondria were detected in RPE cells after OGD. MSCs’ mitochondria were separately stained with Mitotracker prior to co-culture with RPE cells. Confocal images of RPE cells with DAPI (blue), β-tubulin (red), and MSCs’ mitochondria stained with Mitotracker (green). MSCs’ mitochondria were detected within the boundaries of RPE cells. Scale bar 10 µm.
Supplemental Figure III:

Sup. Figure III: MSCs did not restore RPE cells’ Drp1 expression level after OGD. Representative images of Drp1 expression (left columns), DAPI (middle columns) and merged (right columns). OGD caused a significant increase in Drp1 expression. Co-culture with MSC did not decrease the Mfn2 expression compared to OGD. ANOVA with Bonferroni’s post-hoc test * p<0.05; **p<0.01; ***p< 0.001. Scale bar 50 µm
Supplemental Figure IV: In vitro experimental timeline and design for RPE oxygen glucose deprivation and co-culture with MSCs. RPE cells and MSCs were cultured separately. At day 0, RPE cells exposed to OGD for 3 hours. After the 3 hours period, fresh media was reintroduced and the cells were incubated in normoxia condition. At day 1, the RPE cells were allowed to co-culture in a non-contact manner with MSCs by placing the inserts into the wells for 24 hours. At day 2, prior to any assay, the inserts that contained the MSCs were discarded.
Supplemental Tables

Sup. Table I:

|                  | Optic Nerve Width       | Ganglion Cell Count     |
|------------------|-------------------------|-------------------------|
| Day 3            | F(2,15)=13.97, p=0.0004 | F(6,14)=14.36, p<0.0001 |
| Day 14           | F(2,15)=37.33, p<0.0001 | F(6,14)=14.36, p<0.0001 |

Sup. Table I: ANOVA statistical analysis of the optic nerve width and ganglion cell count between sham and MCAO animals at day 3 and day 14.

Sup. Table II:

|                  | ANOVA Analysis         | Control       | OGD           | OGD-MSCs      |
|------------------|------------------------|---------------|---------------|---------------|
| Basal Respiration| F(2,32)=82.03, p<0.0001| 25.1±1.7      | 13.8±2.3      | 22.1±2.5      |
| Spare Respiratory Capacity | F(2,32)=85.51, p<0.0001 | 24.8±2.0      | 13.1±3.0      | 27.4±3.3      |
| ATP production   | F(2,32)=55.69, p<0.0001 | 20.1±1.3      | 12.5±2.0      | 18.2±2.0      |
| Proton Leak      | F(2,32)=80.77, p<0.0001 | 3.9±0.7       | 1.4±0.7       | 5.0±0.7       |
| Footprint        | F(3,167)=8.312, p<0.0001| 52.4±21.0     | 38.9±12.3     | 45.8±14.4     |
| Networks         | F(3,167)=5.774, p=0.0009| 9.00±3.97     | 6.59±2.80     | 8.89±4.24     |
| Individuals      | F(3,167)=9.955, p<0.0001| 83.1±26.8     | 58.8±20.1     | 77.9±30.7     |
| Circularity      | F(3,167)=13.36, p<0.0001| 0.45±0.06     | 0.53±0.07     | 0.48±0.07     |
| Mean Branch      | F(3,167)=10.64, p<0.0001| 0.91±0.13     | 0.79±0.10     | 0.81±0.09     |
| Length           | F(3,167)=4.580, p<0.0001| 5.45±1.42     | 4.74±1.27     | 5.55±1.60     |

Sup. Table II: ANOVA statistical analysis of mitochondrial respiration and network morphology parameters.

Supplemental Videos Legends

Sup. Video I: Live cell imaging of mitochondrial network morphology of Control (left) and OGD (right) groups. Mitochondria were stained with Mitotracker (500 nM/L; M7514; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO2, 95% air). OGD caused visible disorganization of mitochondrial network morphology. Live images were captured at 5-minute interval over 25 minutes using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope. Scale bar 20 μm.

Sup. Video II: Live cell imaging of mitochondrial network morphology of Control-MSC (left) and OGD-MSC (right) groups. RPE cell’s mitochondria were stained with Mitotracker (500 nM/L; M7514; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO2, 95% air). Prior to co-culture, MSC’s mitochondria were stained with Mitotracker (500 nM/L; M22426; Invitrogen) for 30 minutes in an incubator (37°C humidified, with 5% CO2, 95% air). Co-culture with MSCs visibly improved the mitochondrial network of RPE cells. MSC’s mitochondria co-localized with RPE cell’s mitochondria (yellow). Live images were captured at
5-minute interval over 25 minutes using an Olympus FV1200 Spectral Inverted Laser Scanning Confocal Microscope. Scale bar 20 µm.
Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. **This short checklist must be completed, and the answers should be clearly presented in the manuscript.** The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.

This study involves animal models:
Yes

**Experimental groups and study timeline**

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study: Yes

An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated: Yes

An overall study timeline is provided: Yes

**Inclusion and exclusion criteria**

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article: Yes

**Randomization**

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided: Yes

Type and methods of randomization have been described: Yes

Methods used for allocation concealment have been reported: Yes

**Blinding**

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible: Yes

Blinding procedures have been described with regard to masking of group assignment during outcome assessment: Yes

**Sample size and power calculations**

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided: Yes

**Data reporting and statistical methods**

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. Yes
If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:

Baseline data on assessed outcome(s) for all experimental groups have been reported: Yes

Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms: Yes

Statistical methods used have been reported: Yes

Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures: Yes

**Experimental details, ethics, and funding statements**

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described: Yes

Different sex animals have been used. If not, the reason/justification is provided: Yes
Statements on approval by ethics boards and ethical conduct of studies have been provided: Yes
Statements on funding and conflicts of interests have been provided: Yes

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