Diabetic Complications Consortium

Application Title: Cognitive Impairment in Diabetes: Endothelial Mechanisms & Intervention

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1. Project Accomplishments:

The global **hypothesis** of this project is that the entrapment of microemboli (ME) in dysfunctional vessel walls leads to the development of small vessel disease (SVD) ultimately resulting in vascular cognitive impairment (VCID) in diabetic but not control animals. The **objective** of this pilot application is to refine the ME model of VCID and begin preclinical testing with isosorbide mononitrate (ISMN) and cilostazol for the prevention/treatment of SVD and VCID in diabetes. With the support of this DiaComp award, we have accomplished items listed as below:

- Refined the ME model of VCID by increasing the ME dose to 6,000 cholesterol crystals and examined the VCID development over 16 weeks after ME injection.
- Examined the effect of combined treatment with isosorbide mononitrate (ISMN) and cilostazol on the cognitive impairment in the rat ME model.
- Optimized the MRI modalities for the brain microstructure integrity study. Diffusion MRI with multiple metrics (mean diffusivity, axial diffusivity, radial diffusivity, and fraction anisotropy) were used to analyze the results.
- Presented data as posters at the International Stroke Conference 2020 and 2021.
- Presented data as a poster at the Joint Stroke Virtual Conference of European Stroke Organization and World Stroke Organization in November 2020.

2. Specific Aims:

Specific Aim 1. Determine the impact of microemboli (ME) on development of SVD and cognitive impairment in diabetes.

Results:

ME injection aggravated cognitive impairment in diabetic animals, but not in the control animals. We hypothesized that ME injection worsened the endothelial dysfunction in diabetes and exacerbated the cognitive deficits. Male Wistar rats were treated with high fat diet and streptozotocin (STZ, 35 mg/kg) i.p. injection to induce diabetes. ME (6,000 cholesterol crystals/300 μl saline) were injected at 6 weeks after diabetes onset. Control

animals were heavier than the diabetic counterparts, while diabetic animals had significant higher blood glucose levels (Table 1).

The cognitive behavioral tests were performed at baseline before ME injection and at week 8, 12, and 16 after ME injection. In the vehicle groups, diabetic animals had lower recognition index (Fig 1 A) and discrimination index (Fig 1 B) in the novel objection recognition (NOR) test than that of control animals at baseline, while ME worsened both indices after 16 weeks only in diabetic group but not in control group (Fig 1 A and B). The diabetic animals had subordinate performance in the Y maze test over the period of 16 weeks. At the baseline, they showed less interest in entering into each arm of the maze (Fig 1 C) and had less alternations in between the arms (Fig 1 D) comparing to the control rats. By week 16 after ME injection, the diabetic animals had even worse results in the total arm entries (Fig 1 C) and alternation in the arms (Fig 1 D) while the control animals had recovered results similar as baseline. Additionally, there were higher missteps for diabetic animals in the Catwalk test comparing to the control animals at baseline and week 16 after ME injection (Fig 1 E).

Diabetic animals had worsened histopathological damage and increased perivascular space after ME injection. The vacuolization, loss of tissue elements, inflammatory cell infiltration, axonal damage, and white matter (WM) rarefaction were evident in diabetic animals with ME injection (Fig. 2 A and B). Pathology scores based on hematoxylin and eosin (HE, Fig 2 A) and Luxol fast blue (LFB, Fig. 2 B) staining showed that diabetic animals had worse histopathological damage (Fig. 2 C) and demyelination (Fig. 2 D) as well as increased perivascular space (PVS) index (Fig. 2 E) in all brain areas than that of the control group.

Activated microglial cells in diabetic animals with ME injection. Inflammation plays an essential role in cognitive impairment and diabetes, therefore, microglial morphology was examined in the cortical and striatal brain regions in both animal groups. To visualize the activated microglia and macrophages, the expression of ionized calcium-binding adapter molecule 1 (Iba-1) was assessed in the brain sections of control and diabetic animals with ME injection (Fig 3 A). In diabetic animals, cell body swelling was significantly greater in all areas (Fig. 3 B), while number of protrusions (Fig. 3 C), endpoints (Fig. 3 D), and branch length (Fig. 3 E) were all decreased. Collectively, these results indicated that there was an increased activation of microglia in diabetic animals with ME injection.

Diabetic animals had diffuse microstructure damage that exacerbated by ME injection. We hypothesized that ME accelerates the development of SVD in diabetic animals and it is not due to increased microinfarction risk. SVD would contribute to the neurodegenerative damage which showed as axonal damage and demyelination. Diffusion tensor imaging metrics of the MRI scanning showed that there were increased ischemic infarct cases in diabetic group after ME injection (2 out of 6 in diabetes vs. 0 out of 16 in control).

In the cortex, diabetic animals had lower axonal diffusivity (DA) values at baseline (Fig 2 C), which indicates diffuse axonal damage in the cortex. After ME injection, there was a trend to an increase in mean (MD), axial (DA) and radial (DR) diffusivities in the cortex, significant for the DR at 12 weeks after ME injection (Fig 2 C). Fraction anisotropy (FA) had a trend for lower values in the cortex of diabetic rats, which was significant at 12 weeks after

ME injection. The increase in diffusivity and decrease in FA may reflect loss of tissue integrity and edema in the cortex of diabetic animals after ME injection.

In the dorsal hippocampus (Fig 2B), there was no difference in MRI metrics at baseline. However, after ME injection, there was a trend for increase diffusivity that became significant at 12 weeks after ME injection for MD, DA and DR, reflecting abnormal tissue microstructure (loss of cells, loss of axons, or myelin damage) in this region.

In the fimbria (Fig 2C), there was no difference in MRI metrics at baseline. However, it seemed like MD and DR had higher values and DA had lower values in the diabetic rats, which may reflect already some degree of loss in integrity (myelin and axonal damage) in this white matter region. After ME injection, there was a trend for increase diffusivity for all metrics (MD, DA, DR) after 8 weeks for both control and diabetes groups. At 12 weeks after ME injection, MD and DA decreased in both groups, but DR values continued to increase in the diabetic rats, which was significant different from the control rats. FA had a trend for lower values in the fimbria of diabetic rats, which was significant at 12 weeks after ME injection.

In the thalamus (Fig 2D), there was no difference at baseline for all the MRI metrics. At 8 weeks after ME injection, it seemed there was no change for all metrics in diabetic animals, while DR had decreased and FA had increased in control animals. At 12 weeks after ME injection, MD and DA had significantly increased in diabetic animals, while DR and FA had no difference between the two groups.

These changes are reflecting loss of integrity (less fibers) and myelin and axonal damage, which is related to the changes in the dorsal hippocampus, since fibers of the hippocampus are collected together in the fimbria, forming a complex system of fibers closely related functionally and structurally with the hippocampal formation.

Specific Aim 2. Determine the effectiveness of ISMN and cilostazol combination in the prevention of SVD and cognitive decline in diabetes.

Results:

Endothelial protective treatment improved cognitive function in diabetic animals. We hypothesized that correction of endothelial function will prevent development and/or retard progression of VCID in diabetes. Male Wistar rats were treated as described above to induce diabetes. Then both control and diabetes rats were treated with the combination of ISMN (75 mg/kg/day) and cilostazol (60 mg/kg/day) in chow diet for 4 weeks before the ME injection. ME were injected after the treatment period. The cognitive behavioral tests were performed at 16 weeks after ME injection.

In the NOR test, diabetic animals had significant lower recognition index and discrimination index comparing to controls in the vehicle group. After treatment, the diabetic animals had significant recovery on recognition index and discrimination index, while there was no change in control group (Fig. 5 A and B).

In the Y maze test, diabetic animals had significant recovery on the total arm entries (Fig. 5 C) and percentage time spent in novel arm (Fig. 5 E) comparing to the vehicle counterpart. There was a trend for the treated diabetic animals to have more alternations in between the arms than the vehicle diabetic group (Fig. 5 D). There was no difference in the control

vehicle and treated groups. These results indicated that the endothelial protective treatment in diabetic animals may ameliorate the cognitive function exacerbated by ME injection. Further studies are ongoing to explore the possible biomarkers as the crosslink underlying the mechanism of the cognitive impairment developed in these animals.

3. Publications:

1. Chandran R, **Li W***, Ahmed HA, Dong G, Ward RA, He L, Doueiry C, Ergul A. Diabetic rats are more susceptible to cognitive decline in a model of microemboli-mediated vascular contributions to cognitive impairment and dementia. *Brain Res.* 2020 Sep 28;1749:147132. (*corresponding author)
2. Chandran R, Nie X, Voltin J, He L, Jamil S, Falangola M, Ergul A, **Li W**. Magnetic Resonance Imaging-Based Comparison of Temporal Changes in Brain Microstructure After Microemboli Injection in Control and Diabetic Rats: Relevance to Vascular Cognitive Impairment/Dementia. International Stroke Conference 2021, Mar 17-19, 2021, virtual meeting.
3. **Li W**, Chandran R, He L, Doueiry C, Jamil S, Ergul A. Diabetes-mediated Neurovascular Dysfunction Augments Degeneration and Demyelination Leading to Cognitive Decline after Microemboli Injection. ESO-WSO Conference 2020, Nov 7-9, 2020, Vienna, Autria.
4. **Li W**, He L, Abdul Y, Jamil S, Falangola MF, Ergul A. Diabetic Male and Female Rats Are More Susceptible to Infarction and Cognitive Decline in a Microemboli Based Model of Vascular Cognitive Impairment. International Stroke Conference 2020, Feb 19-21, 2020, Los Angeles, CA.

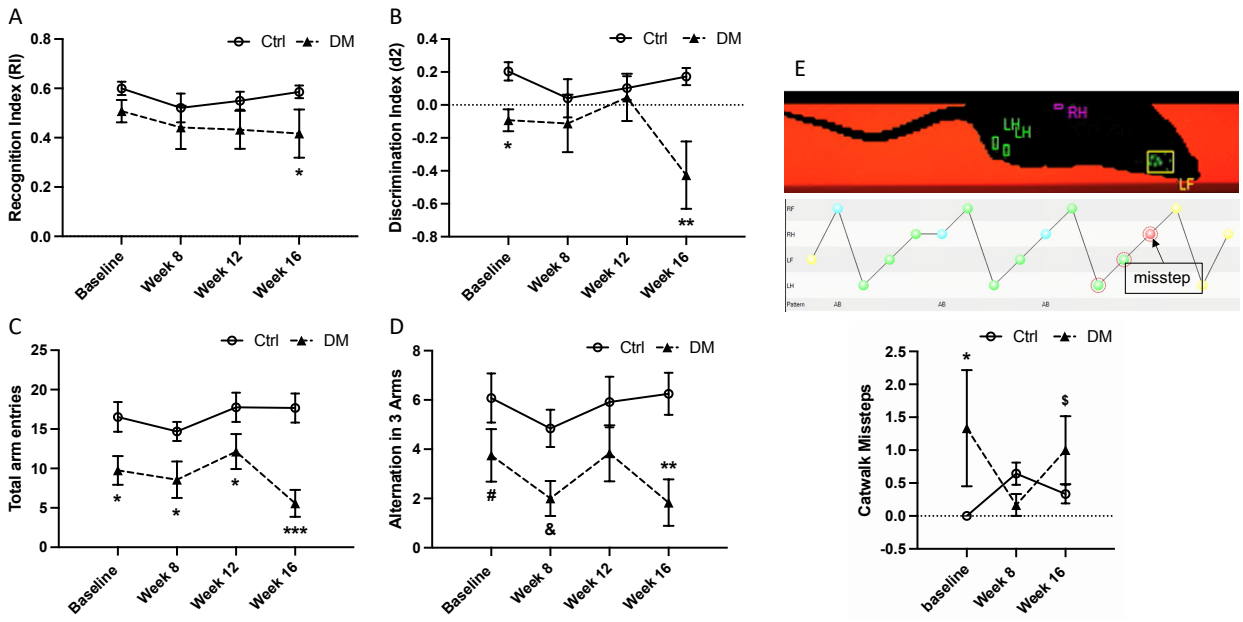

Fig. 1, Long term ME injection caused cognitive impairment in diabetes but not control rats. A: Diabetic rats had a significant lower Recognition Index in NOR test at week 16 after ME injection comparing to the control animals (*p=0.0352 vs. Ctrl). B: Diabetic rats had a lower performance than control group in Discrimination Index of NOR test at baseline (*p=0.0365 vs. Ctrl), which is exacerbated at week 16 after ME injection (**p=0.0004 vs. Ctrl). C: Diabetic rats had an overall less Total arm entries than control animals in Y maze test which is exacerbated at week 16 after ME injection (*p<0.05, ***p<0.0001 vs. Ctrl, respectively). D: There was a trend for diabetic animals had less Alternations than controls in Y maze test at baseline and week 8 ($^{\#}$p=0.0931, $^{\&}$p=0.0797 vs. Ctrl, respectively), while it was significant at week 16 after ME injection (**p=0.005 vs. Ctrl). E: Comparing to control rats, diabetic animals had significant more missteps at baseline (*p=0.0235 vs. Ctrl) and a trend at week 16 after ME ($^{\$}$p=0.0795 vs. Ctrl) in the Catwalk test. Data are mean ± SEM and were compared with corrected two-way ANOVA.

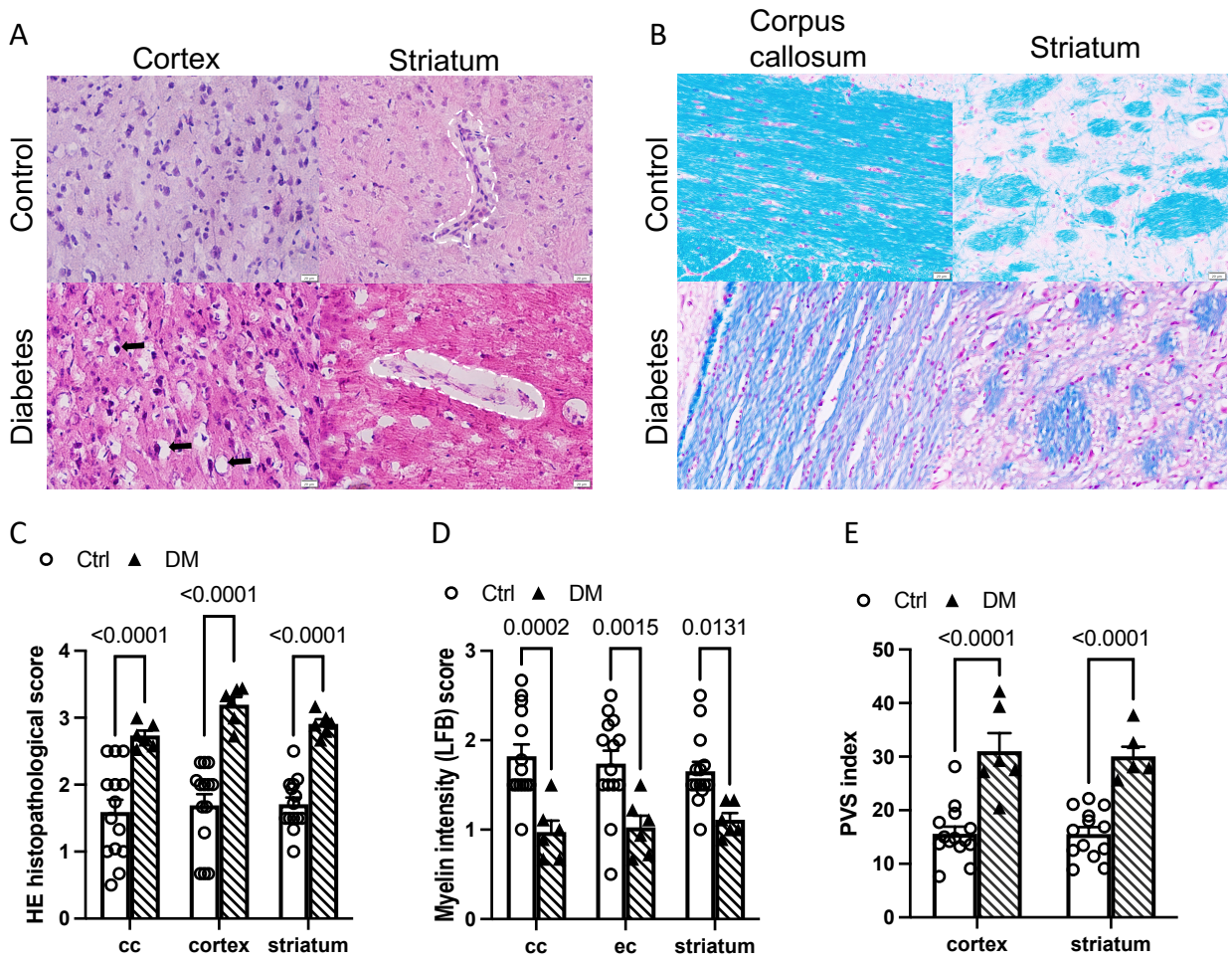

Fig. 2, Increased histopathological damage after ME injection in diabetic animals. A: Representative images of HE staining showing histopathological damage in control and diabetic animals with ME injection, with more vacuole formation (black arrow) and increased perivascular space (dash line) in diabetes. B: Representative images of LFB staining showing increased demyelination, white matter rarefaction, and inflammatory cells immersing in diabetes. C to F: Histopathological score, Myelin intensity score, and PVS index showed significantly worsened histopathological damage in diabetic animals with ME injection. Data are mean ± SEM and were compared with corrected two-way ANOVA.

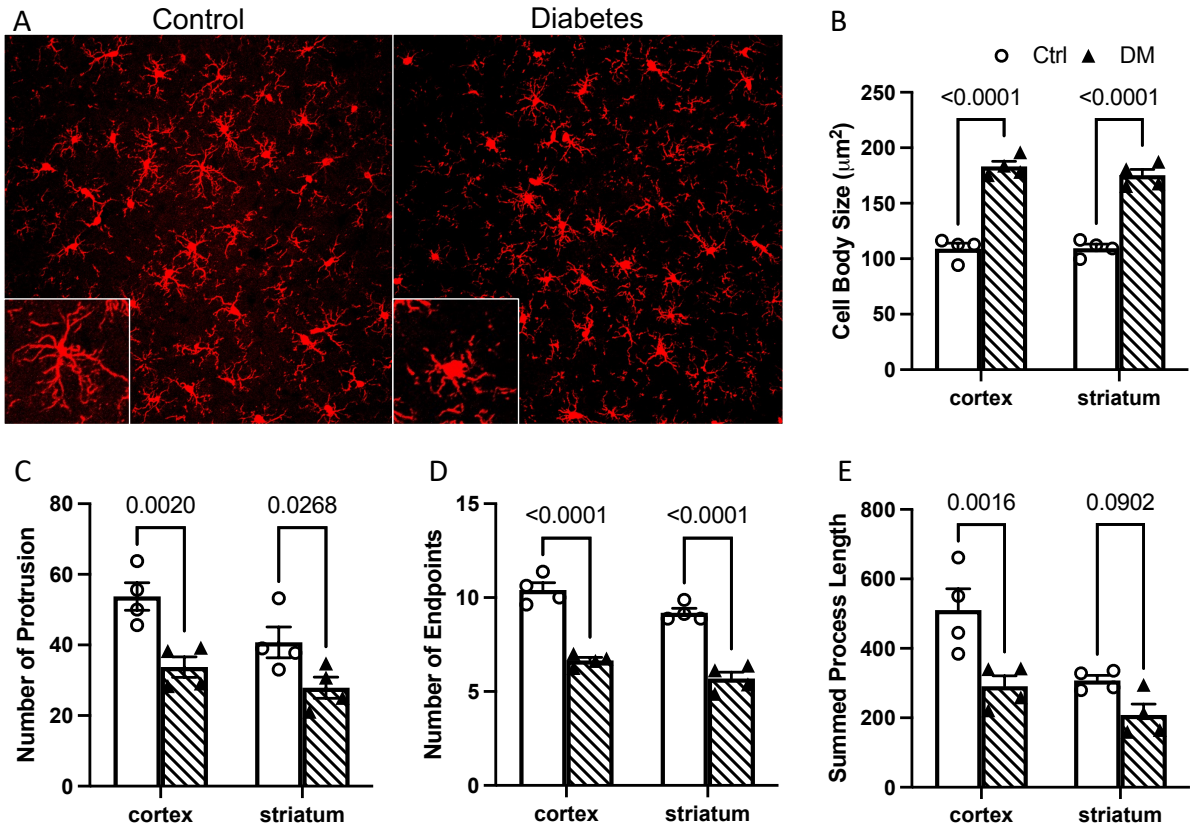

Fig. 3, ME augmented microglial activation in diabetic animals. A: Representative images of Iba-1 positive microglial cells in control and diabetic animals with ME injection. Representative single cells were showed in the inserts. B to E: Increased cell body swelling, and decreased number of protrusions, endpoints, and branch length were seen in diabetic animals, which indicating increases of activated microglia. Data are mean ± SEM and were compared with corrected two-way ANOVA.

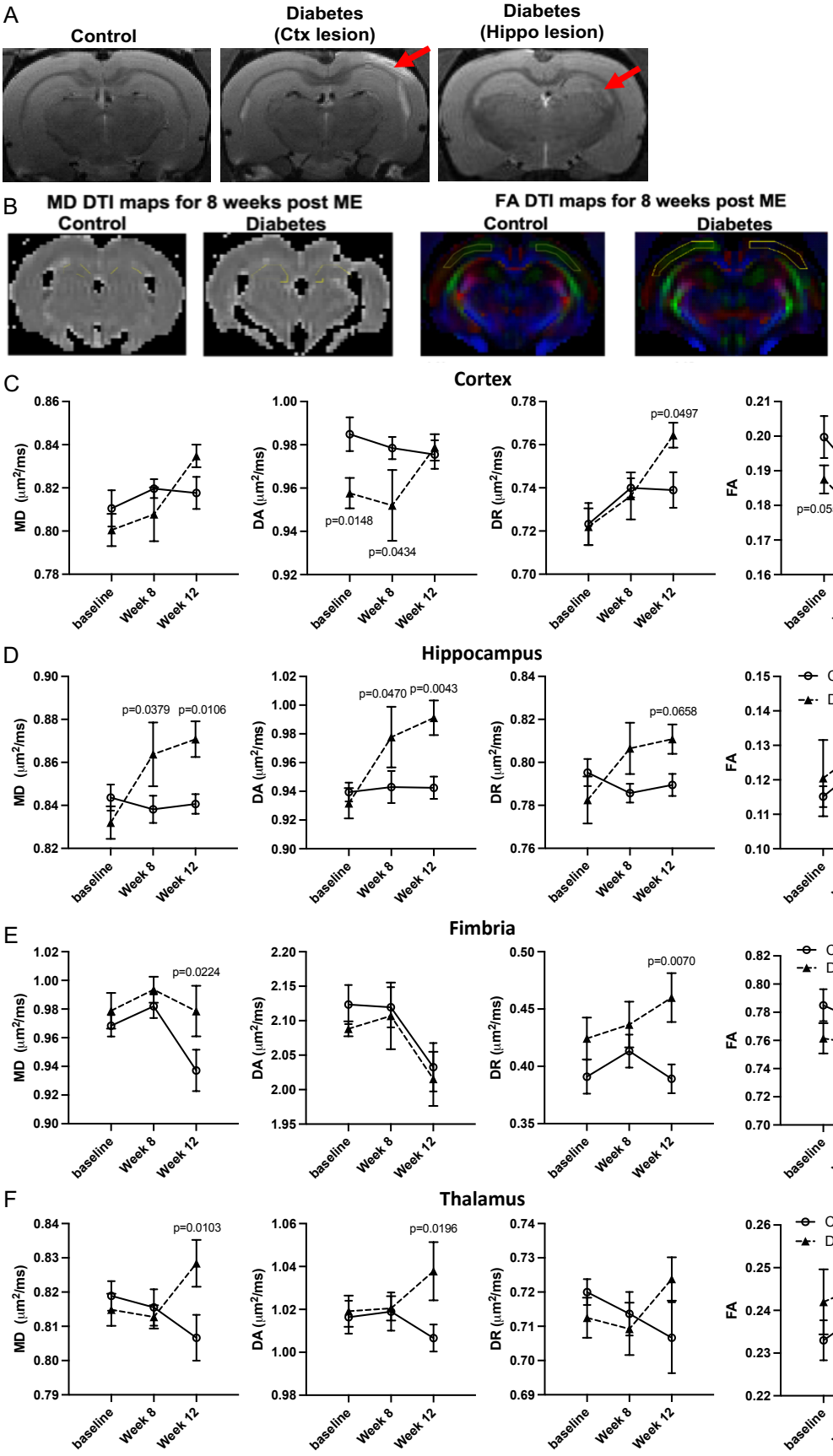
A
Control
Diabetes (Ctx lesion)
Diabetes (Hippo lesion)
B
MD DTI maps for 8 weeks post ME
Control
Diabetes
FA DTI maps for 8 weeks post ME
Control
Diabetes
C
Cortex
D
Hippocampus
E
Fimbria
F
Thalamus
MD (μm²/ms)
DA (μm²/ms)
DR (μm²/ms)
FA
baseline
Week 8
Week 12
Ctrl
DM
p=0.0148
p=0.0434
p=0.0497
p=0.0551
p=0.0279
p=0.0379
p=0.0106
p=0.0470
p=0.0043
p=0.0658
p=0.0224
p=0.0070
p=0.0233
p=0.0103
p=0.0196

Fig. 4, Diabetic animals had diffuse microstructure damage that exacerbated by ME injection. A: Representative images showed the ischemic infarcts (red arrow) in diabetic animals. B: Representative images showed diffusivity maps of diffusion MRI metrics in control and diabetic animals. C: In the cortex, diabetic animals had lower axonal diffusivity (DA) values at baseline. After ME injection, there was a trend to an increase in mean (MD), axial (DA) and radial (DR) diffusivities in diabetes, significant for the DR at 12 weeks after ME injection. Fraction anisotropy (FA) had a trend for lower values in diabetic rats, which was significant at 12 weeks after ME injection. D: In the dorsal hippocampus, there was a trend to an increase of diffusivity that became significant at 12 weeks after ME injection for MD, DA and DR. E: In the fimbria, there was a trend for increase diffusivity for all metrics (MD, DA, DR) at week 8 after ME for both control and diabetes groups. At 12 weeks after ME injection, MD and DA decreased in both groups, but DR values continued to increase in the diabetic rats, which was significant different from the control rats. FA had a trend for lower values in the fimbria of diabetic rats, which was significant at 12 weeks after ME injection. F: In the thalamus, at 8 weeks after ME injection, it seemed there was on change for all metrics in diabetic animals, while DR had decreased and FA had increased in control animals. At 12 weeks after ME injection, both MD and DA had significantly increased in diabetic animals, while DR and FA had no difference between the two groups. Data are mean ± SEM and were compared with corrected two-way ANOVA.

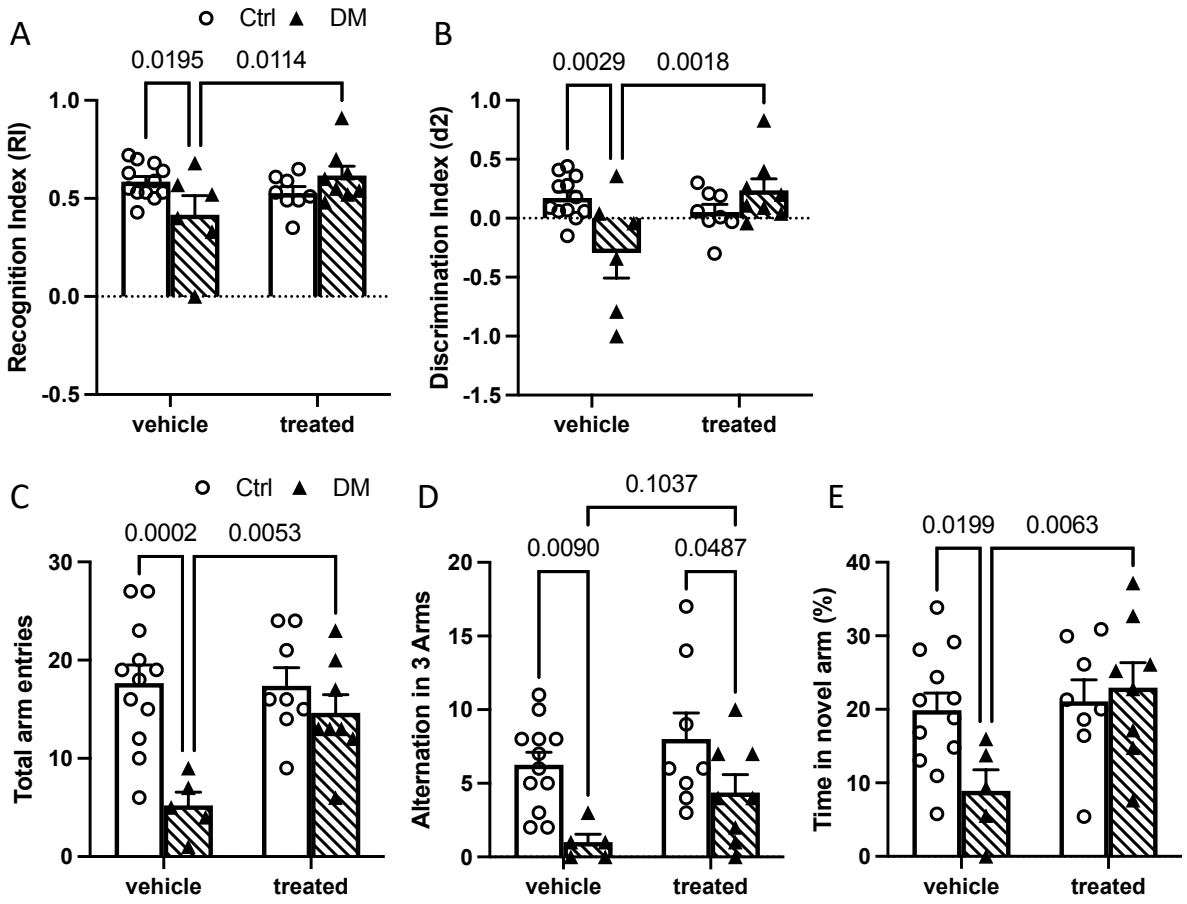

Fig. 5, Endothelial protection treatment with ISMN and cilostazol ameliorated cognitive behavior outcome in diabetic animals. A: Recognitive index of NOR test in treated diabetic animals had significantly been improved comparing to the vehicle diabetic group. B: Discrimination index of NOR test in treated diabetic animals had also been significantly improved. C: Total arm entries showed treated diabetic animals had significantly improved results in Y maze test comparing to vehicle diabetic group. D: There was a trend that treated diabetic animals had more Alternations among the arms in Y maze test comparing to the vehicle diabetic group. E: Comparing to vehicle diabetic group, treated diabetic animals spent more time in the novel arm in Y maze test. Data are mean ± SEM and were compared with corrected two-way ANOVA.

Table 1, Physiological information of the animals in each group. Data are mean ± SEM and were compared with two-tailed unpaired t test. *p=0.0002, **p=0.0019, ***p<0.0001 vs. Ctrl, respectively.

	Ctrl vehicle (n=16)	DM vehicle (n=6)	Ctrl treated (n=8)	DM treated (n=8)
BW (g)	580.7±20.1	408.5±27.4*	493.8±26.4	368.9±19.3**
BG (mg/dL)	76.7±1.9	315.2±32.6***	81.5±2.7	322.9±27.0***