

Original Article

# APX3330 Promotes Neurorestorative Effects after Stroke in Type One Diabetic Rats

Tao Yan<sup>1,2</sup>, Poornima Venkat<sup>2</sup>, Michael Chopp<sup>2,3</sup>, Alex Zacharek<sup>2</sup>, Peng Yu<sup>2</sup>, Ruizhuo Ning<sup>2,4</sup>, Xiaoxi Qiao<sup>5</sup>, Mark R. Kelley<sup>6</sup>, Jieli Chen<sup>1,2,\*</sup>

<sup>1</sup>Gerontology Institute, Neurology, Tianjin Medical University General Hospital; Tianjin Neurological Institute, Key Laboratory of Post-Neurotrauma Neurorepair and Regeneration in Central Nervous System, Ministry of Education and Tianjin City, Tianjin, China

<sup>2</sup>Department of Neurology, Henry Ford hospital, Detroit, MI, USA

<sup>3</sup>Department of Physics, Oakland University, Rochester, MI, USA

<sup>4</sup>Department of Neurology, First Hospital Harbin, Harbin, China

<sup>5</sup>Department of Ophthalmology, Henry Ford Hospital, Detroit, MI, USA

<sup>6</sup>Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA.

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**ABSTRACT:** APX3330 is a selective inhibitor of APE1/Ref-1 redox activity. In this study, we investigate the therapeutic effects and underlying mechanisms of APX3330 treatment in type one diabetes mellitus (T1DM) stroke rats. Adult male Wistar rats were induced with T1DM and subjected to transient middle cerebral artery occlusion (MCAo) and treated with either PBS or APX3330 (10mg/kg, oral gavage) starting at 24h after MCAo, and daily for 14 days. Rats were sacrificed at 14 days after MCAo and, blood brain barrier (BBB) permeability, ischemic lesion volume, immunohistochemistry, cell death assay, Western blot, real time PCR, and angiogenic ELISA array were performed. Compared to PBS treatment, APX3330 treatment of stroke in T1DM rats significantly improves neurological functional outcome, decreases lesion volume, and improves BBB integrity as well as decreases total vessel density and VEGF expression, while significantly increases arterial density in the ischemic border zone (IBZ). APX3330 significantly increases myelin density, oligodendrocyte number, oligodendrocyte progenitor cell number, synaptic protein expression, and induces M2 macrophage polarization in the IBZ of T1DM stroke rats. Compared to PBS treatment, APX3330 treatment significantly decreases plasminogen activator inhibitor type-1 (PAI-1), monocyte chemoattractant protein-1 and matrix metalloproteinase 9 (MMP9) and receptor for advanced glycation endproducts expression in the ischemic brain of T1DM stroke rats. APX3330 treatment significantly decreases cell death and MMP9 and PAI-1 gene expression in cultured primary cortical neurons subjected to high glucose and oxygen glucose deprivation, compared to untreated control cells. APX3330 treatment increases M2 macrophage polarization and decreases inflammatory factor expression in the ischemic brain as well as promotes neuroprotective and neurorestorative effects after stroke in T1DM rats.

**Key words:** Stroke, Type 1 Diabetes Mellitus, APX3330, neuroprotection, neurorestoration

Hyperglycemia and insulin resistance are major risk factors for ischemic stroke, atherosclerosis, and vascular and cardiac complications [1]. Diabetes mellitus (DM) instigates a cascade of events which lead to vascular endothelial cell dysfunction, increased vascular

permeability, a disequilibrium of angiogenesis, and poor recovery after ischemic stroke [2, 3]. Both stroke and diabetes cause extensive damage to white matter (WM) [4, 5] which can potentially lead to long term disability due to the brain's limited capacity of axonal regeneration, and

\*Correspondence should be addressed to: Dr. Jieli Chen, Department of Neurology, Henry Ford hospital, Detroit, MI, USA. Email: JChen4@hfhs.org

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an unfavorable environment for axon regrowth, sprouting and remyelination in the ischemic brain [6]. Therefore, in addition to neuroprotection in the gray matter, therapies that protect WM and promote WM remodeling after stroke, will enable long term neurological functional recovery via strengthening residual neuronal connections, forging new connections, and axonal rewiring in the injured brain [7]. Approximately 30% of all stroke patients suffering from diabetes, battle worse prognosis at least in part due to high inflammation and exacerbated WM injury in the ischemic diabetic brain. Hence, there is a critical need to develop therapeutic strategies specifically to improve neurological functional outcome after stroke in the DM population.

In diabetic stroke, exacerbated blood brain barrier (BBB) disruption and inflammation are key factors that contribute to poor outcome [4, 8, 9]. Matrix metalloproteinases (MMP) are associated with BBB disruption, and known to aggravate WM injury [4, 8, 9]. Myelin degradation following ischemic stroke is attenuated in MMP9 knockout mice [10], implicating MMP's in post-stroke WM injury. Inflammatory factors such as the receptor for advanced glycation endproducts (RAGE) play a vital role in promoting inflammation and diabetic complications [11, 12]. In our previous study, we have reported that RAGE is increased in diabetic stroke animals [13]. Increased RAGE expression has been implicated in promoting inflammation in the ischemic brain, and has been associated with neurological deficits in DM animals after stroke [13]. A permeable BBB facilitates the infiltration of peripheral macrophages into the ischemic brain [14, 15], and these macrophages initially assume an anti-inflammatory M2 phenotype and aid in clearing cellular debris from the injured brain [16, 17]. These macrophages can also assume/transition into a neurodegenerative M1 phenotype and hinder recovery by releasing proinflammatory factors. These pro-inflammatory molecules can in turn worsen BBB damage, initiate the recruitment of peripheral immune cells into the cerebral microcirculation and ischemic brain, as well as induce systemic inflammation [18]. Therefore, in diabetic subjects, it is important to control post stroke inflammatory responses and extend the M2 phase of macrophages and delay their transition into the pro-inflammatory M1 phenotype in to improve stroke outcome.

APX3330 is a small molecule inhibitor of apurinic apyrimidinic endonuclease redox effector factor-1 (APE1/Ref-1) redox activity [19]. Alterations in the expression, subcellular localization and activity of APE/Ref-1 have been associated with several diseases such as neurodegeneration, cancer and cardiovascular diseases. The role of APE1/Ref-1 in disease pathology and its potential as a novel therapeutic target has been

reviewed elsewhere [19-21]. Several studies have demonstrated the specificity of APX3330 for APE1 [19]. APE1 activates nuclear factor kappa B (NF $\kappa$ B) and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), which are involved in apoptosis, inflammation and angiogenesis [20, 22]. NF $\kappa$ B is induced in neurons following stroke and is a key mediator of inflammatory responses and infarction development/expansion after stroke [23]. NF $\kappa$ B is also known to critically regulate resident inflammatory brain cells and microglia that are activated in response to ischemic injury [24]. In T1DM stroke rats, suppressing NF $\kappa$ B signaling pathway significantly decreases inflammatory factors such as RAGE and TLR4 expression in the ischemic brain and improves stroke outcome [25]. HIF-1 $\alpha$  is a transcriptional activator that maintains oxygen homeostasis and functions as a master regulator of tissue responses to hypoxic conditions. While neuroprotective therapies that up regulate HIF-1 $\alpha$  activity have been reported to promote pro-angiogenic responses and improve stroke outcome in diabetic mice [26], several reports also indicate that DM up regulates HIF-1 $\alpha$  protein levels and increases HIF-1 $\alpha$  transcriptional activity in endothelial cells, which in turn can adversely affect tight junction proteins and BBB permeability [27, 28]. DM drives increased but dysfunctional neovascularization in the cerebrovasculature, which upon ischemic stress can become leaky and disrupt/aggravate BBB rupture [29]. Hypoxia stimulates angiogenesis mainly via activation of HIF-1 $\alpha$  and vascular endothelial growth factor (VEGF). In T1DM stroke rats, an abnormal and persistent increase in VEGF correlated with increased BBB permeability in the ischemic brain [30], and inhibiting VEGF improved tight junction proteins and decreased endothelial leakage i.e. BBB leakage [28].

APX3330 inhibits NF $\kappa$ B and HIF-1 $\alpha$  activity [31], and can potentially attenuate neuroinflammatory responses and post ischemic angiogenesis, which makes APX3330 a suitable candidate for stroke therapy in DM rats. In this study, we are the first to investigate whether APX330 treatment could improve functional outcome after stroke in Type one diabetic (T1DM) rats, whether APX3330 induces neurorestorative effects, and what mechanisms are involved in APX330 treatment induced beneficial effects in T1DM stroke rats.

## MATERIALS AND METHODS

All experiments followed the standards of the American Council on Animal Care and Institutional Animal Care and Use Committee of Henry Ford Health System.

### *T1DM induction*

Adult male Wistar rats (225-250g, Jackson Laboratory)

were induced with T1DM using a single intraperitoneal injection of Streptozotocin (STZ, 60 mg/kg) [32]. After 2 weeks, rats with fasting blood glucose >300 mg/dl were defined as T1DM and included in the study.

#### ***Middle cerebral artery occlusion (MCAo) model and experimental groups***

T1DM rats were anesthetized with 2% isoflurane in a chamber for pre-anesthetic, and spontaneously respired with 1.5% isoflurane in 2:1 N<sub>2</sub>O:O<sub>2</sub> mixture using a facemask connected and regulated with a modified FLUOTEC 3 Vaporizer (Fraser Harlake). Rectal temperature was maintained at 37°C throughout the surgical procedure using a feedback regulated water heating system. T1DM rats were subject to transient (2 hour) MCAo via intraluminal vascular occlusion as previously described [33, 34]. T1DM stroke rats were randomized (n=7/group) and treated with phosphate buffered saline (PBS-vehicle control) or APX3330 (10 mg/kg, gavage) initiated at 24 hours after MCAo daily for 14 days. Rats were sacrificed at 14 days after MCAo for immunostaining quantification analysis.

#### ***Functional tests***

An investigator blinded to the experimental groups performed a battery of functional tests including the modified neurological severity score and foot-fault tests prior to MCAo, and on days 1, 7 and 14 after MCAo, as previously described [35]. The modified neurological severity score is a composite of motor, sensory, reflex and balance tests, with scores ranging between 0 (no deficit) to 18 (maximum deficit). Hence, a higher score indicates greater neurological severity. Rats with scores <6 (possibly small to no lesion, their condition improves regardless of treatment) and >13 (very large lesion, poor survival, their condition deteriorates regardless of treatment) were excluded before treatment. The foot-fault test is routinely employed to evaluate sensorimotor function, motor coordination and limb placement deficits during locomotion [36]. The rats were placed on a 2.5 cm × 2.5 cm grid floor (45 cm × 30 cm) placed 2.5 cm above the base floor. A foot-fault was defined as a fall or slip through a grid opening due to an inaccurate forelimb placement. Data are presented as the percentage of foot-faults of the left paw over a 100-forelimb movement.

#### ***Immunohistochemical assessment***

All animals were transcardially perfused with 0.9% saline, and brains were rapidly removed, and immersion fixed in 4% paraformaldehyde and then embedded in paraffin. Seven coronal brain sections were processed and a series of 6 μm thick sections were cut from blocks which were

obtained from the center of the lesion (bregma -1 mm to +1 mm). Every 10<sup>th</sup> coronal section for a total of 5 sections was used for immunohistochemical staining. Hematoxylin and Eosin (H&E) staining was used for ischemic lesion volume calculation and presented as a percentage of the lesion compared with the contralateral hemisphere [37]. Antibody against α-smooth muscle actin (αSMA, smooth muscle cell marker, mouse monoclonal IgG 1:800, Dako), Von Willebrand Factor (vWF, an endothelial cell marker, 1:400; Dako), VEGF (1:50, Santa Cruz), synaptophysin (synaptic protein marker, 1:500, Chemicon), neuron-gial antigen 2 (NG2, oligodendrocyte progenitor cell marker, 1:800, Chemicon) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, oligodendrocyte marker, 1:200, Chemicon), ED1 (microglia/macrophages marker, 1:30; AbD Serotec), and CD163 (M2 macrophage marker, 1:500, Abcam) were employed. FITC-Albumin (1:500, Abcam) staining was employed to evaluate BBB permeability, and Luxol fast blue (LFB) staining was used to demonstrate myelin. In control experiments, primary antibody was replaced by non-immune serum.

#### ***Quantification analysis***

An investigator blinded to the experimental groups performed all immunostaining quantification analysis. Five slides from each brain (each slide containing 8 fields from striatum of the ischemic boundary zone (IBZ)) were digitized under a 20× objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research). The number of arteries stained with αSMA were counted and analyzed with regard to small and large vessels (≥10μm diameter). For other immunostaining measurements, positive cell numbers were counted for each field of view or the positive stained areas were measured using the densitometry function (MCID image analysis system) with a density threshold above unstained set uniformly for all groups [38-40].

#### ***Angiogenic ELISA array***

An additional set of rats (n=4/group) were sacrificed at 14 days after MCAo and brain tissue was extracted from the ischemic brain for angiogenic ELISA assays and Western blot assays. An angiogenesis array kit (R&D Systems, mouse angiogenesis array kit) was used to simultaneously assess the relative levels of angiogenesis-related proteins in ischemic brain tissue which had been treated with or without APX3330. Briefly, samples were mixed with detection antibodies for 1 hour at room temperature and added to the array membrane. Then, the membranes were incubated over night at 2-8°C on a shaker. Following a

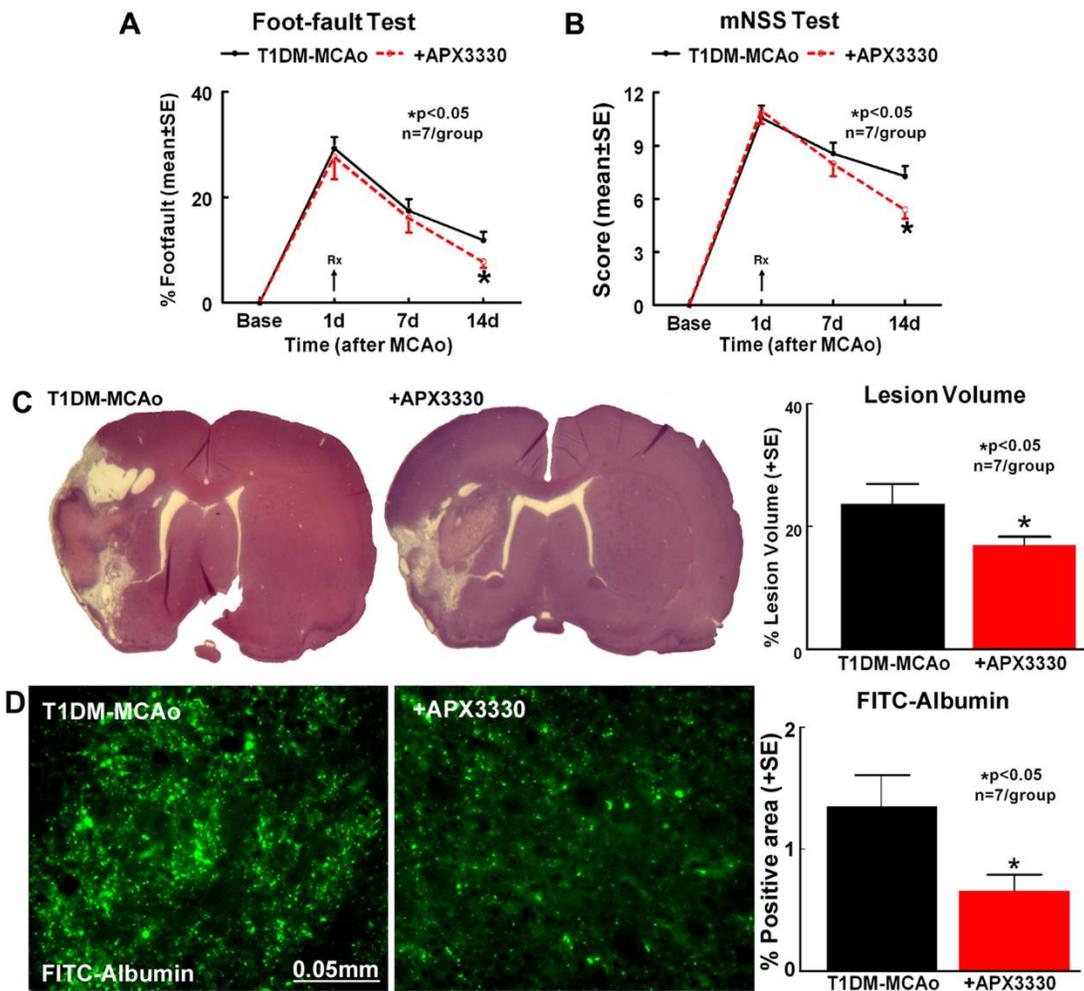
washing step, horseradish peroxidase-conjugated streptavidin was added and incubated for 30 minutes. After washing, X-ray film and ImageJ analysis software were used to quantify array signals.

**Western blot assay**

Equal amounts of cell lysate were subjected to Western blot analysis which was performed as previously described [41]. Specific proteins were visualized using a Super Signal West Pico chemiluminescence kit (Pierce). Protein concentration was measured using the BCA kit (Thermo Scientific). The following primary antibodies were used: anti-β-actin (1:2000; Santa Cruz Biotechnology), anti-MMP9 (1:500; Santa Cruz Biotechnology) and anti-RAGE (1:500, R&D Biosystems).

**Primary cortical neuron (PCN) culture**

PCNs were harvested from pregnant (day 18) embryonic Wistar rats (Charles River). The cultures were prepared as previously described with some modifications [42, 43]. Briefly, the embryo cerebral cortex was dissected and dissociated in Ca<sup>2+</sup> and Mg<sup>2+</sup> free HBSS with 0.125% trypsin. The cells were placed on poly-d-lysine (Sigma) coated dishes (35 mm, Corning) and initially cultured in DMEM media (Life Technologies) with 5% fetal bovine serum for 5 hours, then neurobasal growth medium (Life Technologies) with 2% B-27 (Life Technologies), 2 mM GlutaMax, and 1% antibiotic-antimycotic was used.



**Figure 1. APX3330 improves stroke outcome and decreases ischemic burden and BBB permeability in T1DM rats.** Treatment with APX3330 significantly improves neurological functional outcome after stroke in T1DM rats as indicated by (A) Foot-fault test and (B) modified neurological severity score (mNSS). APX3330 treatment also significantly decreases (C) ischemic lesion volume and (D) BBB disruption after stroke in T1DM rats.

### High glucose and oxygen glucose deprivation (OGD) conditions

To subject cells cultures to OGD, serum and glucose free media was used. Cells were placed in a hypoxia chamber (Forma Anaerobic System; Thermo Scientific) with 37°C incubator for 2 hours. After 2 hours, the cells were removed and replaced in high glucose cultured media (37.5  $\mu$ M).

### Lactate dehydrogenase (LDH) assay

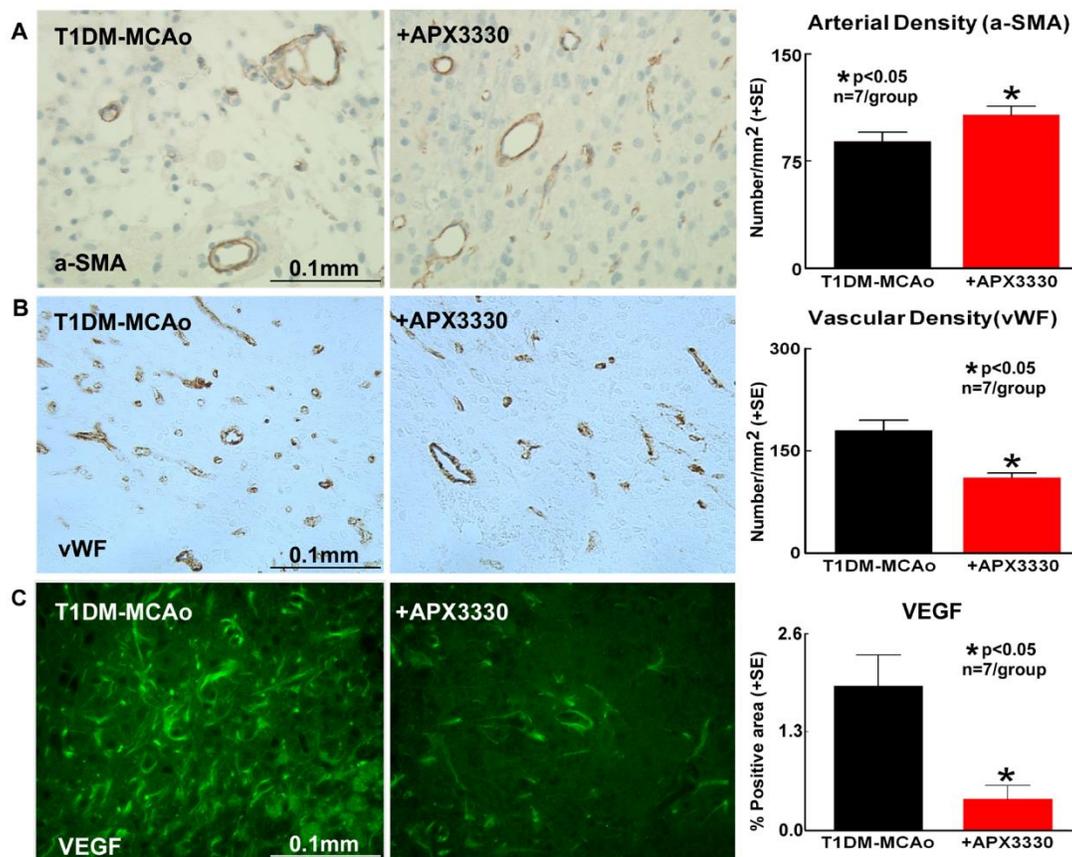
The CytoTox 96 non-radioactive cytotoxicity assay kit (Promega) was used following standard protocol. Briefly, cells were cultured in a 96 well plate for 24 hours and then the cells in half the wells for each experimental group were lysed to give total LDH, while media only was collected from the other set of wells for secreted LDH. The media was added to a fresh 96 well plate, followed by the addition of substrate solution. Following incubation, absorbance was recorded at 490 nm.

### MTS assay

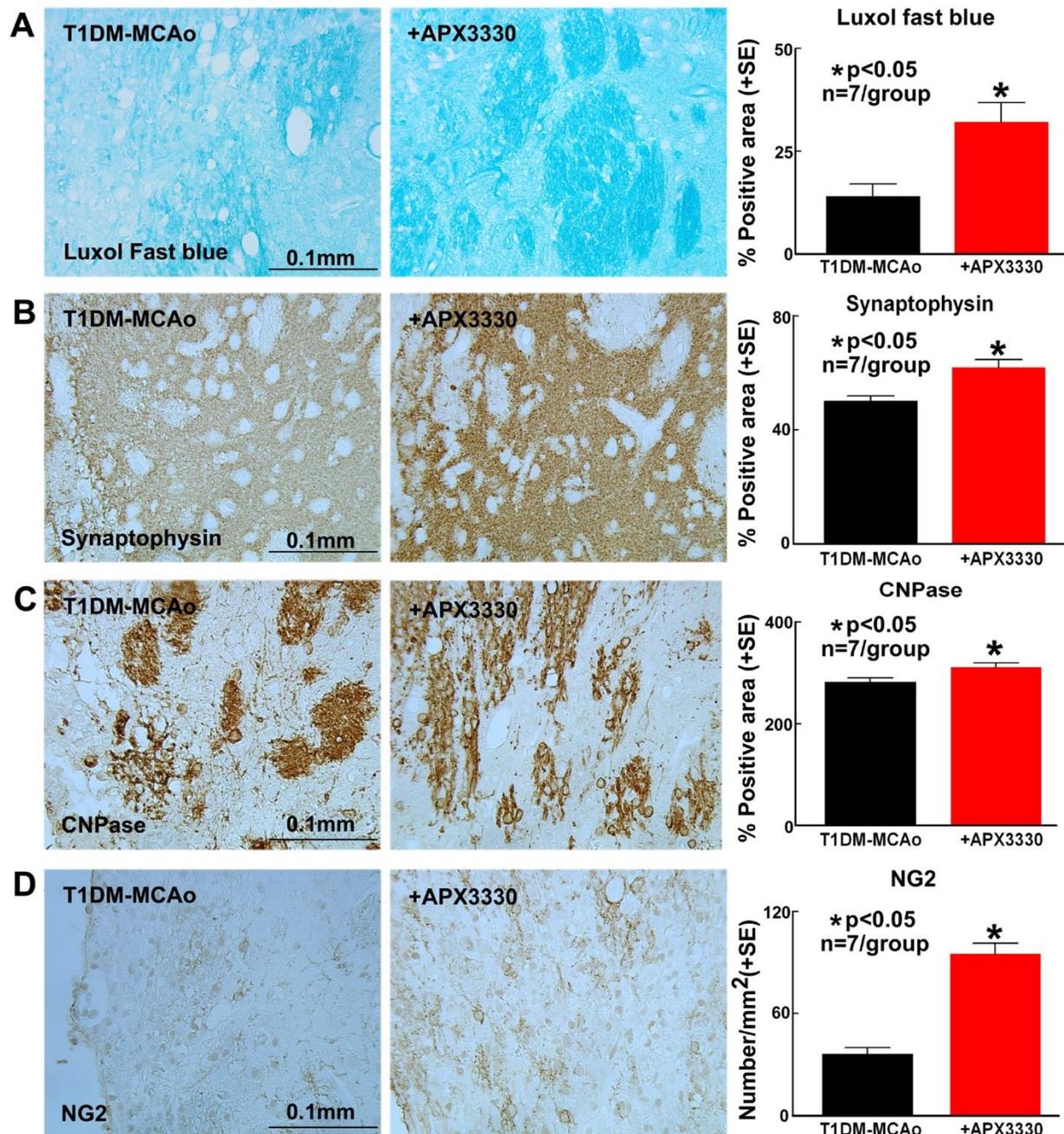
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). 5000 cells were plated and 20  $\mu$ L MTS was added to each well and incubated after treatment. Absorbance was recorded at 490 nm.

### Statistical analysis

All measurements and analyses were performed by normality of distribution. One-way Analysis of Variance (ANOVA) was used for the evaluation of functional tests, immunostaining, respectively. “Contrast/estimate” statement was used to test the group difference. All data are presented as mean  $\pm$  standard error (SE).



**Figure 2.** APX3330 increases arterial density and decreases dysfunctional angiogenesis after stroke in T1DM rats. APX3330 significantly increases (A) arterial density and decreases (B) vascular density and (C) VEGF expression in the ischemic border zone after stroke in T1DM rats, as indicated by  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), Von Willebrand Factor (vWF) and vascular endothelial growth factor (VEGF) immunostaining and quantification data respectively.



**Figure 3. APX3330 significantly promotes white matter remodeling after stroke in T1DM rats.** APX3330 significantly increases (A) myelin density (Luxol fast blue), (B) synaptic protein expression (Synaptophysin), (C) oligodendrocyte cell number (CNPase), and (D) oligodendrocyte progenitor cell (NG2) number in the ischemic border zone of T1DM stroke rats.

## RESULTS

*APX3330 treatment significantly improves neurological outcome, and decreases lesion volume and BBB permeability after stroke in T1DM rats*

To evaluate the therapeutic effects of APX3330 treatment in T1DM stroke rats, mNSS and foot-fault tests were used to evaluate neurological function. APX3330 treatment significantly improves functional outcome after stroke in T1DM rats and the data show that the overall group effect

was significant on day 14 after stroke ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 1A-B). APX3330 significantly decreases ischemic lesion volume ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 1C) and decreases BBB leakage ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 1D) compared to PBS treated control T1DM stroke rats.

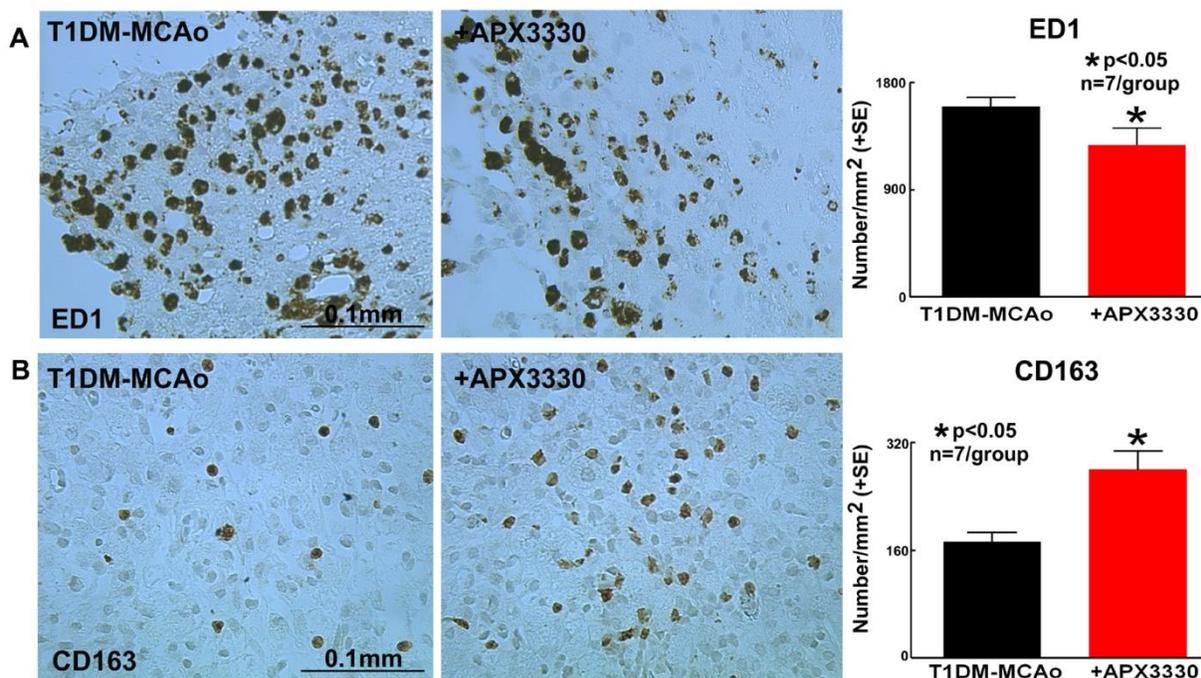
#### ***APX3330 treatment decreases dysfunctional angiogenesis and VEGF expression in T1DM stroke rats***

To test whether APX3330 treatment regulates post stroke angiogenesis, vascular and arterial density was measured in the IBZ. Figure 2A shows that APX3330 significantly increases arterial density ( $p < 0.05$ ,  $n = 7/\text{group}$ ) compared to PBS treated control T1DM stroke rats. However, total vascular density was significantly reduced in APX3330 treatment rats compared with PBS treated control T1DM stroke rats ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 2B). APX3330 also significantly decreases VEGF expression in the IBZ ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 2C) compared to PBS treated

control T1DM stroke rats.

#### ***APX3330 treatment significantly promotes WM and axonal remodeling after stroke in T1DM rats***

To test whether APX3330 treatment regulates WM and axonal remodeling, Luxol fast blue (myelin density) and Synaptophysin (synaptic protein expression) expression were quantified in the IBZ. APX3330 significantly promotes myelin density ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 3A) and synaptophysin expression ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 3B) in the IBZ compared to PBS treated control T1DM stroke rats. APX3330 treatment also significantly increases oligodendrocyte (CNPase,  $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 3C) and oligodendrocyte progenitor cell density (NG2,  $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 3D) in the IBZ compared to PBS treated control T1DM stroke rats.



**Figure 4. APX3330 treatment significantly promotes M2 macrophage polarization in the ischemic brain of T1DM stroke rats.** APX3330 significantly decreases (A) ED1 (M1 macrophage marker, and significantly increases (B) CD163 (M2 macrophage marker) in the ischemic border zone of T1DM stroke rats.

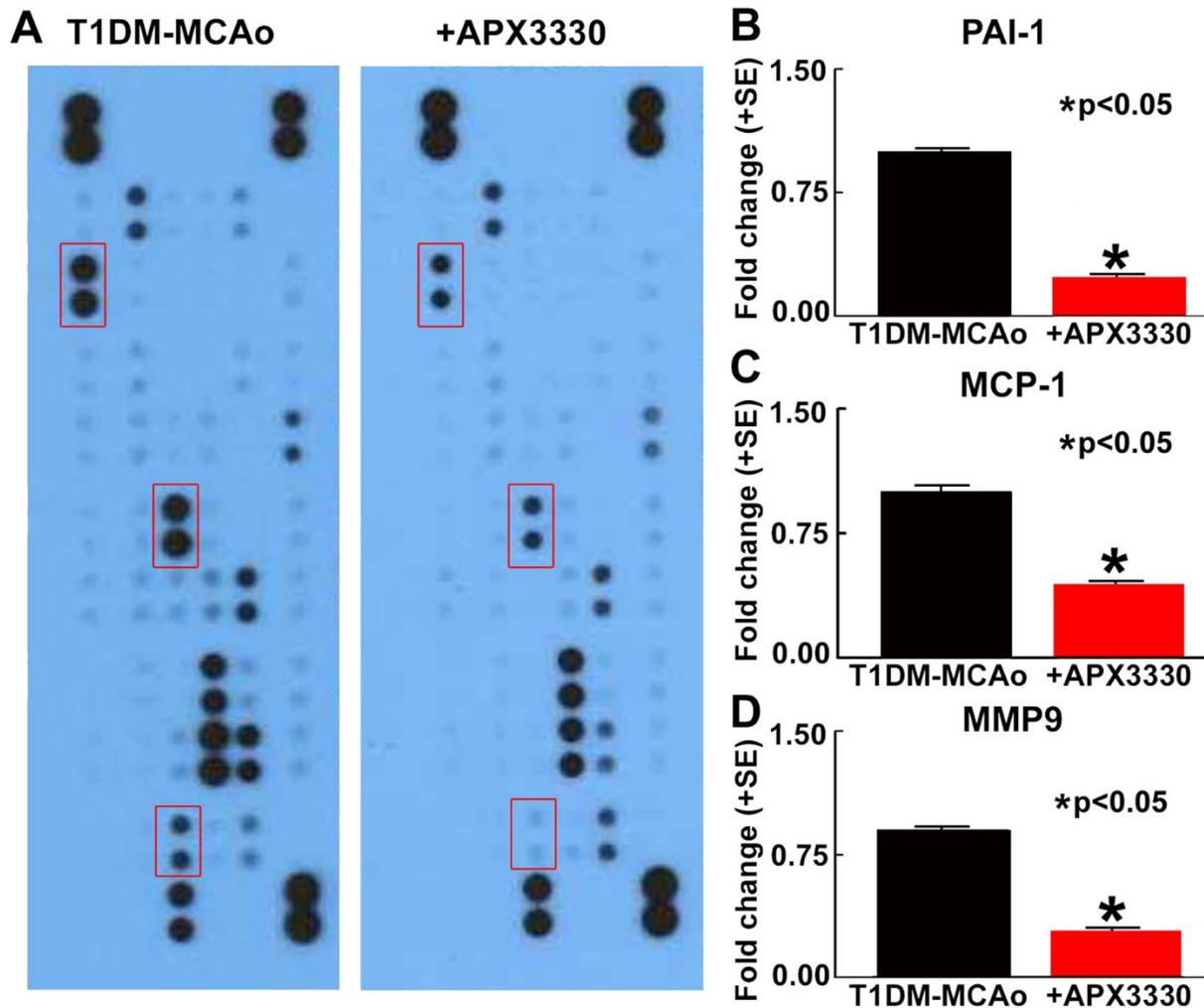
#### ***APX3330 treatment significantly decreases inflammatory factor expression after stroke in T1DM rats***

To investigate mechanisms of APX3330 induced neuroprotective and neurorestorative effects in T1DM stroke, we evaluated inflammatory factor expression in

the ischemic brain. APX3330 treatment significantly promotes M2 macrophage polarization as indicated by decreased ED1 (M1 macrophage marker) and increased CD163 (M2 macrophage marker) in the IBZ of T1DM stroke rats ( $p < 0.05$ ,  $n = 7/\text{group}$ , Fig. 4A-B). Angiogenic ELISA assay (Fig. 5A) shows that APX3330 decreases plasminogen activator inhibitor type 1 (PAI-1, 5-fold, Fig.

5B), monocyte chemoattractant protein-1 (MCP1, 3-fold, Fig. 5C) and MMP9 (3-fold, Fig. 5D) compared to T1DM-MCAo control rats. We also measured the ischemic brain expression level of MMP9 and RAGE by Western blot. APX3330 significantly decreases ischemic brain tissue expression of MMP9 and RAGE compared to PBS treated

control T1DM stroke rats ( $p < 0.001$ ,  $n = 4/\text{group}$ , Fig. 6A). These data indicate that APX3330 treatment decreases inflammatory factor and pro-thrombotic factor expression in the ischemic brain after stroke in T1DM rats.

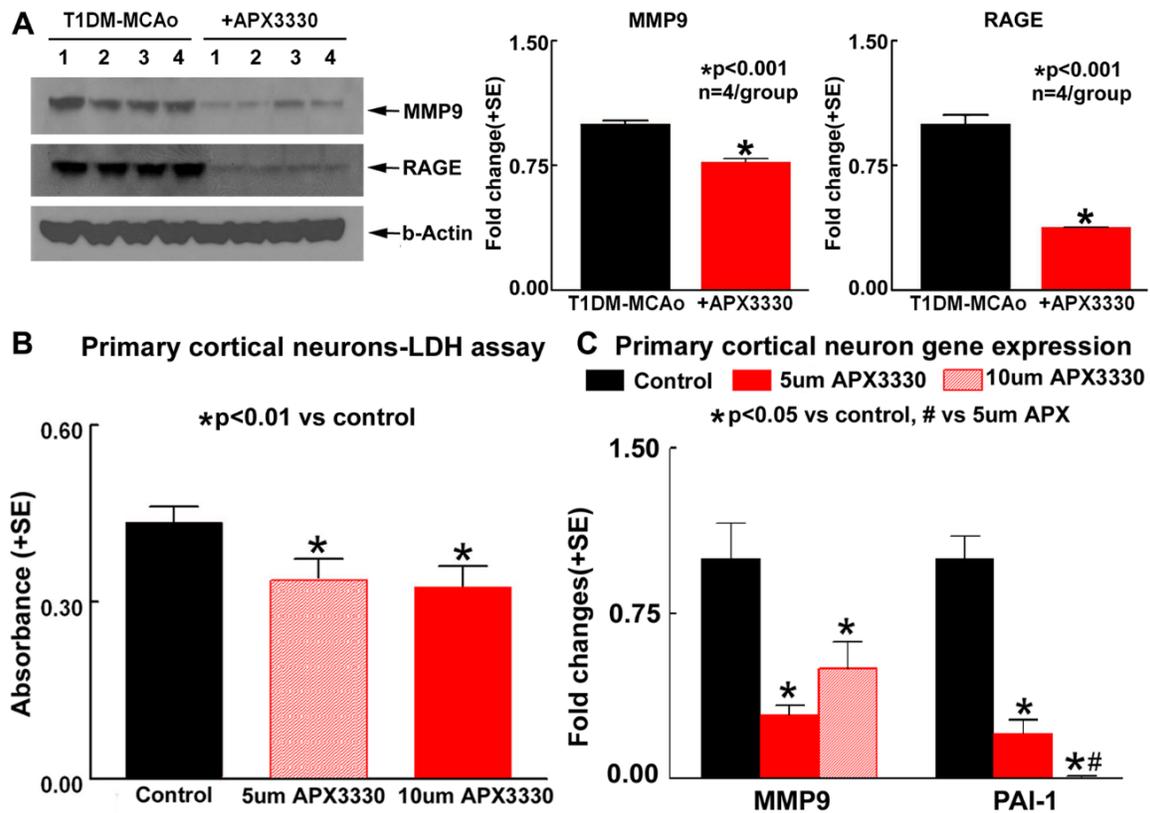


**Figure 5. APX3330 treatment significantly decreases inflammatory factors after stroke in T1DM rats.** (A) An ELISA array was employed to measure inflammatory protein expression in the ischemic brain. APX3330 significantly decreases (B) plasminogen activator inhibitor type 1 (PAI-1), (C) monocyte chemoattractant protein-1 (MCP1) and (D) MMP9 compared to control T1DM stroke rats.

**APX3330 significantly decreases cell death and inflammatory factor gene expression in primary cortical neurons subjected to high glucose and OGD conditions**

To test the effects of APX3330 on primary cortical neurons cell death and proliferation, LDH and MTS assays were performed. APX3330 treatment does not increase primary cortical neuron proliferation, but

significantly decreases cell death compared to control (untreated) cells as measured by MTS (data not shown) and LDH (Fig. 6B) assays, respectively, which is consistent with the myelin protective effect observed in vivo. APX3330 treatment significantly decreases MMP9 and PAI-1 gene expression in primary cortical neurons subjected to high glucose and oxygen glucose deprivation, when compared to control (untreated) cells (Fig. 6C).



**Figure 6. APX3330 treatment decreases inflammatory factor expression in the ischemic brain of T1DM stroke rats and significantly decreases primary cortical neurons cell death and inflammatory factor expression after stroke.** (A) Western blot assay shows that APX3330 significantly decreases ischemic brain tissue expression of MMP9 and RAGE compared to control T1DM stroke rats. APX3330 treatment significantly decreases (B) primary cortical neuron cell death compared to control (untreated) cells as measured by LDH assay and (C) significantly decreases MMP9 and PAI-1 gene expression in primary cortical neurons subjected to high glucose and oxygen glucose deprivation, when compared to control (untreated) cells.

## DISCUSSION

In this study, we are the first to demonstrate that APX3330 treatment significantly improves neurological functional outcome and decreases lesion volume and BBB permeability after stroke in T1DM rats. APX3330 promotes WM remodeling and exerts myelin protective effects in the IBZ of T1DM stroke rats. By employing an angiogenic ELISA assay and Western blot analysis, we also confirmed that APX3330 treatment significantly decreases the expression of inflammatory factors such as MCP1, MMP9 and RAGE and pro-thrombotic factor PAI-1, in the ischemic brain of T1DM stroke rats. APX3330 significantly decreases gene expression of inflammatory factor MMP9 and pro-thrombotic PAI-1 in cultured primary cortical neurons subjected to high glucose and oxygen glucose deprivation conditions. These data indicate that APX3330 may be a novel treatment for T1DM stroke.

DM stroke induces extensive vascular damage, and our previous study has shown that there is a significant increase in vascular density in the ischemic border zone after stroke in T1DM rats compared to wild type non-DM rats [44]. However, the increase in vascular density is often an increase in non-functional blood vessels that contribute to brain hemorrhage and worse functional outcome after stroke [44]. Our present study shows that APX3330 treatment significantly increases arterial density but decreases total vascular density, indicating the potential of APX3330 to reverse DM stroke induced growth of unnecessary and non-functional blood vessels. BBB function is widely attributed to proper function and integrity of endothelial cells, tight junction protein and pericytes [45]. Aggravated BBB disruption has been demonstrated in T1DM stroke rats compared to non-DM stroke rats [44]. Protecting/improving BBB integrity is important for recovery after stroke, and we found that

APX3330 decreases BBB leakage compared to control T1DM stroke rats.

In addition to massive vascular destruction, DM stroke also induces significant damage to the WM in the ischemic brain. Extensive WM after stroke has been associated with greater mortality, worse neuronal dysfunction and greater residual sensory-motor deficits after DM stroke [5, 46-48]. Promoting WM remodeling essentially initiates rewiring of the neural circuits thereby improving intercellular connectivity and communication. Since APX3330 significantly improves neurological function after stroke in T1DM rats, we investigated APX3330 effect on WM remodeling. Our data show that APX3330 significantly increases myelin density in the ischemic border zone of T1DM stroke rats compared to control T1DM stroke rats. In the central nervous system, oligodendrocyte and oligodendrocyte progenitor cells form myelin and exert crucial roles in WM function and homeostasis [49]. In several neurological diseases including stroke, WM damaged has been associated with oligovascular uncoupling [49]. Therefore, maintenance/increase of oligodendrocytes and oligodendrocyte progenitor cells number may contribute to WM recovery and decrease neurological functional deficits [49-51]. We found that APX3330 treatment in T1DM stroke rats significantly increases the number of oligodendrocytes and oligodendrocyte progenitor cells in the ischemic border zone, which may contribute to functional recovery after stroke in T1DM rats. Taken together, it is most likely that APX3330 improves neurological functional outcome via promoting WM remodeling after stroke in T1DM rats.

Neuroinflammatory responses play an important role in post-stroke recovery. While mild to moderate inflammation can be beneficial to brain repair, uncontrolled inflammation can create an inhospitable environment for brain repair and hinder/worsen recovery [7, 52]. In our study, Western blot analysis and an angiogenic ELISA array show that APX3330 treatment significantly decreases inflammatory factors such as MCP1, MMP9 and RAGE compared to control T1DM stroke rats. APX3330 also significantly decreases MMP9 and PAI-1 gene expression in cultured primary cortical neurons subjected to high glucose and OGD conditions. Both in-vivo after ischemic stroke and in-vitro using OGD model of stroke with primary rat cortical neurons, it has been reported that MMP9 is activated and expressed in neuronal nuclei and associated with delayed neuronal death [53]. Other studies have also reported that MMP9 inhibition in cultured primary cortical neurons subject to OGD decreases apoptosis and induces neuroprotective effects [54]. DM is associated with diminished fibrinolytic capacity, increased coagulability and increased PAI-1 concentration in blood [55]. Increased PAI-1 levels in the DM population can cause formation of

intravascular thrombi and atherosclerotic lesions which in turn can cause stroke [56, 57]. Increased PAI-1 levels can trigger several cytokines and chemokines, and promote pro-inflammatory responses [58]. In addition, proinflammatory cytokines can disturb vascular endothelial function and increase PAI-1 level [59]. Elevated PAI-1 levels has also been suggested to be a true indicator of metabolic syndrome [59]. DM patients also have elevated MCP levels [60], which has been implicated in DM induced vascular calcification and diabetic nephropathy [61, 62]. Rats with nonfunctional MCP1 have smaller infarct volume and lesser macrophage infiltration post stroke, which suggests that attenuation of MCP1 may exert neuroprotective effects [63].

Inflammatory factors such as MMP9 and RAGE are typically increased in diabetic stroke animals [13]. In addition to regulating post stroke inflammatory responses, MMP9 also plays an important role in post stroke angiogenesis and BBB disruption [5, 64]. Increased MMP9 expression in the acute phase of stroke in DM patients creates a pro-inflammatory state that worsens WM damage. Neuroinflammation plays a major role in WM damage and oligodendrocyte death, in particular increased MMP9 expression has been associated with myelin breakdown [65, 66]. RAGE, as a proinflammatory factor, plays a critical role in neurodegenerative pathology, necrotic cell death and inflammatory responses [67]. The RAGE signaling pathway is involved in numerous pathological process following ischemia, such as inflammation, vascular injury, and brain damage [68, 69]. Our previous studies have implicated increased RAGE expression in worsening the outcome of DM stroke [13]. In this study, we found that APX3330 significantly decreases PAI-1, MCP-1, MMP9 and RAGE levels in the ischemic brain which may contribute to APX3330 induced neuroprotective and neurorestorative effects in T1DM stroke rats.

Following ischemic injury, macrophages promote both injury and repair depending on their phenotype. M2 macrophage polarization is a potential target for stroke therapy since anti inflammatory M2 macrophages exert neuroprotection, improve neuronal survival and decrease the expression of inflammatory factors in the ischemic brain [17, 70]. M2 macrophage polarization has been associated with improved functional recovery after stroke [71], as well as decreased neuroinflammation and enhanced axon growth in injured mouse spinal cord [72]. APX3330 promotes M2 macrophage polarization, marked by increased M2 macrophage marker CD163 and decreased M1 macrophage marker ED1 expression in the IBZ of T1DM stroke rats. MCP-1 is a chemokine that regulates the migration and infiltration of macrophages in pathological tissue [73], and MCP-1-deficient mice subjected to stroke exhibit a reduction of phagocytic

macrophage accumulation in the infarcted brain tissue [74]. Macrophage differentiation into proinflammatory M1 phenotype is mediated at least in part by RAGE/NF- $\kappa$ B pathway activation [75]. M1 Macrophages (in addition to monocytes and leukocytes) are a prolific source of inflammatory factors like MMP9 [76]. Therefore, it is likely that APX3330 induced attenuation of MCP-1 and RAGE/NF- $\kappa$ B pathway decreases M1 macrophage and promotes M2 macrophage polarization, which then in turn decreases neuroinflammatory factor production. However, the interconnections between M2 polarization and APX3330 induced regulation of neuroinflammation and WM remodeling leading to beneficial effects after stroke in T1DM rats are not completely understood and further studies are warranted.

We acknowledge that APX3330 may have conflicting results in DM versus non-DM stroke. For instance, using an APE1 conditional knockout mouse line, it has been recently demonstrated that the deletion of APE1 significantly increases ischemic lesion volume, worsens neurological functional recovery, impairs neuronal communication and aggravates demyelination as well as neuronal and oligodendrocyte degeneration in non-DM mice subjected to stroke [77]. However, the present study shows that APE1/Ref-1 redox inhibition via APX3330 treatment in DM stroke rats exerts neuroprotective and neurorestorative effects. These differences could be attributed at least in part to the role of VEGF signaling which differs between DM and non-DM stroke [30]. A robust increase in VEGF post ischemic injury to the brain correlated with aggravated BBB disruption in DM stroke mice [30]. Attenuation of VEGF after stroke significantly decreased BBB permeability, prevented the loss of synaptic structure in the IBZ, and promoted neurological functional recovery after stroke in DM mice, however, in non-DM stroke mice; attenuation of VEGF had adverse effects [30]. It has been previously demonstrated that in the acute phase of stroke in non-DM rodents, administration of VEGF enhances cerebral microvascular perfusion, increases BBB leakage, induces cerebral hemorrhage and expands infarction volume after stroke [78]. Our previous study has reported that at 1 day after stroke, VEGF is significantly increased in the ischemic brain of DM rats compared to non-DM rats [79]. Therefore, particularly in DM stroke, VEGF signaling can potentially induce extensive vascular damage [80, 81]. In this study, our data indicate that APX3330 decreases VEGF expression in the IBZ compared to control T1DM stroke rats. Since APE1/Ref-1 redox inhibition can affect multiple signaling pathways including VEGF, effects of APX3330 are likely to be different in DM stroke compared to non-DM stroke and further studies are warranted.

While the molecular mechanisms of APX3330 induced neuroprotective and neurorestorative effects in T1DM stroke rats are still unclear, this proof-of-concept study shows that APX3330 can significantly improve neurological functional outcome, decrease lesion volume, decrease BBB permeability, regulate inflammatory responses and promote white matter remodeling after stroke in T1DM rats.

## Conclusions

APX3330 treatment of stroke significantly improves neurological functional outcome and decreases ischemic lesion volume and BBB permeability in T1DM rats. APX3330 treatment significantly decreases VEGF, PAI-1, RAGE, MCP1 and MMP9 levels in ischemic brain which may contribute to decreasing dysfunctional angiogenesis, inflammation in T1DM stroke rats. APX3330 significantly increases myelin density, oligodendrogenesis and synaptic plasticity after stroke in T1DM rats. Therefore, APX3330 treatment derived neuroprotective and neurorestorative effects in T1DM stroke rats may be attributed at least in part to attenuation of dysfunctional angiogenesis, decrease in pro-inflammatory factors and increased white matter remodeling.

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## Conflicts of interest

Mark R. Kelley has licensed APX3330 through Indiana University Research and Technology Corporation to Apexian Pharmaceuticals LLC. Apexian Pharmaceuticals had neither control nor oversight of the studies, interpretation, or presentation of the data in this manuscript. The other authors have no conflicts of interest to disclose.

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