Mito-TEMPO, a mitochondria-targeted antioxidant, improves cognitive dysfunction due to hypoglycemia: An association with reduced pericyte loss and blood-brain barrier leakage

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Research Article

Keywords: Mito-TEMPO, blood-brain barrier, cognitive dysfunction, hypoglycemia, pericyte, oxidative stress

Posted Date: July 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1759711/v1

Abstract

Hypoglycemia is associated with cognitive dysfunction, however, the exact mechanisms have not been elucidated. Our previous study found that severe hypoglycemia can lead to cognitive dysfunction in a type 1 diabetes (T1D) mouse model after severe hypoglycemia. Thus, the aim of this study was to further investigate whether the mechanism of severe hypoglycemia leading to cognitive dysfunction is related to oxidative stress-mediated pericyte loss and blood-brain barrier leakage. A streptozotocin T1D model, using male C57BL/6J mice, was used to induce hypoglycemia. The brain tissue was stained, ROS and ATP were determined, and cognitive function was tested using the Morris water maze. Also, an *in vitro* glucose deprivation model was constructed. The results show that blood-brain barrier (BBB) leakage after hypoglycemia is associated with excessive activation of oxidative stress and mitochondrial dysfunction due to glucose deprivation/reperfusion. Interventions using the mitochondria-targeted antioxidant Mito-TEMPO in both *in vivo* and *in vitro* models reduced mitochondrial oxidative stress, decreased pericyte loss and apoptosis, and attenuated BBB leakage and neuronal damage, ultimately leading to improved cognitive function.

1. Introduction

In type 1 diabetes mellitus (T1DM), in the context of insulin deficiency, hypoglycemia may act as triggers for cognitive impairment [1]. A study that included 718 older patients with T1DM found significantly lower global cognitive scores, language, executive function, and memory for recent episodes of severe hypoglycemia, suggesting that the cognitive status of patients with T1DM is strongly associated with a history of severe hypoglycemia [2]. However, the mechanisms by which hypoglycemia causes cognitive dysfunction have not been fully elucidated.

Previous studies have shown that neuronal death is caused, to some extent, by hypoglycemia itself [3]. Clinically, the first treatment for patients with T1DM who develop severe hypoglycemia is a rapid increase in blood glucose levels, known as 'glucose reperfusion', which is essential for saving lives. However, glucose reperfusion allows patients to recover rapidly from severe hypoglycemia, leading to secondary damage that can lead to more severe neuronal death [4]. This is called "glucose reperfusion injury" following hypoglycemia. The mechanisms by which this injury leads to neuronal cell death may include the production of reactive oxygen species (ROS), disruption of the blood-brain barrier (BBB) and activation of inflammatory factors [5]. Thus, it seems likely that diabetic neuropathy is caused by oxidative stress induced by recurrent hypoglycemia.

Our previous study reported that severe hypoglycemia in diabetes can increase BBB leakage and pericyte loss, leading to cognitive dysfunction [6]. Therefore, protecting pericyte function and reducing BBB leakage may be key targets for the treatment of cognitive dysfunction. Interventions to reduce vascular oxidative stress have been shown to restore microvascular function and cognitive performance [7]. One study demonstrated that the antioxidant edaravone protects microvascular pericytes from apoptosis,

which is essential for the induction and maintenance of the BBB [8]. The study hints that it is possible to find a suitable antioxidant to rescue brain damage caused by hypoglycemia.

Mito-TEMPO is a mitochondria-targeted antioxidant that acts in ischemic tissues [9]. Previous studies by our team have confirmed that Mito-TEMPO can attenuate severe hypoglycemia-induced cardiac damage [10]. In terms of brain damage, animal experiments have shown that Mito-TEMPO not only inhibited mitochondrial ROS production and rescued mitochondrial respiratory function but also effectively reduced the accumulation of tau oligomers in mouse cortical neurons and improved cognitive function [11]. *In vitro* tests have shown that Mito-TEMPO protects neurons from bupivacaine-induced toxic damage [12].

Therefore, this study is the first to investigate whether the mechanism of action of severe hypoglycemia on cognitive dysfunction is related to pericyte dysfunction and BBB leakage caused by oxidative stress at both animal and cellular levels. Whether the mitochondria-targeted antioxidant Mito-TEMPO can reverse these disruptions. The results of this study will help elucidate the mechanisms of severe hypoglycemia and cognitive dysfunction. Simultaneously, it will provide new ideas and methods for the prevention and treatment of diabetic brain injury and the development of novel drugs.

2. Materials And Methods

2.1 Experimental animals and treatment

Male C57BL/6J mice (20–25 g) were purchased from Shanghai Slacker Laboratory Animal Co., Ltd (Shanghai, China) and randomly divided into normal (NC), diabetic (DM), severely (DH), and severely + Mito-TEMPO-treated (DHT) groups. DHT mice were treated with 0.7 mg/kg/d Mito-TEMPO (SML0737; Sigma-Aldrich) [10, 13]. T1D was induced with a single intraperitoneal (i.p.) injection of streptozotocin (150 mg/kg; STZ, S0130, Sigma-Aldrich, St Louis, MO, USA). On the 3rd day post-injection, mice had blood glucose levels of > 16.7 mmol/L and presented with polyuria, polydipsia, polyphagia, and wasting, suggesting successful induction of T1D. After fasting overnight, DH and DHT mice were intravenously injected with regular insulin (15 mU/g; Wanbang, Jiangsu, China) to maintain tail vein glucose levels of < 2.0 mmol/L for 90 min [10]. To terminate episodes, mice were allowed to inject glucose (1 mg/kg i.p.)after severe hypoglycemia to ensure that their blood glucose level was > 10 mmol/L. All experimental procedures were performed in accordance with the Chinese Society for Experimental Animal Care and Use guidelines and were approved by the Fujian Animal Research Ethics Committee (approval number: FJMU IACUC 2021 -0029).

2.2 Morris water maze (MWM) task

We tested cognitive function by performing a water maze test on the remaining mice (n = 10 per group) one week after severe hypoglycemia. Subsequent assessment of cognitive function allows for better measurement of clinical outcomes and assessment of neuroprotection, as it considers a complete and comprehensive assessment of ongoing brain damage and possible recovery from severe hypoglycemia

[14, 15]. In the hidden platform experiment, a transparent platform of approximately 7 cm in diameter is fixed 1.5 cm below the water surface. The mice's swimming trajectory was tracked by a video camera. In the hidden platform experiment, mice were trained to swim four times a day for five days. The time it took for the mice to find the hidden platform was the latency period of the hidden platform. On day 6, a memory retention experiment was performed. The camera tracking system recorded the time spent swimming in each quadrant, the number of times the mouse crossed the original platform and the number of swimming paths.

2.3 Hematoxylin-eosin stain

After 24-h of severe hypoglycemia modeling, some of the mice were euthanized for histological testing. Mice were anesthetized using an intraperitoneal injection of 2% sodium pentobarbital (2 ml/kg) and the fresh brain tissue was removed and fixed using a fixative for 24h. The tissue was dehydrated and waxed, followed by embedding and sectioning. This tissue was subjected to hematoxylin and eosin staining, followed by dehydration sealing of the sections in sequence. Microscopic examination, image acquisition, and analysis.

2.4 Nissl staining

Nissl-stained specimens were embedded in paraffin and cut with a microtome (RM2016, Leica, Wetzlar, Germany) to obtain 4-mm thick sections. Tissue slices were treated with toluidine blue for 2–5 minutes, rinsed with tap water, and treated with 1% glacial acetic acid. The degree of differentiation was determined using a microscope. The samples were then washed with tap water, dried in an oven, cleared in xylene for 10 min, and sealed with neutral gum. Microscopy inspection, image acquisition, and analysis were observed.

2.5 Evaluation of BBB permeability and measurement of brain water content

Evans Blue is a commonly used azo dye. When the BBB structure is disrupted, plasma proteins bound to Evans Blue can penetrate the barrier into the tissue interstitial space, and this assay is commonly used to test the structural integrity of the BBB [16]. The brain tissue was removed after 6 hours of in vivo circulation using a 2% concentration of Evans Blue solution (Sigma, E2129, 4 ml/kg); the heads were photographed using a camera after cardiac perfusion. Afterward, the mouse brain tissue was homogenized in 50% trichloroacetic acid solution (Sigma, T5159) and Evans Blue was quantified in the heads by measuring the fluorescence intensity (620 nm excitation wavelength, 680 nm emission wavelength).

The water content of brain tissue was used to assess the degree of brain edema and the BBB functional disruption. Mice were sacrificed by anesthesia and weighed to obtain wet weights (W). They were then oven-dried (65°C, 72 h) and weighed to obtain a dry weight (D). The brain water content was calculated using the following formula: brain water content (%) = (W - D) / W x 100%.

2.6 Transmission electron microscopy

The CA1 region of the hippocampus was identified as the site of extraction and a culture dish with an electron microscope fixative was prepared in advance. The small tissue blocks were removed *in vitro* and immediately placed into the culture dish with fixative and cut into 1 mm^3 piece using a scalpel. The small tissue blocks were then transferred to EP tubes containing a new electron microscope fixative for further fixation at 4°C. The samples were rinsed three times using 0.1 M phosphate buffer PB (PH 7.4), followed by fixation, room temperature dehydration, osmotic embedding, polymerization, ultra-thin sectioning, and staining. The samples were subjected to transmission electron microscopy and the images collected were analyzed.

2.6 Brain frozen section ROS assay

Frozen sections of mouse brain were rewarmed at room temperature and dried. The cells were incubated at 37°C for 30 min in a light-proof incubator, nuclei stained with DAPI, sealed, and the sections were observed under a fluorescent microscope. The nuclei of the DAPI-stained cells were blue under UV excitation (excitation wavelength 465 nm, emission wavelength 515 nm) and red for ROS-positive expression (excitation wavelength 510, emission wavelength, 590 nm).

2.8 Enzyme-linked immunosorbent assay (ELISA)

The levels of ROS, malondialdehyde (MDA), and total superoxide dismutase (SOD) in the mouse brain tissue were measured using ELISA. Brain tissue was weighed and added to nine times the volume of saline at a ratio of weight (g):volume (ml) = 1:9. The tissue was cut up and homogenized in an ice water bath, centrifuged at 2500–3000 rpm for 10 min, and 10% of the supernatant of the homogenate was collected. Repeated measurements were made using ROS, MDA, and SOD kits (Mlbio, Shanghai, China).

2.9 ATP assay

ATP levels were measured in the mice's brain tissue using the Enhanced ATP Assay Kit (Sigma, MAK190). The ATP levels were determined by adding 20 mg of tissue to 100 μL of lysate, homogenizing, and centrifuging at 12,000 g for 5 min at 4ºC. An ATP standard curve was generated and the ATP levels were measured using a microplate reader (SpectraMax i3x; Molecular Devices, CA, USA).

2.10 Western blot analysis

Brain tissue ground on ice for 20 min using a glass homogenizer, centrifuged at 12000 rpm at 4°C for 15 min in a low-temperature ultracentrifuge, and the supernatant was carefully aspirated into centrifuge tubes. This was followed by protein concentration determination, protein denaturation, SDS-PAGE electrophoresis, and membrane transfer. The membranes were incubated overnight at 4°C with the following primary antibodies: PDGFR-β (1:1000, Abcam, ab32570), α-SMA (1:1000, Abcam, ab7871), Occludin (1:1000, Abcam, ab236127), Claudin5 (1:1000, Abcam, ab131259), MMP-9 (1:1000, ab76003), and GAPDH (1:2000, Abcam, ab8245). Finally, blot imaging was performed using a ChemiDoc imaging system (Bio-Rad).

2.11 Cell culture and intervention

Human brain microvascular perivascular (HBVP) cells were purchased from ScienCell (Cat. 1200; Carlsbad, CA, USA). When cell density reached 80–90%, cells were digested down using 0.25% trypsin (Cat. 25200072; Gibco, Waltham, MA, USA) using different pretreatments and divided into 5.5 mM normal glucose group (NC), 35 mM high glucose group (HG), 0 mM glucose deprivation group (GD) and glucose deprivation + Mito-TEMPO intervention group (Mito-T). Cells in the GD group were treated with high glucose for 12 h, followed by glucose deprivation for 24 h, and then re-treated with high glucose at 35 mM for 4 h. The Mito-T group was supplemented with 100 μM Mito-TEMPO based on the premise of the GD group.

2.12 Cell viability

The activity of the HBVP cells was assayed using the CCK-8 Cell Activity Kit (Cat. BS350B; Biosharp, Hefei, China). After each group of cell samples was subjected to the corresponding intervention, 10 μl of CCK-8 solution was added to the 96-well plate and the cells were incubated for 1 h, while protected from light, and the absorbance was measured at 450 nm using a spectrophotometer (MultiskanGO; Thermo Fisher Scientific, WA, USA).

2.13 Fluorescence detection of apoptosis

At the end of treatment, the Hoechst staining solution (Cat. 33342; Beyotime), Annexin V-FITC staining solution, and propidium iodide (PI) were added to the medium and the cells were incubated at 37◦C for 15 min, while protected from light. After staining, the cells were carefully washed and observed under a conventional fluorescence microscope (Dmi8; Leica). Apoptotic cells exhibited dense or fragmented nuclei under green fluorescence.

2.14 Detection of mitochondrial ROS in HBVP cells

Mitochondrial ROS production was detected using the MitoSOX stain (Cat. 40778ES50; YEASON). After treatment with different concentrations of glucose, the cells were incubated for 30 min at 37◦C in the dark using MitoSOX (5 μM). After washing the cells three times with PBS, the cellular mitochondrial ROS were observed under a fluorescence microscope. The intensity of the red fluorescence was calculated using the Image J software.

2.15 Detection of mitochondrial morphology

Mitochondrial morphology was observed using the fluorescent probe MitoTracker Red (Cat. C1048B; Beyotime). After different interventions, the mitochondrial morphology was analyzed using a confocal laser scanning microscope (Leica SP8, Germany) after incubation for 30 min in the dark using 250 nM of MitoTracker Red.

2.16 Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) was measured using the MMP Assay Kit for JC-1 (Cat. C2006; Beyotime). Cell culture medium and JC-1 staining solution were mixed in a 1:1 ratio, added to the cells, and the cells incubated at 37◦C for 20 min. The supernatant was removed and an appropriate amount of cell culture medium was added, the cells were then observed under a fluorescence microscope (DMi8; Leica). The formation of J-aggregates by JC-1 indicated a high mitochondrial membrane potential whereas if JC-1 remained in its monomeric form, it indicated a low mitochondrial membrane potential.

2.17 Statistical analyses

All data were statistically analyzed using SPSS 25.0 and are expressed as mean ± SD. Data from the MWM test were analyzed using repeated-measure two-way analysis of variance (ANOVA). Sample means of groups meeting the requirements of the chi-square test were compared using one-way ANOVA and the LDS test, and data not meeting the requirements of the chi-square test were compared using the Kruskal-Wallis rank sum test. Differences were indicated as statistically significant at $P < 0.05$.

3. Results

3.1 Mito-TEMPO improves cognitive impairment due to severe hypoglycemia

Changes in general indicators in mice are shown in Fig. 1A-C. Compared with the NC group, the mice in the DM, DH, and DHT groups showed a significant decrease in body weight after STZ injection (Fig. 1A), whereas blood glucose increased significantly on day 3 (Fig. 1B).

To verify the effect of severe hypoglycemia on cognitive dysfunction in T1D mice and the ameliorative effect of Mito-TEMPO on cognitive function, the MWM test was administered in each group of mice. No significant differences were found in the swimming speeds of the four groups during MWM test ($P > 0.05$, Fig. 1D). The escape latency (time spent searching for a hidden platform) for each group of mice is shown in (Fig. 1E). As the number of training days increased, the escape latency increased in the DM group compared to that in the NC group, but no significant difference was observed ($P > 0.05$), while the escape latency was significantly longer in the DH group ($P < 0.01$). Escape latency was shorter in the DHT group than in the DH group ($P < 0.05$). In the spatial exploration experiment with the platform removed (Fig. 1F-G), mice in the DH group passed through the original platform location significantly less often than those in the NC and DM groups ($P < 0.01$). The number of times the platform was crossed was higher in the DHT group than in the DH group ($P < 0.05$). This indicates that severe hypoglycemia in the diabetic state can significantly impair cognitive function in mice, which can be reversed by Mito-TEMPO.

3.2 Mito-TEMPO attenuates neuronal damage in cortical and hippocampal CA1 regions caused by severe hypoglycemia

We used HE staining to assess the histological alterations of neurons in the CA1 region of the cortex and hippocampus (Fig. 2A). The structure of the neurons in the cortical and CA1 regions of the NC group was regular, and 4–5 layers of cone cells were neatly and closely arranged, surrounded by red cytoplasm. Large round nuclei were visible inside the vertebral cells, with 1–2 nucleoli clearly visible inside the nuclei. Some degree of damage was visible in the DM group, but the overall condition was better than that in the DH group. In contrast, neuronal pyramidal cells in the DH group were disorganized, with atrophied cell bodies and shrunken or absent nuclei, particularly in the hippocampal CA1 region. Compared with the DH group, the morphology and number of pyramidal cells in the cortical and hippocampal CA1 regions of the DHT group were improved, with clearer and neatly arranged nuclei.

Neuronal damage was observed in each group of mice by Nisslr staining. Nisslr staining is specific for neurons. As shown in Fig. 2B-D, the number of neurons in the hippocampus and cortex was significantly reduced in the DH group compared with the NC group (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$), while the staining of Nisinia vesicles was reduced, light, and blurred, suggesting neuronal damage. After Mito-TEMPO treatment, the number of neurons in the hippocampus and cortex of mice in the DHT group was significantly increased ($P < 0.05$), while the staining of nisin microsomes was darker and clearer, suggesting a protective effect of Mito-TEMPO on neurons.

3.3 Mito-TEMPO reduces blood-brain barrier leakage and brain edema due to severe hypoglycemia

As shown in Figure (Fig. 3A), the NC and DM groups showed almost no staining of the mouse brain with Evans Blue, whereas the DH group showed significant penetration of Evans Blue dye. Compared with the DH group, the DHT group showed reduced Evans blue leakage. Further quantification of Evans Blue in the brain (Fig. 3B) showed that severe hypoglycemia could lead to BBB leakage in diabetic mice and that Mito-TEMPO could reverse this damage. The water content of the brain tissue of each group of mice was calculated by weighing the weight of the brain tissue before and after drying. The results (Fig. 3C) showed that the water content of the brain in the DH group was significantly higher than that in the NC and DM groups (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$). There was a decrease in the brain water content in the DHT group compared to that in the DH group ($P < 0.05$).

As shown in Fig. 3D, the BBB was normal in NC mice. Compared to the NC group, the DH group showed severe damage to the BBB, with marked dilatation of the rough endoplasmic reticulum (RER) and local degranulation. The capillary lumen (Cap) is markedly atrophied and collapsed. The basement membrane (BM) structure was blurred. The endothelial cells (EC) are heavily edematous, with loss of intercellular tight junctions (TJ) and narrowing of the intercellular spaces. Astrocyte footplate (Ast) is markedly oedematous with sparse stroma. At further magnification, the pericytes (P) with intracellular low electron density edematous areas. The nucleus (N) is irregularly shaped, with an intact nuclear membrane, a slightly widened perinuclear gap and a heterochromatin border set. The mitochondria (M) are heavily swollen, with lysis of the intra-membrane matrix and reduction and loss of cristae. In contrast, the DM group showed mild damage to these indicators and the BBB and pericytes. After Mito-TEMPO treatment,

compared with the DH group, the mice in the DHT group showed significantly reduced BBB damage, an increased number of TJ, reduced pericyte edema, and near-normal mitochondrial morphology.

3.4 Mito-TEMPO attenuates oxidative stress and impaired mitochondrial energy metabolism in the brain of T1D mice caused by severe hypoglycemia

Brain tissue ROS immunofluorescence staining images are shown in Fig. 4A, and the ROS fluorescence intensity was significantly increased in the DM and DH groups compared to the NC group ($P < 0.01$). The brain ROS fluorescence intensity was further increased in mice after severe hypoglycemia (DH group) compared with that in the DM group ($P < 0.01$). Brain ROS fluorescence intensity was significantly reduced in mice in the DHT group compared to that in the DH group ($P < 0.01$). The brain ROS, MDA, and SOD levels of mice in each group were measured using ELISA. The results showed that the DH group had the highest ROS and MDA levels, which were statistically significant compared with the NC and DM groups ($P < 0.01$; Fig. 4B-C), and Mito-TEMPO treatment improved these indices ($P < 0.01$). In contrast, SOD levels were significantly lower in the DH group than in the NC and DM groups ($P < 0.01$; Fig. 4D). SOD levels were higher in the DHT group than in the DH group ($P < 0.01$; Fig. 4D). Evaluation of mitochondrial ATP content in the brain tissue showed that severe hypoglycemia significantly reduced brain mitochondrial ATP production in diabetic mice (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$; Fig. 4E), whereas Mito-TEMPO ameliorated this impairment ($P < 0.01$).

3.5 Mito-TEMPO improves pericyte dysfunction and loss of TJ proteins due to severe hypoglycemia

As shown in the figure (Fig. 5A-C), the mouse pericyte-specific proteins PDGFR-β and α-SMA expression was significantly decreased in the DH group compared to the NC and DM groups (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$), suggesting that severe hypoglycemia could cause a decrease in the number of pericytes. This damage was reversed by Mito-TEMPO (DH vs. DHT, $P < 0.05$). Similarly, the expression of the tissue TJ proteins occludin and claudin-5 was significantly decreased after experiencing severe hypoglycemia compared to that in the NC and DM groups (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$; Fig. 5A, Fig. 5D-E). There was a greater increase in occludin and claudin-5 expression in the DHT group than in the DH group ($P < 0.05$). MMP-9 responds to pericyte inflammatory status. MMP-9 expression was higher in the DH group than that in the NC and DM groups (NC vs. DH, $P < 0.01$; DM vs. DH, $P < 0.05$; Fig. 5A, Fig. 5F). The expression of the inflammatory factor MMP-9 decreased after treatment with Mito-TEMPO ($P < 0.05$).

3.6 In vitro, Mito-TEMPO ameliorates glucose deprivation-induced decrease in HBVP cells viability and apoptosis

As shown in the figure (Fig. 6A), the activity of HBVP cells decreased significantly after 24 h of glucose deprivation compared to high glucose, and further decreased after 36 h. Compared with glucose deprivation (Fig. 6B), the cell viability of HBVP cells increased after treatment with 75 μM and 100 μM

Mito-TEMPO ($P < 0.05$, $P < 0.01$, respectively), but the effect of 100 μM Mito-TEMPO was better than that of 75 μM ($P < 0.01$). As shown in Figure (Fig. 6C), compared with the NC group (normal sugar group), HBVP cell viability was decreased in the HG group ($P < 0.05$), the most significant decrease in HBVP cell viability was observed in the GD group ($P < 0.01$), and Mito-TEMPO improved cell viability ($P < 0.01$). Hoechst staining showed the effect of different concentrations of glucose in culture conditions on the apoptosis of HBVP cells. As shown in the figure (Fig. 6D), the NC group had large nuclei and darker fluorescence with a dark blue color. The number of apoptotic cells in the HG group was between those in the NC and GD groups. After treatment with Mito-TEMPO, apoptosis was reduced in the Mito-T group.

3.9 Glucose deprivation can cause increased ROS production, mitochondrial disruption and decreased mitochondrial membrane potential (MMP) in HBVP cells, which can be reversed by Mito-TEMPO

As shown in Fig. 7A, red fluorescence was significantly increased in the HG and GD groups compared to the NC group (NC vs. HG, $P < 0.05$; NC vs. GD, $P < 0.01$; Fig. 7A-B), but more significantly in the GD group (HG vs. GD, $P < 0.05$; Fig. 7A-B), suggesting that glucose deprivation caused ROS accumulation in HBVP cells. Red fluorescence intensity was lower in the Mito-T group than in the GD group ($P < 0.01$; Fig. 7A-B). The effects of different glucose concentrations on the mitochondrial morphology of HBVP cells were compared. As shown in Fig. 7C, the mitochondria in the NC group were round tubes or thin filaments and interlocked to form a complex meshwork. After glucose deprivation, the mitochondria in the GD group were heavily fragmented, with short rods, small circles, and broken dots in a dispersed distribution. Mito-TEMPO intervention reversed mitochondrial breakage in HBVP cells.

JC-1 red fluorescence represents normal membrane potential, green fluorescence represents reduced membrane potential, and a decrease in the ratio of red to green fluorescence represents a decrease in MMP. As shown in Fig. 7D-E, the intracellular red fluorescence intensity of HBVP cells in the GD group was significantly reduced, the green fluorescence intensity was increased, and the aggregate/monomer ratio (i.e., red/green fluorescence ratio) was reduced compared to that in the NC group ($P < 0.01$). The above indices in the HG group were between the two groups, with statistically significant differences between the groups (NC vs. HG, $P < 0.01$; HG vs. GD, $P < 0.01$). The Mito-T group had an increased aggregate/monomer ratio compared with the NC group ($P < 0.01$).

4. Discussion

This study showed that severe hypoglycemia in a T1D mouse model can lead to BBB leakage, pericyte dysfunction, and neuronal damage, causing the onset of cognitive dysfunction, which is consistent with the results of our previous study [6]. Further findings in this study revealed that the above mechanism of injury may be related to the excessive activation of oxidative stress and mitochondrial dysfunction due to glucose reperfusion after hypoglycemia. Interventions using the mitochondria-targeted antioxidant Mito-TEMPO in both *in vivo* and *in vitro* models have revealed that it may improve cognitive function in mice by resisting mitochondrial oxidative stress, reducing pericyte loss and apoptosis, attenuating BBB leakage, and neuronal damage.

Severe hypoglycaemia is a common and serious complication of insulin therapy in patients with T1DM [17]. The close association between T1DM and cognitive dysfunction, the cause of which may be related to recurrent episodes of hypoglycemia [1, 2]. The brain is most vulnerable to hypoglycaemia because it is highly dependent on glucose as its primary fuel and has little storage capacity for glucose [18]. Studies in rodents have shown that severe hypoglycemia leads to significant brain damage through a variety of mechanisms, including ROS production, mitochondrial permeability shifts, and oxidative DNA damage [19]. Among these, glycemic recovery after hypoglycemia (i.e., glucose reperfusion injury) is thought to be one of the direct causes of exacerbated brain damage in patients with diabetes [5]. In this study, we constructed a T1D mouse model of severe hypoglycemia to observe the effects of severe hypoglycemia on cognitive function in mice experiencing a rapid rise in blood glucose to a high glucose state. Our results revealed that glucose reperfusion after hypoglycemia could cause increased levels of oxidative stress and mitochondrial dysfunction in the brain. We found significant damage to hippocampal and cortical neurons in histology, and impaired cognitive function was observed in mice. Intervention with the mitochondria-targeted antioxidant Mito-TEMPO significantly reduced neuronal death and improved cognitive function in mice while reducing mitochondrial ROS production and improving mitochondrial morphology and function. Therefore, the role of oxidative stress in cognitive dysfunction due to hypoglycemia remains unclear.

Oxidative stress is a severe imbalance between ROS and reactive nitrogen species (RNS) production and antioxidant defenses [20]. Mitochondria are a major source of intracellular ROS [21] and are considered one of the major targets of ischemia-reperfusion injury and a key regulator of neuronal cell life and death [22]. Hypoglycemia, a source of oxidative stress, may lead to neuronal damage in the CA1 region of the hippocampus and accelerate cognitive decline by exacerbating hyperglycemia-induced oxidative stress and inflammation [23]. Among these, glucose reperfusion following severe hypoglycemia is considered a period of marked ROS production and oxidative stress. The scavenging capacity of SOD, an effective free radical scavenger, plays a key role in maintaining the dynamic balance between ROS production and mitochondrial integrity [24]. MDA is a product of the reaction of lipids with oxygen-free radicals, and its content represents the degree of lipid peroxidation. SOD and MDA are important indicators for evaluating oxidative stress in terms of antioxidant and oxidative capacity, respectively [25], and are often used together in the field of research related to oxidative stress. In this study, increased brain tissue ROS and MDA levels were found in the DH group of mice, accompanied by a decrease in SOD activity, confirming that severe hypoglycemia can enhance oxidative stress in the brains of T1D mice.

Several studies have shown the beneficial effects of natural mitochondrial antioxidants such as Okamoto maple [26] and melatonin [27] in neurodegenerative diseases. During induced acute hypoglycemia, vitamin C infusion (as an antioxidant) may reduce oxidative stress and inflammation in patients with T1DM [28]. Mito-TEMPO is a specific scavenger of the mitochondrial superoxide [13]. Mito-TEMPO was reported to cross the blood-brain barrier and prevent nicotine-induced ischemic brain injury [29], and also improved cognitive function by reducing the accumulation of tau oligomers in the cortical neurons of mice [11]. Therefore, Mito-TEMPO may have therapeutic potential in hypoglycemia-induced brain injury. In this study, intervention with Mito-TEMPO, a mitochondria-targeted antioxidant, reduced ROS and MDA

expression, increased SOD activity in mouse brain tissue, and improved neuronal death and cognitive dysfunction in the cortical and hippocampal CA1 regions of mice caused by severe hypoglycemia, a mechanism of action that may be mediated by the targeted scavenging of mitochondrial ROS and attenuation of oxidative stress. Therefore, we suggest that by enhancing antioxidant defenses, for example, through the administration of antioxidants, it may be possible to reduce oxidative stress-induced damage and improve synaptic dysfunction and neuronal damage caused by hypoglycemia and glucose reperfusion.

Brain endothelial cells interact with astrocyte endfeet, pericytes, and basement membranes to form neurovascular units (NVUs), which are essential components of the BBB [30]. The BBB controls the composition of the neuronal internal environment and is essential for normal neuronal and synaptic functions [31]. Degeneration of the BBB and dysregulation of blood vessels can be detected in the early stages of patients with Alzheimer's disease (AD) [32]. Furthermore, damage to the BBB is now considered to be one of the key mechanisms leading to diabetic encephalopathy [33]. Hypoxia/glucose deprivation can go so far as to lead to mitochondrial dysfunction and a subsequent reduction in ATP production, leading to destruction of the BBB and exacerbating brain damage [34]. Our results show that severe hypoglycemia could cause a decrease in ATP content in the brain of mice, and treatment with Mito-TEMPO resulted in a significant increase in ATP content, suggesting an improvement in mitochondrial function. A previous study by our team [6] found that severe hypoglycemia can cause TJ protein deficiency and increased BBB leakage in diabetic mice; however, the exact mechanism has not been clarified. It has been found that TJ can be disrupted by oxidative stress, and changes in TJ protein levels and/or in the cellular localization/transport of TJ proteins are among the factors contributing to BBB disruption [35]. In this study, mice experiencing severe hypoglycemia and glucose reperfusion were found to have reduced TJ protein expression and increased blood-brain barrier leakage, and electron microscopic findings also showed disruption of the BBB and loss of TJ. This damage was reversed by the Mito-TEMPO intervention. Based on the above, we hypothesized that BBB leakage and TJ loss are related to the oxidative stress caused by severe hypoglycemia. Therefore, there are two main questions that must be addressed: at which target does oxidative stress generated by hypoglycemia primarily cause BBB leakage? What cellular functions need to be focused on?

There is evidence that BBB disruption in patients with AD is associated with pericyte dysfunction and that pericyte loss occurs early in the AD disease process, at the stage of mild cognitive impairment [36]. Pericytes are highly sensitive to oxidative stress and in many diseases such as diabetes and AD, pericytes are found to be the first NUV cells to die [37]. BBB disruption can be prevented by reducing oxidative stress and protecting pericyte function [38]. Matrix metalloproteinases (MMPs) are protein-degrading enzymes that degrade extracellular matrix proteins and are common culprits of oxidative stress-induced BBB damage. MMP-9 is the major MMP that is most closely associated with barrier permeability following oxidative injury [39]. It has been reported that oxidative stress can contribute to the secretion and activation of MMP-9 by pericytes [40], which in turn leads to TJ disruption and increased BBB leakage. Based on the importance of pericytes in the maintenance of BBB structure and function, we focused on pericytes as the cause of BBB leakage due to hypoglycemia. In our previous study, we found

that hypoglycemia could induce cell loss and increase MMP-9 expression in the perivascular brain of diabetic mice [6]. The present study further confirmed these results and constructed an *in vitro* hypoglycemic model, and found that glucose deprivation followed by re-hyperglucose (simulating glucose reperfusion after severe hypoglycemia in the diabetic state of T1DM) on top of high glucose cultures could cause increased mitochondrial ROS production in HBVP cells, mitochondrial disruption, and decreased mitochondrial membrane potential, ultimately leading to apoptosis of HBVP cells. Furthermore, an increase in pericyte numbers and an improvement in pericyte function after intervention with Mito-TEMPO were observed in both *in vivo* and *in vitro* trials. Therefore, we suggest that mitochondrial oxidative stress induced by hypoglycemia and/or glucose reperfusion leads to pericyte dysfunction and reduced numbers, causing BBB leakage and, ultimately, cognitive impairment.

Chronic hyperglycemia plays an important role in BBB function and cognitive dysfunction in the brain in diabetes [41], and may contribute to neuronal damage by increasing polyol pathway fluxes, late glycosylation end-product formation, and oxidative stress [42]. In this study, increased brain ROS production was observed in the T1D group. *In vitro*, high glucose decreased HBVP cell viability, increased cellular ROS production, mitochondrial disruption, and decreased mitochondrial membrane potential. However, no further effects of hyperglycemia on reduced pericyte numbers, BBB leakage, neuronal damage, or cognitive dysfunction were observed. We hypothesize that this is due to the short duration of hyperglycemia in the mouse model used in this study (3 days of hyperglycemia followed by execution for histological testing and 10 days of hyperglycemia followed by cognitive behavioral testing). The acute short duration of hyperglycemia was insufficient to cause damage to the BBB and neurons in mice, leading to significant cognitive impairment, which is not contradictory to the conclusion of the current study that hyperglycemia is an important risk factor for cognitive impairment. Our studies have demonstrated that acute short-term hyperglycemia can cause a rapid increase in oxidative stress (e.g., 3 days of hyperglycemia in mouse experiments resulted in a significant increase in ROS in the mouse brain, and in cellular assays, 40 h of hyperglycemia incubation resulted in a significant increase in oxidative stress indicators in HBVP cells).

This study had some limitations. The model of insulin-induced severe hypoglycemia used in this study is less accurate than the use of the glucose clamp in terms of simulating the duration and degree of hypoglycemia. However, the insulin-induced hypoglycemia model has the advantages of being simple, easy to perform, and equally efficient, and is now widely used as a test method, second only to the glucose clamp, in studies related to acute and chronic complications associated with hypoglycemia.

Conclusion

In summary, this innovative study investigated the mechanism of the effect of severe hypoglycemia on cognitive dysfunction at both the animal and cellular levels and found that hypoglycemia leads to increased mitochondrial oxidative stress through blood glucose reperfusion, causing pericyte dysfunction, and BBB leakage, ultimately leading to neuronal death and cognitive dysfunction. More importantly, the use of the mitochondria-targeted antioxidant Mito-TEMPO reversed these

pathophysiological changes and improved cognitive dysfunction caused by hypoglycemia by scavenging the overproduction of mitochondrial ROS and improving pericyte mitochondrial function.

Declarations

Funding: This work was supported by the Fujian Science and Technology Innovation Joint Fund Project (2017Y9060), the Financial Department Special Funds of Fujian Province (2018B041), the Joint Funds for the innovation of science and Technology, Fujian province (2019Y9062), Shanghai Health and Medical Development Foundation (DMRFP_I_03), and the Startup Fund for Scientific Research of Fujian Medical University (2020QH2022).

Competing Interests: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lu Lin, Zhou Chen and Cuihua Huang. The first draft of the manuscript was written by Lin Lu and all authors commented on previous versions of the manuscript. Yubin Wu, Lishan Huang, Lijing Wang and Sujie Ke participated in the separation experiment. Libin Liu conceived and designed the study. All authors read and approved the final manuscript.

Consent to Participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Ethics approval

The animal study protocol was approved by the Fujian Animal Research Ethics Committee (protocol code FJMU IACUC 2021 -0029).

Acknowledgments

We acknowledge the Public Technology Service Center Fujian Medical University for providing technical support in laser confocal microscope. Thanks to the support of my family, especially my husband Fu

yang, who gave me a lot of encouragement during this study and let me finish the experiment without worries.

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Figures

Figure 1

Mito-TEMPO improves cognitive impairment due to severe hypoglycemia. (A) Glucose levels. (B) Body weight trajectory. (C) Glucose levels during the severe hypoglycemia episode (< 2.0 mmol/L). (D)

Swimming speed. (E) Escape latency. (F) Representative traces of swimming paths of each group in the probe test. (G) Crossings of the target quadrant during the probe trial. N = 10 per group. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}$P < 0.05 vs. DM, $^{\&\&}$P < 0.01 vs. DM, $^{\#}$P <0.05vs DH, $^{\#\#}$P <0.01vs DH. NC, normal control; DM, type 1 diabetes mellitus; DH, severe hypoglycemia; DHT, diabetic mice pretreated with Mito-TEMPO experiencing hypoglycemia.

Figure 2

Mito-TEMPO attenuated neuronal damage in the cortical and and hippocampal CA1 regions caused by severe hypoglycemia. (A) HE staining to assess the histological changes of neurons in the CA1 region of the cortex and hippocampus. Scale bars: 500 μm (left), 100 μm (right). (B) Nissler staining to assess the histological changes of neurons in the cortical and hippocampal CA1 regions. Scale bars: 500 μm (left), 100 μm (right). Quantitation of neuron numbers in the hippocampi (C) and cortices (D). N = 3 per group. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. DM, $^{\&\&}P$ < 0.01 vs. DM, $^{\#}P$ < 0.05 vs DH, $^{\#\#}P$ < 0.01 vs DH.

Figure 3

Mito-TEMPO reduces BBB leakage and brain edema due to severe hypoglycemia. (A) Naked-eye view of Evans blue exocytosis in mouse brain. (B) Quantification of Evans blue extravasation. (C) Mouse brain water content. (D) Representative images of hippocampal BBB and pericyte morphology under transmission electron microscopy are shown. Scale bars: 2 μm (left), 1 μm (right). Ast, astrocyte; P, pericyte; N, nucleus; Nu, nucleolus; RER, rough endoplasmic reticulum; Cap, capillary lumen visible as a

myelin-like structure; BM, basement membrane; EC, endothelial cell; TJ, tight intercellular junction M, mitochondria. N = 3 per group. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. DM, $^{\&\&}P$ < 0.01 vs. DM, $^{\#}P$ < 0.05 vs DH, $^{\#\#}P$ < 0.01 vs DH.

Figure 4

Mito-TEMPO attenuates oxidative stress and impaired mitochondrial energy metabolism in mouse brain caused by severe hypoglycemia. (A) Immunofluorescent images of brain tissues, including nuclear staining with DAPI and ROS staining with DCFH-DA. Scale bars: 2000 μm. (B) Determination of ROS content in brain tissues using an ROS ELISA kit. (C) Determination of MDA content in brain tissues using an MDA ELISA kit. (D) Determination of SOD content in brain tissues using an SOD ELISA kit. (E) Determination of ATP content in brain tissues using an ATP colorimetric/fluorometric assay kit. N = 3 per group. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. DM, $^{\&\&}P$ < 0.01 vs. DM, $^{\#}P$ < 0.05 vs DH, $^{\#\#}P$ < 0.01 vs DH.

Figure 5

Mito-TEMPO improves pericyte dysfunction and loss of TJ proteins due to severe hypoglycemia. (A) Representative immunoblot images of relative protein expression. Western blot analysis was performed for the protein levels of PDGFR-β (B), and α-SMA (C), Occludin (D), Claudin-5 (E), MMP-9 (F). N = 3 per group. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. DM, $^{\&\&}P$ < 0.01 vs. DM, $^{\#}P$ < 0.05 vs DH, $^{\#\#}P$ < 0.01 vs DH.

Figure 6

***In vitro*, Mito-TEMPO improves glucose deprivation-induced decrease in HBVP cell viability and apoptosis.** (A) Assessment of HBVP cells at different glucose deprivation times (6, 12, 24, and 36 h) and cell viability using the CCK-8 kit. (B) Assessment of HBVP cells at different Mito-TEMPO concentrations (50 μM, 75 μM, 100 μM, and 125 μM) and cell viability using the CCK-8 kit. (C) Assessment of HBVP cells under different interventions (normal glucose, high glucose, no glucose, and no glucose + Mito-TEMPO) and cell viability using the CCK-8 kit. (D) Effect on apoptosis of HBVP cells under different group interventions (Hoechst 33342 staining, 10×). Scale bars: 50 μm. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. HG, $^{\&\&}P$ < 0.01 vs. HG, $^{\#}P$ < 0.05 vs GD, $^{\#\#}P$ < 0.01 vs GD. NC, normal control (normal glucose); HG, high glucose; GD, glucose deprivation; Mito-T, GD + Mito-TEMPO.

Figure 7

Improved glucose deprivation by Mito-TEMPO can cause increased ROS production, mitochondrial disruption, and decreased mitochondrial membrane potential in HBVP cells. (A) Detection of intracellular ROS with MitoSOX staining; Scale bar = 50 μm. (B) Quantification of fluorescence intensity of ROS based on Image J. (C) Mitochondrial staining with MitoTracker Red; Scale bar = 5 μm. (D) Mitochondrial membrane potential assayed with JC-1 kit; Scale bar = 50 μm. (E) Quantification of relative fluorescence intensity of JC-1. Data are presented as the means ± SD. *P < 0.05 vs. NC, **P < 0.01 vs. NC, $^{\&}P$ < 0.05 vs. HG, $^{\&\&}P$ < 0.01 vs. HG, $^{\#}P$ < 0.05 vs GD, $^{\#\#}P$ < 0.01 vs GD

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