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Research report

Potential role of *myo*-inositol to improve ischemic stroke outcome in diabetic mouse

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HIGHLIGHTS

- Glucose inhibits *myo*-inositol brain transport mechanisms.
- *Myo*-inositol improves astrocyte cell viability after oxygen glucose deprivation.
- Oxygen glucose deprivation increases *myo*-inositol transporters.
- *Myo*-inositol treatment reduces stroke damage in both diabetic and non-diabetic animals.

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ABSTRACT

Brain edema is one of the critical factors causing hightened disability and mortality in stroke patients, which is exaggerated further in diabetic patients. Organic osmolytes could play a critical role in the maintenance of cytotoxic edema. The present study was aimed to assess the role of *myo*-inositol, an organic osmolyte, on stroke outcome in diabetic and non-diabetic animals. *In situ* brain perfusion and acute brain slice methods were used to assess transport of *myo*-inositol across the blood-brain barrier and uptake by brain cells using non-diabetic (C57BL/6) and diabetic (streptozotocin-induced) mice, respectively. *In vitro* studies were conducted to assess the role of *myo*-inositol during and after ischemia utilizing oxygen glucose deprivation (OGD) and reperfusion. Further, the expression of transporters, such as SGLT6, SMIT1 and AQP4 were measured using immunofluorescence. Therapeutic efficacy of *myo*-inositol was evaluated in a transient middle cerebral artery occlusion (tMCAO) mouse model using non-diabetic (C57BL/6) and diabetic (*db/db*) mice. *Myo*-inositol release from and uptake in astrocytes and altered expression of *myo*-inositol transporters at different OGD timepoints revealed the role of *myo*-inositol and *myo*-inositol transporters during ischemia reperfusion. Further, hyperglycemic conditions reduced *myo*-inositol uptake in astrocytes. Interestingly, in *in-vivo* tMCAO, infarct and edema ratios following 24 h reperfusion decreased in *myo*-inositol treated mice. These results were supported by improvement in behavioral outcomes in open-field test, corner test and neurological score in both non-diabetic and *db/db* animals. Our data suggest that *myo*-inositol and *myo*-inositol transporters may provide neuroprotection during/following stroke both in non-diabetic and diabetic conditions.

Abbreviations: BBB, blood-brain barrier; OGD, Oxygen Glucose Deprevation; SGLT6, sodium dependent myo-inositol transporter; SMIT1, sodium myo-inositol transporter 1; DCI, D-chiro-inositol; AQP4, Aquaporin Transport 4; GFAP, Glial fibrillary acidic protein; HMIT, H+dependent myo-inositol transporter; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; tMCAO, transient MCAO; t-PA, tissue-plasminogen activator; MIOAX, Myo-inositol oxygenase enzyme; NVU, neurovascular unit; CNS, central nervous system; HMIT, H+dependent myo-inositol transporter; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; STAIR, Stroke Therapy Academic Industry Roundtable; STZ, Streptozotocin; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; EBSS, Earle's balanced salt solution; DPBS, Dulbecco's Phosphate Buffered Saline Solution; PFA, paraformaldehyde; TTC, 2,3,5-Triphenyltetrazolium chloride; ANOVA, One-way Analysis of Variance; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; BCA, bicinchonicic acid assay; PVDF, polyvinylidene difluoride membrane; TBS, Tris-Buffered Saline

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1. Introduction

Ischemic stroke, characterized by a temporary or permanent obstruction of cerebral blood flow affects an area of the brain, causing a series of brain pathologies including cytotoxicity, oxidative stress, and edema. The extent of brain edema is an initial determinant of whether a person will survive beyond an ischemic injury and worsened brain edema can be associated with worse clinical outcomes (Dirnagl et al., 1999). Cytotoxic edema results in increased intracellular ion accumulation causing increased water content in cells including glia and neurons. Although improvement in the imaging techniques and reduction of time to administer t-PA have been increasing the number of patient eligible to administer t-PA (Schwamm et al., 2013), there is still a need for new stroke therapies to target important pathogenic events that occur during an ischemic injury, such as edema.

Myo-inositol (MI) is a cytosolic, organic osmolyte found in either its free form or bound to phospholipids (Fisher et al., 2002). Osmolytes play a vital role in the maintenance and regulation of osmotic pressure in cells. *Myo*-inositol is also a precursor to second messengers in the inositol triphosphate/diacylglycerol pathway (IP_3/DAG). There are reports of cirrhotic patients with hepatic encephalopathy who have low levels of *myo*-inositol (Cordoba et al., 1996; McConnell et al., 2017), which was associated with brain edema due to the imbalance between the accumulation and loss of osmolytes. *Myo*-inositol has also been reported to have potential neuroprotective actions in brain disorders such as epilepsy in which status epilepticus was induced by kainic acid, an analog of glutamate. In a cell assessment study reported by Kortoria et al., pretreatment with *myo*-inositol preserved the structure of neurons and neuronal cell loss/damage (Kotaria et al., 2013), after an epilepticus event.

With regard to stroke and brain ischemia, in a preliminary study, ^{1}H-MRS images between control and stroke survivors showed higher *myo*-inositol levels in both ipsilesional and contralateral motor cortex areas of the brain, which was related to arm motor impairment. Their findings suggest that *myo*-inositol may play a role in post-stroke plasticity in glial cells, especially since astrocytes are known to release trophic factors promoting neuronal survival (Cirstea et al., 2011). Therefore, understanding the role of *myo*-inositol during an ischemic injury may provide insight into improving quality of life after an ischemic injury. Moreover, diabetes is a well-known risk factor for stroke. Studies have reported that patients with hyperglycemia within the first 48 h of an ischemic injury were associated with worse clinical recovery (Fuentes et al., 2009). Further, diabetic patients excrete more *myo*-inositol in urine and have lower *myo*-inositol plasma levels (Chang et al., 2015). Lower levels of *myo*-inositol may play a role in poor clinical outcomes seen in stroke patients with hyperglycemia in type 2 diabetes and could provide an insight into potential therapeutic treatments.

The blood-brain barrier (BBB) does not work independently; rather, it functions as a unit, known as the neurovascular unit (NVU), which includes pericytes, neurons, endothelial cells, and astrocytes which exhibit dynamic interactions. It has been reported that in the human brain, astrocytes outnumber neurons by seven fold, are key factors in control of edema formation, and have been observed to survive a prolonged time during ischemic injury (Chen and Swanson, 2003; Zhao and Rempe, 2010). Studying the astrocytic component of the NVU is essential in understanding how these cells function in cerebrovascular diseases such as stroke. Cells in the NVU, such as astrocytes and neurons, express important transporters of organic osmolytes, including *myo*-inositol. These include the sodium-dependent glucose transporter (SGLT6 or sodium dependent *myo*-inositol transporter 2 (SMIT2)) (*myo*-inositol affinity in astroglial cells, k_m = 120 μM) (Sasseville et al., 2014) and sodium-dependent *myo*-inositol transporter (SMIT1) (k_m = 25 μM) (Coady et al., 2002; Wiesinger, 1991). Both are co-transporters for transporting glucose, ions such as Na^+, K^+ and osmolytes such as *myo*-inositol, as well as water (Szablewski, 2017). Aquaporin (AQP4) channel protein is highly expressed at the end-feet of astrocytes and plays an important role in water transport in the brain (Badaut et al., 2002). When cellular mechanisms or transporters fail to respond properly, cytogenic and vasogenic edema occurs in response to loss of ATP production and failure of Na^+/K^+ ATPase leads to improper balance of Na^+ and water accumulation leads to edema formation, along with disruption of ions and osmolyte concentrations (Dirnagl et al., 1999; Simard et al., 2007). Fortunately, organic osmolytes, like *myo*-inositol, are highly enriched in the central nervous systems (CNS), especially in astrocytes (Brand et al., 1993) compared to neurons (Urenjak et al., 1993), and utilization of these “non-perturbing” osmolytes during ionic shifts helps to minimize changes in the membrane potential and can even provide stabilization of signalling complexes, such as inositol lipids, and improve cell viability (Harvey et al., 2002).

Given the possible multi-modal effects of *myo*-inositol related to brain cell viability, the present study was aimed to investigate the beneficial effects of *myo*-inositol in neurons and astrocytes during and after OGD conditions and test the therapeutic potential of *myo*-inositol for improved stroke outcome.

2. Results

2.1. Glucose competes with myo-inositol to transport across the BBB

In situ brain perfusion method was used to assess the transport of *myo*-inositol and the effect of hyperglycemia on the transport of *myo*-inositol across the BBB. Initial transport (k_{in}) of *myo*-inositol was found to be $3.88 \pm 0.33 \times 10^{-5}$ and $3.05 \pm 0.50 \times 10^{-5}$ mL/s/g in non-diabetic and diabetic animals, respectively. However, when *myo*-inositol transport was measured in presence of 15 mM D-glucose in perfusion buffer, K_{in} of *myo*-inositol was reduced more than 50% in both diabetic and non-diabetic brains. Study suggested that *myo*-inostol is transported across BBB utilizing glucose transporters and that hyperglycemia could alter *myo*-insotol uptake across the BBB (Fig. 1A).

2.2. Role of SGLT6 and SMIT1 in Myo-inositol uptake by brain cells

To assess the role of different transporters and their contribution in the uptake of *myo*-inostiol by brain cells, acute brain slice method was performed in the presence and absence of substrates for SGLT6 and SMIT1. *Myo*-inositol uptake was reduced by 80% in presence of D-chiro-inositol (DCI), a substrate for SGLT6 (0.04 ± 0.001 vs 0.14 ± 0.01 nmoles/min/g without DCI) and 30% in presence of L-fucose, a substrate for SMIT1, (0.09 ± 0.001 vs 0.14 ± 0.01 nmoles/min/g without fucose) suggesting the majority of *myo*-inositol transport in the brain is mediated by SGLT6 (Fig. 1B). Further, it was also observed that *myo*-inositol uptake was reduced by 79% (0.03 ± 0.001 vs 0.14 ± 0.01 nmoles/min/g) and 87% (0.03 ± 0.001 vs 0.22 ± 0.01 nmoles/min/g) in the presence of higher (20 mM) amounts of *myo*-inositol in non-diabetic and diabetic brains respectively, suggesting that the transport systems are saturable. Moreover, reduction in the transport of *myo*-inositol in the presence of a Na^+ free buffer suggests major uptake of *myo*-inositol is mediated by SGLT6 and SMIT1. Residual uptake in the presence of Na^+ free buffer condition could be attributed to HMIT and diffusion mediated uptake.

2.3. Myo-inositol release from astrocytes increases during ischemia

The study was conducted to assess the release of *myo*-inositol during ischemia. Primary mouse astrocytes were incubated with ^{3}H *myo*-inositol (0.1 μCi/ml) for 1 h followed by exposure to normoxic and OGD conditions. Following exposure, release of radioactive *myo*-inositol was assessed by liquid scintillation counts. Results of the study (Fig. 2A) demonstrated that *myo*-inositol release was significantly higher ($P < 0.05$) during OGD, compared to release in normoxic controls.