Summary & Conclusion
The present study was carried out to evaluate the potential protective effect of GSNO in preventing hyperglycemia induced altered BBB permeability causing neurobehavioural deficits in mice model of hyperglycemia. Keeping in view the above objective, diabetes was induced in male laca mice by intraperitoneal injection of streptozotocin (40mg/kg body weight) for 5 days and GSNO was administered orally (100µg/kg body weight) daily for 8 weeks after the induction of diabetes. Important findings of this investigation are as follows:

i. STZ induced mice demonstrated marked increase in plasma glucose levels, water and food intake suggesting polydipsia and polyphagia along with marked reduction in body weight. GSNO administration was able to partially reverse hyperglycemia in diabetic animals.

ii. Diabetic mice showed a significant decline in neurobehavioural functions related to memory, learning, anxiety as well as in motor coordination assessed by morris water maze, elevated plus maze, radial arm maze and rotarod test. However, GSNO supplementation was able to ameliorate these neurobehavioral deficits in diabetic animals.

iii. A significant increase in ROS, protein carbonyls, 3-NT and lipid peroxidation in terms of MDA and 4-HNE were observed in cortex and hippocampus of diabetic animals suggesting increased oxidative/nitrosative stress. GSNO administration was able to normalize the diabetes induced oxidative/nitrosative stress in both the brain regions.

iv. Diabetic animals also showed a significant reduction in redox ratio (GSH/GSSG) as well as enzymatic antioxidants (CAT, SOD) suggesting compromised antioxidant defense system in cortex and hippocampus. GSNO proved to be beneficial in ameliorating both non-enzymatic and enzymatic antioxidants in diabetic animals.

v. A significant decrease in activities of mitochondrial electron transport chain enzymes and aconitase were observed in diabetic brain contributing to mitochondrial impairment which can have serious implications on CNS. GSNO administration was able to mitigate these changes thereby preventing mitochondrial dysfunction.

vi. Ultrastructural analysis of mitochondria of cerebral cortex and hippocampus also revealed that diabetic animals showed mitochondrial swelling and aberrant cristae formation. GSNO supplementation to diabetic mice results in restoration of
mitochondrial structure as evident by reduced mitochondrial swelling and improved cristae structure.

vii. Diabetic animals showed a significant decrease in norepinephrine, dopamine and serotonin levels in cortex and hippocampus while epinephrine levels remained unchanged. GSNO supplementation to diabetic animals was able to restore neurotransmitter levels in both the regions of brain thereby preventing CNS deficits to some extent.

viii. BBB permeability measured in terms of sodium fluorescin, Evans blue and FITC dextran uptake was found to be significantly increased in cortex and hippocampus of diabetic mice. GSNO administration to diabetic animals was able to alleviate BBB disruption suggesting that it might be beneficial in chronic diabetics exhibiting BBB dysfunction and related pathological features.

ix. Increased BBB permeability in diabetic animals was assessed by edema and ion imbalance depicted by increased water content and sodium levels in cortex and hippocampus. However, GSNO supplementation was able to restore water and ion imbalance in brain tissue in diabetic animals that may improve neuronal functions and related cognitive deficits.

x. Diabetic mice demonstrated significantly increased activation of MMP-9 with no changes in MMP-2 as observed in cortex and hippocampus. mRNA and protein expression of MMP-9 was also found to be increased in both the brain regions. GSNO supplementation was able to attenuate MMP-9 through modulation of MMP-9/TIMP-1 expression suggesting its beneficial role in improving BBB dysfunction by regulating MMP and TIMP levels in diabetic animals.

xi. In situ zymography in cortex, hippocampus and microvessels was performed to measure gelatinase activity of MMPs. It was observed that cerebral cortex and hippocampus had discrete areas exhibiting high levels of gelatinase activity in diabetic animals. Microvessels also showed increased gelatinase activity suggesting that MMPs were increased in BBB after diabetes. GSNO supplementation to diabetic mice lowered MMP activity in both the brain regions as well as microvessels suggesting its protective role in maintaining BBB integrity.

xii. A significant decrease in relative mRNA and protein expression of tight junction proteins; Occludin and ZO-1 were observed in isolated microvessels obtained from
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cortex and hippocampus of diabetic animals, while no change was observed in claudin-5. GSNO treatment to diabetic mice was able to restore hyperglycemia mediated loss of occludin and ZO-1 in both the regions.

xiii. Immunofluorescence of tight junction proteins was performed in isolated microvessels to confirm their reduced mRNA and protein expression. The fluorescent intensity for both ZO-1 and occludin appeared to be reduced in microvessels of cortex and hippocampus obtained from diabetic animals while, no change appeared in fluorescent intensity of claudin-5. However, GSNO supplemented diabetic animals showed an increased fluorescent intensity for both ZO-1 and occludin.

xiv. Ultrastructure of microvessels from cortex and hippocampus of diabetic animals revealed endothelial cell pyknosis, lumen stenosis, basement membrane thickening, perivascular edema and heterochromatin. Moreover, the endothelial cell layer was found to be loosely attached to the basement membrane suggesting damaged neurovascular unit that comprises of BBB. However, the ultrastructure of microvessels from the brain regions of GSNO supplemented diabetic animals exhibited a relatively unobstructed capillary lumen, clear pericytes, continuous basement membrane quite firmly attached to endothelial. GSNO administration to diabetic animals improved the ultrastructure of the damaged microvessels signifying its protective role in improving BBB disruption.

xv. Relative mRNA and protein expression of cell adhesion molecules like ICAM-1 and VCAM-1 was found to be significantly upregulated in cortex and hippocampus of diabetic animals which may be associated with activation of endothelial cells and infiltration of leukocytes resulting in BBB disruption. GSNO significantly lowered the upregulation of ICAM-1 and VCAM-1 in a similar manner thereby rendering protection to BBB in diabetic animals.

xvi. Protein and mRNA relative expression for iNOS was found to be significantly increased in diabetic brain suggesting crucial role in secondary damage to brain. However, no change was observed in the mRNA expression of eNOS in diabetic animals indicating its preventive effect in attenuating BBB mediated secondary injury.

xvii. Diabetic animals showed a significant decrease in NO levels in cortex and hippocampus suggesting reduced bioavailability of NO in diabetic condition. GSNO
supplemented diabetic mice showed significantly increased NO levels in both the brain regions.

xviii. Hematoxylin and eosin stained cortex and hippocampus from diabetic animals revealed marked neuronal degeneration and cell death and GSNO supplementation was observed to improve the neuronal architecture in diabetic animals.

These findings clearly demonstrate that hyperglycemia induced biochemical abnormalities like oxidative stress, mitochondrial dysfunction, defective neurotransmitter release and BBB disruption following hyperglycemia. GSNO on the other hand was able to ameliorate these abnormalities thereby acting as a neuroprotective agent. Also it becomes evident that aberrant BBB permeability plays a pivotal role in diabetes induced neuronal dysfunction. In addition, BBB permeability is a result of complex interplay of molecules like MMPs (MMP-2, MMP-9), TIMPs (TIMP-1, TIMP-2), tight junctions proteins (Occludin, ZO-1, Claudin-5), cell adhesion molecules (ICAM-1, VCAM-1) that are altered in diabetic condition leading to secondary damage involving neuro-inflammation. These changes may eventually be responsible for the neurobehavioral deficits in diabetic animals. Thus, it could be concluded from the present study that diabetes exerts its neurotoxic effects by augmenting oxidative stress and mitochondrial dysfunction thereby altering BBB permeability. These changes were mitigated by GSNO treatment showing its potential therapeutic effect in preventing diabetic complications.
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Figure: Hypothetical model representing altered BBB permeability in STZ induced model of diabetes.