EFFECT OF β₂ ADRENERGIC AGONIST AND HIF1α ANTAGONIST ON NEUROPROTECTION IN CEREBRAL HYPOPERFUSION MODEL

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Submitted By

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<u>CERTIFICATE</u>

This is to certify that the dissertation work entitled **"EFFECT OF β_2 ADRENERGIC AGONIST AND HIF1α ANTAGONIST ON NEUROPROTECTION IN CEREBRAL HYPOPERFUSION MODEL"** submitted by University **Reg no. 261925907** is a bonafide work carried out by the candidate under my guidance and submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2020-2021.

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DECLARATION

I do hereby declare that the dissertation work entitled **"EFFECT OF β_2 ADRENERGIC AGONIST AND HIF1α ANTAGONIST ON NEUROPROTECTION IN CEREBRAL HYPOPERFUSION MODEL"** submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology, was done by myself under the guidance of **Dr. M. Ramanathan D.Sc.** Principal & Head of the Department, Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2020-2021.

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<u>EVALUATION CERTIFICATE</u>

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Examination centre: PSG College of Pharmacy, Coimbatore

Date:

Internal examiner   **External Examiner**

<u>ACKNOWLEDGEMENT</u>

Dedicated

to

Respected guide

My lovable parents,

Teachers, God

&

My dear friends

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ABBREVIATIONS

VaD -Vascular Dementia

CBF - Cerebral Blood Flow

NVU-Neurovascular Unit

NFkB- Nuclear factor kappa-light-chain-enhancer of activated B cells

HIF1α-Hypoxia-inducible factor 1α

CREB-cAMP response element binding protein

JNK -c-Jun N-terminal kinases

GSK3 - Glycogen Synthase Kinase 3

mTOR-Mammalian Target of Rapamycin

ROS-Reactive Oxygen Species

β2AR - β2 Adrenergic Receptors

CDK5-Cyclin-dependent kinase 5

MCI-Mild Cognitive Impairment

Aβ- Amyloid β

FTD- Frontotemporal Dementia

TIA-Transient Ischaemic Attack

CAA - Cerebral Amyloid Angiopathy

MRI- Magnetic Resonance Imaging

PET-Positron Emission Tomography

CT- Computerized Tomography

CADASIL- Cerebral Autosomal Dominant Arteriopathy with Subcortica Infarcts and Leukoencephalopathy

ATP-Adenosine triphosphate

EVA- Encephalic Vascular Accident

NO- Nitric Oxide

BBB- Blood Brain Barrier

GDNF- Glial cell derived neutrophic factor

IGF1- Insulin-like growth factor 1

PHD- Prolyl hydroxylase

pVHL -Von Hippel-Lindau tumour suppressor gene

HPH- HIF prolyl hydroxylase

Iκβ- Inhibitor of κβ

OGD- Oxygen-glucose deprivation

WB-Western Blot

t-BAR-thio-Barbuturic acid

NED-N-(1-naphthyl) ethylenediamine

TBST - Tris Buffer Saline TWEEN 20

HRP- Horseradish Peroxidase

SDS-PAGE- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

ANOVA-Analysis of Variance

SD- Standard Deviation

GPCR- G- protein couple receptor

SO- Sham-operated

1. Introduction

Vascular dementia (VaD) is the second-largest cause of dementia. Around 50 million people worldwide have dementia with almost 60% living in low- and middle-income countries. Annually there are nearly 10 million new cases. VaD is more common in India compared to the West. VaD results due to neurodegenerative changes and cognitive decline. The major risk factor for VaD is stroke [1]. Ischemic stroke occurs due to occlusion of the cerebral artery resulting in reduced blood flow leading to hypoxia and infarction & this neuronal impairment due to hypoxia is a causative factor for VaD [2].

Global cerebral ischemia differentially affects the brain regions like the cortex, striatum, and hippocampus where cortex and striatum were found to be late ischemic tolerant while hippocampus showed delayed neuronal death leading to cell death and cellular dysfunction [3].

Cerebral blood flow (CBF) determines the severity of ischemia the normal CBF ranges from 50ml/100g/min. The central core of the infarcted region has CBF of <7ml/100g/min and the penumbra surround the core region where the CBF ranges from 7 to 15ml/100g/min, this penumbra region is metabolically active but functionally inactive and does not mediate cell-cell or neuronal communication this disruption is due to ischemia and hypoxia. The neurons in the penumbra regions are viable and can be made functionally active and rescued employing proper neuroprotective therapy to overcome further insult [4].

The neurovascular unit (NVU) regulates cerebral blood flow. NVU consists of astrocytes, pericytes, interneurons, and other glial cells that interact with each other and communicate with neurons to regulate CBF according to neural activity [5].

During hypoxic conditions, many transcription factors and molecular pathways are activated in neurons. Transcription factors like NFκβ and HIF1α have dual roles in neuronal survival and neuronal death. Other factors like CREB, NRF are also activated [6]. NFκβ aids in the transcription of pro-inflammatory cytokines, cell survival, and cell proliferation whereas HIF1α aids in the expression of several growth factors, cell survival factors needed for neuroprotection [7, 8]. HIF1α also plays a detrimental role in neuronal apoptosis [9]. The cellular pathways like JNK, ERK, GSK3β, p38, PI3K/AKT are also well studied for their defined roles in neuronal survival and death [4].

NFκβ is bound with Iκβ to stay in an inactive form. Cellular pathways such as JNK, p38, PI3K/AKT activates NFκβ. NFκβ is also directly activated during hypoxic conditions by modulation of PHD. NFκβ after activation translocates to the nucleus to activate the expression of several pro-inflammatory genes like IL-6 and TNFα which induces neuroinflammation and apoptosis. On the contrary, NFκβ also stimulates several survival genes like Bcl-2, Bcl-XL **[10, 11]**.

HIF1α is a transcription factor triggered during hypoxia in neurons and this may be the factor responsible for the expression of neuroprotective factors **[12, 13]**.

HIF1, a crucial regulator of gene expression during hypoxia that regulates pro-survival genes than pro-apoptotic genes which aid in neuroprotection **[8, 14]**. The neuroprotection of the HIF1 mimetic agent depends on the cell type conferred and the magnitude of ischemic damage **[15]**.

The neuroprotective and detrimental effect of HIF1 depends on the duration of the hypoxic insult. HIF1 may prevent apoptosis through inhibition of caspase, cytochrome c, and PARP cleavage during cerebral ischemia **[16, 17]**.

NFκβ and HIF1α have a controversial function of regulating proliferative, survival, apoptotic, inflammatory genes aiding in neuroprotection as well as neuronal death. This controversial action of these transcription factors depends upon the pathway activating these transcription factors, the genes regulated by them, cell type, and the duration of hypoxic stress insult **[8-10]**.

cAMP-regulated PKA has a role in the activation of hypoxic controlled NFκβ and HIF1α transcription factors. PKA also has a role in the activation of MAPK pathways and inhibition of pro-apoptotic genes **[18]**.

Protein Kinase A (Pka) is found to be involved in HIF1α phosphorylation. HIF1 is also controlled by stressors and growth factors other than hypoxic stimuli **[19]**.

β2 adrenergic receptors are G-protein coupled receptors which upon activation stimulate cAMP and PKA **[20]**.

β2 adrenergic receptors are expressed in neurons **[21]** and are found to regulate NFκβ and increase HIF1 levels in pancreatic cancer cells under normoxic conditions **[22]**. Salbutamol

and propranolol are well-documented drugs known for their stimulation and inhibition of β2 adrenergic receptors respectively **[23, 24]**.

As of now, there is no current effective treatment to attenuate neuronal impairment in VaD and so elucidating the molecular mechanistic pathways behind neuroprotection may provide a clear insight on neuroprotective therapy and help solve the crisis **[1]**.

Hypothesis Framed:

Elucidating the molecular mechanistic pathways of salbutamol and propranolol on neurons acting via β2 adrenergic receptors or direct modulation of MAPK, NFκβ, and HIF1α after hypoxic insult may provide a clear insight of their function on neuroprotection and it may also be a choice of treatment.

The study has been designed **to elucidate the neuroprotective effect of salbutamol and propranolol through β₂ adrenergic receptor and to study the behavioural, biochemical, molecular and pathological changes induced by ischemic injury in different brain regions.**

2. Literature review

2.1 Dementia

Dementia is a syndrome characterized by progressive cognitive decline. This cognitive impairment is caused due to neuroinflammation, neurodegeneration, synaptic loss, and impaired neurotransmission. Dementia is characterized by a decline in defined cognitive functions such as learning and memory, language, complex attention, executive function, perceptual-motor, agnosia, apraxia, communication while intelligence remains unchanged. Mild cognitive impairment (MCI) is the stage between normal cognition and dementia. Symptoms include mild memory loss, difficulty in planning and organization, trouble finding words, frequently losing things, forgetting names. People with MCI are at greater risk of dementia but MCI does not always progress to dementia. The degree of memory impairment is significant in dementia compared to MCI. According to the severity of symptoms, the cognitive disorder is classified into mild and major. Mild cognitive disorder patients are characterized by modest cognitive decline whereas patients with major cognitive decline are characterized by severe cognitive decline **[25]**.

2.1.1 Subtypes of dementia

Alzheimer's disease- occurs due to the accumulation of Aβ and tau proteins.

Vascular dementia occurs due to sudden-onsets cerebrovascular disease (stroke, lacunar infarcts, and aneurysm) and atherosclerotic comorbidities (diabetes, hypertension, coronary heart disease).

Lewy body dementia -occurs due to parkinsonism and abnormal deposits of alpha-synuclein (Lewy bodies).

Fronto-temporal dementia- affects the frontal and temporal lobes of the brain which affects the ability to reason and make a decision, prioritize the task. They are classified into several types like behavioral variant FTD, primary progressive aphasia, pick's disease, corticobasal degeneration **[25]**.

2.2 Vascular dementia

Vascular Dementia (VaD) is the second-largest cause of dementia. Around 50 million people worldwide suffer from dementia with almost 60% living in low- and middle-income countries. Annually there are nearly 10 million new cases. VaD is more common in India compared to the west. VaD results due to neurodegenerative changes and subsequent cognitive decline.

VaD is a heterogeneous and progressive neurocognitive disorder caused due to a reduction in cerebral blood flow to the brain. Hemorrhagic, ischemic, and hypoxic injuries contribute to VaD [1].
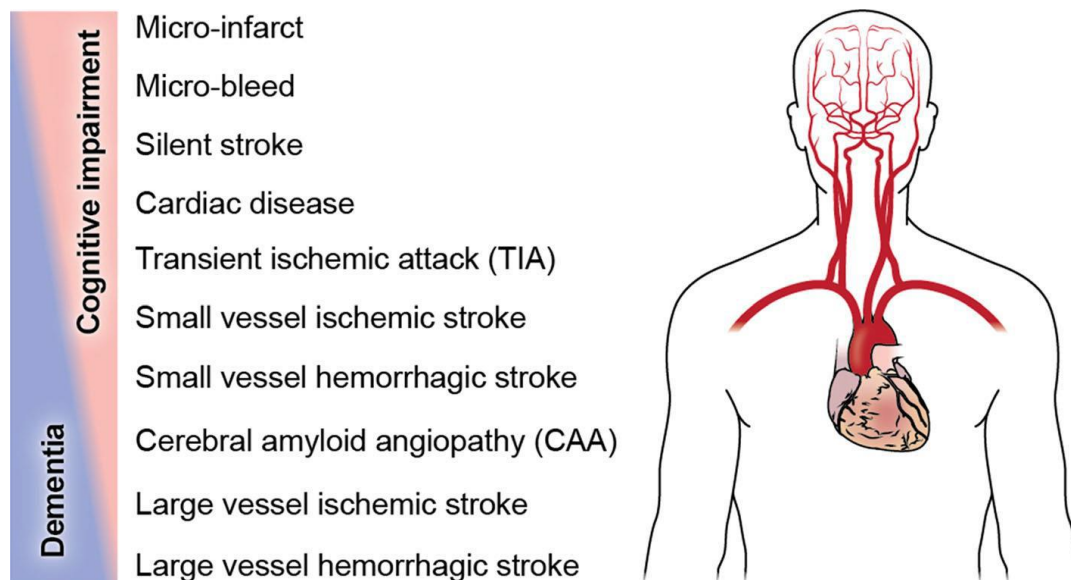
Fig 1: Science of vascular contributions to cognitive impairment and dementia [1]

2.2.1 History

In 1549, De Cerebri Morbi a book published by Jason Pratnesis first described the link between dementia and stroke. In 1658, Johann Wepfer theorized that a broken blood vessel causes apoplexy (stroke). The correlation between dementia and atherosclerotic disease was precisely identified during the 20th century. Alois Alzheimer and Otto Binswanger contributed to the field of neurodegeneration during the 20th century. In the 1960s VaD was classified as a unique type of dementia from Alzheimer's due to its altered mechanism of cerebral vascularization and decreased cerebral blood flow contributing to neuronal loss.

In 1975, Vladimir Hachinski described the "ischemic score", used for clinical diagnostic of VaD. From 1980 to the 2000s with a new revolution in imaging technologies like MRI, PET, CT scans the diagnosis for VaD became clear & it distinguished between the various types of dementia [26].

2.2.2 Subtypes

Vascular cognitive impairment is briefly defined as the impairment in cognition arising due to vascular deformities and vascular insults that serve the brain. They are majorly classified into two types depending on the severity of cognitive impairment and cognitive decline. One is vascular dementia and the other is vascular mild cognitive impairment. VaD occurs due to severe cognitive decline manifested by neuronal loss and synaptic dysfunction. The disability of more than one cognitive function that is of sufficient severity to affect daily activities outlines the clear feature of VaD.

VaD is further classified into several types depending upon the vascular deformities. Vascular deformities can be majorly classified into two large vessel diseases and small vessel diseases serving the brain.

Post-stroke dementia - develops months after a major stroke

Subcortical ischemic vascular dementia (Binswanger disease) - microscopic damage to small blood vessels and nerve fibers.

Multi-infarct dementia (cortical dementia) -results due to combined effects of many small infarcts.

Strategic single infarct dementia- caused by single infarcts in strategic regions i.e., in regions that cause significant cognitive deficits when destroyed by an infarct.

Mixed dementia -dementia related to the coexistence of Alzheimer's and vascular dementia.

Cerebral amyloid angiopathy- amyloid plaque deposition in walls of a blood vessel resulting in decreased blood flow.

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) - a rare inherited disorder caused by thickening of small and medium-sized blood vessels [27, 28].
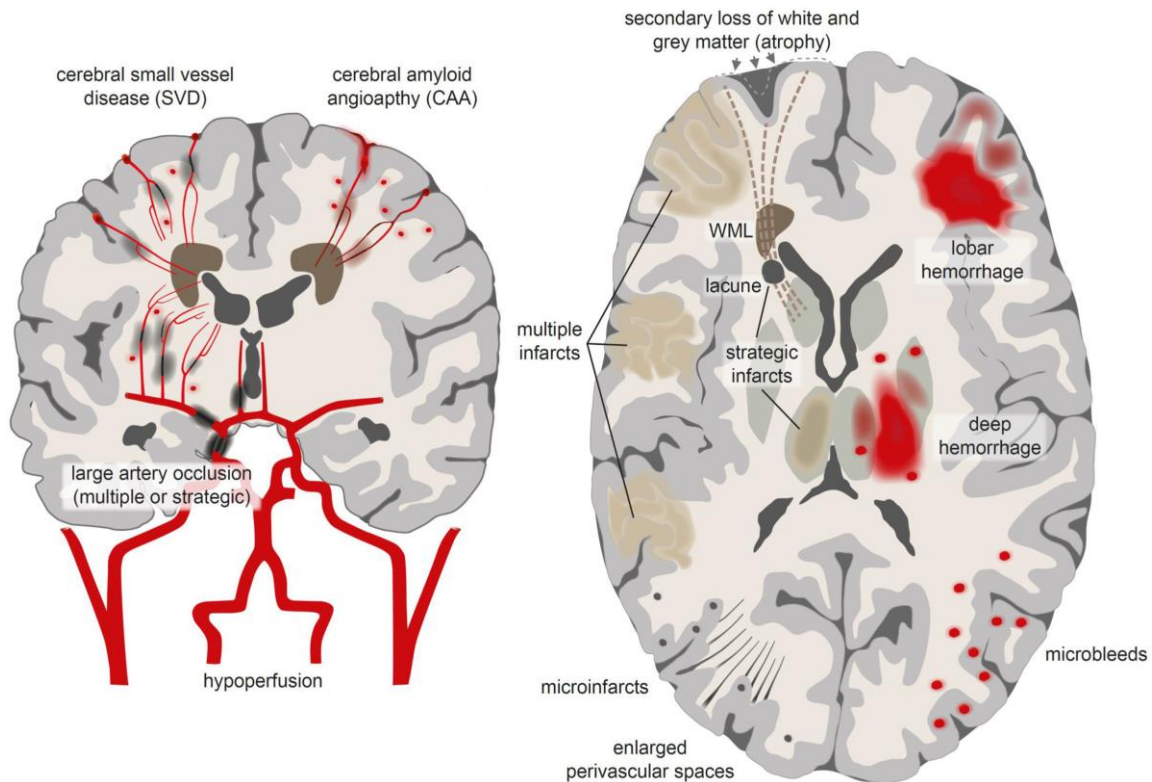
Fig 2: Mechanism underlying VaD [27]

2.2.3 Diagnosis

Diagnosing VaD is not simple. Currently, there is a lack of validated criteria for establishing a diagnosis of VaD, and many of the various pathologies that reduce the brain's blood supply are complex.

CT scans, MRI, PET scans are used to diagnose the nature of infarct.

2.2.4 Risk factors

VaD is contributed by several risk factors affecting the blood vessels serving the brain. The major one is stroke. Other risk factors include brain traumatic injuries, aneurysm, hypertension, diabetes mellitus, cerebral amyloid angiopathy, age, gender, atherosclerosis, genetic abnormalities (e.g., ApoE4 genetic variant) **[29]**.

2.2.5 Pathogenesis

A decrease in cerebral blood flow, ischemia, hemorrhage caused due to vascular insults and deformities describe the pathogenesis for VaD. Hippocampus and cerebral cortex are the main areas affected. Large and small vessel insults cause white matter destruction,

microinfarct, microhemorrhages, and large infarcts which may lead to atrophy and neuronal loss resulting in vascular cognitive impairment.
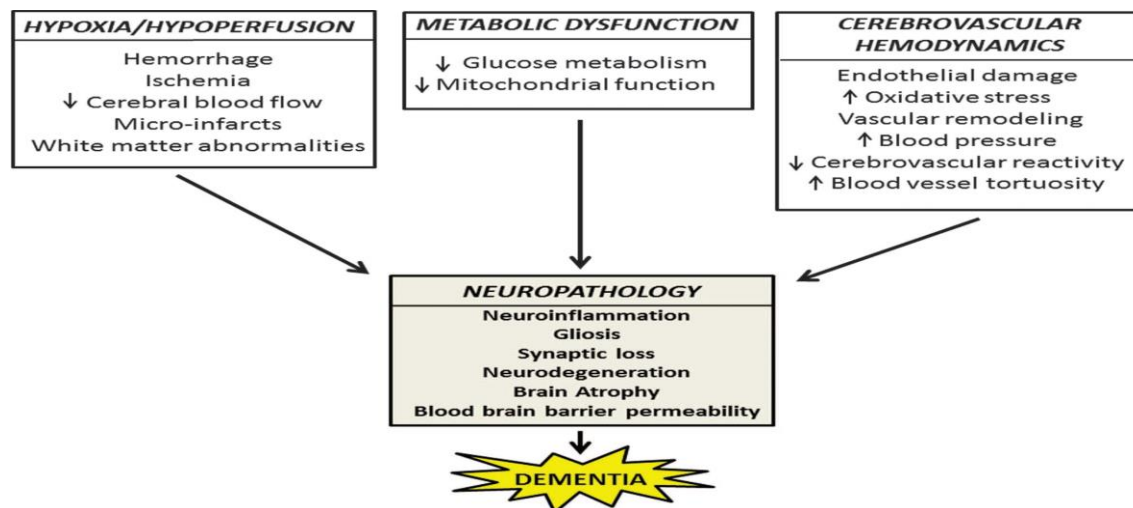
Fig 3: Neurovascular factors driving neuropathology and dementia [28]

Decreased cerebral blood flow creates a hypoxic state and nutrient deprivation in neuronal cells. Prolonged hypoxia results in decreased ATP synthesis in the neuronal cells. Due to decreased ATP, there is an influx of sodium and calcium ions inside the neuronal cells. Accumulation of these ions triggers glutamate excitotoxicity and activates various cellular pathways resulting in apoptosis, neuroinflammation, and free radical production. Neuroinflammation and free radicals further activate several cascade pathways finally resulting in necrosis and apoptosis of the neuronal cells. Intermittent hypoxia may trigger cell adaptability pathways for cell survival and combat stress. Loss in synaptic neurotransmission and neuronal loss in the hippocampus, cortex, and other brain regions creates a dementia state.

Normal aging is associated with histological alterations in the vessel wall like thickening of the basement membrane, loss of pericytes which relates to changes in cerebral blood flow. An increase in HIF1α during hypoxia was documented in aged animals **[28, 30]**.

2.2.6 Treatment and Management

The current treatment for VaD is directed towards treating the risk factors. There is no established treatment either to cure or control neurodegenerative changes. Cholinesterase inhibitors (donepezil, galantamine) may have a brief role in symptomatic relief. Serotonin

reuptake inhibitors are the treatment of choice for managing psychosis and depression. Anti-platelet and thrombolytic drugs are prescribed depending upon the patient's vascular pathology [30].

2.3 Stroke and Vascular dementia

Stroke is one of the major risk factors leading to vascular dementia. The development of dementia after stroke depends on several factors such as the location, volume, degree of related neuronal damage, and presence of pre-existing cognitive impairment. Patients affected by stroke necessarily don't get dementia but the frequency of acquiring dementia is higher. Dementia mostly occurs within three months of stroke onset. Ischemic stroke patients have a higher survival rate compared to hemorrhagic stroke which proves that ischemic stroke can cause cognitive impairment. The region of the brain affected by stroke plays a major role in the development of dementia [31].

2.3.1 Stroke

Stroke is a focal neurologic deficit caused by an alteration in circulation in the encephalon. The most common type of stroke is atherothrombotic brain infarction, embolic stroke. Stroke survivors develop physical and intellectual limitations. Encephalic vascular accident (EVA) is a term recently used for stroke. EVA occurs in four different forms: 1. Ischemic and transitory, with decreased blood flow and possible recovery after 24h; 2. Ischemic and complete, with neurologic deficits caused by vascular disturbance for a day or more that remains stable; 3. Progressive, with an intermittent increase in deficits caused by embolism and thrombus; 4. Hemorrhagic, ruptured vessels, and blood overflow caused by increased intracranial pressure. The pathophysiology of cerebral ischemia has been studied and they have shown that metabolic and chemical alterations during ischemia and reperfusion lead to cellular lesions in specific brain regions, depending on the duration of ischemia. Regional destructions of brains may lead to alteration in motor activity and other cognition-related activities.

The recovery process may happen but full recovery may not be observed due to the lesions and neuronal loss. Strokes vary from mild to severe and the consequences faced can be temporary or permanent. Most strokes are caused due to cerebral ischemia [32].
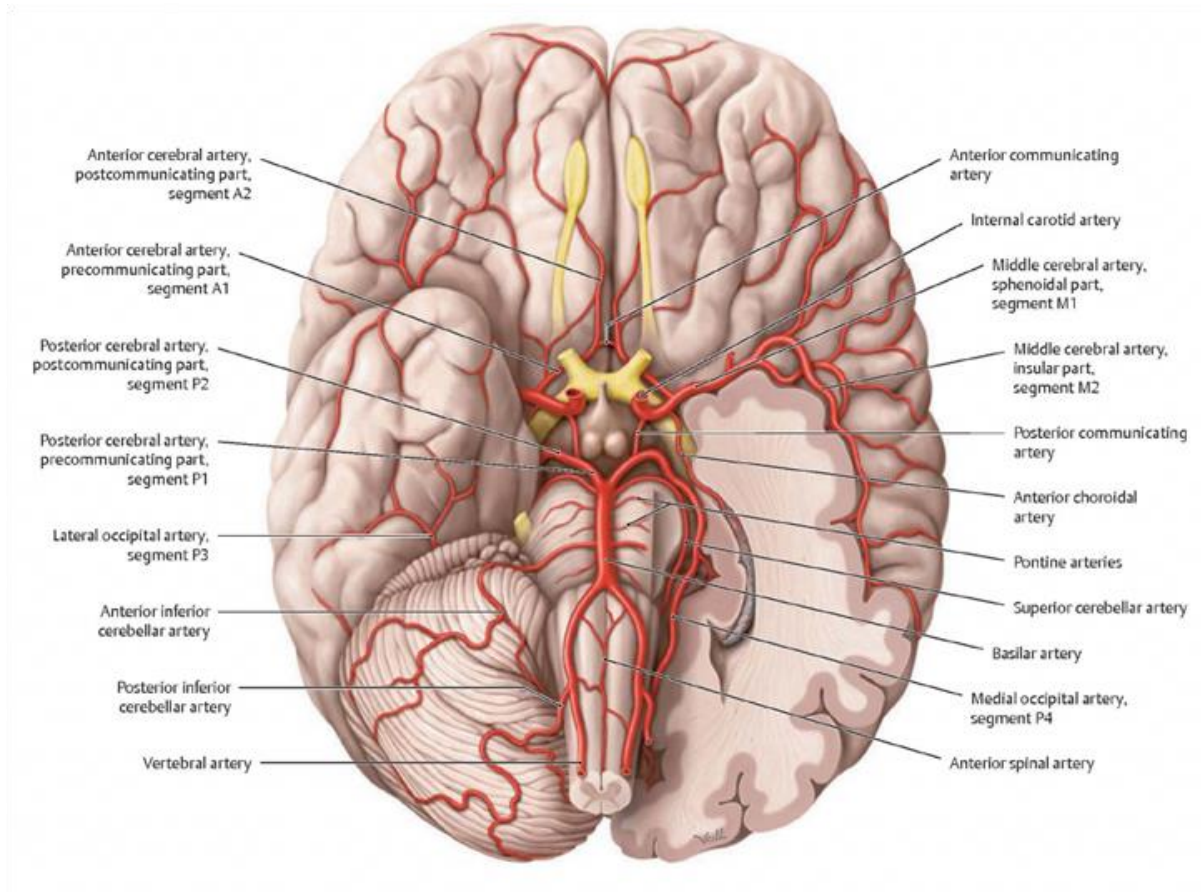
Fig 4: Arteries of the brain [63]

2.3.2 Cerebral Ischemia

Cerebral ischemia occurs due to the occlusion of the arteries supplying the brain or due to a reduction in blood flow to the brain. Occlusion occurs mainly due to thrombosis or embolus formation in the arteries. Cerebral ischemia is majorly classified into two 1. Focal cerebral ischemia and 2. Global cerebral ischemia. In focal cerebral ischemia, there is a cessation of blood flow to localized areas of the brain. Focal cerebral ischemia occurs when a blood clot has blocked the cerebral blood vessel. It can be caused due to thrombosis or embolism. The case of global cerebral ischemia occurs when there is a decreased flow of blood to the brain which occurs due to hypotension, hypoperfusion, cardiac arrest. If adequate circulation is restored within a short period the symptoms may be brief.

However, if it takes a long period to restore the circulation then the symptoms may become permanent. In global cerebral ischemia, the brain regions affected are widespread and as with the neuronal death **[33]**.

2.3.3 Cellular mechanisms of ischemic cellular injury

The brain is a highly aerobic tissue and so requires a constant supply of glucose and oxygen for its metabolic and neurotransmission activities. over a wide range of blood pressures and intracranial pressure. The neurovascular unit plays a major role in controlling the CBF and maintaining its constant levels.

The brain may be deprived of oxygen by several mechanisms. Hypoxia is of several types. Hypoxemic type is where hypoxia is caused due by the low partial pressure of oxygen, Anemic type is when the amount of hemoglobin is less and hence the capacity of blood to carry oxygen is low, Stagnant type where there is a reduction in blood flow to tissues as in ischemic conditions which occurs due to several factors like arterial occlusion, Histoxic type is when the tissues are not able to properly utilize the supplied oxygen.

Interruption of blood flow leads to hypoxia and glucose deprivation; ATP levels are decreased due to attenuation of oxidative phosphorylation in mitochondria. Decrease ATP levels lead to failure of sodium-potassium-ATP pump which causes alterations of cell membrane ionic gradients and sodium influx accompanied with water influx. Ionic and water influx leads to cell edema. Ca2+ influx and increased Ca2+ concentrations due to destruction of intercellular calcium stores lead to activation of several cellular pathways directing the cell to apoptosis, necrosis, and triggers neuroinflammation. Increased calcium also leads to glutamate excitotoxicity and nitric oxide production which further triggers various pathways leading to neuronal cell death. Increased ROS and peroxynitrites are other factors released during hypoxic stress, they are formed due to dysfunction of mitochondria during hypoxia and lack of antioxidative enzymes. Calcium, ROS, NO, and cytokines activate MAPK (ERK, JNK, p38), PI3K/AKT, NOTCH, Wnt signaling cascade pathways which lead to cell death and few pathways may aid inadaptability of the cell to withstand hypoxic conditions for some duration of time by expression and release of growth factors, cell survival factors and anti-inflammatory molecules.

Hypoxia also activates several transcription factors like HIF1α and NF-κB directly. The affected neurons release several cytokines and chemokines that trigger the nearby glial cells, endothelial cells, and other vascular cells. These glial cells and vascular cells aid in both neuroprotection and neuroinflammation. All these biochemical changes in neurons and nearby cells depend upon the duration, severity of the stress condition.

A neuron may try to adapt and survive if it experiences stress for less duration and low severity whereas if the duration and severity are high the cell may undergo apoptosis, necrosis.

So, Alterations in biochemical changes/pathways for adaptability, cell survival, and cell death depend upon the nature of the neuron and the stress condition it experiences **[2, 4, 33, 34]**.
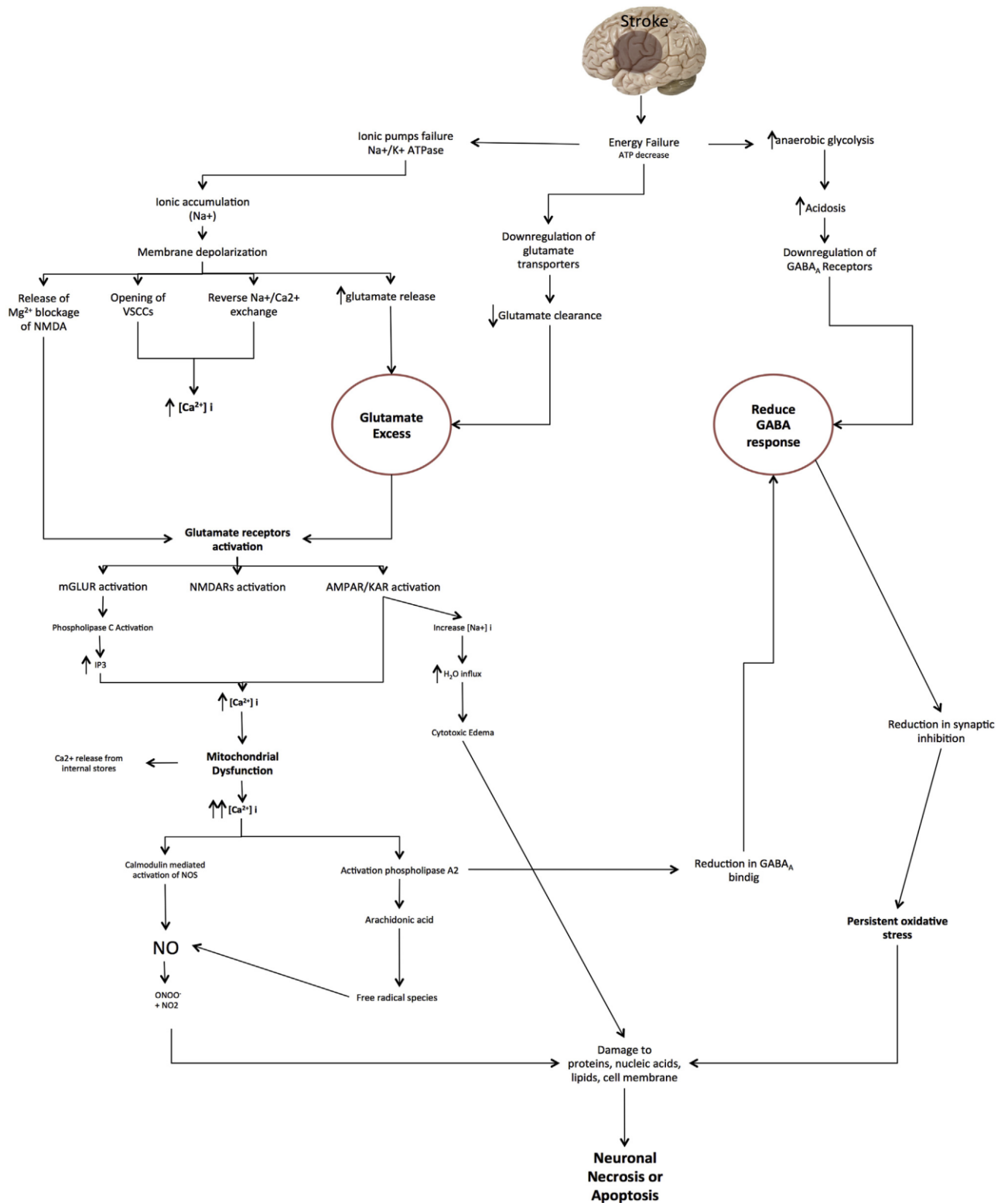
Fig 5: General Mechanism in neurons after occlusion of the artery [35]

2.3.4 Neurovascular unit (NVU)

An efficient blood supply is of great importance for the normal functioning of the brain. Cerebral blood flow (CBF) is regulated by the interplay between various cell types of the NVU. CBF is controlled according to brain neural activity. BBB protects the brain from leakage of blood to the parenchyma tissues and it protects from toxins and infections. Neurons are not in direct connection with the blood. They are structurally and functionally connected to a network of cell types called NVU. NVU regulates CBF in response to neuron's metabolic activity in a relationship termed neurovascular coupling. Neurovascular coupling ensures the brain has proportionally matched CBF in response to neural activity. Dysfunction of neurovascular coupling causes neurologic disorders. NVU facilitates hemodynamic changes known as hyperemia.

NVU is comprised of neurons, astrocytes, microglia, interneurons, pericytes, vascular smooth muscle cells, glial cells, and endothelial cells. Neurons directly or indirectly interact with the NVU through complex neurogliovascular signaling pathways **[5, 36-38]**.
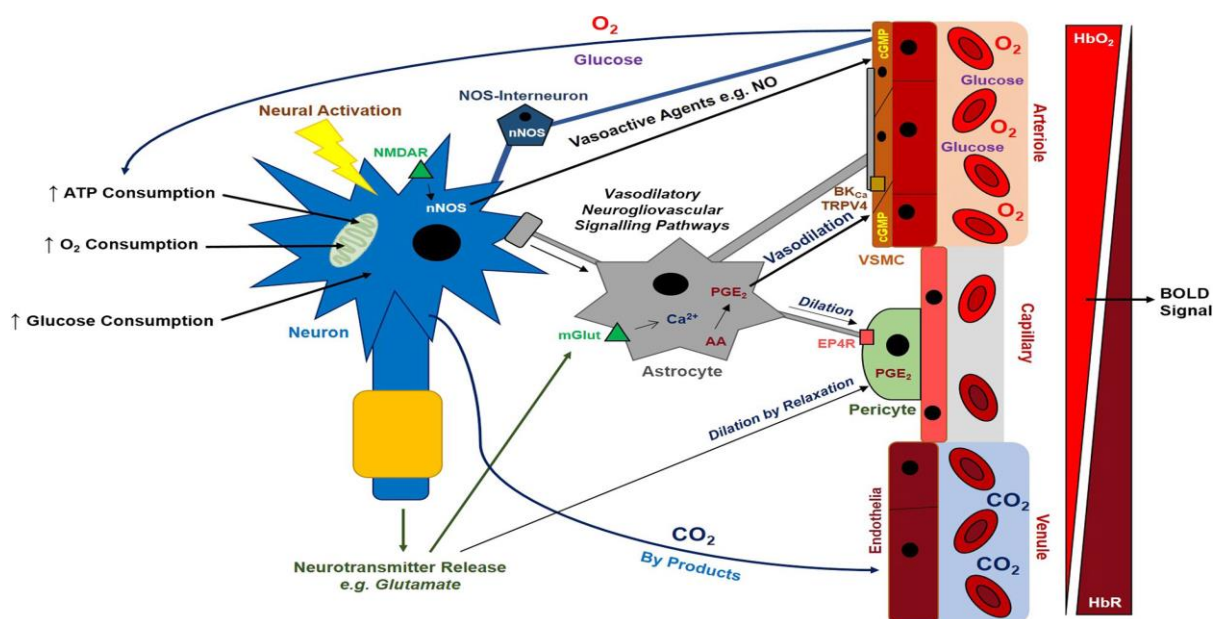

Fig 6: Neurophysiology of neurovascular coupling [5]

Astrocytes: Star-shaped cells that aid neurons in the formation of synapses, give considerable strength, selective permeability of substances, control vascular tone, regulate CBF, communicate with other cells, remove excess neurotransmitters, and regulate pericyte tone.

Oligodendrocytes: They are responsible for the formation of the myelin sheath, signal to neurons via myelin-axon interactions, oligodendrocyte-derived trophic factors, such as IGF1, and GDNF, promote neuron survival.

Microglia: They are immune cells and function as phagocytes/macrophages, helping in removing cellular debris and damaged tissues. They get activated during the stroke and release inflammatory cytokines. Endothelial cells: Cerebral endothelial cells with astrocytes and pericytes from BBB. They nourish neighboring neurons, guide developing axons, protect neurons against stress, help in vasodilation and provide a niche for supporting neural stem cells. Provide a supportive framework for oligodendrocyte lineage cells. Pericytes: They are located in the capillary network surrounding endothelial cells. They play a key role in the BBB function. They help in regulating CBF, secrete various factors for neuronal development, source of pluripotent stem cells, provoke immune cells to regulate neuroinflammatory conditions **[36, 39]**.

In physiologic conditions, the normal CBF is 50-60 ml/ 100mg/ min during cerebral ischemia the CBF falls to < 7 ml/ 100mg/ min in the central ischemic core and 7-17ml/ 100mg/ min in penumbra region. The Penumbra region is the region surrounding the ischemic core where the tissues are metabolically active but electrically silent.

During cerebral ischemia, NVU is disrupted which causes BBB permeability and neuroinflammation. In hypoxic conditions, NVU secretes biochemical factors which have a dual role in neuroprotection and neurodegeneration. Elucidating the mechanistic pathways and biochemical alterations of NVU during ischemia and intervening with drugs may help protect neurons **[4, 37]**.

2.4 Hypoxia

Eukaryotic cells are often subjected to various kinds of stress. Hypoxia is one such stress which affects neurons. Hypoxia regulates and activates several transcription factors to combat stress.HIF-1α, NF-κβ, CREB, STAT, Myc, Nrf2 are some of the transcription factors activated during stress conditions **[6]**.

HIF-1α

HIF1 is an oxygen-dependent transcriptional activator. HIF1 functions as a master regulator of oxygen and undergoes conformational changes in response to oxygen concentrations. HIF1 consists of the two subunits alpha and beta, alpha subunit are found to be highly expressed during hypoxia and expression is low in normoxia conditions. In normoxia conditions, the HIF1α subunit is rapidly degraded via the von Hippel-Lindau tumor suppressor gene product (pVHL)-mediated ubiquitin-proteasome pathway. The association of HIF-1α with pVHL is triggered by post-translational hydroxylation of proline residue mediated by prolyl hydroxylase (PHD) or HIF prolyl hydroxylase (HPH) [8].

Growth factors activate PI3K, MAPK pathways which in turn activate and regulate the transcriptional and translational synthesis of HIF-1α. This activation is irrespective of hypoxia and occurs in normoxia conditions [40].

HIF1 has multiple roles in cellular proliferation, survival, apoptosis, motility, cytoskeletal structure, adhesion, erythropoiesis, inflammation, vascular tone, angiogenesis, pH, iron, amino acids, energy, and glucose metabolism [8, 19].

NF-κβ

NF- κβ is a family of transcription factors that is composed by RelA, RelB, cRel, NF- κβ1 (p105/p50) and NF- κβ2 (p100/p52). These transcription factors are usually held captive in the cytoplasm by a family of inhibitors called Inhibitor of Inhibitor of κβ (Iκβ). During stress conditions, several pathways activate NF- κβ and can be classified into canonical, non-canonical pathways. In the canonical pathway, NF- κβ is activated via the TAK1-IKK axis whereas NF- κβ can also be activated via PKA, PKC, Ca2+, HIF1, PI3K & MAPK pathways [7, 10, 34, 41].

Hypoxia can directly activate NF- κβ by PHD & FIH. NF- κβ can induce HIF1α [7].

NF- κβ is found to aid in the transcription of pro-inflammatory and cell survival genes [11].

CREB

CREB is a transcription factor phosphorylated by PKA, PKC, Akt, Ca2+, MAPK. The phosphorylation of CREB is found in hypoxic states. The hypoxic response is important for survival in both neurons and astrocytes. HIF-1 and CREB are activated during hypoxic

treatment and PKA plays a key role in the activation of these molecules. CREB plays a major role in cell survival and neuroprotection by expressing survival and proliferative genes [6].

JNK3 & GSK3β

JNK3 is highly expressed in neurons. JNK3 activates c-jun and other apoptotic transcription factors leading to apoptosis. JNK3 is also directly activated during hypoxic conditions. JNK3 after phosphorylation activates c-jun which translocates to the nucleus to initiate apoptosis [42].

GSK-3β has been found to express in all brain regions and is elevated in neurodegenerative diseases prominently inactive state. GSK-3β plays a role in cellular function and is controlled by complex mechanisms that are dependent upon specific signaling pathways like PI3K/AKT/GSK-3, p53, JNK, c-jun, p38, CDK5, calpain, mTOR, hedgehog, notch, and ERK-1,2. In its unphosphorylated inactive state, β-catenin gets stabilized and accumulates in cytosol and translocates into the nucleus, where it initiates transcription of target genes which helps in neuronal survival. The neuroprotective effect of GSK-3β is attributed to restoration of neurobehavioral functioning, mitochondrial biogenesis, prevention of reactive oxygen species (ROS), reducing infarct size and cerebral damage, and promoting hippocampus neurogenesis [43].

cAMP-regulated PKA has a role in the activation of hypoxic controlled JNK3 and GSK3β transcription factors. PKA also has a role in the activation of MAPK pathways and inhibition of pro-apoptotic genes [18].

2.5 Beta 2 Adrenergic receptors

Beta 2 adrenergic receptors are found to be expressed in neurons particularly in hippocampus regions [47]. Activation of these receptors stimulates ERK, PI3K, CREB whereas the cAMP-derived PKA is found to regulate HIF1α and NFκβ expression. These receptors are said to regulate synaptic plasticity and may aid in cognition and neuroprotection.

Studies suggest that endogenous β2 AR agonists and NE depletion lead to increased microglial-induced neuroinflammation. Further, it also states that NE dose-dependently blocks microglial expression of inflammatory mediators this effect is reversed by coapplication of β2 AR specific antagonist. β2 AR agonists can directly interact with the receptor or indirectly do not stimulate receptor activation.

β2 AR agonists are found to upregulate neurotrophic growth factors and astrocyte activation which mediate neuroprotection which was evident from studies in DA neurons, Parkinson and traumatic brain injury. In the TBI model, the β2 AR agonist decreases neuronal impairment and helps in recovery by decreasing the level of glutamate levels. β2 AR antagonists are found to block IL-6 production and acute inflammatory response providing neuroprotection in the hemorrhagic stroke model whereas the β2 AR antagonist did not provide neuroprotection in the focal cerebral ischemic model **[44, 45]**.

β2 AR signaling pathways involved in mediating neuroprotection

β2 AR agonist mediates signals through two pathways one is through Gαs mediated pathway activating cAMP and PKA which can activate CREB. The other pathway is through association with β-arrestin which can also activate other pathways like MAPK cascade and other kinase complexes that help in the regulation of NF-κB.

β2 AR agonists at high concentrations were found to induce inflammatory effect through increased cAMP production in contrast low concentration of β2 AR agonists inhibits LPS induced inflammatory mediators, such as ROS, NO, TNFα which is mediated through activation of β-arrestin and independent of cAMP. This activation of β-arrestin helps in neuroprotection.

β-arrestin binds with Iκβa which is an inhibitor of NF-κB. Activation of this Iκβa helps in negative regulation of NF-κB.

β2 AR agonists can stimulate MAPK signaling cascade via β-arrestin. The low dose of β2 AR agonist reduced the activation of p38& JNK.

β2 AR in neurons directly promotes the synthesis and release of cAMP/PKA/CREB which promotes the synthesis and release of NGF, BDNF, IGF-1, and pro-survival pathways and also inhibits glutamate toxicity which helps in neuroprotection. Further, it can also activate the PI3K-Akt-mTOR pathway.

β2 AR antagonist is found to downregulate HIF1α during ischemic insults and this may help in neuroprotection, whereas in contrary β2 AR agonist is found to upregulate HIF1α.

β2 AR has multiple roles and multiple pathways which on activating and deactivating at different doses of β2 AR agonist and β2 AR antagonist help in neuroprotection and this area has to be studied mechanistically to explore the neuroprotective benefits of β2 AR in neurons.

Many clinical trials are undergoing on β2 AR-mediated neuroprotection

β2 AR agonists have been said to have an anti-inflammatory response. β2AR agonists block the NFκβ expression thereby inhibiting the expression of pro-inflammatory cytokines. This NFκβ inhibition may be independent of PKA. Low doses of salmeterol were found to decrease the activation and upregulation of JNK, p38, ERK1/2 **[44-46]**.

β2AR agonists were said to have a neuroprotective action in Parkinson's disease.

β2AR was found to upregulate the expression of HIF1α in neurons and pericytes **[46]**.

β2AR agonists and antagonists were found to modulate emotional memory and decrease neuronal death in the rat hippocampus **[44, 47-49]**.

Salbutamol a β2AR agonist widely used for symptomatic treatment of asthma and Propranolol a β2AR antagonist used for treating hypertension may modulate and have various roles in neuroprotection **[23, 24]**.

Molecular pathways behind β2AR on neuroprotection during cerebral hypoxia and ischemia have not been widely studied. Hence, elucidating the molecular mechanisms of salbutamol a β2AR agonist, and propranolol a β2AR antagonist on neurons may provide a clear insight on neuroprotection in VaD.

To study the molecular pathways during cerebral ischemia on neuroprotection a stable model has to be selected which are specific to neurons and which can mimic cerebral ischemia.

2.6 Stroke induction and animal models

The majority of stroke experiments are carried out in small animals (e.g., mice, rats, rabbits). The use of small animals presents clear advantages – lower cost and greater acceptability from an ethical perspective compared to larger animals. The rat is one of the most commonly used animals in stroke studies for many reasons: the cerebral vasculature and physiology of the rat are similar to that of humans **[50]**.

Induction method		Species	Age	Infarct size
Embolism				
	Preformed fibrin clot into right internal carotid artery	Rat	Unknown	Unknown
	Fibrin microemboli	Mice	Adult	Unknown
	Photocoagulation of internal carotid artery	Mice	Range	7% to 15% brain volume
	Use of small clot	Rabbit	Unknown	Unknown
	Single clot injection in internal carotid artery	Rabbit	Unknown	Greater stroke volume in anterior cerebral artery; greater percentage of stroke occurrence in middle cerebral artery
Middle cerebral artery occlusion				
		Rat	Adult	4% to 27% of hemisphere
		Rat	Adolescent	High number of nervous system structure were damage
Perforating artery occlusion				
	Cortical photocoagulation	Rat	Adult	Depth, 1.7 to 4.2 mm
	Cortical pial vessel crush	Rat	Adult	Diameter, 1629 ± 261 μm
		Rat	Adult	1.09 mm^3
Endothelin 1 injection				
	Into striatum and subcortical white matter	Rat	Adult	17.5 μL per 20 μm^2
	Into striatum	Rat	Unknown	Unknown
	Into internal capsule	Rat	Unknown	Diameter, ~ 500 μm
Spontaneous stroke				
		Rat (hypertensive)	Range	Unknown
		Dog	Range	Unknown
		Dog	Range	Diameter, 9–21 mm
Miscellaneous				
	2 injections of sodium laurate into internal carotid artery	Rat	Adult	8% of slice area
	Genetic model of CADASIL syndrome	Mice	Range	0.2–0.6 μm

Fig 7: Stroke models

2.7. Diagrammatic representation of molecular pathways taking place during hypoxia
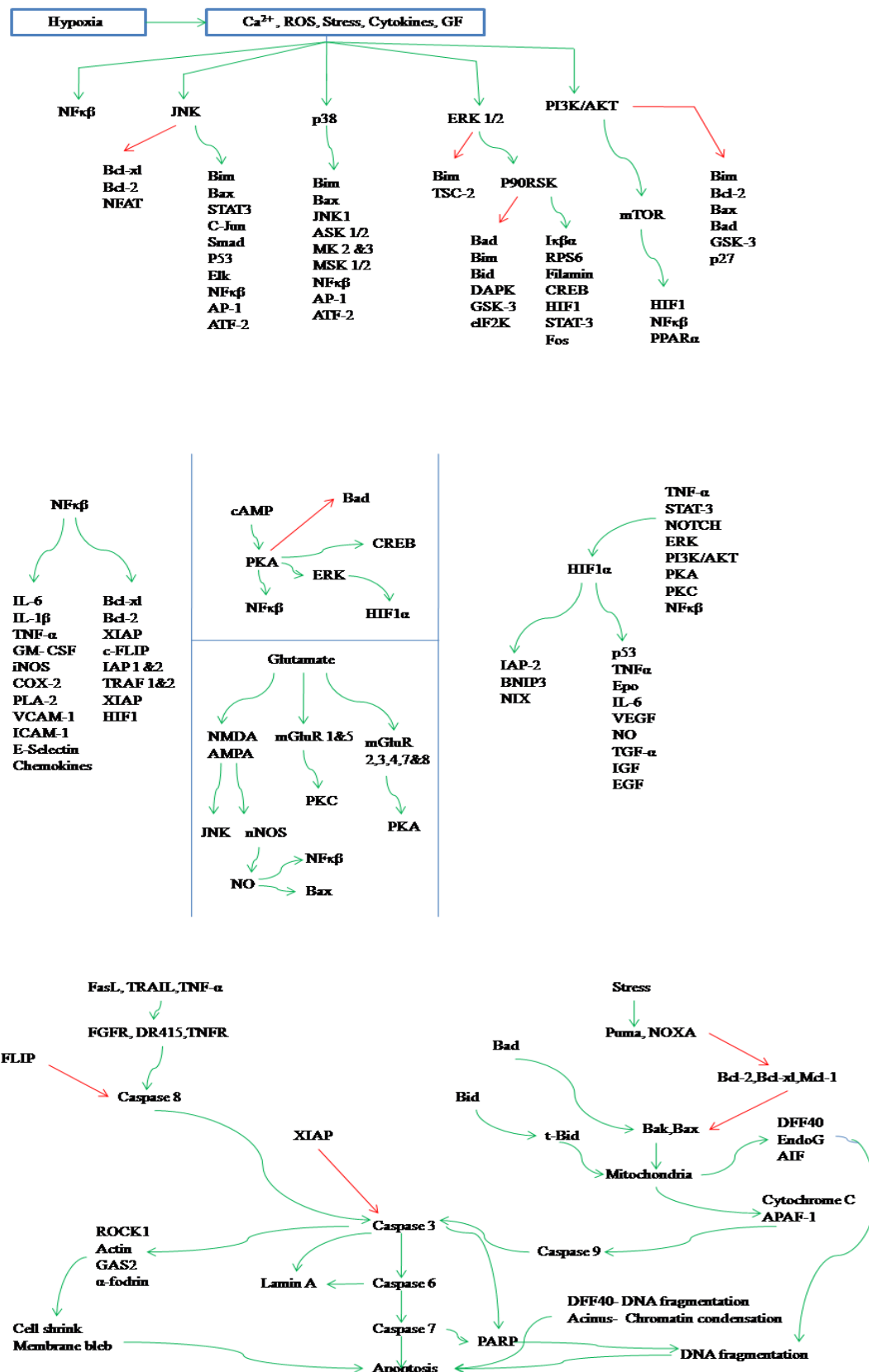

Fig 8: Upstream and downstream signaling during the hypoxic insult

There is no current effective treatment to attenuate neuronal impairment in VaD and so elucidating the molecular mechanistic pathways behind neuroprotection may provide a clear insight into neuroprotective therapy and help solve the crisis

With the reviewed literature in mind, we postulated a hypothesis on elucidating molecular mechanistic pathways during cerebral ischemia which results in VaD.

Hypothesis

Salbutamol a β2 adrenergic agonist and Propranolol β2 adrenergic antagonist may aid in neuroprotection by modulating the hypoxic regulated gene expression thereby upregulating the cell survival factors and downregulating the pro-apoptotic and pro-inflammatory cytokines.

3. Aim and Objective

3.1 Aim

- To elucidate the effect of β2 adrenergic drugs (agonist & antagonist) & HIF1α antagonist interaction on neuroprotection in cerebral hypoperfusion model

3.2 Objective

- To assess the neuroprotective effect of $β_2$ adrenergic receptor-mediated response through administration of salbutamol and propranolol and also with phenylephrine to confirm that there is no involvement of α mediated response.

- To elucidate the $β_2$ agonist response through HIF1α blockade with YC-1 (HIF1α antagonist)

- To assess the functional recovery and cognitive functions of ischemic rats through behavioral studies.

- To assess the restoration of cerebral blood flow and reversal of ischemic injury.

- To estimate the level of biochemical parameters and molecular markers for elucidating its pathway.

- To examine the neuroprotection of $β_2$ agonist and antagonist through histopathological studies in hippocampus and cortex region of ischemic rats.

4. Plan of Study

❖ **Phase I**
- ➢ Review of literature
- ➢ Optimization of model and anesthesia dose
- ➢ Animal ethical approval

❖ **Phase II**
- ➢ BCCAo Surgery and drug treatment for 7 days
- ➢ Behavioral assessment and laser perfusion imaging
  - ✓ Neurological deficit score
  - ✓ Grip strength measurement
  - ✓ Transfer latency using the elevated plus-maze

❖ **Phase III**
- ➢ Brain tissue harvesting and micro-dissection of cortex, striatum and hippocampus
- ➢ Biochemical assays
  - ✓ Griess assay (NO)
  - ✓ GSH assay
  - ✓ Thiobarbituric acid reactive substances (TBAR's) assay
  - ✓ ATP colorimetric assay
- ➢ Molecular studies
  - ✓ ELISA - cytokines (IL1β, IL6, TNFα)

❖ **Phase IV**
- ➢ Histopathology and immunohistochemistry
  - ✓ Hematoxylin and Eosin staining
  - ✓ Glial fibrillary acidic protein (GFAP)
  - ✓ Synaptophysin IHC

❖ **Phase V**
- ➢ Statistical analysis and data interpretation

Timeline of the study
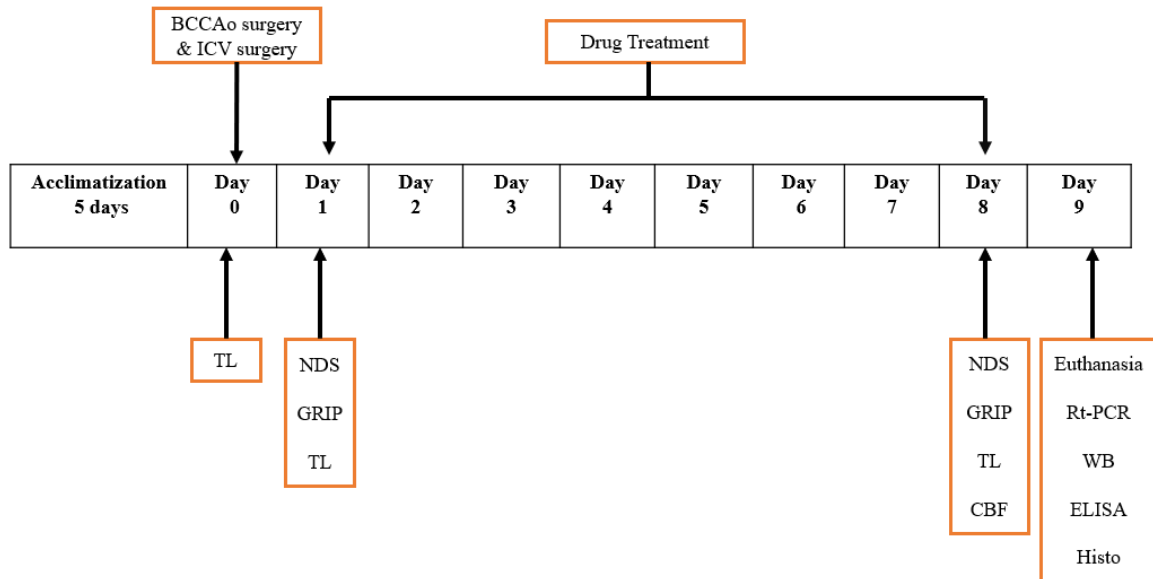
BCCAo – Bilateral Common Carotid Artery Occlusion
ICV – Intracerebro Ventricular Surgery
NDS – Neurological Deficit Score
TL – Transfer latency
CBF – Cerebral Blood Flow
WB – Western Blotting
ELISA – Enzyme Linked Immunosorbent Assay
Rt – PCR – Real time Polymerase Chain Reaction

Fig 9: Timeline of the study

5. Materials & Methods

Equipment's	qPCR	Western Blot
Dissection microscope	SYBR-green premix	Casting frame
Microvascular clip	RNA conversion kit	Casting stand
Scalpels	Thermocycler	Nano-drop
Scissors	Applied Biosystems qPCR	Vortex mixer
Needle driver	DEPC water	Thermo mixer
FLPI	Micropipettes	Buffer tank
ICV stereotaxic apparatus	Vortex mixer	Electrophoresis Chamber
Hamilton syringe	Centrifugal tubes	Electric voltage supply
Bioseb grip strength meter	PCR tubes	TEMED, Kaleidoscope
Elevated plus-maze	Primer set	Bis-Acrylamide
Gel rocker	Nano-drop	APS
Semi-dry apparatus		SDS
Gel Doc analyzer	**Primers set**	Milli-q-Water
	β_2 AR	Blot paper
	PI3K	Nitrocellulose membrane
	NT3	Semi-dry blot equipment
	BDNF	Forceps
	GDNF	Micropipettes
	eNOS	Lamelli buffer
	iNOS	Washing buffer
	nNOS	Running buffer
	Bax	Transfer buffer
	Bcl2	luminescence kit
		Primary antibody
		Secondary antibody
		TBS
		Bovine serum albumin
ELISA	**Drugs**	**Protein and RNA isolation**
Primary antibody	Ketamine	TRI reagent
Secondary antibody	Xylazine	Chloroform
Nunc-96 well plate	Atropine	Iso-propanol
Reagent diluents	Salbutamol	Ethanol
Wash buffer	Propranolol	DEPC water
PBS	Phenylephrine	Centrifuge
Sulphuric acid	YC-1	Micropipette
HRP-Conjugate	Betadine solution	RIPA buffer
BSA	Zinc phosphate cement	Nano-drop
Standard	Xylocaine jelly	**Kits**
Normal goat serum	Cipladine ointment	ATP colorimetric assay kit
Multiskan ELISA reader	Neosporin ointment	
	DMSO	
	Reduced glutathione	
	Annexin V	
	Primary antibodies	
	HIF1α	
	HIF PHD2	
	STAT3	
	ERK ½	
	β-arrestin	
	mTOR	
	JNK3	
	GSK 3β	
	Caspase 3	
	Secondary antibodies	
	IgG-goat/mouse antibody	

Table 1: List of Equipment's and Drugs

5.1. Animals

A total of 36 male Sprague-Dawley rats (200-250g) was used in the study and the rats were housed in individual polypropylene cages in a well-ventilated room under an ambient temperature of $25\pm2°$ C with 50-55% RH and a 12:12 h light/dark cycle. They were provided with food and purified water. All the animals were acclimatized for 7 days to the laboratory conditions before experimentation. They were randomly divided into 6 different groups. After surgery, the rats were placed in individual cages and post-operative care was maintained. Ethics were followed as per Indian Council Medical Research guidelines: "Guide for the care and use of laboratory animals". The study protocol had been approved by the Institutional Animal Ethics Committee vide number 464/IAEC/2020 dated 03.09.2020.

5.2. Grouping and Treatment

Groups	Treatment	No of SD rats
Group – 1	Sham operated	3
Group – 2	Negative control	3
Group – 3	Salbutamol (30mg/kg)	6
Group – 4	Propranolol (10mg/kg)	6
Group – 5	Phenylephrine (10mg/kg)	6
Group – 6	YC-1 (30μM)	6
Group – 7	YC-1 (30μM) + Salbutamol (30mg/kg)	6

Table 2: Animal grouping and treatment

5.3. Surgical procedure

BCCAo – Induction of ischemia using bilateral common carotid artery occlusion was done following previously reported procedure with slight modification. Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), intraperitoneally. A midline incision was made to expose both the right and the left common carotid arteries. Cerebral ischemia was induced by clamping both the arteries using microvascular clips for 20 min. The clips were removed from both the carotid arteries to produce IR injury. The incision was then sutured with a surgical suture. The sutured area was cleaned with 70% ethanol, sprayed with antiseptic powder, and treated with a local analgesic ointment. After completion of the surgical procedure, the rats were shifted individually to their home cage and allowed to

recover. Throughout the surgical procedure, the body temperature was maintained at 37°C using a surgery plate connected to a temperature stabilizer **[51, 52]**.

ICV - Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), intraperitoneally. Sedation is monitored using a gentle toe pinch withdrawal reflex. The Head is shaved of fur and cleaned with iodine before incision After skin incision and removal of all soft tissue from the surface of the skull, placement of the guide cannula is determined concerning bregma. A 6 mm hole is drilled through the skull with a battery-operated driller designed for rodent surgery. Care is taken so that the drill bit does not penetrate through meningeal membranes or blood vessels. Skull is implanted with bilateral 5 mm 21-gauge stainless steel guide shafts leading to the posterior nucleus accumbens, dorsal striatum, or prefrontal cortex. The stereotaxic coordinates are established as per Paxinos and Watson, 2006 (The Rat Brain in Stereotaxic Coordinates, Academic Press). Implants are secured by dental cement. A bolus of Lactated Ringers of the 0.9% saline is given at the end of surgery (5mls SC in rats after fluids are warmed to normal body temperature) to prevent dehydration. Buprenorphine (0.1-0.5mg/kg SC) is administered twice daily and then, on an as-needed basis, if the animal appears to be in pain. Local antibiotic treatment (bacitracin ointment) and systemic antibiotic treatment (penicillin 100,000 IU/kg IM every 12 hours for the first 48 hours post-op) are administered if post-operative infections occur. Following surgery, animals are individually housed with food and water available ad libitum. Throughout the surgical procedure, the body temperature was maintained at 37°C using a surgery plate connected to a temperature stabilizer **[53]**.

5.4. Bodyweight assessment

The body weight of the rats was monitored before surgery and after seven days of drug treatment.

5.5. Behavioural assessment

5.5.1. Neurological deficit score

Neurological deficit in all the treatment groups was assessed by the following score pattern at 24h and on the 7th day after bilateral common carotid artery occlusion. The scores were assigned from 0-5 based on the observed neurological deficit, 0 = No neurological deficit, 1 = Failure to extend left forepaw fully, 2 = Circling behavior, 3 = Falling to the right, 4 = No spontaneous walking with depression, 5 = Death **[54]**.

5.5.2. Grip strength measurement

A modified forelimb grip strength test was used to evaluate the neuromuscular functions in rats. Loss of muscle strength and function is a severe pathology in post-ischemic conditions. Modified grip strength was performed using Bioseb's grip strength meter. The motor coordination was measured by determining maximal peak force developed after BCCAo at 24hr and on 7th-day surgery. Measurements are recorded in grams during the study [55, 56].

5.5.3. Transfer latency

Transfer latency was measured using an elevated plus-maze which consists of two open arms (16×5 cm) and two enclosed arms (16×5×12 cm). The maze was elevated to a height of 25 cm. Rats were placed individually at end of an open arm facing away from a central platform and the time it took to move from the end of the open arm to either closed arm (Transfer latency, TL) was recorded. If the animal did not enter into one of the enclosed arms within 90 sec, a gentle push was given towards one of the two enclosed arms, and the TL was assigned as 90 sec [57].

The inflexion ratio was calculated using the formula

$$\text{Inflexion ratio} = \frac{(L1 - L0)}{L0} \times 100$$

5.6. Laser perfusion imaging

After the behavioral assessment, the rat was subject to mild anesthesia. A skin incision was made above the skull region and the skull was exposed to measure the cerebral blood flow using the Laser Doppler image analyzer (Moor-FLPI, UK) technique. The blood flow intensity was expressed as a flux mean. The color patterns in the brain images are represented as red – high blood flow; yellowish-orange – low blood flow and blue – no blood flow respectively [51].

5.7. Preparation of brain samples

The animals were sacrificed after 8 days of drug treatment. The brain was removed and different regions of brain tissues like the cortex, striatum, and hippocampus were dissected. The brain samples were kept at −80°C [54].

5.8. Biochemical assays

5.8.1. Griess assay (NO)

Nitric oxide (NO) was indirectly measured in the form of nitrates and nitrites taking 0.2 ml of 10% homogenate followed by the addition of 1.8 ml of saline and 0.4ml of 35% sulphosalicylic acid for protein precipitation. The precipitate was removed by centrifugation at 4000 rpm for 10 min. Griess reagent was prepared by dissolving 100mg of sulphanilamide and 10mg of naphthyl ethylene-diamine (NED) in 10ml of 5%Hcl. 50µl of standard (sodium nitrite)/ sample supernatant is added to each well (96 well flat bottom plate) and then add 50µl of Griess reagent. This reagent forms a violet color in the presence of nitrite. The plate was incubated in dark for 10 mins and absorbance was measured at 540nm. The unknown concentration of samples was determined by interpolating the absorbance values in the standard linear graph [54, 58].

5.8.2. GSH assay

GSH content was estimated by the following method. Homogenize the tissue in 0.6% sulfosalicylic acid – Triton -X solution and centrifuge at 8000g for 10 min at 2-4ºC. Transfer the clear supernatant and use it for the total GSH assay. 20 µl of standard/sample is added to each well (96 well flat bottom plate) and then add 120 µl of equal volumes of freshly prepared DTNB and GR solutions. Allow 30s for the conversion of GSSG to GSH and then add 60 µl of β- NADPH and read the absorbance at 412 nm in a microplate reader and take measurements every 30s for 2 mins. Calculate the rate of 2-nitro-5-thiobenzoic acid formation (change in absorbance min^{-1}) and determine the actual total GSH concentrations in the samples by using linear regression to calculate the values obtained from the standard curve. Total GSH concentration is expressed as µM or nM mg^{-1} protein [54, 59].

5.8.3. Thiobarbituric acid reactive substances (TBAR's) assay

Lipid peroxidation was evaluated by measuring the TBAR's content. TBA reagent was prepared by adding 5.76mg of Thiobarbituric acid in 10ml of glacial acetic acid after addition it was sonicated for 10 mins for uniformity. 150µl of standard/ sample was added to each well (96 well flat bottom plate) and then 75µl of TBA reagent is added to it. Optical density/ absorbance was measured as a pre-read at 532nm. After the pre-read, the 96 well plates were incubated at 50ºC for 3hours. After incubation, the absorbance was measured at 532nm. The pre-read absorbance was subtracted with the final absorbance value to get the corrected final

absorption value. A standard graph was plotted and the unknown concentration of samples was determined by interpolating the absorbance values from the linear graph **[54, 60]**.

5.9. ATP colorimetric assay

The amount of ATP present in the brain tissue was measured using ATP colorimetric assay kit (Sigma Aldrich). The measurement of ATP was performed step-by-step as per the ATP kit protocol. ATP present in the sample is expressed as ng/µl.

5.10. Quantification of inflammatory cytokines by ELISA

The levels of cytokines (TNF-α, IL-β, and IL-6) in the brain tissue were measured using an ELISA kit (Diaclone, France) specific for rats. The measurement of cytokines was performed step-by-step as per ELISA kit protocol. The cytokines contents were expressed as pg/ml total protein **[54]**.

5.11. Histopathology and Immunohistochemistry

5.11.1. Hematoxylin and Eosin staining

Hematoxylin and Eosin staining is done to study the details of the nuclei of the cell. In H&E-stained tissues, the nucleic acids stain dark blue and the proteins stain red to pink or orange. After 7 days of drug treatment, euthanasia on the experimental animals was done. The brains were dissected out quickly, fixed in 10% formalin, and 5 µm thick sections were taken. The sections were processed and stained in hematoxylin and eosin. The stained sections were observed under a binocular light microscope and photographed and compared with control groups **[61]**.

5.11.2. Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein is an intermediate filament protein present in astrocytes. GFAP expression is increased in severe mechanical or osmotic stress, hypoxia, brain, and spinal cord injury. Brain samples were stained with monoclonal mouse anti-GFAP (Dako, Denmark) (1:300) using the immunoperoxidase technique. The paraffin sections of the rat brain were deparaffinized and hydrated with distilled water. The sections containing antigenic sites were exposed by incubating them in a microwave oven with antigen retrieval solution (trisodium citrate, pH 6.2) for 20 min at 96ºC. Following retrieval, the slides were cooled with distilled water for 5 min. Subsequently, the slides were washed with PBS in each step.

Then the slides were treated with 3% hydrogen peroxide to decrease the endogenous peroxidize activity. The slides were then incubated with 1.5% bovine serum albumin (BSA) for blocking the non-specific binding sites. The immune reactivity was detected with an Immunocruz mouse ABC staining kit using biotinylated goat anti-mouse IgG (for GFAP) secondary antibodies at 1:100 dilution and evaluated under a binocular light microscope (40x) and photographed **[62]**.

5.11.3. Synaptophysin IHC

Synaptophysin is an integral membrane glycoprotein present in the presynaptic vesicles of neurons. The integrity of the synapse in ischemic brain regions was measured by performing immunohistochemistry staining of synaptophysin. After isolation of the brain, it was fixed with 10% formalin. Paraffin sections of 5 μm thick were made and IHC was done by using a monoclonal mouse anti-synaptophysin clone SY38 antibody kit (Dako, Denmark). The active synapse was evaluated under a binocular light microscope (40x) and photographed **[62]**.

5.12. Statistical analysis

Data were tabulated and statistics were performed using statistical analytical tool software Graph pad Prism v9.3. Data were expressed as mean ± standard deviation (SD). The significance level was set to $p<0.05$ and is analyzed using one-way and two-way ANOVA followed by Bonferroni's multiple comparison test for posthoc analysis.

6. Results

6.1 Surgical observations
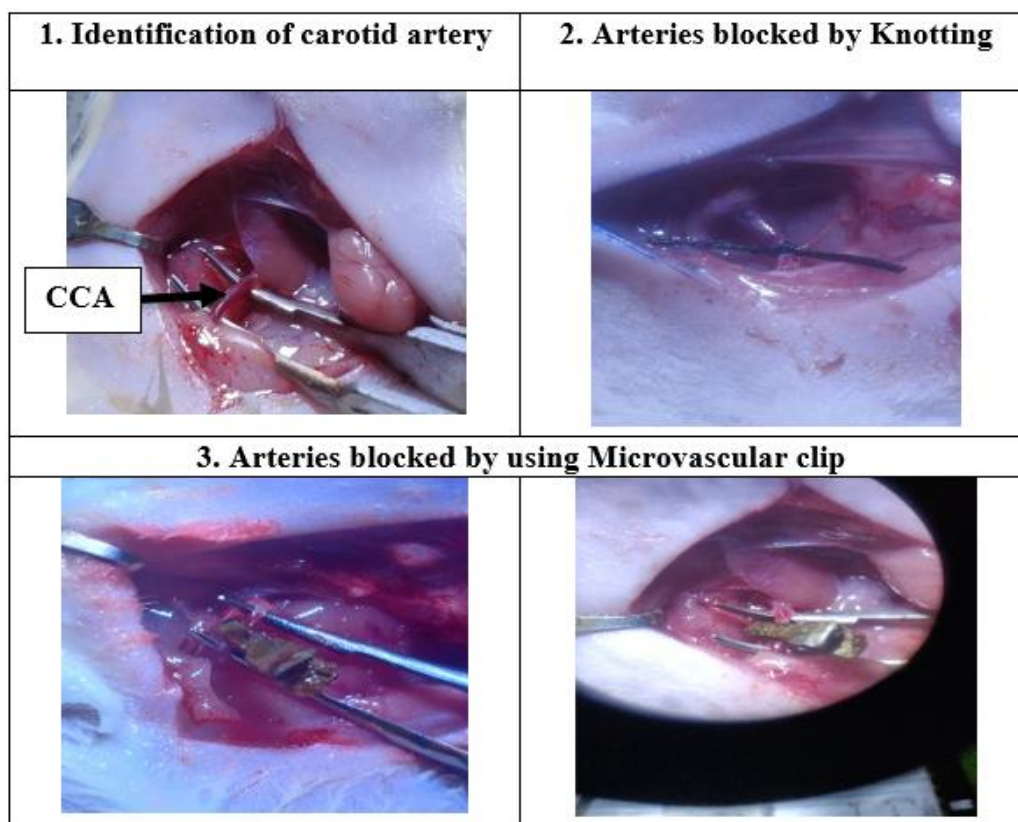
CCA- Common Carotid Artery

Fig 10: Surgical procedure of BCCAo model

6.2 Body weight assessment

The body weight of all rats was measured before surgery (day 0) and after drug treatment (day 8). No significant changes in the body weight were observed in comparison to the sham-operated rats. (Figure.11)

Data were expresses as mean ± SD. Statistical analysis were carried out using two-way ANOVA followed by Bonferroni's multiple comparison test.
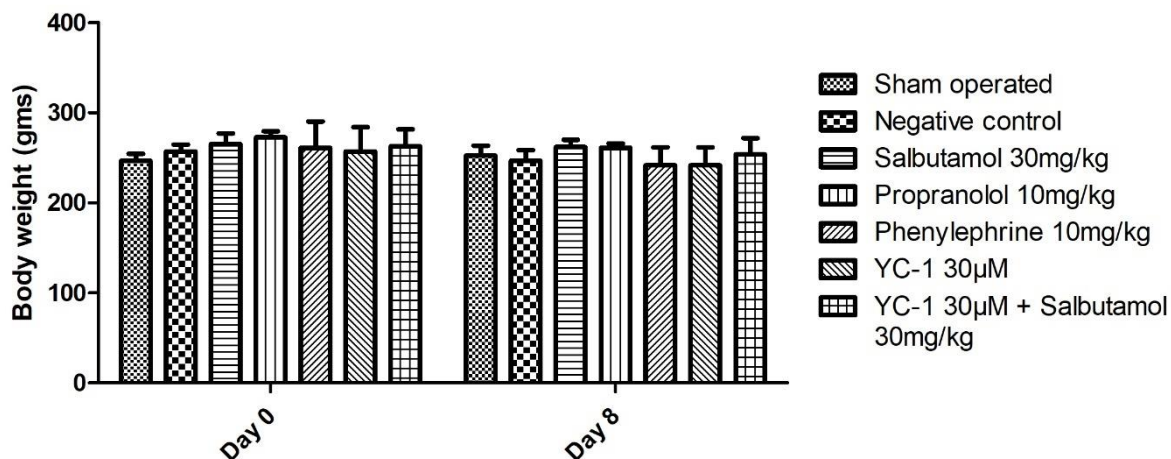
Fig 11: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg at day 0 & day 8 on body weight in global ischemic rats. Data were expressed as mean ± SD (n=3, 6).

6.3 Behavioural assessment

6.3.1 Neurological deficit score

The neurological function of all the rats were measured before surgery and was found to be normal. After induction of global ischemia, the neurological function was measured on day 1 and day 8 of the drug treatment.

Inducement of global cerebral ischemia in rats produced significant increase in the neurological score at day 1 and day 8 respectively (Figure.12). Drug treatments with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg showed significant increase ($p < 0.001$) in neurological function as noted by better neurological score in comparison to vehicle treated ischemic rats on day 8.

Data were expresses as mean ± SD. Statistical analysis were carried out using two-way ANOVA followed by Bonferroni's multiple comparison test.
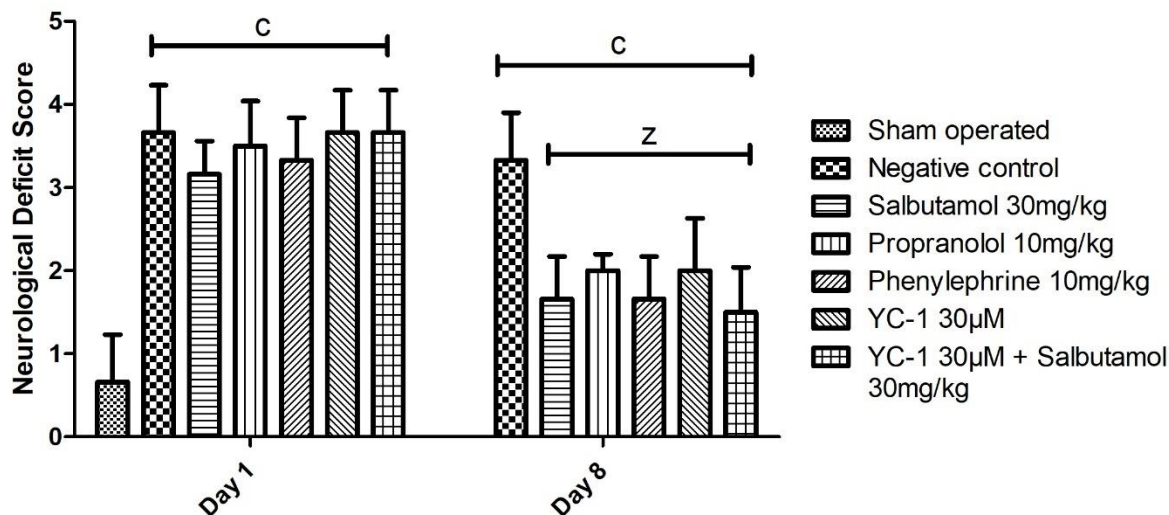
Fig 12: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg at day 1 & day 8 on neurological score in global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at $p < 0.001$ respectively.

6.3.2 Modified forelimb grip strength test using bioseb grip strength apparatus

Inducement of cerebral ischemia in rats exhibited significant loss of muscle coordination as observed by significant decrease in the force applied in grip strength activity (Figure.13). Drug treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg showed significant improvement ($p < 0.001$) in grip strength activity in comparison to vehicle treated ischemic rats on day 8.

Data were expresses as mean ± SD. Statistical analysis were carried out using two-way ANOVA followed by Bonferroni's multiple comparison test.
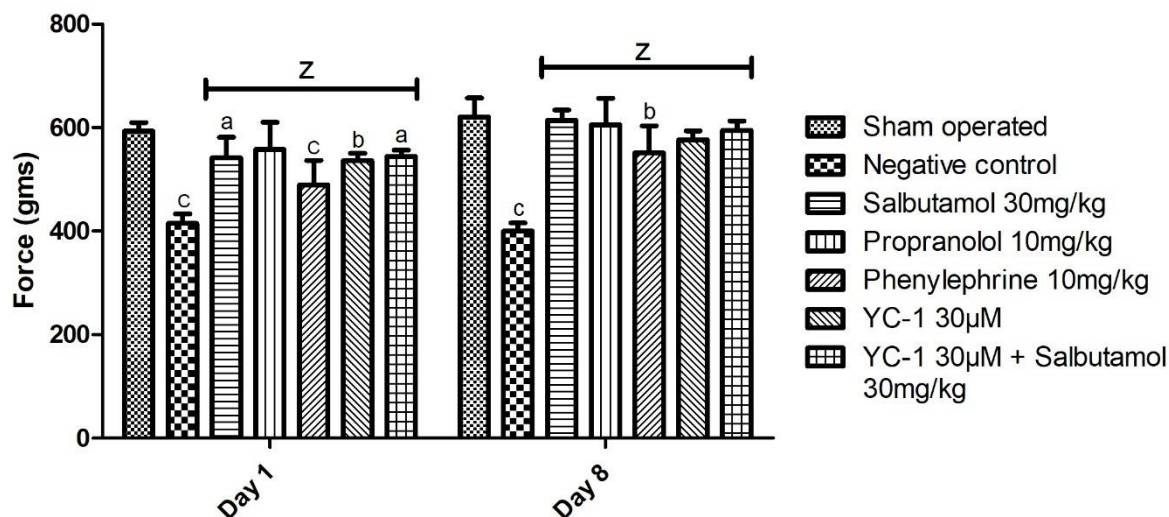
Fig 13: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg at day 1 & day 8 on grip strength in global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts a, b, c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.05, p<0.01 and p<0.001 respectively.

6.3.3 Transfer latency using Elevated Plus Maze

The day 1 and day 8 memory retention activity for different treatment groups were represented as transfer latency in table 3 and inflexion ratio in figure 14. Transfer latency at day 0 is considered as acquisition (learning) and memory retention is assessed on day 1 and day 8. Treatment with YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg significantly (p<0.001) improved the (day 1) short term memory as observed by increased inflexion ratio in comparison to vehicle treated ischemic rats. On day 8, salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg treated rats exhibited significant (p<0.001) increase in inflexion ratio indicating memory retention in transfer latency test.

Data were expresses as mean ± SD. Statistical analysis were carried out using two-way ANOVA followed by Bonferroni's multiple comparison test.

S.no	Groups	Transfer latency (sec)		
		Day 0	Day 1	Day 8
1	Sham operated	27.83±2.66	20.69±1.95	14.2±1.51
2	Negative control	44.22±4.38	39.81±4.1	41.51±3.96
3	Salbutamol 30mg/kg	32.18±3.18	28.55±2.91	19.3±1.87
4	Propranolol 10mg/kg	33.8±3.25	31.14±3.05	22.19±2.36
5	Phenylephrine 10mg/kg	41.82±4.28	37.53±3.62	29.04±3.1
6	YC-1 30μM	57.05±4.47	44.57±3.22	31.71±2.22
7	YC-1 30μM + Salbutamol 30mg/kg	55.9±4.89	39.24±3.73	28.03±2.72

Data were expresses as mean ± SD.

Table 3: Transfer latency period
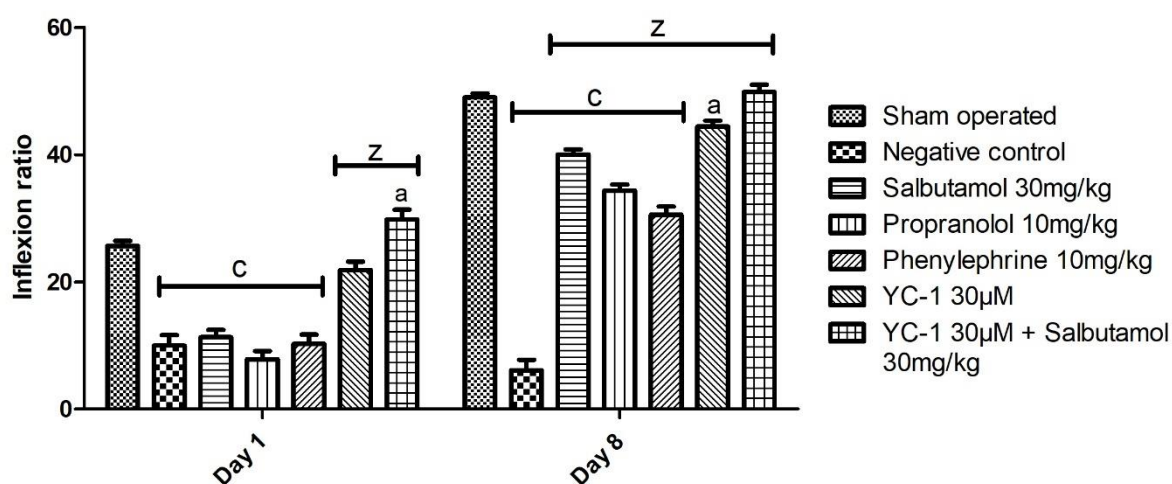
Fig 14: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg at day 1 & day 8 on inflexion ratio in global ischemic rats. The inflexion ratio was calculated in percentage using transfer latency period. Data were expressed as mean ± SD (n=3, 6). Superscripts a, c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at $p<0.05$ and $p<0.001$ respectively.

6.4 Laser Perfusion Imaging – Cerebral blood flow

The cerebral blood flow intensity was expressed as flux mean and it was measured in the midbrain region. The colour pattern in the brain images is represented as red – high blood flow, yellowish orange – low blood flow and blue – no blood flow respectively.

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
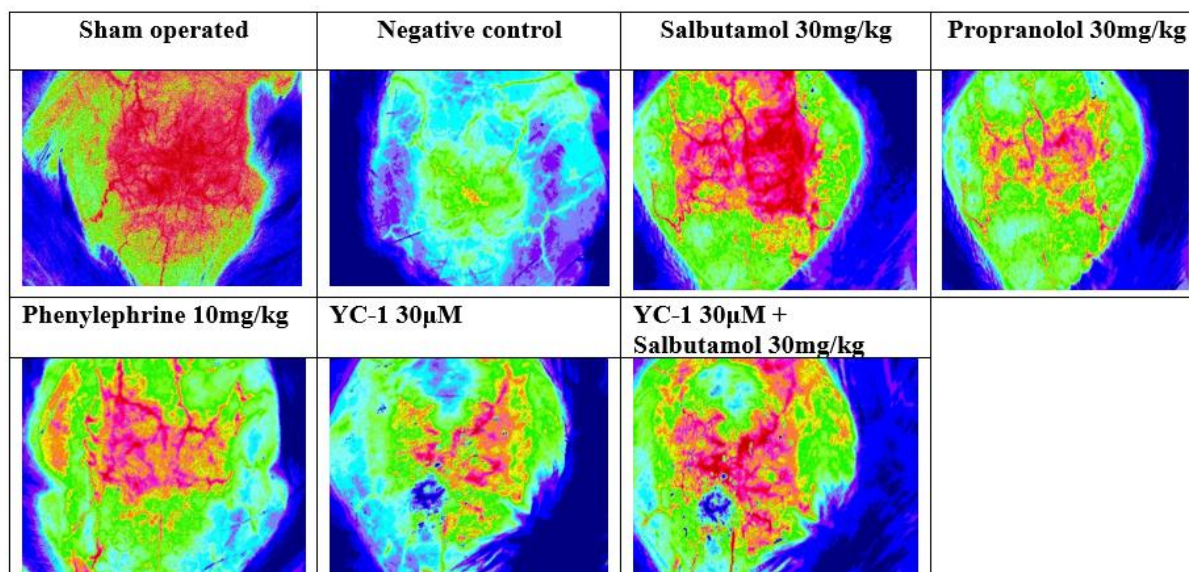

Fig 15: Full-field laser perfusion imaging

Induction of ischemia through BCCAo model resulted in decreased cerebral blood flow in the mid-region of the brain as indicated by significantly decreased flux mean ($p<0.001$) in negative control rats in comparison to sham-operated rats. On day 8, propranolol 10mg/kg showed significant increase ($p<0.01$) in blood flow in comparison to vehicle treated ischemic rats. Salbutamol 30mg/kg, phenylephrine 10 mg/kg and YC-1 30μM + salbutamol 30mg/kg treated rats have demonstrated significant increase in cerebral blood flow after ischemic induction in comparison to vehicle treated ischemic rats (Figure.16).
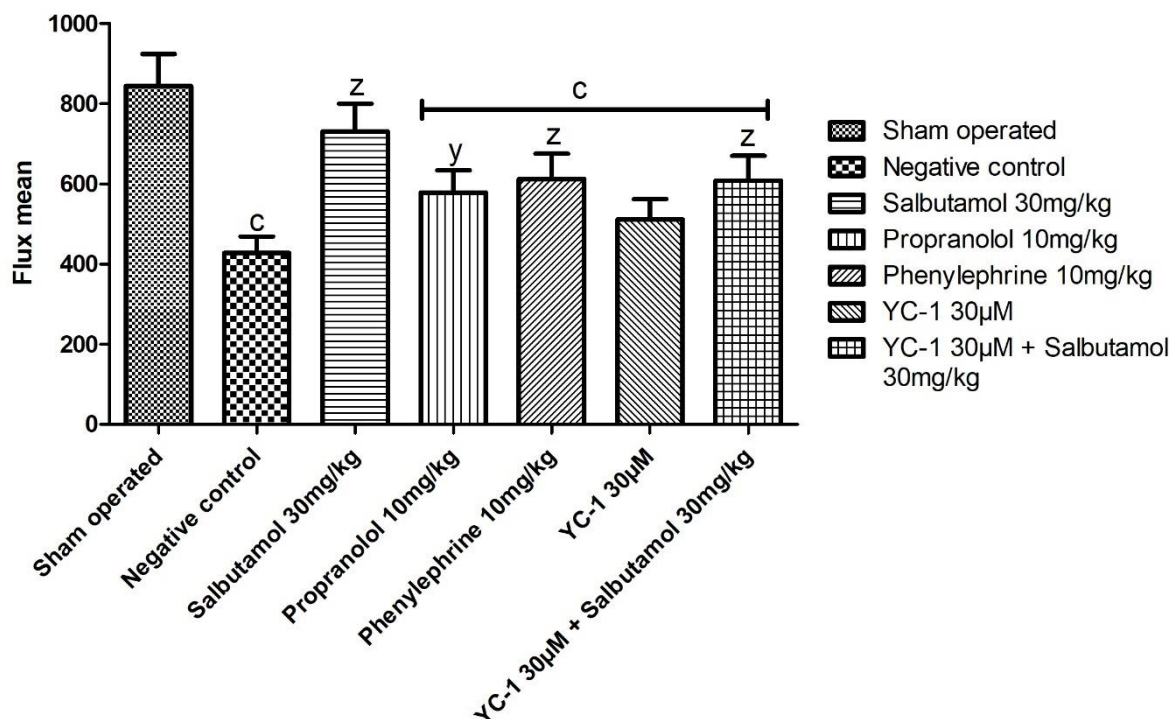
Fig 16: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg at day 8 on cerebral blood flow in global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and y, z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.01 and p<0.001 respectively.

6.5 Biochemical assays

6.5.1 Griess assay (NO)

The occlusion of bilateral common carotid artery followed by reperfusion resulted in elevated Nitrite levels (p<0.001) in the cortex, hippocampus and striatum regions in comparison to the Sham-operated rats.

There was a significant decrease (p<0.001) in nitrite levels with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg in all three regions were observed in comparison to vehicle treated ischemic rats. The maximum increase in NO release was observed in the cortex region of the ischemic rats. (Figure. 17a, b, c)

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
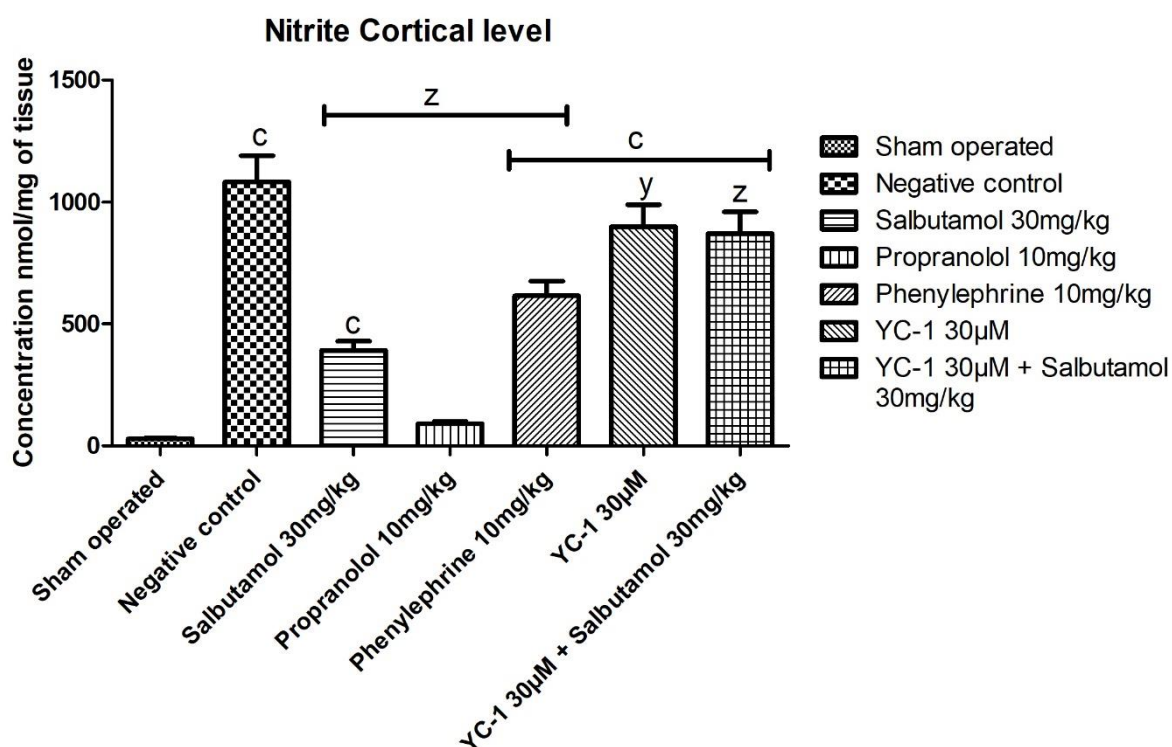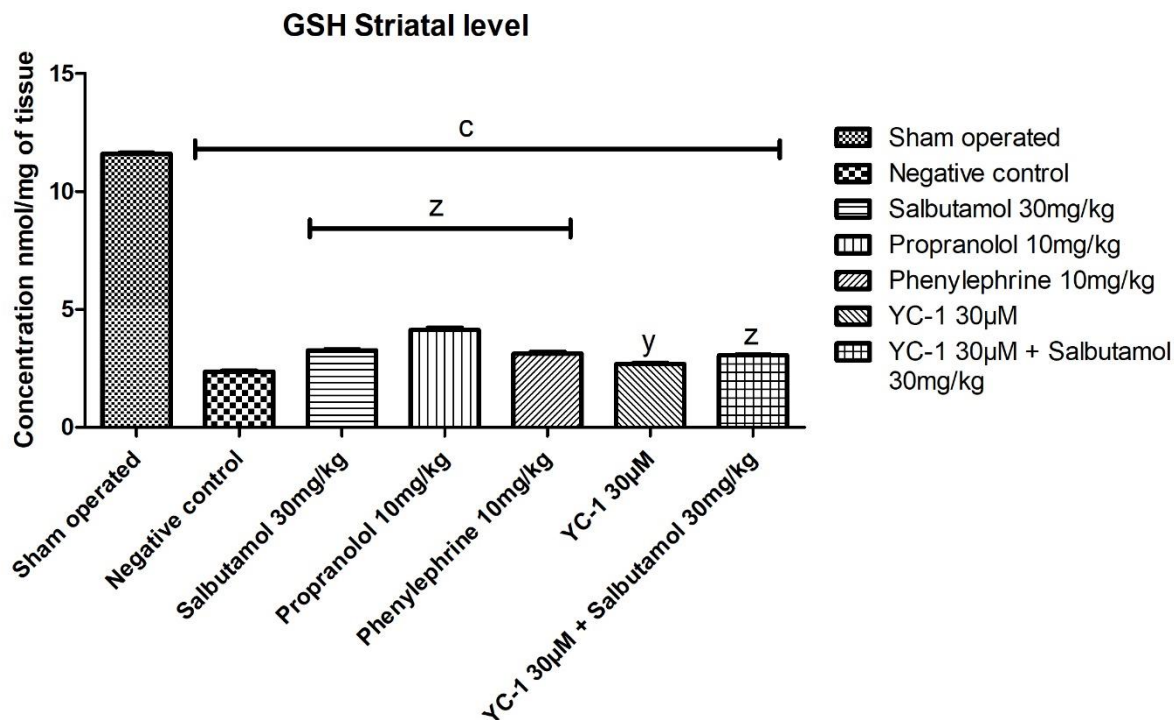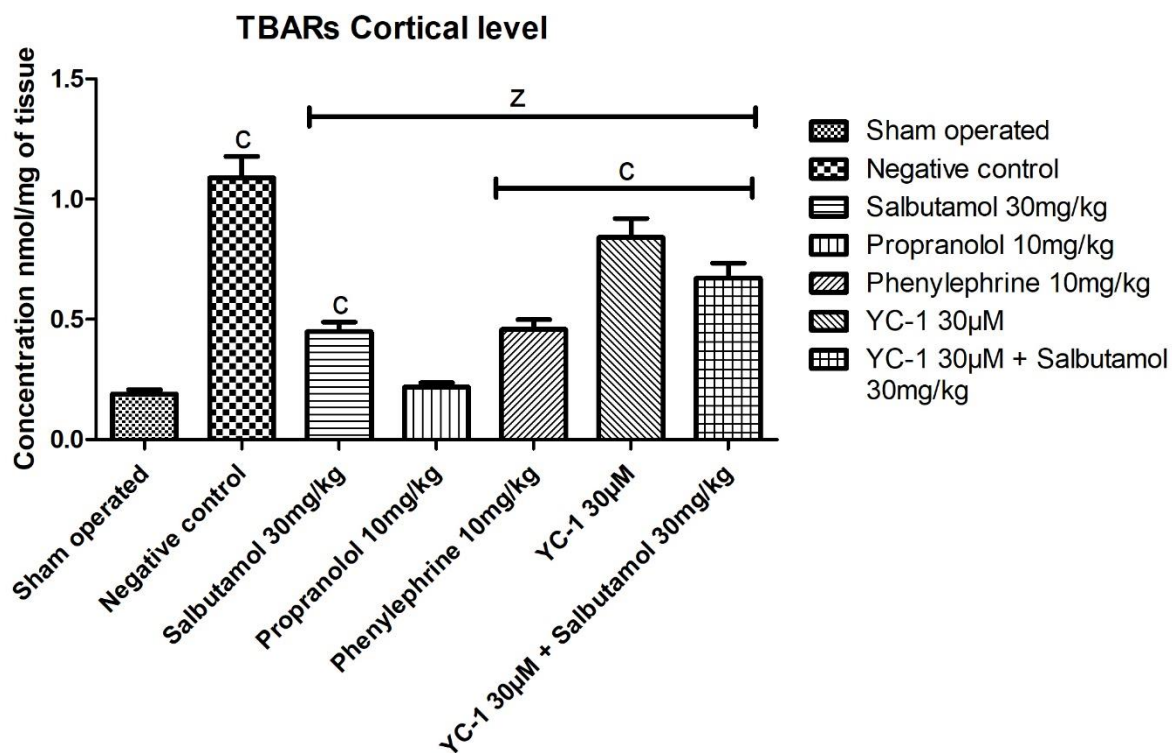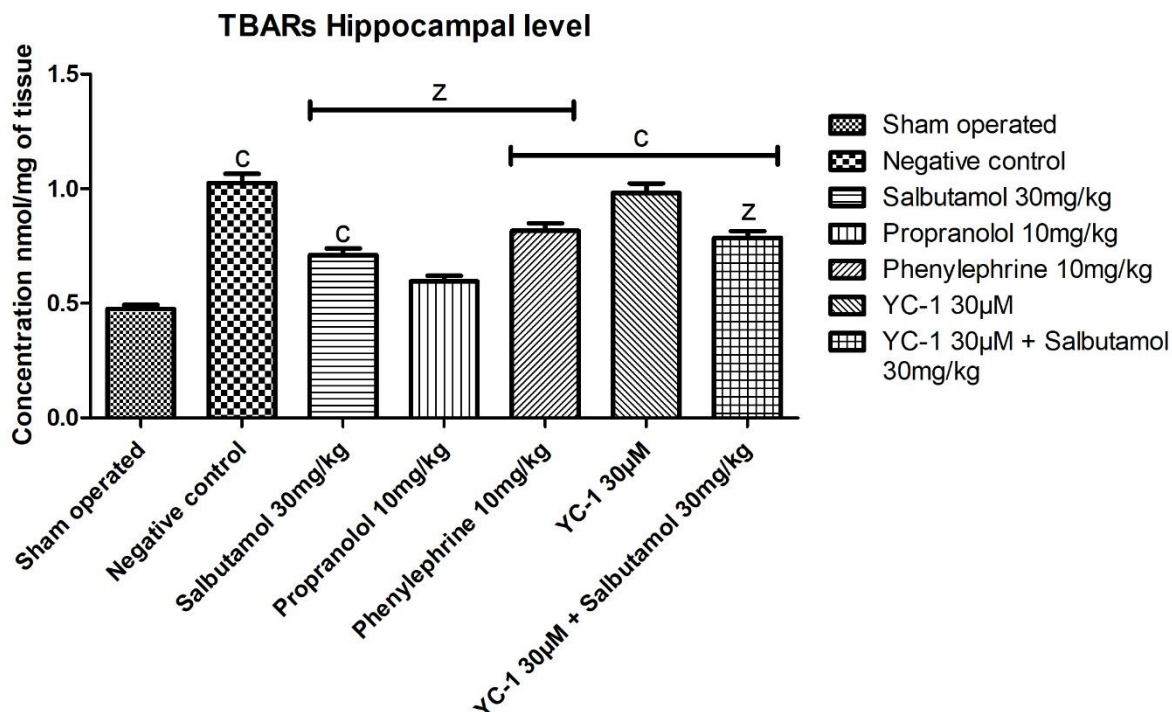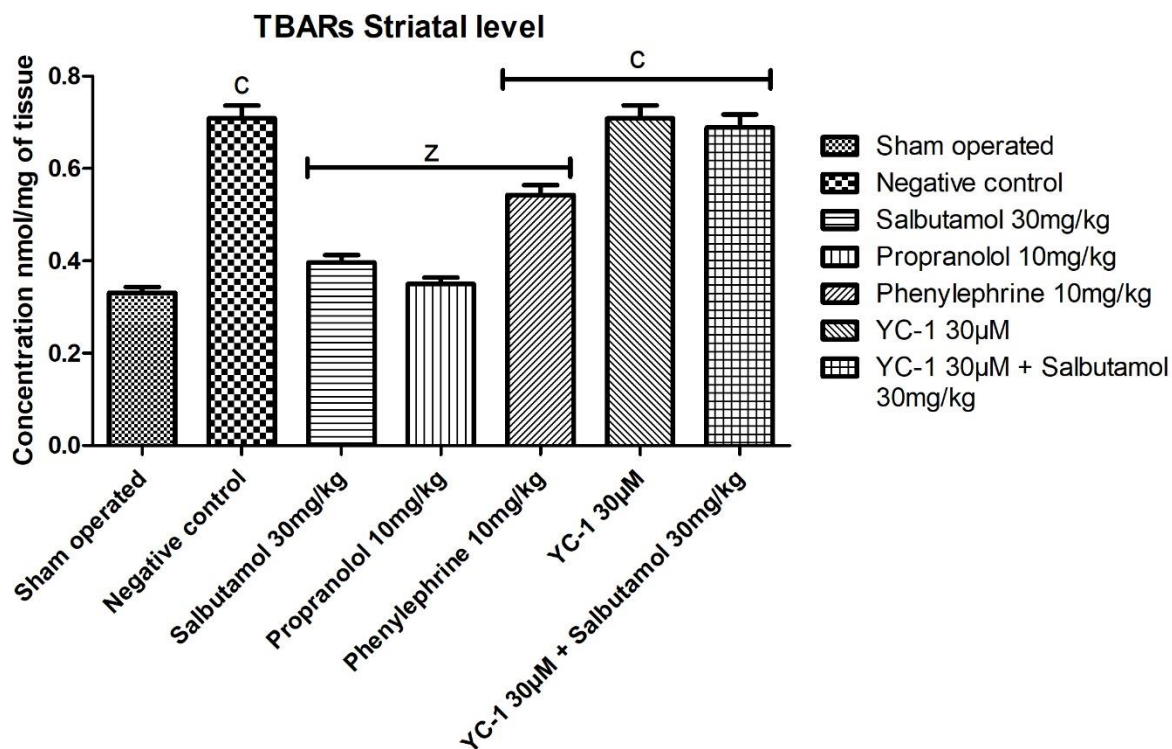

Fig 17a: Nitrite cortical level
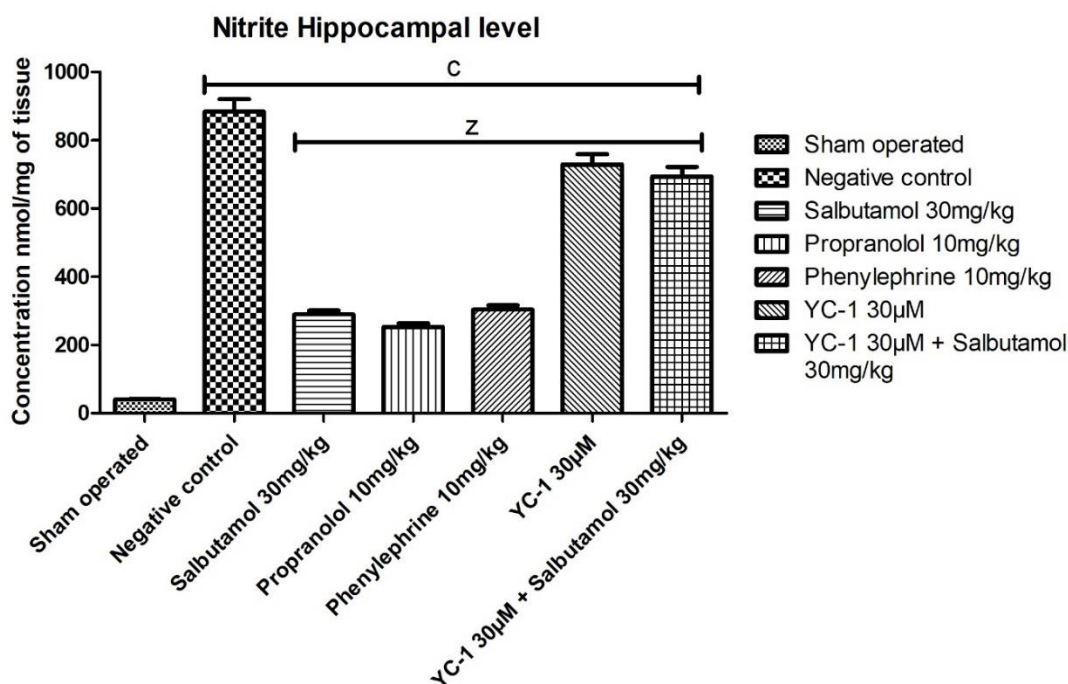
Fig 17b: Nitrite Hippocampal level
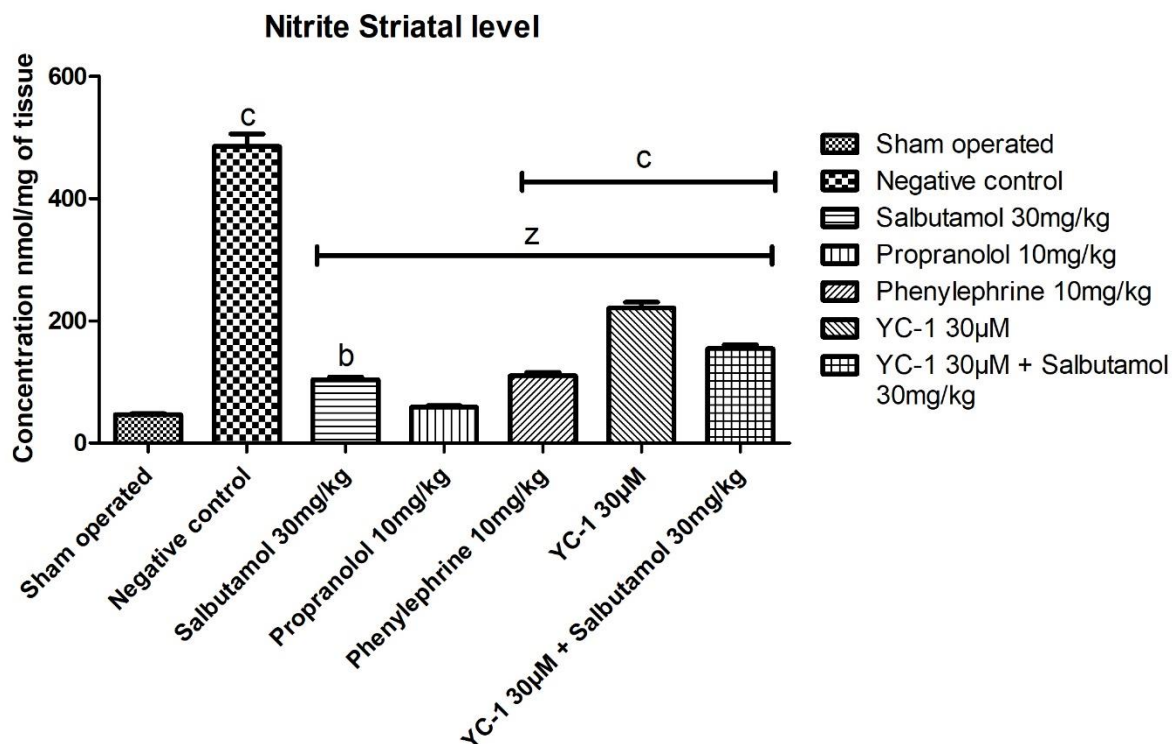
Fig 17c: Nitrite striatal level

Fig 17a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on nitric oxide levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts b, c and y, z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at $p < 0.01$ and $p < 0.001$ respectively.

6.5.2 GSH assay

In comparison to sham-operated rats the reduced glutathione levels in cortex ($p < 0.001$), hippocampus ($p < 0.001$) and striatum ($p < 0.001$) was found to be significantly reduced in the vehicle treated ischemic rats. Treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg and YC-1 30μM + salbutamol 30mg/kg led to significant increase in the cortex, hippocampus and striatum regions GSH levels in comparison to vehicle treated ischemic rats.

 YC-1 30μM treatment showed significant decrease ($p < 0.01$) in the striatum GSH level in comparison to vehicle treated ischemic rats. (Figure. 18a, b, c)

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
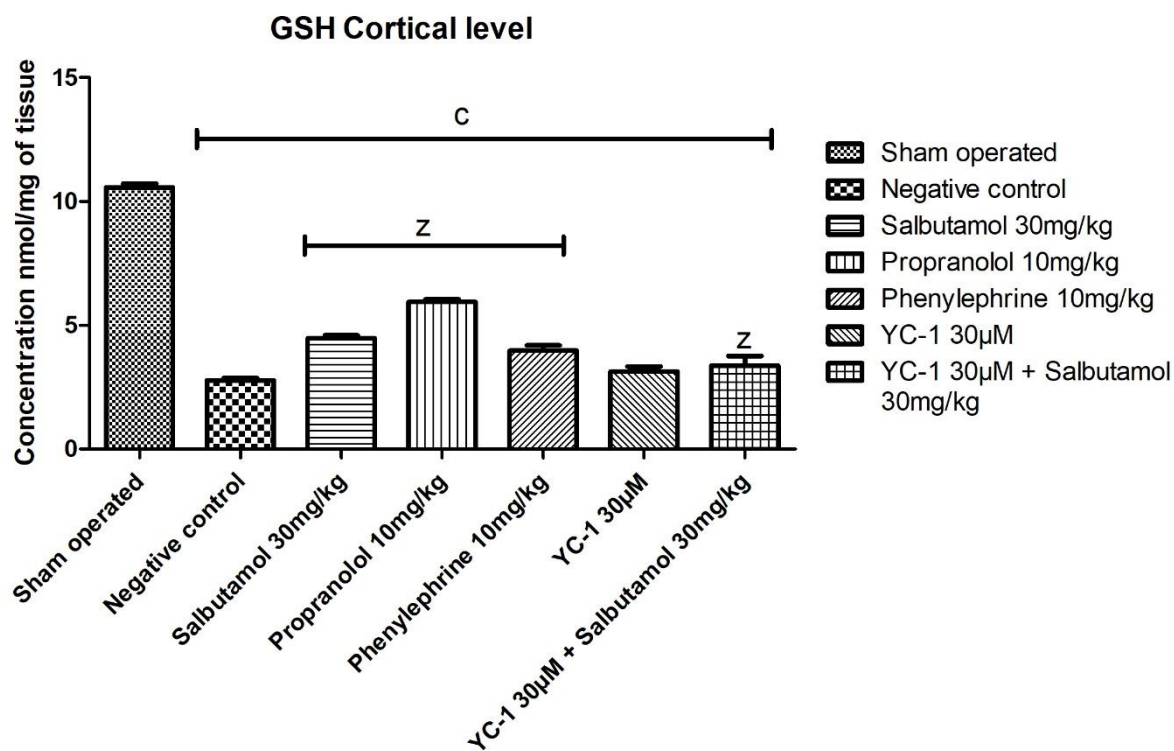
Fig 18a: GSH cortical level
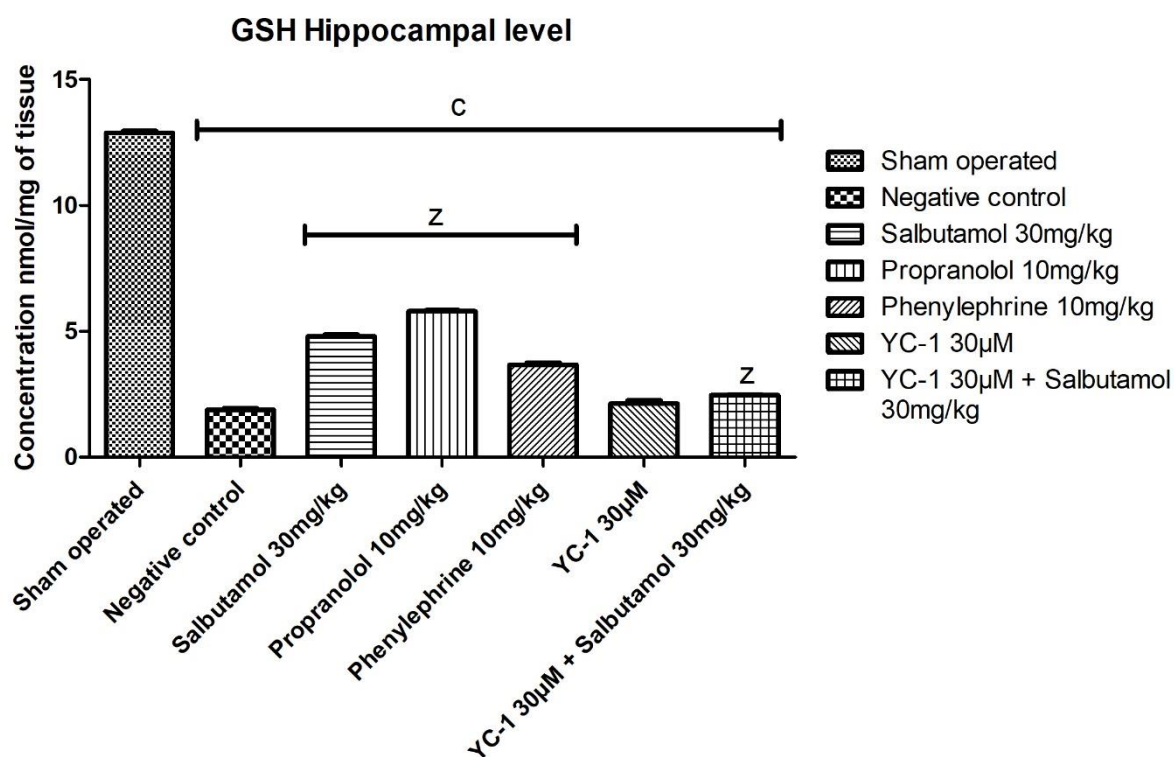
Fig 18b: GSH Hippocampal level

Fig 18c: GSH striatal level

Fig 18a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on GSH levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and y, z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.01 and p<0.001 respectively.

6.5.3 Thiobarbituric acid reactive substance (TBAR's) assay

The circulating lipid peroxide levels were significantly elevated (p<0.001) in the cortex, hippocampus and striatum regions in vehicle treated ischemic rats in comparison to sham-operated rats.

Treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg significantly attenuated the lipid peroxide levels (p<0.001) in the cortex region, Salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10mg/kg treatment showed significant decrease (p<0.001) in the lipid peroxide levels in hippocampus and striatum regions. (Figure. 19a, b, c)

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
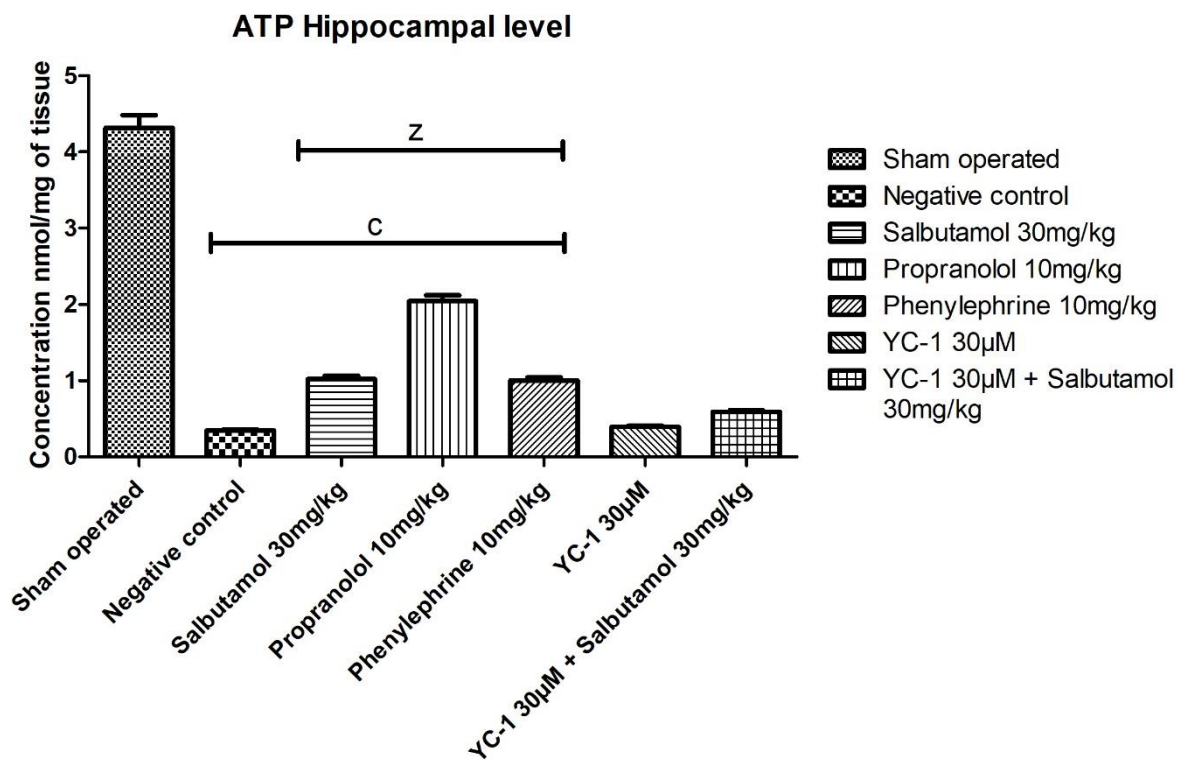
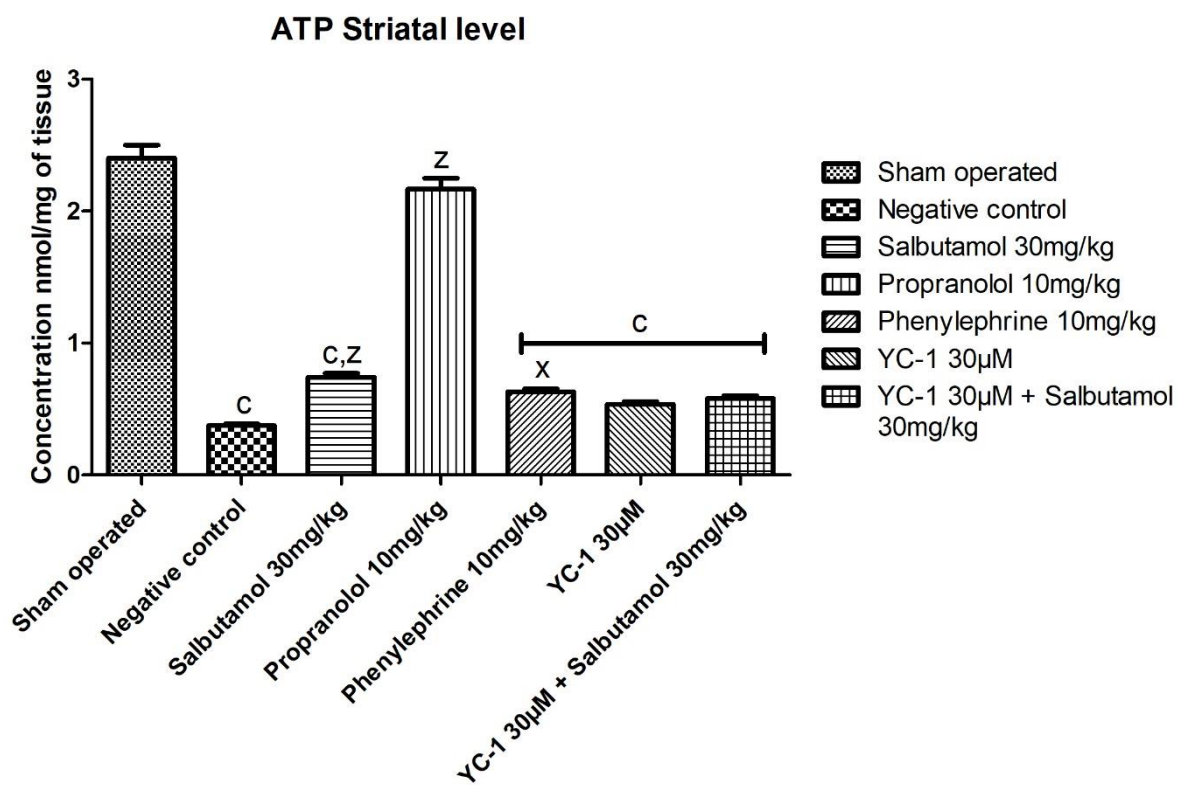**Fig 19a:** TBARs cortical level

Fig 19b: TBARs Hippocampal level

Fig 19c: TBARs striatal level

Fig 19a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on TBARs levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.001 respectively.

6.5.4 ATP colorimetric assay

Inducement of global ischemia in rats depleted brain ATP levels significantly(p<0.001) in the cortex, hippocampus and striatum regions in comparison to SO rats which indicates energy depletion after reperfusion neuronal injury. The ATP levels in the cortex region were significantly elevated (p<0.001) following salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg treatment in comparison to vehicle treated ischemic rats. (Figure. 20a)

The ATP levels were significantly increased (p<0.001) in hippocampus of the ischemic group with treatment of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg in comparison to vehicle treated ischemic rats. (Figure.20b)

The ATP levels were found to be significantly increased in striatum of the ischemic rats salbutamol 30mg/kg (p<0.001), propranolol 10mg/kg (p<0.001) and phenylephrine 10 mg/kg (p<0.05) treatment in comparison to vehicle treated ischemic rats. (Figure.20c)

Among the regions studied in comparison to the cortex, the hippocampal and striatal ATP level was significantly elevated with salbutamol 30mg/kg and propranolol 10mg/kg treatment.

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
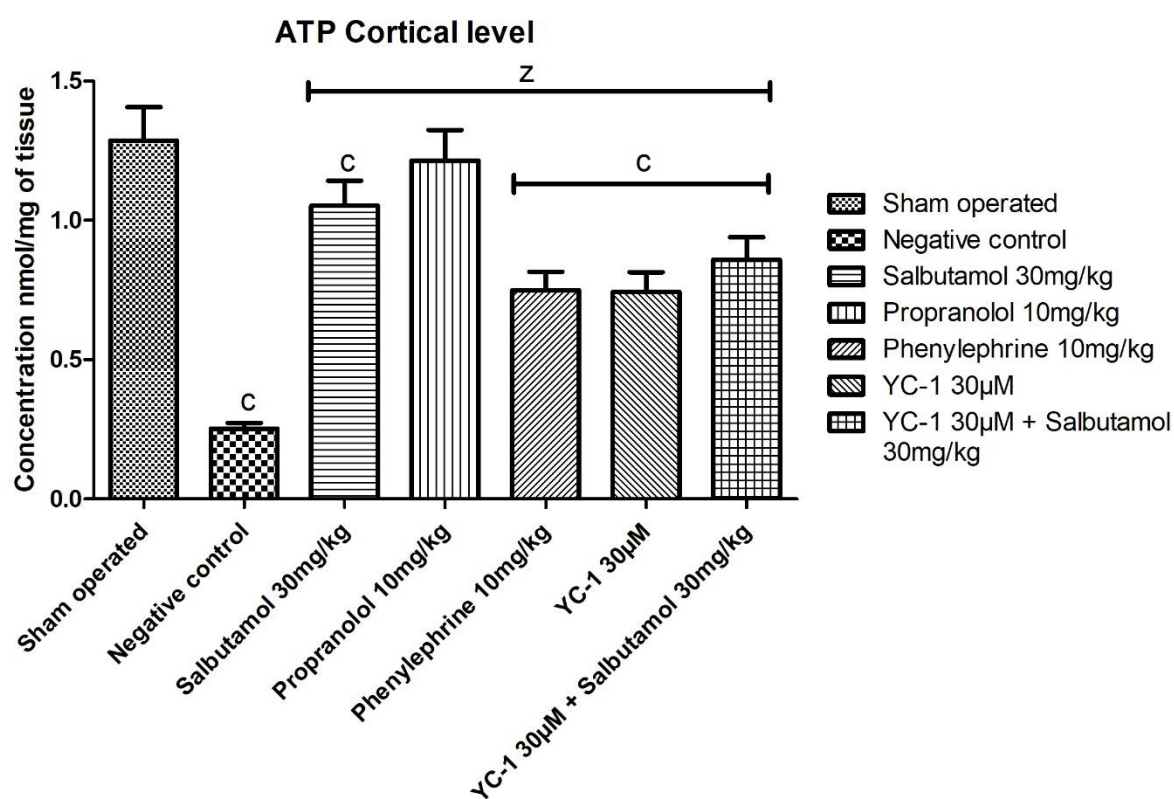
Fig 20a: ATP cortical level

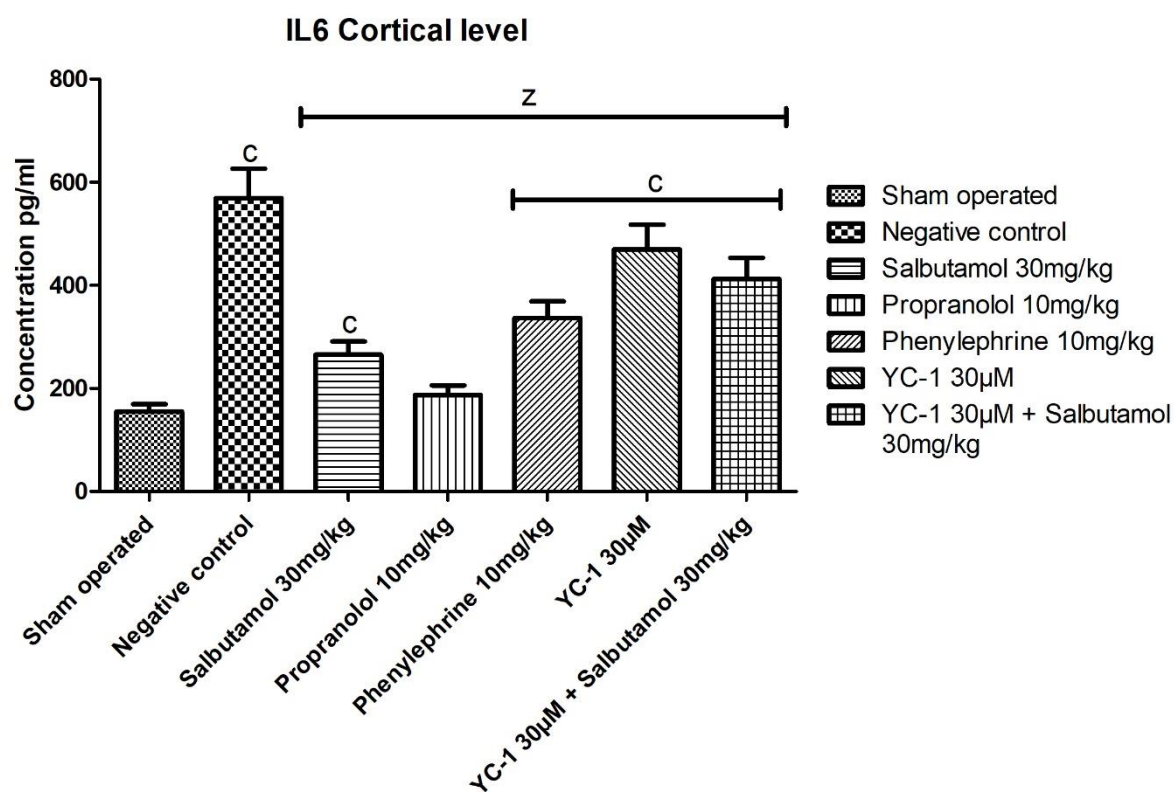**Fig 20b:** ATP Hippocampal level

Fig 20c: ATP striatal level

Fig 20a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on ATP levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and x, z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at $p < 0.05$ and $p < 0.001$ respectively.

6.6 Quantification of Inflammatory cytokines by ELISA

6.6.1 Effect of drug treatment on IL1β

Inducement of global cerebral ischemia in rats elevated the pro-inflammatory cytokine levels in the cortex ($p < 0.001$), hippocampus ($p < 0.001$) and striatum ($p < 0.001$) in comparison to SO rats. Treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg significantly reduced the level of IL1β in all the three regions in comparison to vehicle treated ischemic rats ($p < 0.001$).(Figure.21a, b, c)

Among the regions studied in comparison to cortex, hippocampal and striatal levels of IL1β was significantly elevated upon ischemia induction.

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
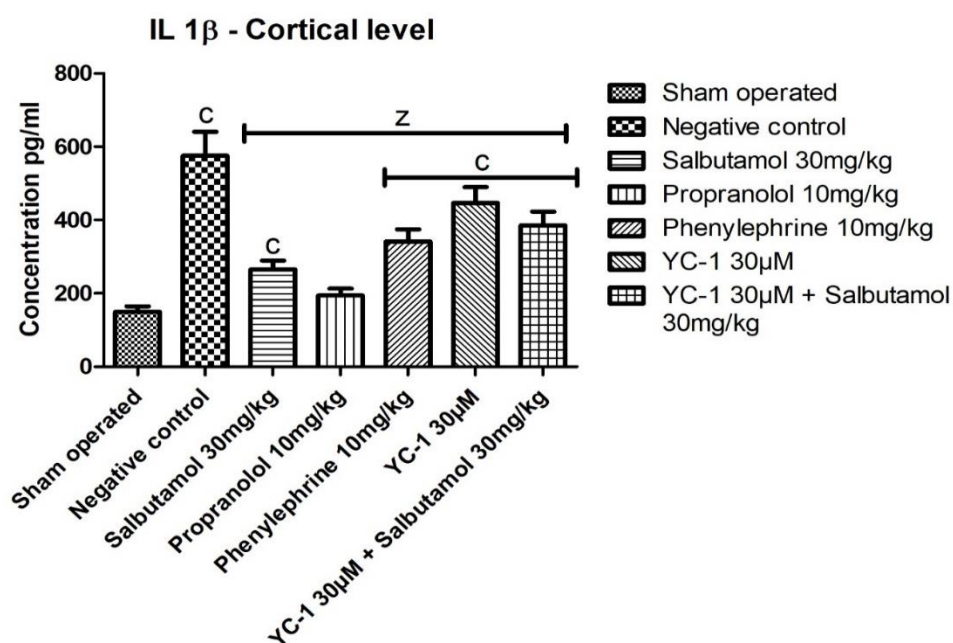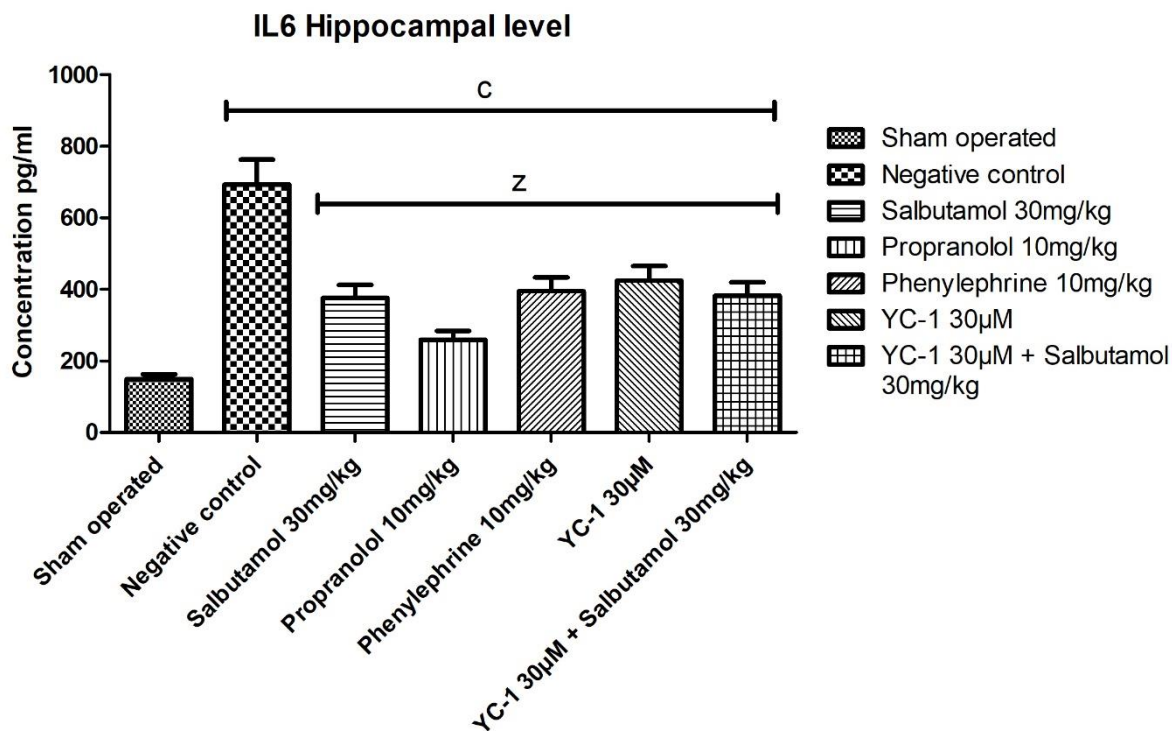

Fig 21a: IL1β cortical level
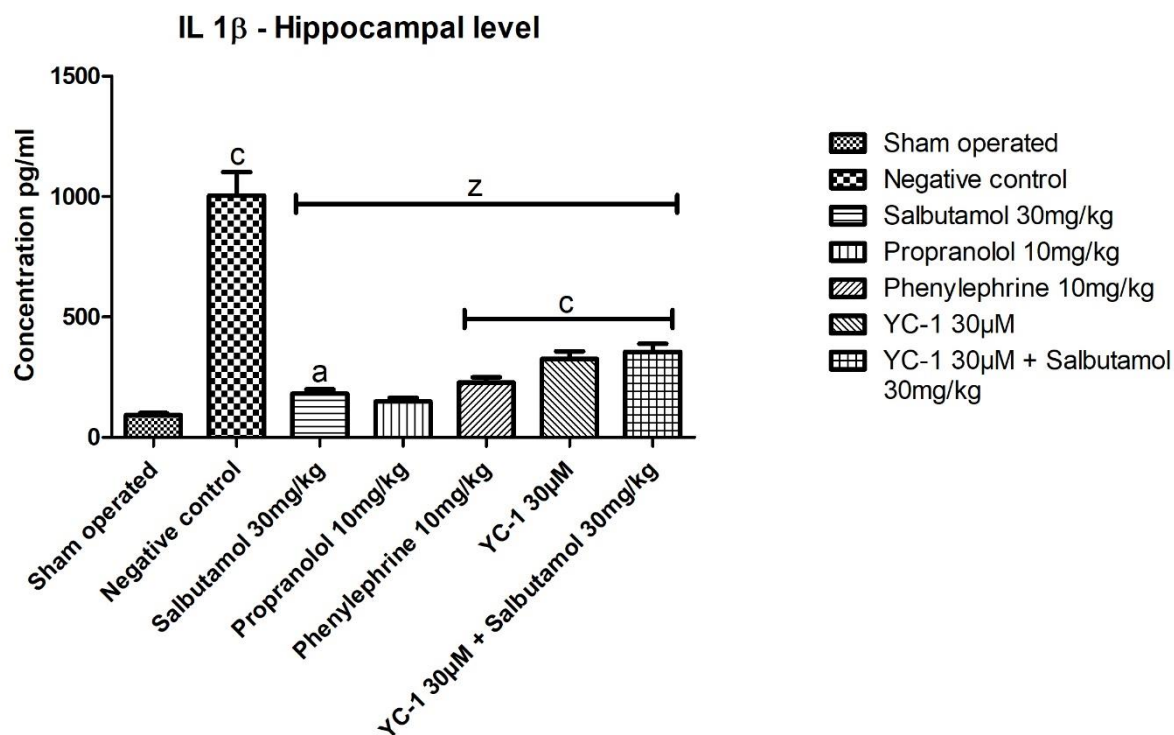
Fig 21b: IL1β Hippocampal level
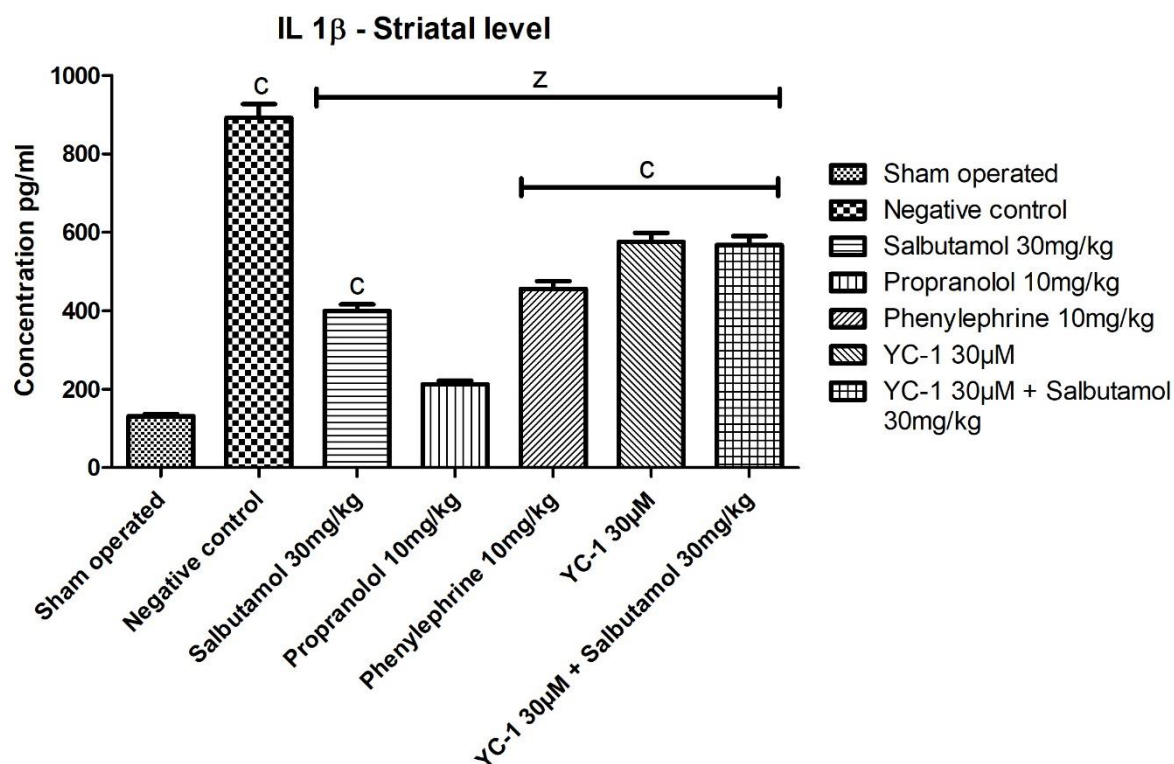
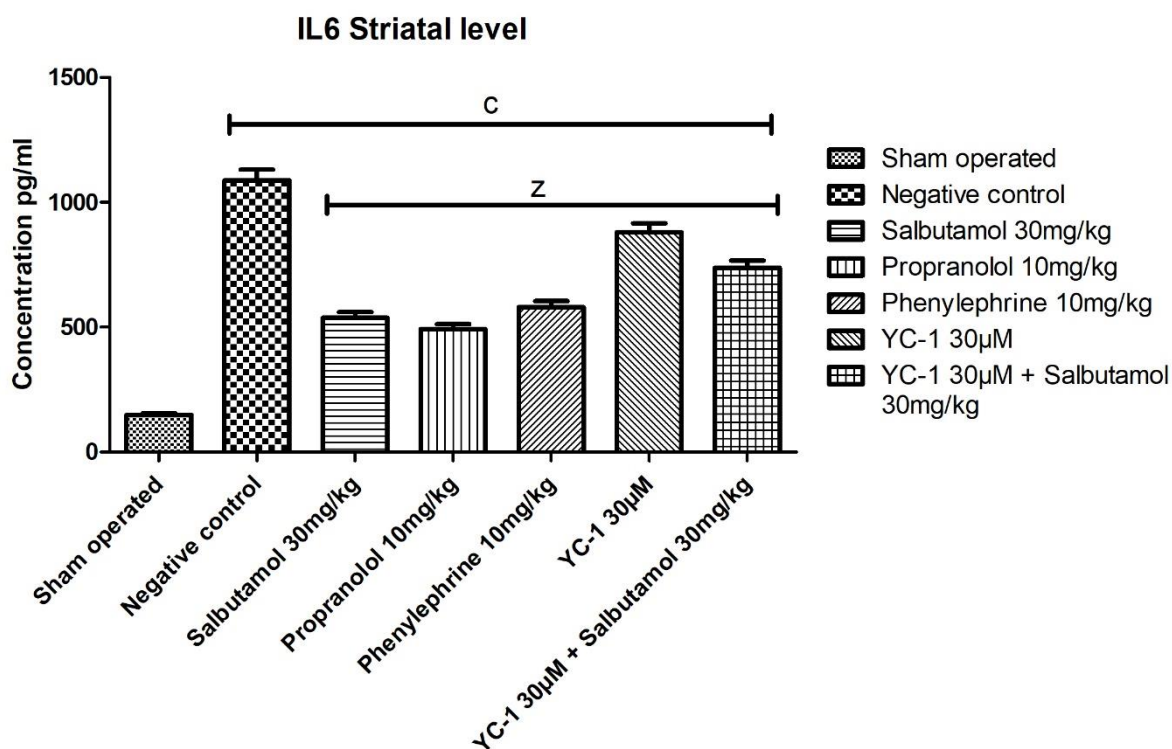**Fig 21c:** IL1β striatal level

Fig 21a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on IL1β levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts a, c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.05 and p<0.001 respectively.

6.6.2 Effect of drug treatment on IL6

Induction of cerebral ischemia elevated the pro-inflammatory cytokine IL6 levels in the cortex (p<0.001), hippocampus (p<0.001) and striatum (p<0.001) in comparison to SO rats. Treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg significantly reduced the elevated IL 6 in all the three regions in comparison to vehicle treated ischemic rats (p<0.001). (Figure.22a, b, c)

Among the regions studied striatal IL6 level was significantly elevated upon ischemia induction.

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.

Fig 22a: IL6 cortical level

Fig 22b: IL6 Hippocampal level

Fig 22c: IL6 striatal level

Fig 22a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on IL6 levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts c and z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at $p < 0.001$ respectively.

6.6.3 Effect of drug treatment on TNFα

TNFα cytokine levels was found to be elevated in the cortex ($p < 0.001$), hippocampus ($p < 0.001$) and striatum ($p < 0.001$) region of ischemic rats in comparison to SO rats.

No significant difference in the TNFα was observed with the treatments in the cortex region. Propranolol (10mg/kg) showed significant decrease ($p < 0.01$) in the TNFα level in comparison to vehicle treated ischemic rats. (Figure.23a)

Treatment with salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg and YC-1 30μM + salbutamol 30mg/kg showed significant decrease ($p < 0.001$) in the hippocampal TNFα level was observed in comparison to vehicle treated ischemic rats. (Figure.23b)

TNFα level in the striatum region remains unaltered with different treatments. Propranolol 10mg/kg significantly decreased ($p < 0.05$) the TNFα level in striatum in comparison to vehicle treated ischemic rats. (Figure.23c)

Data were expresses as mean ± SD. Statistical analysis were carried out using one-way ANOVA followed by Bonferroni's multiple comparison test.
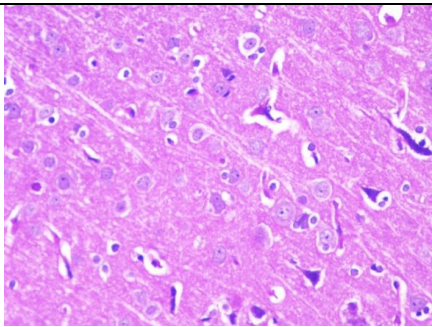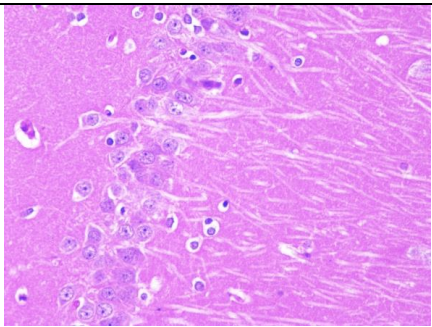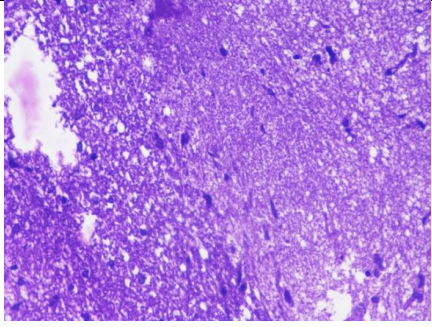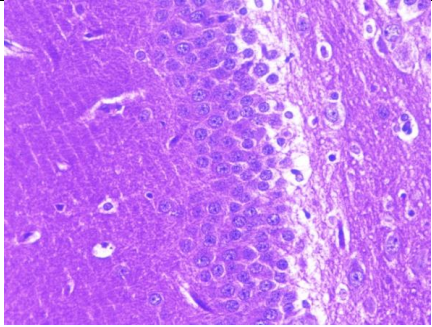
TNF α Cortical level

Fig 23a: TNFα cortical level
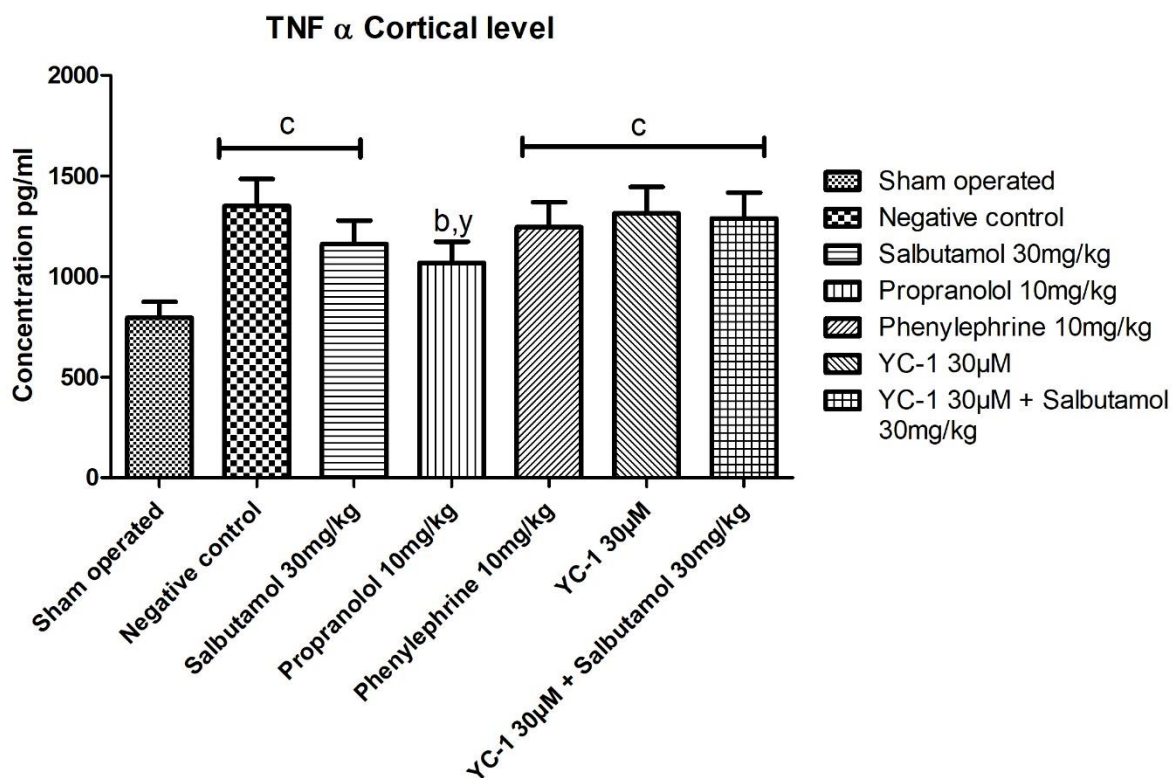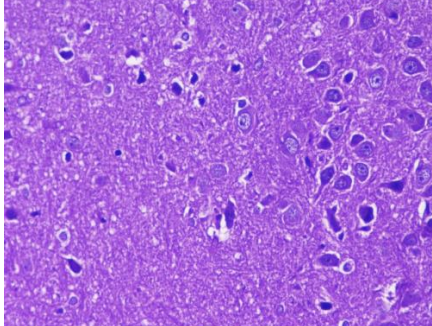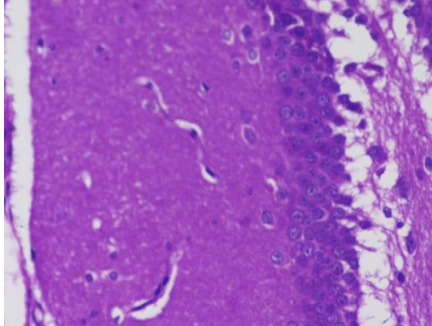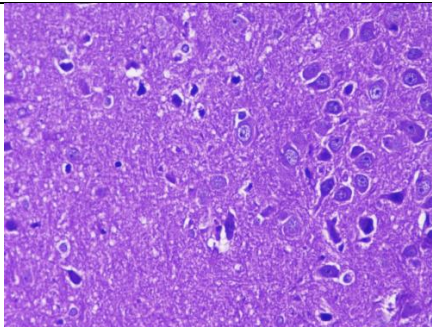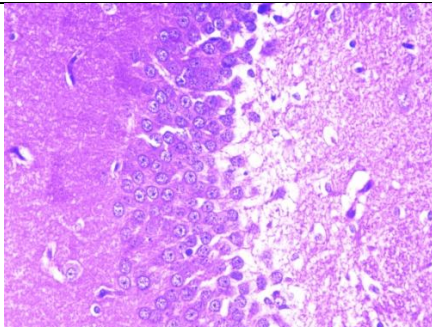
## TNF α Hippocampal level
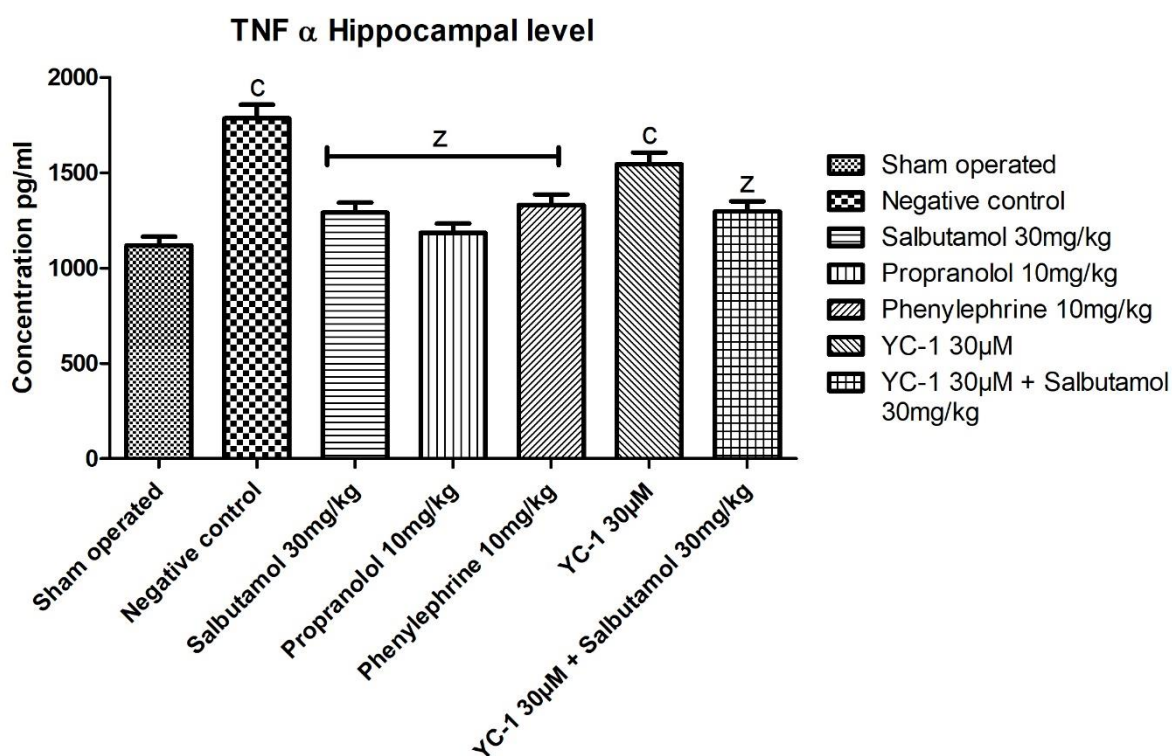
Fig 23b: TNFα Hippocampal level
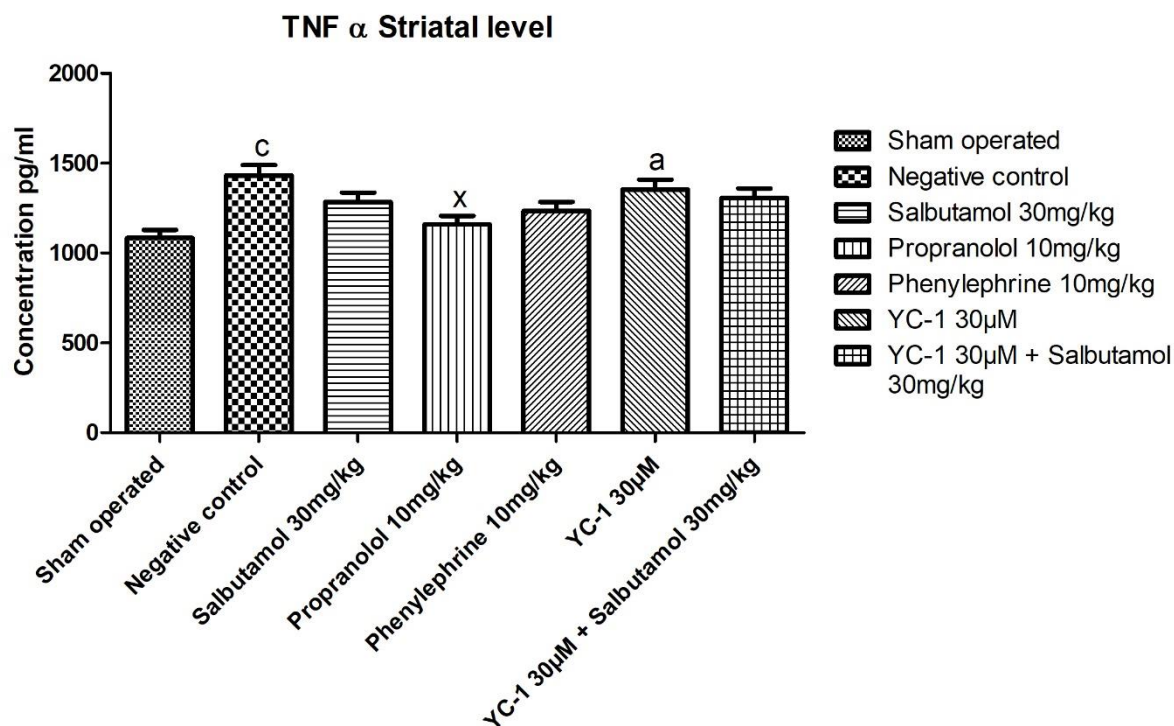
Fig 23c: TNFα striatal level

Fig 23a, b, c: Effect of salbutamol 30mg/kg, propranolol 10mg/kg, phenylephrine 10 mg/kg, YC-1 30μM and YC-1 30μM + salbutamol 30mg/kg on TNFα levels in cortex, hippocampus and striatum regions of global ischemic rats. Data were expressed as mean ± SD (n=3, 6). Superscripts a, b, c and x, y, z denotes statistical significance in comparison to sham-operated and vehicle treated ischemic rats at p<0.05, p<0.01 and p<0.001 respectively.

6.7 Histopathology and Immunohistochemistry
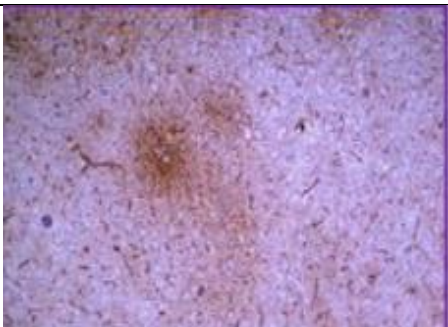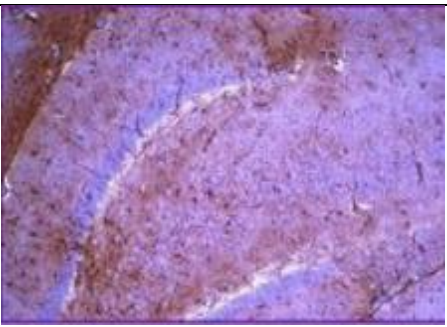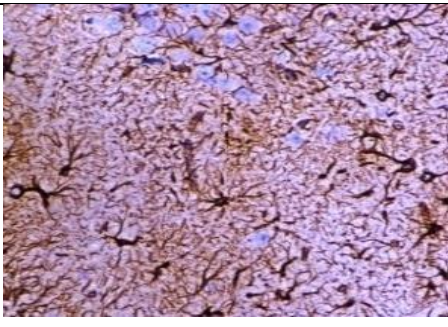
6.7.1 Hematoxylin and Eosin staining
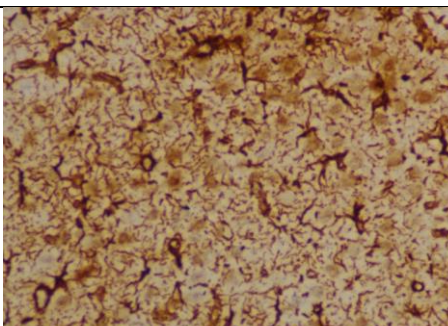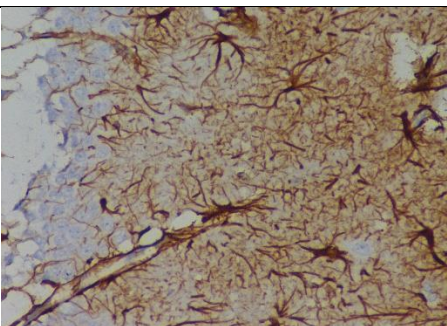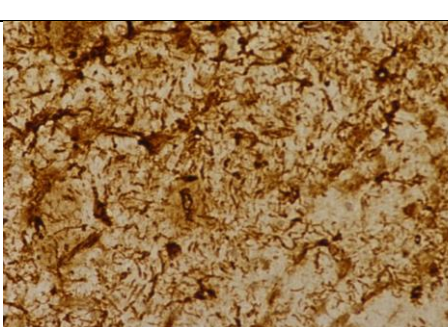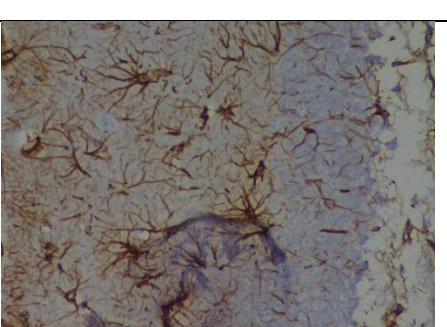
Group	Cerebrum 40x	Hippocampus 40x
Sham operated		
Negative control		
Salbutamol (30mg/kg)		
Propranolol (10mg/kg)		

Phenylephrine (10mg/kg)		
YC-1 (30μM)		
YC-1 (30μM) + Salbutamol (30mg/kg)		
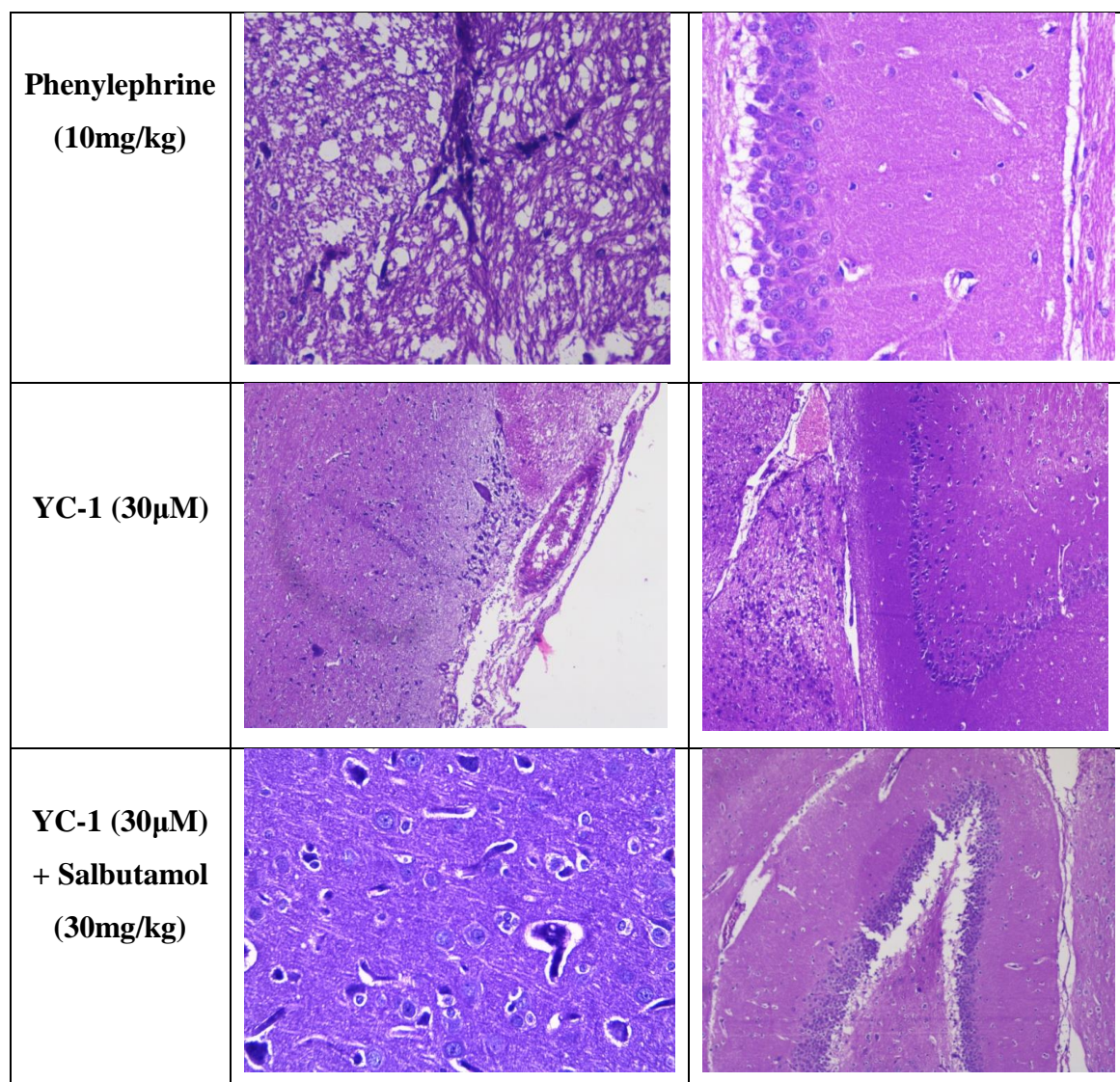
Fig 24: H & E staining

No histopathological changes were found in cerebrum, meninges and cerebellum for sham operated, salbutamol 30mg/kg, propranolol 10mg/kg and YC-1 30μM + salbutamol 30mg/kg treated rats whereas Cerebrum with area of ischemic necrosis and focal shrunken eosinophilic neuron and gliosis was observed in hippocampal CA1 region vehicle treated ischemic rats, phenylephrine 10mg/kg and YC-1 30μM treated rats.

6.7.2 Glial fibrillary acidic protein (GFAP)
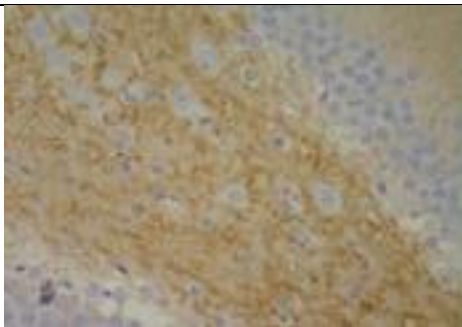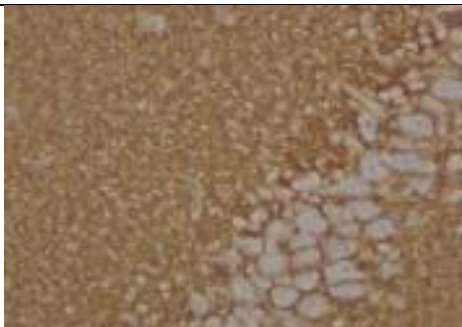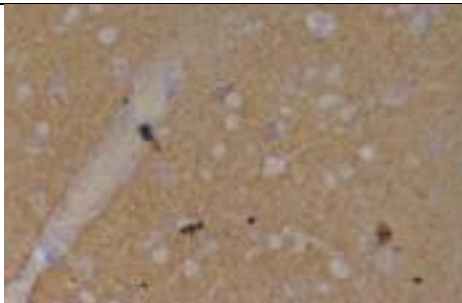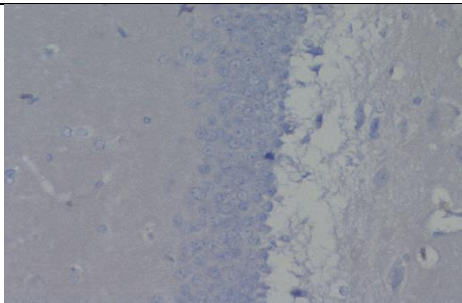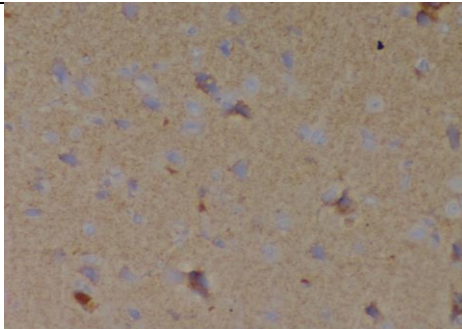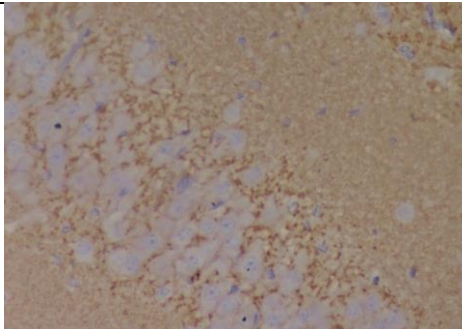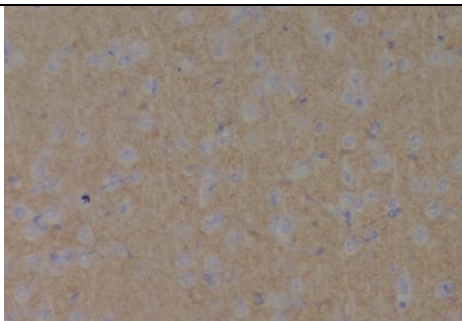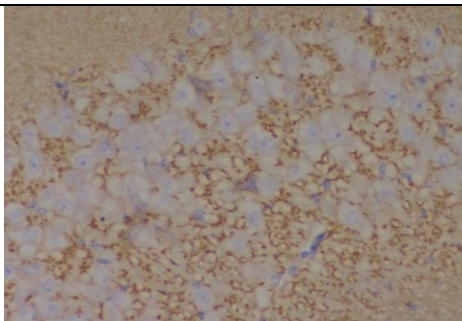
Group	Cerebrum 40x	Hippocampus 40x
Sham operated		
Negative control		
Salbutamol (30mg/kg)		
Propranolol (10mg/kg)		

Phenylephrine (10mg/kg)		
YC-1 (30µM)		
YC-1 (30µM) + Salbutamol (30mg/kg)		
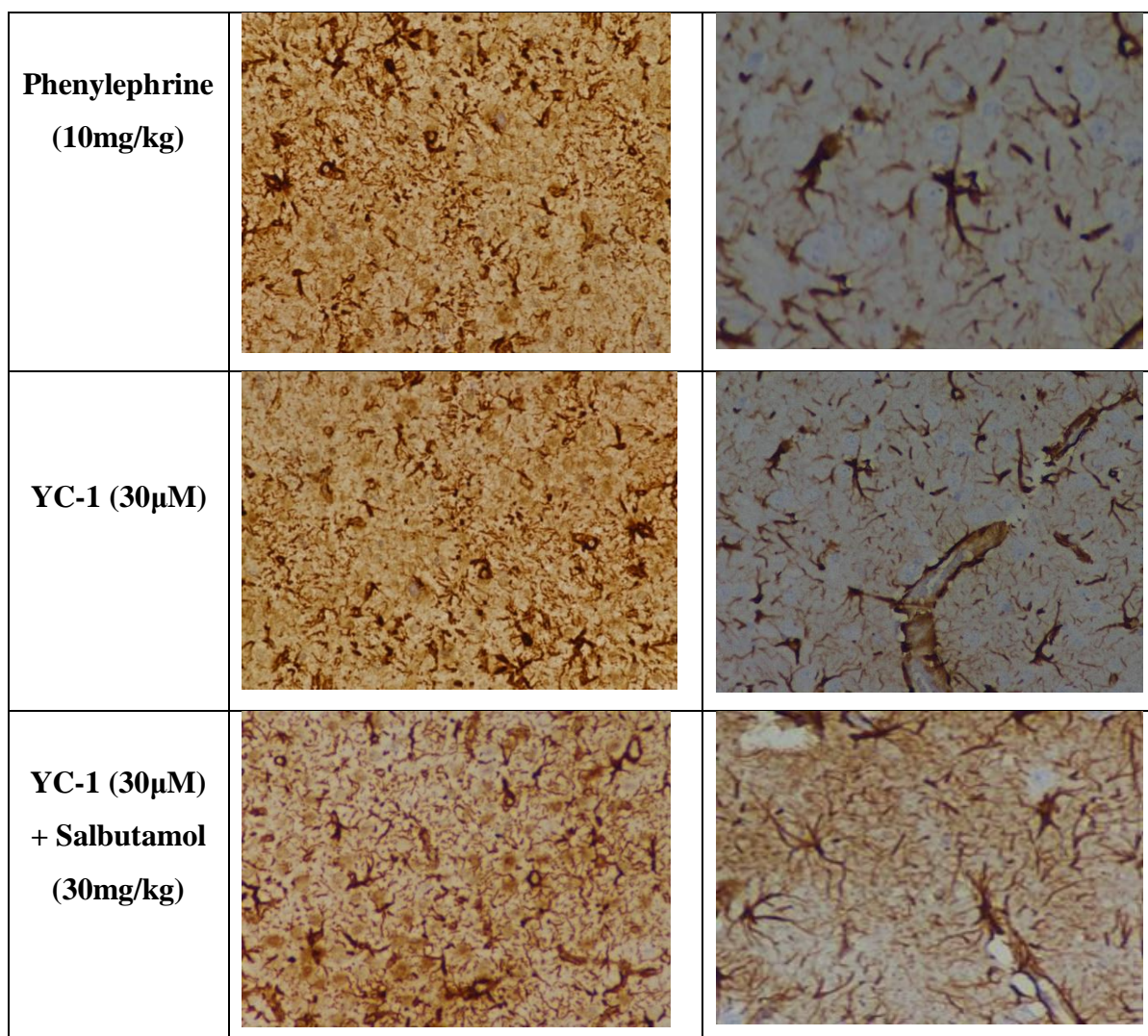
Fig 24: GFAP

The expression of GFAP was found to be increased in ischemic rats, indicating the reactive changes of glial cells in response to damage. Salbutamol 30mg/kg, propranolol 10mg/kg and YC-1 30µM + salbutamol 30mg/kg treated rats showed increased expression of GFAP which was indicated by brown colour.

6.7.3 Synaptophysin IHC

Group	Cerebrum 40x	Hippocampus 40x
Sham operated		
Negative control		
Salbutamol (30mg/kg)		
Propranolol (10mg/kg)		

Phenylephrine (10mg/kg)		
YC-1 (30µM)		
YC-1 (30µM) + Salbutamol (30mg/kg)		
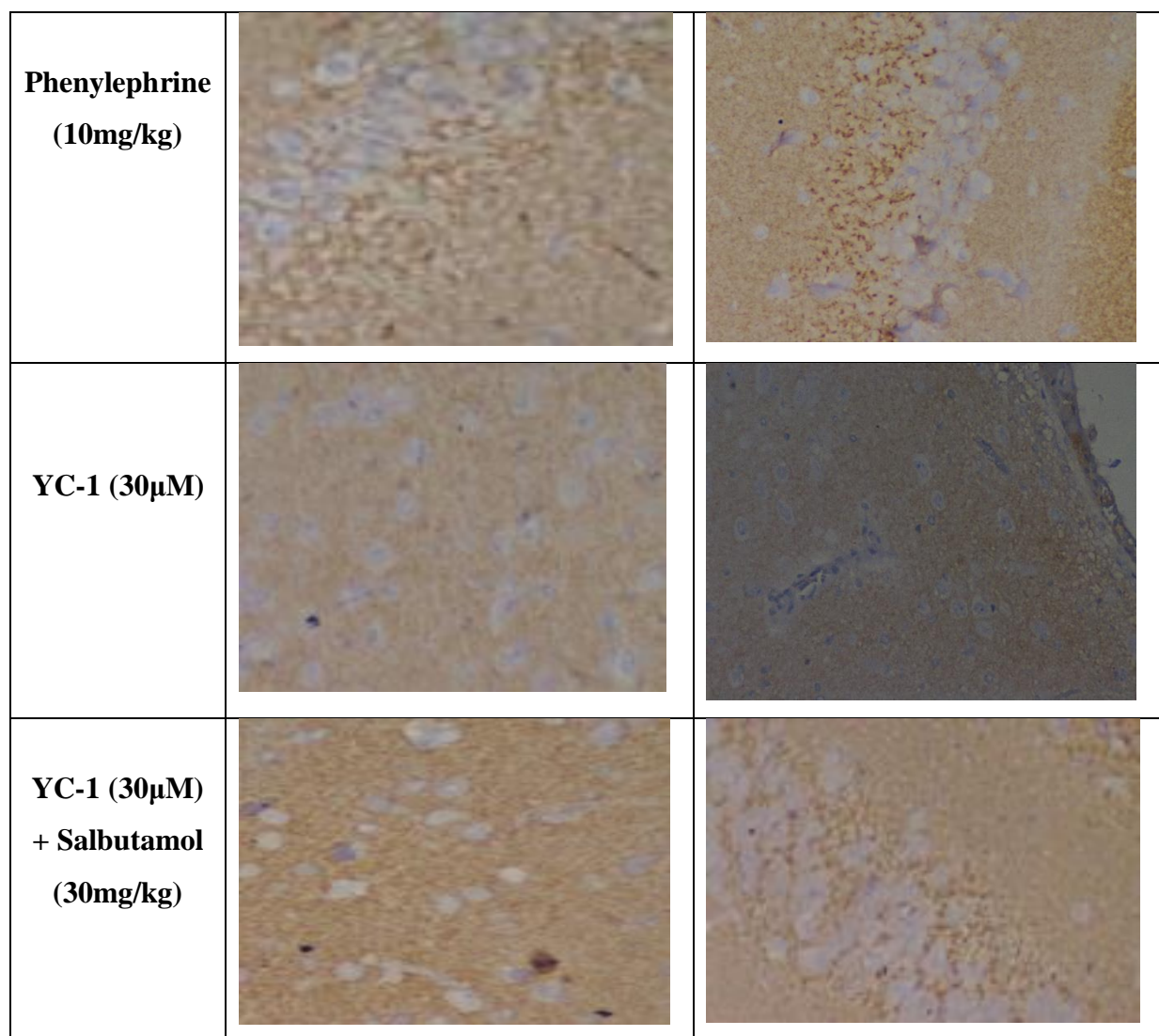
Fig 24: Synaptophysin IHC

The onset of ischemia is evidenced by the loss of synaptophysin protein expression in the hippocampal region of the brain which is observed by the loss of brown colour stain. Salbutamol 30mg/kg, propranolol 10mg/kg and YC-1 30µM + salbutamol 30mg/kg treated rats showed expression of synaptophysin which was indicates the restoration of synapse integrity.

7. Discussion

The study was designed to address the role of β2 adrenergic receptor agonist and antagonist on neuroprotection and its interaction with HIF1α antagonist (YC-1). β2 adrenergic receptors are widely expressed in hippocampus regions and helps in synaptic plasticity and neurogenesis. Salbutamol is a selective β2 adrenergic receptor agonist whereas propranolol is a non-selective β2 adrenergic receptor antagonist which has selectivity towards both β1 & β2 receptors. β2 AR are GPCR receptors coupled to Gαs which when stimulated activates PKA. PKA in turn stimulates MAPK pathways.

Literature studies states that β2 AR has multiple pathway activation related to dose concentration. β2 AR agonist leads to activation of two different pathways depending upon the concentration. β2 AR agonist activates cAMP/PKA which regulates NF-κB activation & MAPK activation and it also can act through activation of β-arrestin. β-arrestin is a binding partner of Iκβ which in turn inhibits NF-κB activation. Low doses of β2 AR agonist are found to activate β-arrestin and found to reduce the production of p38, JNK, NO thereby aiding in neuroprotection. Conversely, at high doses, β2 AR agonist is found to upregulate cAMP, and may decrease the neuroprotection by increased NADPH oxidase activity **[45].** These literatures supported with our results representing that salbutamol 30mg/kg showed significant increase in neuroprotection by reducing NO level, lipid peroxidation level and increased GSH and ATP levels which aided in neuroprotection.

β2 AR antagonist was found to downregulate HIF1α and helped in neuroprotection in OGD induced SHSY5Y cells whereas β2 AR agonist was found to upregulate HIF1α. β2 AR antagonist was also found to increase neuroprotection by inhibiting cAMP mediated effects **[45].** These studies support our results indicating that propranolol showed significant neuroprotection by restoration of cerebral blood flow and downregulation of NO and TBARs level.

Phenylephrine 10mg/kg (α1 AR agonist) treatment was done to confirm that there is no involvement of the α1 receptor on neuroprotection, which can be seen from the results that due to its vasoconstrictive property, the cerebral blood flow in the region was minimal which might be aggravated to ischemia. This effect of phenylephrine could see in the decreased muscle coordination and cognitive dysfunction and also the restoration of ATP levels was low when compared to salbutamol and propranolol treated groups. It clearly indicated that the neuroprotective response is more β receptor mediated.

In combination treatment of salbutamol with YC-1 (HIF1α antagonist) (pre-treatment) showed synergistic effect on restoring cognitive function, cerebral blood flow and attenuation of pro-inflammatory cytokines.

In the present study BCCAo resulted in degeneration of the different brain regions due to ATP depletion, biochemical alterations resulting in behavioural dysfunction indicates neurodegeneration. The alterations in the behavioural parameters in the ischemic animals indicate an elevation in EAA like glutamate [64]. Body weight was measured before and after surgery. The drug treatment did not show difference in the body weight between the treatment groups. In the present study, memory impairment was assessed using transfer latency as a function of inflexion ratio in an elevated plus-maze. The higher inflexion ratio indicates the nootropic effect of the drug [57]. The memory loss would have occurred due to a low level of blood supply to the brain, which results in neuronal damage, loss in synaptic integrity and plasticity in the hippocampal region [65]. Loss of muscle strength and function is a severe pathology in post-ischemic conditions [55]. Grip strength is an essential test to evaluate the neuromuscular function in a diseased condition such as cerebral ischemia. Grip strength of ischemic rats was found to be low which was progressively improved upon treatment.

The reduction of blood supply could have contributed towards the neurodegeneration induced by ischemia. The reduced blood supply could be the cause of ischemic neuronal death resulting from the formation of ischemic core infarct regions [66, 67]. Studies showed that BCCAo model produces 32% reduction in the cerebral blood flow in hippocampus and 21% in the cortex [65]. In the present study, decreased CBF along with loss of grip strength and memory deficit was observed indicating a successful inducement of cerebral ischemia in rats.

During cerebral ischemia, neurons are exposed to hypoxia, reduced mitochondrial function, increased intracellular cytosolic calcium level (Ca^{2+}), excitatory amino acids and toxic free radicals [68, 69]. The depletion of GSH could be because of the oxidative stress induced by cerebral ischemic induction because earlier studies have suggested the release of free radicals during ischemic insult could deplete the GSH content in neuronal cells [70]. The released glutathione acts as an anti-oxidant and protects the cell from radical mediated cell injury which was observed due to decreased energy utilisation of mitochondrial hypoxic status. In this condition cells will undergo necrosis rather than apoptosis, resulting in more inflammatory mediator response. Interestingly, treatment with salbutamol, propranolol,

phenylephrine significantly increased the GSH levels in the cortex, hippocampus and striatum suggesting that these drugs could antagonize ischemia-induced oxidative stress and also reduced the elevation of NO and TBARs in the penumbra region, further supporting the attenuation of oxidative stress. NO, a major free radical has been widely reported during ischemia. The initial phase of release from eNOS is helpful in vasodilation and can control inflammation. The NO release through iNOS can precipitate inflammation.

Further, current experimental findings have shown the role of inflammatory events in ischemia as indicated by increased levels of pro-inflammatory cytokines like IL1β, IL6 and TNFα in cortex, hippocampus and striatum regions of ischemic rats **[54].** Pro-inflammatory cytokines (IL1β, IL6, TNFα) released during ischemic phase in the neuronal cells exhibit various effects on neurons, microglia and astrocytes **[71]** leading to inflammation and reduced glutamate clearance in the extracellular space due to impaired glutamate transporter activity **[72].** Our results showed that post-ischemic condition enhanced the expression of pro-inflammatory cytokines which was downregulated upon treatment showing their role in neuro-inflammation in cerebral ischemic condition.

Neuroprotective effect of drugs was assessed by histopathology and immune-histochemistry using H&E staining, GFAP and synaptophysin IHC. The vehicle treated ischemic rats showed loss of expression of GFAP and synaptophysin and the synaptophysin expression was significantly increased in salbutamol, propranolol and YC-1 + salbutamol treated rats indicating its neuroprotective property.

Further studies have to be conducted on certain pathways β-arrestin/ NFkβ, cAMP/PKA/CREB, cAMP/PKA/NFkβ, neurotrophic factors levels and HIF1α regulation during ischemic insults which might help in providing a clear insight on neuroprotection. This elucidation can help in treating ischemic related stroke which in turn helps protect neurons in VaD.

Hence, our current study clearly reveals the neuroprotective effect of salbutamol and propranolol and its synergistic effect in combination treatment with YC-1 during ischemic insult in cerebral hypoperfusion model. The study also states that the molecular mechanistic pathways have to be further elucidated with different set of proteins to get a clear insight on the neuroprotective molecular mechanism.

8. Conclusion

The present study identified that salbutamol and propranolol showed significant neuroprotection by restoring muscle coordination, cerebral blood flow and downregulating nitrite level, lipid peroxidation and pro-inflammatory cytokines levels and also by upregulating the GSH and ATP levels in cortex, hippocampus and striatum regions of ischemic rats. Combination of salbutamol and YC-1 showed synergistic effect on salbutamol neuroprotection.

Hence, our current study clearly reveals the neuroprotective effect of salbutamol and propranolol and its synergistic effect in combination treatment with YC-1 during ischemic insult in cerebral hypoperfusion model. The study also states that the molecular mechanistic pathways have to be further elucidated with different set of proteins to get a clear insight on the neuroprotective molecular mechanism.

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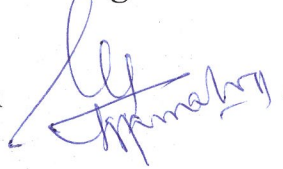
This is to certify that the project proposal no. **464/IAEC/2020** entitled **"*Effect of β2 adrenergic & HIF 1α antagonist on pericyte induced neuroprotection in cerebral hypoperfusion model.*"** submitted by **Mr.A.Karthik Aravinda Rajan & Mr. B. Naveen Raj** has been approved/recommended by the IAEC of PSGIMS&R (Organization) in its meeting dated **03.09.2020** and **200-250g, 6 months old, 36 Male SD rats** (No. of animals) have been sanctioned under this proposal for a duration of next 12 Months.

Authorized by	Name	Signature	Date
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Member Secretary:	Dr.N.Ramanujam		03.09.2020
Main Nominee of CPCSEA:	Dr.C.Kathirvelan		03.09.2020

The Chair Person, CPCSEA
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Dr. N. RAMANUJAM,
MEMBER - SECRETARY,
PSG-INSTITUTIONAL ANIMAL ETHICS
COMMITTEE (PSG-IAEC)

Main Nominee, CPCSEA
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(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

PP 28

Effect of echinacoside in thrombotic ischemic model in rats

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Focal cerebral ischemia occurs when a blood clot has blocked a cerebral vessel, where it reduces blood flow to the particular region, increases the risk of cell death in that area. Echinacoside is a phenylethanoid glycoside that is isolated and purified from stems of *Cistanche salsa*, a parasitic plant native to Northwest China that possesses bioactivities such as antioxidant, anti-inflammation, anticancer, hepatoprotective and neuroprotective effects. *Centella asiatica* (Linn.) Urban sys. commonly known as Indian pennywort, belongs to the family Apiaceae (Umbelliferae), contains Asiatic side, centelloside, madecassoside, Asiatic acid and possesses various activities like wound healing, cytotoxic and antitumor, memory enhancing, cardioprotective, immunomodulatory. The current study is to assess the neuroprotective effect of echinacoside and fractionated asiaticoside enriched extract in a thrombotic ischemic model. The neuroprotective effect of echinacoside and asiaticoside enriched extract was compared with streptokinase. The parameters like body weight, cerebral blood flow, neurologic deficit score, grip strength, inflammatory mediators, excitatory and inhibitory amino acid levels were measured to elucidate the possible mechanism of the selected phytochemicals in focal ischemic damage. The H&E and GFAP images showed normal histology in the CA1 region of the hippocampus treated with asiaticoside 200mg/kg and echinacoside 5mg/kg. The results show that after 7 days of treatment of echinacoside and asiaticoside enriched extract cerebral blood flow was restored and it was evident through flux mean measurement. Similarly, muscle coordination was also found to be recovered with 7 days of drug treatment. The inflammatory markers TNFα, IL 1β, IL 6 levels were found to be significantly decreased which indicates echinacoside has more efficacious to control inflammation than asiaticoside enriched extract. The

excitatory amino acid levels were found to be decreased confirms echinacoside had better neuroprotective activity. Expression of synaptophysin indicates treatment of echinacoside and asiaticoside enriched extract restores synaptic integrity with 7 days of drug treatment. To conclude, treatment with echinacoside protects the brain from ischemic degeneration through restoration of cerebral blood flow and controls the levels of cytokines indicating its neuroprotective activity.

Keywords: Centella asiatica, Stroke, Cytokines, Excitotoxicity, Asiaticoside, Neurodegeneration

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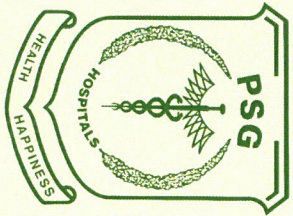
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