Investigating the Molecular Mechanisms Involved in Neuroprotective Effect of Remote Limb Ischemic Post Conditioning against Cerebral Ischemic Reperfusion Injury and Associated Cognitive Deficits

THESIS

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By
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CERTIFICATE

This is to certify that the thesis entitled "**Investigating the Molecular Mechanisms Involved in Neuroprotective Effect of Remote Limb Ischemic Post Conditioning against Cerebral Ischemic Reperfusion Injury and Associated Cognitive Deficits**" and submitted by **SRUTHI RAMAGIRI** ID No. **2013PHXF0405P** for award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

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THIS THESIS IS AFFECTIONALTELY
DEDICATED TO MY PARENTS

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List of abbreviations

ATP   : Adenosine Tri Phosphate

BCCAO   : Bilateral Common Carotid Artery Occlusion

BDNF   : Brain Derived Neurotrophic Factor

BSA   : Bovine Serum Albumin

CA1   : Cornus Ammonis 1

CREB   : cyclic AMP response Element Binding Protein

CNS   : Central Nervous System

DMSO   : Dimethyl Sulfoxide

ELISA   : Enzyme Linked Immunosorbent Assay

EPM   : Elevated Plus Maze

FDA   : Food and Drug Administration

GSK-3β   : Glycogen Synthase Kinase-3β

HAT   : Histone Acetyl Transferase

HDAC   : Histone Deacetylases

HO-1   : Heme Oxygenase-1

I/R   : Ischemic Reperfusion

ICV   : Intra Cerebro Ventricular

IL-6   : InterLeukin-6

IPC   : Ischemic Pre-Conditioning

IPOC   : Ischemic Post Conditioning

MCAO : Middle Cerebral Artery Occlusion

mPTP : Mitochondrial Permeability Transition Pore

NAD : Nicotinamide Dinucleotide

NAMPT : Nicotinamide Phosphoribosyl Transferase

NMDA : N-Methyl-D-Aspartate receptor

NR2B : NMDA Receptor subunit 2B

PA : Passive Avoidance

PI3K-Akt : Phosphatidylinositol 3-Kinase

RIPC : Remote Ischemic Pre-Conditioning

RIPOC : Remote Ischemic Post Conditioning

RIPEC : Remote Ischemic Per Conditioning

ROS : Reactive Oxygen Species

SIRT1 : Sirtuins 1

tPA : Tissue Plasminogen Activator

TNF-α : Tumor Necrosis Factor α

TUNEL : Terminal deoxynucleotidyl transferase dUTP nick end labeling

WHO : World Health Organisation

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Abstract

According to World Health Organisation (WHO), vascular disorders accounts for the major cause of the death worldwide, where stroke ranks second position in frequency and importance as the leading cause of mortality and associated inabilities like speech, anxiety, depression and cognition deficits. Following an acute ischemic attack, early and successful cerebral reperfusion with the use of thrombolytics/mechanical endovascular procedures is the available strategy to reduce the pathological outcomes. However, the process of restoring blood flow to ischemic brain tissue may induce reperfusion injury i.e. cerebral ischemic reperfusion (I/R) injury. Although the molecular mechanisms of cerebral I/R injury are not clear, the depletion of energy during ischemia and the induction of oxidative stress during reperfusion have been reported to initiate a cascade of energy failure/deficit, ionic imbalance, calcium overload, excitotoxicity, increased neuroinflammation, mitochondrial dysfunction and apoptosis that lead to cell death followed by severe organ failure. Moreover, all these processes may concurrently lead to significant changes in gene expression leading to epigenetic modifications.

The thrombolytic, tissue Plasminogen Activator (tPA) is the only Food and Drug Administration (FDA) drug with a narrow therapeutic window of 4-6 hrs and may result in serious side effects like intracerebral hemorrhage. In decades of past research on cerebral I/R injury, numerous preclinical investigations with promising results, could not provide any effective treatment in clinical trials. Thus, there is a pressing need to develop effective treatment strategy with fruitful clinical translation.

The ongoing research is therefore directed towards retrieving the underlying endogenous mechanisms to induce ischemic tolerance. Ischemic Preconditioning (IPC) refers to non-injurious cycles of ischemia and reperfusion applied to vital arteries to provide protection against prolonged I/R injury. Moreover, ischemic Postconditioning (IPOC) offers an advantage over IPC as it can be applied after prolonged I/R injury. However, to overcome the limitation of mechanical intervention of vital arteries by IPC and IPOC, remote ischemic post conditioning (RIPOC) where non-injurious cycles of ischemia and reperfusion applied to non-vital arteries was developed. Some earlier studies revealed the potential of RIPOC, however a complete

understanding of its mechanisms has remained elusive. Therefore, we hypothesize to explore the molecular mechanisms involved in RIPOC protection against cerebral I/R injury and associated cognitive deficits.

Cerebral I/R injury can be induced by focal and global model of cerebral ischemia. In this study, focal model of cerebral I/R injury with 90 min of ischemia followed by 24 hrs reperfusion was validated using laser doppler technique. We observed that induction of focal I/R injury by middle cerebral artery occlusion (MCAO) resulted in significant neurological deficits, an elevation in oxido-nitrosative markers and reduction in anti-oxidants like GSH and SOD. Moreover, we observed an increase in neuroinflammatory cytokine levels like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) along with increased cerebral infarction and neuronal damage as evidenced in TTC and HE staining respectively. However, focal I/R injury induced sensory and motor abnormality that may interfere with cognitive assessment.

Therefore, we standardised global I/R injury model with increasing ischemic duration and constant reperfusion of 72 hours. We found that 20 min of ischemia followed by 72 hours of reperfusion provided consistent neuronal damage as evidenced by elevated oxidative stress, neuroinflammation, significant neuronal cell loss in hippocampal CA1. Further, cerebral I/R injury resulted in cognitive dysfunction as assessed by elevated plus maze (EPM), y-maze task and passive avoidance following global I/R injury. Therefore, we preferred this model for further investigation as this study was focused to assess cerebral I/R injury and its associated cognitive dysfunction. Likewise, during RIPOC standardisation we found that RIPOC induced by non-invasive bilateral femoral artery occlusion with 3 cycles of 10 min I/R at early onset of reperfusion was highly effective against cerebral I/R injury.

There is growing evidence that cerebral I/R injury induced oxidative stress and neuroinflammation may result in excitotoxicity via prolonged activation of NR2B receptors to promote cell death. To explore the potential of RIPOC in cerebral I/R injury induced excitotoxicity, we administered NR2B agonist, Quinolinic acid (QA) prior to RIPOC intervention. We found that the protective effects of RIPOC were abolished by QA as evidenced by elevated oxidative stress, neuroinflammation, pyknotic neuronal count in hippocampus along with poor performance in cognitive parameters that indicates RIPOC mediates protection via NR2B

inhibition. This was supported by protective effects of NR2B antagonist, Ifenprodil treatment against cerebral I/R injury and associated cognitive deficits.

NR2B-R mediates pro-survival or pro-death signals by posttranslational modification such as phosphorylation/dephosphorylation of survival kinases. Moreover, dysregulation of PI3K-Akt (survival kinase) is implicated in various neurological disorders. Therefore, to investigate the downstream regulators involved in protective effect of NR2B inhibition, we treated rats with PI3K-Akt inhibitor, LY294002 prior to Ifenprodil treatment. The protective effects of Ifenprodil in reducing oxidative stress, neuroinflammation and improving cognition and healthy neuronal count in hippocampus were abolished in presence of LY294002.This indicates that NR2B inhibition requires PI3K-Akt activation to mediate its neuroprotection. Strikingly, we found that RIPOC protection is abolished by LY294002, this indicates that RIPOC mediates neuroprotection by NR2B inhibition and subsequent activation of PI3K-Akt. Glycogen synthase kinase-3β (GSK-3β) and hemeoxygenase-1 (HO-1) were emerged as integration points of many survival pathways to mediate its protective signals. Growing experimental evidence supports the importance of GSK-3β and HO-1 in pathogenesis of neuro-degenerative diseases. In this study, we found RIPOC inhibit GSK-3β expression and increased the expression of HO-1, cAMP response element binding protein (CREB) and brain derived neurotrophic factor (BDNF), while this was abolished in presence of LY294002. This indicates that RIPOC protection is mediated via PI3K/Akt/GSK-3β and PI3K/Akt/HO-1 pathways.

Given the diversity of upstream pathways involved in disease progression of cerebral I/R injury, they integrate and may convey death signal to the end-effector mitochondrial permeability transition pore (mPTP). To confirm this, we administered mPTP opener, atractyloside prior to RIPOC intervention. We observed that the RIPOC protection was abolished in presence of atractyloside as indicated by increased oxidative stress and neuroinflammation along with up-regulation of apoptotic marker cytochrome c and neuronal cell death as assessed by TUNEL staining.

The pathological events during cerebral I/R injury including oxidative stress, neuroinflammation, excitotoxicity and apoptosis may lead to epigenetic modifications particularly histone acetylation. In this study, we observed a reduction in global histone H3

acetylation following cerebral I/R injury, while RIPOC intervention improved global histone H3 acetylation levels. Histone acetylation is a dynamic process maintained by histone acetyltransferases (HAT)s and histone deacetylases (HDAC) enzymes. Recent studies reveal the involvement of nicotinamide adenine dinucleotide (NAD) and its dependent class III HDAC particularly sirtuins 1 (SIRT1) in neurological disorders. Moreover, Nicotinamide phosphoribosyl transferase (NAMPT) is the rate limiting enzyme in NAD synthesis, therefore, SIRT1 activity may be dependent on NAMPT expression. Therefore, we further study to explore the role of NAMPT/NAD/SIRT1 pathway in cerebral I/R injury and RIPOC protection.

We observed that RIPOC protection was abolished by NAMPT inhibitor, FK866 as evidenced by increased oxidative stress, impaired learning and memory along with hippocampal CA1 damage, this indicates that RIPOC mediates protection via NAMPT activation. In addition, RIPOC reduced NAD and SIRT1, and concurrently attenuated CREB and BDNF levels in presence of FK866, this suggested the role of NAMPT mediated NAD and SIRT1 activation in RIPOC neuroprotection. Further, to support role of NAMPT activation mediated neuroprotection against cerebral I/R injury and its associated cognitive deficits, we treated I/R injured rats with P7C3-A20 (NAMPT activator) that reduced the oxidative stress, improved the cognitive performance and decreased hippocampal CA1 damage. Moreover, P7C3-A20 treatment significantly improved NAD, SIRT1, CREB and BDNF expression.

Based on the above results, it is understood that SIRT1 activation, induction of HO-1 and inhibition of GSK-3β could be protective against cerebral I/R injury and associated cognitive deficits. We observed two different molecular pathways i.e modulation of epigenetics (SIRT1 activation), and excitotoxicity (GSK-3β inhibition and HO-1 up-regulation) involved in neuroprotection of RIPOC against cerebral I/R injury and associated cognitive deficits. Therefore, we used sub-effective dose combination of resveratrol (SIRT1 activator)+lithium chloride (GSK-3β inhibitor) and resveratrol+hemin (HO-1 agonist) in cerebral I/R injury and associated cognitive deficits. These combinations showed significant reduction in oxidative stress, neuroinflammation and markedly improve the memory parameters and hippocampal healthy neuronal count.

Altogether, the findings of the present study suggest that ischemia induced energy failure by NAD depletion and excitotoxicity by NR2B activation may act as triggers to mediate disease progression by elevated oxidative stress, neuroinflammation and modulation of PI3K-Akt/GSK-3β/HO-1 pathway that may converge in mitochondrial dysfunction by mPTP opening. The pathological events in the ischemic cascade may induce epigenetic modifications via reduced H3 acetylation and NAMPT/NAD/SIRT1 pathway that strongly impact neuro-pathological process of cerebral I/R injury. We observed that RIPOC at early onset of reperfusion altered the activity of various molecular targets involved in trigger, mediator and end-effector pathways to induce neuroprotection against cerebral I/R injury and associated cognitive deficits. Therefore, RIPOC may be developed as therapeutic intervention to treat unpredictable conditions like cerebral I/R injury. Based on analysis of our results, we suggest that NAMPT activators upon reperfusion may produce beneficial effects against cerebral I/R injury and its associated cognitive deficits. Further, sub-effective combination of lithium chloride or hemin along with resveratrol may be used for long term therapy of cerebral I/R injury associated cognitive deficits.

1. Introduction

1.1 *Definition of Stroke:*

Cerebral stroke remains a major leading global health problem and is the fifth leading cause of adult mortality and second largest contributor of adult disability, embracing 7,95, 000 people annually. In United Nations (UN), stroke accounts for every 1 out of 20 deaths. WHO defines Stroke as, "*rapidly developing clinical signs of focal disturbance of cerebral function, with symptoms lasting 24 hours or longer leading to death, with no apparent cause other than of vascular origin*" (Bakhai, 2004; Mukherjee and Patil, 2011; Truelsen et al., 2000). Approximately, 87% of strokes are ischemic stroke (IS), arising due to thromboembolic occlusion of cerebral artery, while the remaining 10% includes hemorrhagic stroke (HS) and 3% Subarachnoid Hemorrhage (SAH). According to Global Burden Disease (GBD) stroke 2013 study, stroke burden increased greater than threefold with 6.4 million deaths and 112 million DALYs (Disability Adjusted Life Years) in developing and high-income countries. Over a span of 23 (1990-2013) years, significant reduction in stroke deaths were noticed in developed countries than developing countries, thus further intensifying the discrepancy of global burden of stroke and reminding the pressing need of attention from government, health insurance providers, private hospital care units and clinicians, as 59% of stroke deaths occur outside hospital care (Feigin et al., 2015)

1.2 *Epidemiology and Prevalence:*

The global epidemiology of stroke was estimated according to malmgren *et al*, wherein the key note was to include both fatal and non –fatal events in hospitalized and non-hospitalized cases. The data of ideal stroke study not only defines the incidence, but also include detailed data on its prevalence, mortality, morbidity, disability and survival. WHO MONICA (Multinational **MONI**toring of trends and determinants in **CA**rdiovascular disease) was initiated in early 1980s to continuously monitor the global burden of myocardial infarction and stroke (Investigators and others, 1988; Thorvaldsen et al., 1995). The WHO MONICA Collaborating Centers (MCC) provided the best knowledge regarding the stroke occurrence and its trends in 11 different countries like China, Denmark, Finland, Germany, Hungary, Italy, Lithuania, Poland,

Russia, Sweden, Yugoslavia. The limitation of the WHO MONICA project study was excluding the age group with higher risk of stroke, (while 35-64 years were considered) and the study was terminated in mid-1990s (Thorvaldsen et al., 1997).

The GBD 2013 study covered a broad spectrum of 23 years (1990-2013) years to provide new data on stroke epidemiology along with daily adjusted life years (**DALY**)s and Years lived With Disability (YLD) for IS and HS at both national (regional) and global levels. This updated report on stroke global burden facilitated us to update our knowledge on stroke epidemiology and to arrange for adequate stroke care units. The study indicated that, there were almost 25.7 million stroke survivors (71% with IS), 6.5 million deaths from stroke (51% died from IS), 113 million DALYs due to stroke (58% due to IS) and 10.3 million of people with new strokes (67% where IS). They also found that death and DALYs due to stroke has increased from 1990 (3.54% (95% UI 3.11–4.00) and 9.66% (95% UI 8.47–10.70), respectively) to 2013 (4.62% (95% UI 4.01–5.30) and 11.75% (95% UI 10.45–13.31), respectively) with a diverging trend in developed and developing countries, i.e., rise in stroke death and DALYs in developing countries while reduction was noticed in developed countries. This study also emphasized the effect of co-morbidities, which have a major impact on health system of stroke community (Feigin et al., 2015). The main drawback of the study is lack of differentiation between first lifetime stroke and recurrent stroke.

The prevalence, incidence and mortality data on stroke has been studied by various organisations at regional level. A study by National Institute of Neurological Disorders and Stroke (NINDS) indicates that stroke symptoms were seen in participants with higher Framingham stroke risk score and in blacks. Further, Framingham study also suggests that in 55-75 years aged population and women (20-21%) are at higher risk of stroke then men (17-18%). An extrapolation to 2030 indicates that an additional 3.4 million population with greater than or equal to 18 years will have stroke, especially in the elderly women. Based on stroke mortality geographical discrepancies arise in US, southeastern US is termed as Stroke belt with higher stroke rate, wherein the coastal region of few countries (North and South Carolina, Georgia) were considered as Stroke Buckle (Ayala et al., 2002; Kelly-Hayes et al., 2003; Thorvaldsen et al., 1997).

1.3   Types of Stroke:

Based on the pathological differences the stroke can be ischemic or hemorrhagic which ultimately leads to disturbance in the normal cerebral blood flow (Fig.1.1).

1.3.1   Ischemic Stroke:

For the Trial of Org 10172 in Acute Stroke Treatment (TOAST), classified the IS into five categories: 1) large-artery atherosclerosis (embolus or thrombus), 2) cardiovascular associated embolism (medium risk or high risk), 3) small-artery occlusion (lacunae), 4) stroke of other determined etiology, and 5) stroke of undetermined etiology (Adams et al., 1993). IS, which accounts for 87% of all strokes, usually results from the cerebral blood vessel obstruction due to development of fatty deposits lining the vessel wall, which is referred as atherosclerosis. Further, the fatty deposits in the vessel wall may result in the formation of thrombus (developed at the clogged part of the cerebral blood vessel) or embolus (generally formed at another part of circulatory system including heart and large arteries of neck and upper chest region) that may finally results in ischemia for whole or part of brain. Embolic IS majorly arises from the cardiovascular disturbances due to irregular heart beat (atrial fibrillation) or cardiac lesions. Lacunar IS are associated with deep infarcts in the small perforating arteries due to unknown pathology (Adams et al., 1993).

1.3.2   Hemorrhagic Stroke (HS):

HS occurs when a weakened cerebral blood vessel ruptures and bleeds that may further result in blood clot compromising the surrounding brain tissue. The blood vessel damage may arise due to aneurysm (can be congenital or developed later in life due to drugs, lifestyle, infection or trauma) or arteriovenous malformations (AVM). Aneurysm usually develop at the weak branching point of the cerebral arteries, due to constant cerebral blood flow that gradually progress into balloon like structure in turn leads to internal hemorrhage. AVM is a cluster of abnormal or tangled blood vessel that diverts the blood from arteries to veins bypassing the normal brain tissue. Based on the location of blood vessel rupture, HS is subcategorized into intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH).
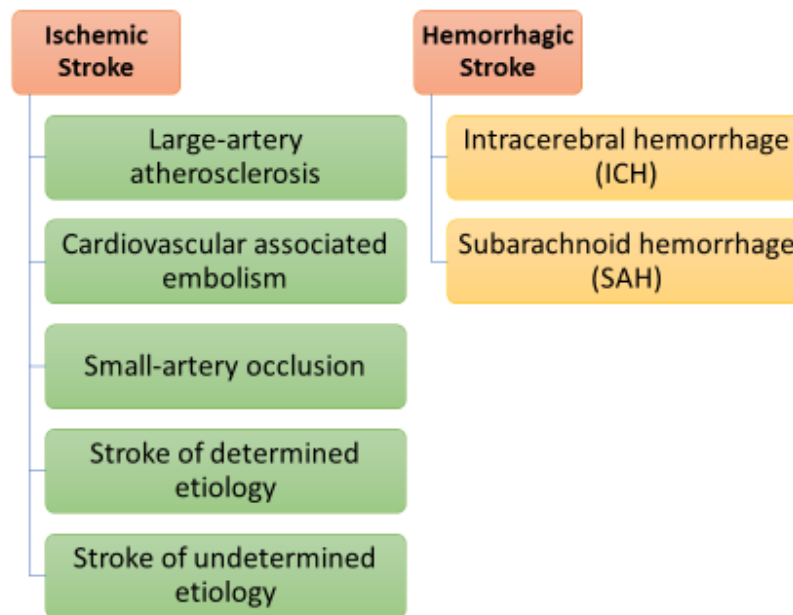
Fig. 1.1. Pictorial representaion of types of stroke

1.4   *Risk factors:*

The most effective way of preventing the stroke burden should involve identification and modification of vascular risk factors in early stages by creating patient awareness and education regarding treatment of stroke and multiple risk factors. To overcome this, Framingham Stroke Risk Profile (FSRK) has been developed, for providing an illustration for patients to understand impact of risk factors and possible therapeutic interventions. The FSRP provides sex specific estimation of the probability of stroke within ten years using clinical information (Romero et al., 2008; Vokó et al., 2004).

Risk factors have been classified as modifiable and non-modifiable risk factors (Fig.1.2). Modifiable risk factors were further classified into conventional and novel. Modifiable conventional risk factors include atrial fibrillation, hypertension, diabetes, hyperlipidemia, obesity, smoking and carotid artery occlusion. Modifiable novel risk factors include hypercoagulable states and hyperhomocysteinemia (H. S. Collaboration and others, 2002). Non-modifiable risk factors include sex, age, race and family history (Romero et al., 2008).
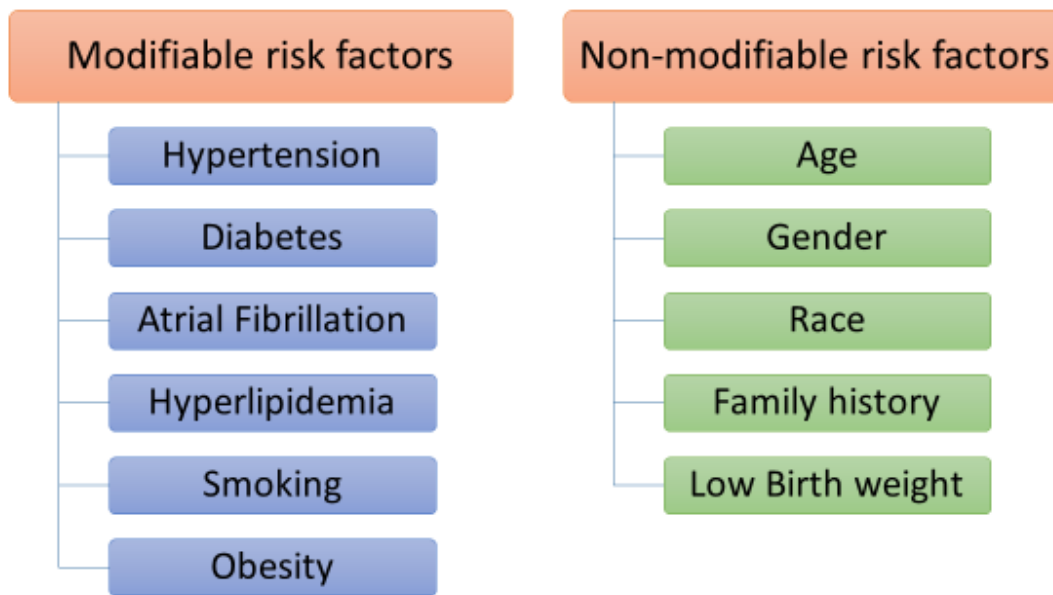
Fig. 1.2. Diagrammatic representation of modifiable and non-modifiable risk factors of stroke

1.4.1   Modifiable risk factors:

Hypertension (HT) emerges as the most prevalent modifiable risk factor from Framingham study. The risk of HT increases with age, elevated systolic and diastolic values. A meta-analysis of one million adults revealed that the risk of stroke increases linearly with systolic blood pressure (P. S. Collaboration and others, 2002). They found an increase in mortality for every 20mm Hg systolic or 10mm Hg diastolic blood pressure increase, while 40% lower risk of mortality would result from 10mm Hg reduction of systolic or 5mm Hg reduction of diastolic blood pressure. Therefore, there is a need to treat HT to reduce the risk of stroke. However, there is no clear idea regarding the best choice of anti-hypertensive intervention. The following studies were conducted to identify the choice of drug Heart Outcomes Prevention Evaluation (HOPE), Losartan Intervention for Endpoint reduction in hypertension (LIFE), Perindopril Protection against recurrent stroke study (PROGRESS), and found that ACE inhibitors or ARB along with diuretics has been demonstrated to be favorable for stroke patients (Gorelick, 2002; Romero et al., 2008).

Currently two on-going trials Secondary Prevention of Small Subcortical Strokes (SPS3) and Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET)

were being performed for assessing the effect of different blood pressures and to establish the drug of choice respectively (Redon et al., 2012).

Diabetes is a known risk factor for stroke, data from earlier studies have demonstrated the association of impaired glucose tolerance or insulin resistance with risk of stroke. Moreover, diabetes worsens the post stroke mortality and outcomes when compared to patients with no diabetes. DREAM trial and UK Prospective Diabetes study could not provide a clear idea on the effect of rosiglitazone and glycemic control on stroke outcome respectively (Investigators and others, 2004). Therefore, future trials like Insulin Resistance Intervention after Stroke (IRIS) were conducted to evaluate the effect of pioglitazone on secondary stroke prevention (Hsieh and Chiou, 2014; van Wijk et al., 2005). However, the drug of choice in insulin resistance induced stroke is not clear

Atrial Fibrillation (AF) is a vital risk factor for stroke with increasing age. European Atrial Fibrillation Trial (EAFT) has shown the benefit of anti-coagulant, warfarin in stroke prevention for patients with AF. Although, warfarin remains a gold standard for stroke prevention in AF patients, but hemorrhagic complications and rigorous monitoring schedule remain as major challenges. Therefore, the clinical research is focused more on novel targets like clotting factor Xa inhibitors, reversible thrombin inhibitor and pharmacogenomic based warfarin dosing (Gage et al., 2001; Grau et al., 2001; Hart et al., 2003).

Prospective studies have demonstrated a close relation between high cholesterol levels and stroke incidence. Randomized trials on statins have concluded that, reduction in stroke is associated with lowering lipid levels (Amarenco et al., 2009, 2002). Smoking is an independent risk factor of stroke with an increase 50% stroke attack proportionally with number of cigarettes per day. Based on randomized clinical trials, Food and Drug Administration (FDA) approved varenicicline for smoking cessation. However, patient education and strict regulatory control of tobacco will have an impact on smoking cessation (Harrison-Woolrych, 2012; Spence, 2010). Obesity is also a major risk factor for stroke and is associated with other metabolic syndromes including HT, hyperlipidemia and diabetes. Current guidelines for stroke prevention in obese individuals include dietary recommendations such as high vegetable and fruit content, low salt and saturated oil content (Romero et al., 2008).

1.4.2   Non-modifiable risk factors:

Traditionally the prevalence of stroke is higher in men than in women in linearity with age. However, recent statistics suggest that in the United States and Europe, 1 in 6 women die out of stroke, while in Spain it is the most frequent cause of mortality, morbidity and severity in women (Phan et al., 2017; Roquer et al., 2003). Till date, the treatment regimen of stroke was unaffected by the gender. According to clinical data based on Acute Stroke Treatment (TOAST) classification, women are more prone to cardio-embolic stroke (Weimar et al., 2002). Although no difference was observed based on gender in anti-coagulation therapy during hospitalization. Therefore, the aim of the preventive strategies to treat stoke in women should streamline towards HT and AF management.

The stroke frequency in blacks is twice when compared to US and UK population. Whereas, UK black and US black population differ in behavioral risk factors for stroke. Another population based study also suggest that the stroke incidence is high in black and Hispanics compared to whites (White et al., 2005). The Greater Cincinnati and Northern Kentucky Stroke Study (GCNKSS) also concluded that blacks are more prone to stroke when compared to white population. The Brain Attack Surveillance in Corpus Christi (BASIC) project found no significant difference in ischemic subtypes among whites and Mexican Americans (Hajat et al., 2001; Kleindorfer et al., 2010). There are significant race-ethnic differences in incidence of ischemic stroke subtypes. The possibility of this discrepancy may be due to variations in genetic make-up and modifiable risk factors.

1.5   *Signs and symptoms of Stroke:*

The well recognized symptoms to identify stroke includes a sudden weakness or numbness of face, arm or leg on one side of the body or sudden confusion or trouble in speech, sudden visual disturbances, sudden motor dysfunction like walking or loss of coordination, dizziness, sudden headache with unknown cause were considered to be the warning symptoms of stroke (Fussman et al., 2010; Mosley et al., 2014).

1.6   *Current Treatment options (reperfusion strategies):*

1.6.1   *Recombinant tissue plasminogen activator (rt-PA):*

The thrombolytic or clot bluster rt-PA (alteplase) is the only Food and Drug Administration (FDA) approved therapeutic intervention for ischemic stroke till date (Kwiatkowski et al., 1999; Lindgren et al., 1996). The thrombolytic therapy, alteplase upon intravenous administration dissolves the clot in the cerebral blood vessels, thus permits recanalization to ischemic tissue. In the clinical trial (NINDS rt-PA) alteplase was administered between 0-90 and 91-180 min after stroke. However, subsequent European Co-operative Acute Stroke Study (ECASS III) was designed to determine the efficacy with an extended therapeutic window of 4.5 hours and was shown to be efficacious in subset of 5% of population when compared to controls (based on modified Rankin Scale) (Bluhmki et al., 2009; Kwiatkowski et al., 1999; Lees et al., 2010). In addition, alteplase has narrow therapeutic window and may lead to intracerebral hemorrhage, hemorrhagic transformation, increase in mortality rate and adverse events in some circumstances (Lees et al., 2010). However, only 5% of diagnosed patients receive this treatment, due to various other reasons like delay of patient's admission to hospital, delay in CT scan to rule out hemorrhagic stroke, availability of atleplase and other co-morbid complications. It has now been 20 years since FDA approved rt-PA, unfortunately there is rapid underutilization globally. It is clear from above facts that a poor percentage of patients are receiving thrombolytics, therefore it is critical to develop faster reperfusion therapy to target majority of patients.

1.6.2   *Endovascular Procedures:*

During stroke, endovascular mechanical therapies are an alternative strategy to extend therapeutic window and open intracranial vessels in patient's ineligible for rt-PA intervention. The endovascular procedures/mechanical recanalization is preferable in a subset of ischemic stroke patient with large vessel occlusion (LVO) where thrombolytics may contribute to greater risk of hemorrhagic complications (Kidwell et al., 2013; Molina and Saver, 2005). In addition, mechanical recanalization works rapidly within a few minutes with less hemorrhagic risk. Mechanical interventions may be broadly classified into two major categories which includes

endovascular embolectomy and mechanical disruption. Two important subcategories of endovascular embolectomy are clot retrieval devices which physically grasp the thrombi (pull them to extra cerebral sites) and suction thrombectomy devices that aspirate clots (Appireddy et al., 2015; Nesbit et al., 2004; Thomassen and Bakke, 2007). The clot retrieval devices commonly applied to clear cerebral thrombi in acute ischemic stroke include Microsnare, Neuronet, and the Merci retriever. The new standard therapy for patients diagnosed with LVO is the combined use of thrombolytics and mechanical embolectomy. Mechanical reperfusion by embolectomy may reduce the thrombolytic dose and improves its effectiveness by increasing the surface area of the clot that is exposed to thrombolytics and thereby reducing the serious hemorrhagic complications. Moreover, due to promising results in Mechanical Embolus Removal in Cerebral Ischemia (MERCI) trial, FDA approved Merci retriever to restore blood flow in acute ischemic stroke in August 2004 (Appireddy et al., 2016; Becker and Brott, 2005; González et al., 2007). However, mechanical embolectomy is limited to patient's ineligible for rt-PA treatment. Suction thrombectomy devices including the AngioJet, the Oasis and the Hydrolyser were developed for extracranial circulation, while Neurojet was developed specially for the intracranial circulation. However, initial trials suggest a lack of feasibility and safety of Neurojet (Lutsep et al., 2002; Molina and Saver, 2005; Raychev and Saver, 2012). Therefore, the development of these devices for ischemic stroke has now been halted. However, further investigations in the development, safety and feasibility of endovascular procedures (in the presence or absence of thrombolytics) to improve the clinical function are recommended. The current treatment options are focused on symptomatic relief rather than molecular mechanism-based approach, therefore, a deep insight into the molecular mechanism involved in the pathological cascade is essential to investigate novel therapeutic targets.

1.7   *Pathophysiology of Stroke-Ischemia reperfusion (I/R) injury:*

Within a distinct course of time, some complex multiple mechanisms are responsible for cell death following cerebral stroke such as energy failure, glutamate excitotoxicity, calcium overload, oxidative stress, inflammation and apoptosis (Fig.1.3).
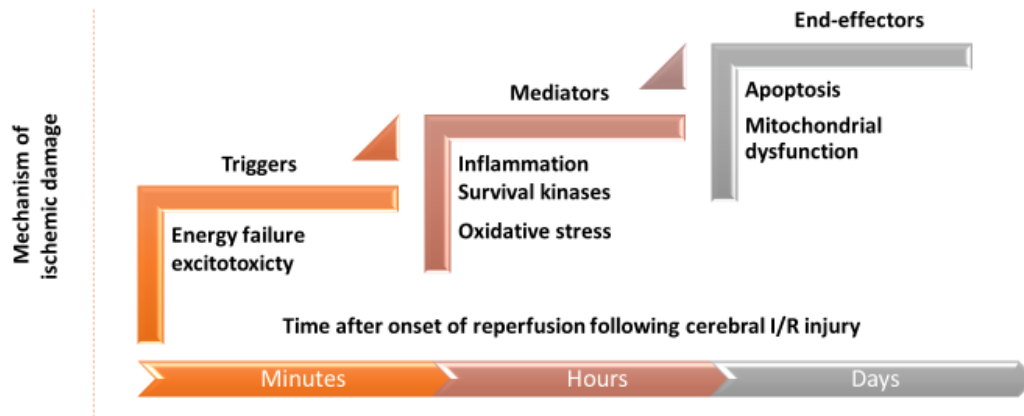
Fig. 1.3. Putative temporal representation of mechanisms of ischemic damage following cerebral ischemic reperfusion injury.
Y-axis of this figure represents the peak activity of particular -mechanism, while the X-axis represents the time after onset of reperfusion

1.7.1   Energy Failure:

The human brain accounts for 2% of the bodyweight, but consumes approximately 20% of the total oxygen. The possible reason for the oxygen demand is oxidative phosphorylation for ATP production to maintain the neuronal ionic homeostasis (Fig.1.4). Therefore, during ischemia neurons are the most sensitive to loss of oxygen supply, thus a minute disturbance in the blood flow and supply of nutrients, may have significant effect on neurons (Doyle et al., 2008; Leker and Shohami, 2002). Energy failure is putatively the most important mechanism of neuronal injury and cell death in the ischemic brain (Astrup et al., 1977).   During an ischemic event (Fig.15), due to compromised oxygen and glucose supply, ATP production by mitochondrial oxidative phosphorylation is severely arrested and cell switches to anaerobic metabolism. However, it must be emphasized that, the brain compensates energy production by producing ATP from glucose stores, ADP and phosphocreatine. Thus, during first two minutes of ischemia induced energy failure, there is a significant decrease in ATP and phosphocreatine, rise in ADP and AMP, along with byproducts like inorganic phosphates, H^+ (acidosis) and lactate. Depletion of neuronal ATP stores result in massive downhill of ionic fluxes (Na^+, Ca^{+2}, Cl^-) and loss of ionic homeostasis leading to anoxic depolarization. Excessive uptake of calcium nearly 90% from the extracellular fluids triggers a battery of interrelated neurotoxic events which includes excitotoxicity, oxidative stress, neuroinflammation and apoptosis (Kristián, 2004). Apart from

ATP, Nicotinamide adenine dinucleotide (NAD)is an another important energy substrate and cofactor involved in multiple metabolic reactions such as glycolysis, tricarboxylic acid cycle, DNA repair processes, and in the function of several NAD dependent enzymes. Moreover, neuronal death is closely related to NAD depletion rather than ATP reduction, as NAD depletion indicates complete failure of glycolysis to restore ATP levels during cerebral ischemic injury (Liu et al., 2009). Thus, during cerebral ischemia the impaired energy metabolism with ATP and NAD depletion may lead to membrane depolarisation that result in excessive release of excitatory amino acid glutamate from the synaptic vesicles of ischemic neurons.

1.7.1.1   Specific role of NAD

Neurons require large amounts of energy to maintain its survival and function. In this context, NAD and its phosphorylated forms NADH and NADPH have crucial role in cellular energy metabolism and energy production. Earlier studies revealed the indispensable role of NAD in metabolic monitoring (Belenky et al., 2007; Houtkooper et al., 2010). This notion roots in the important role of NAD as it is a coenzyme for key enzymes in glycolysis and respiratory chain. Further, NAD participates in the redistribution of electron equivalents generated by the catabolic pathways into macromolecule *de novo* biosynthesis. Studies from the last decade suggest that NAD as a co-enzyme for oxido-reductases and it is also being consumed as a substrate in certain reactions (Houtkooper et al., 2010). Further, NAD plays an important role in maintaining cellular NADH and NADPH levels and in regulation of mitochondrial and cytosolic redox sate. During physiological conditions, NAD is synthesized *de novo* from tryptophan, however, the main source is from salvage pathway with Nicotinamide (NAM) as a precursor. Nicotinamide phosphoribosyl transferase (NAMPT) is involved in the conversion of NAM to nicotinamide mono nucleotide (NMN) which further is converted to NAD by NMN adenyl transferase (NMNAT). NAMPT is considered to be an important enzyme in the regulation of NAD levels (Houtkooper et al., 2010).

During ischemia, when ATP stores gets depleted NAD may serve as a source of adenosine donor and high energy phosphate that is crucial for synthesis of ATP. However, NAD depletion during ischemia may result in inhibition of important ATP synthesizing metabolic reactions including glycolysis, oxidative phosphorylation and citric acid cycle (Liu et al., 2009). Therefore,

preserving NAD levels could be an attractive strategy to treat neuronal damage following cerebral I/R injury. Along this line, the most obvious treatment would be NAD precursors such as NAM. Recently, European Nicotinamide Diabetes Intervention Trial (ENDIT) evaluated the long-term effects of NAM treatment on the development of type 1 diabetes mellitus (T1DM) suggested no positive effect on pancreatic beta cells from the long-term NAM treatment (Gale et al., 2004). Moreover, high doses of NAM are not well tolerated in long term use. Further, the role of NMNAT as a potential target to improve NAD levels to treat various pathological states linked to NAD metabolism is still under investigation. An earlier study suggested the neuroprotective role of NMNAT against neuronal degeneration by inhibiting excitotoxic necrotic cell death. They found that mice overexpressing NNMAT1 in the cytoplasm resulted in markedly less injury and protected the mice against neonatal hypoxic ischemia induced neuronal injury (Gale et al., 2004). Although there is still doubt whether the protective effect on Wallerian neurodegeneration is exerted only through NMNAT or may involve NMAT3 or NMN. Further, the role of NAMPT has been investigated in Type 2 Diabetes Mellitus (T2DM). In this context, a reduction in the NAMPT protein impaired the glucose stimulated insulin secretion by reducing NMN levels (Gale et al., 2004). However, the role of NAMPT in the context of neuronal injury is still not clear.
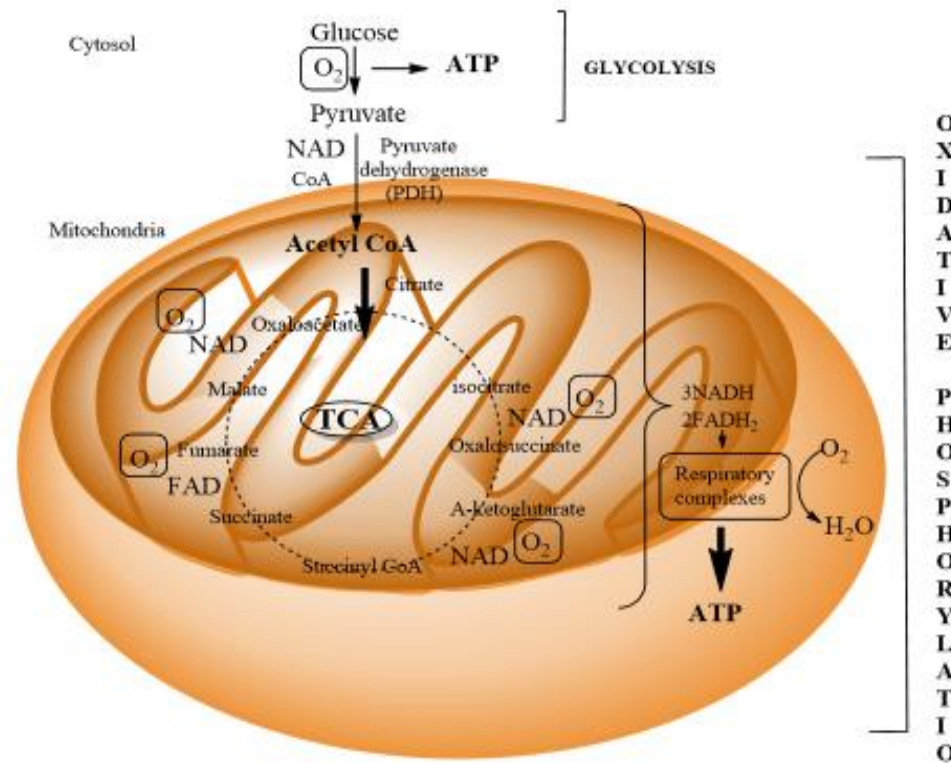
Fig. 1.4.Pictorial representation of ATP production by oxidative phosphorylation during physiological conditions.
NAD-Nicotinamide Adenine Dinucleotide, FAD-Flavin Adenine Dinucleotide, NADH-Nicotinamide Adenine Dinucleotide Hydride, FADH-Flavin Adenine Dinucleotide Hydride, TCA-Tricarboxylic acid cycle
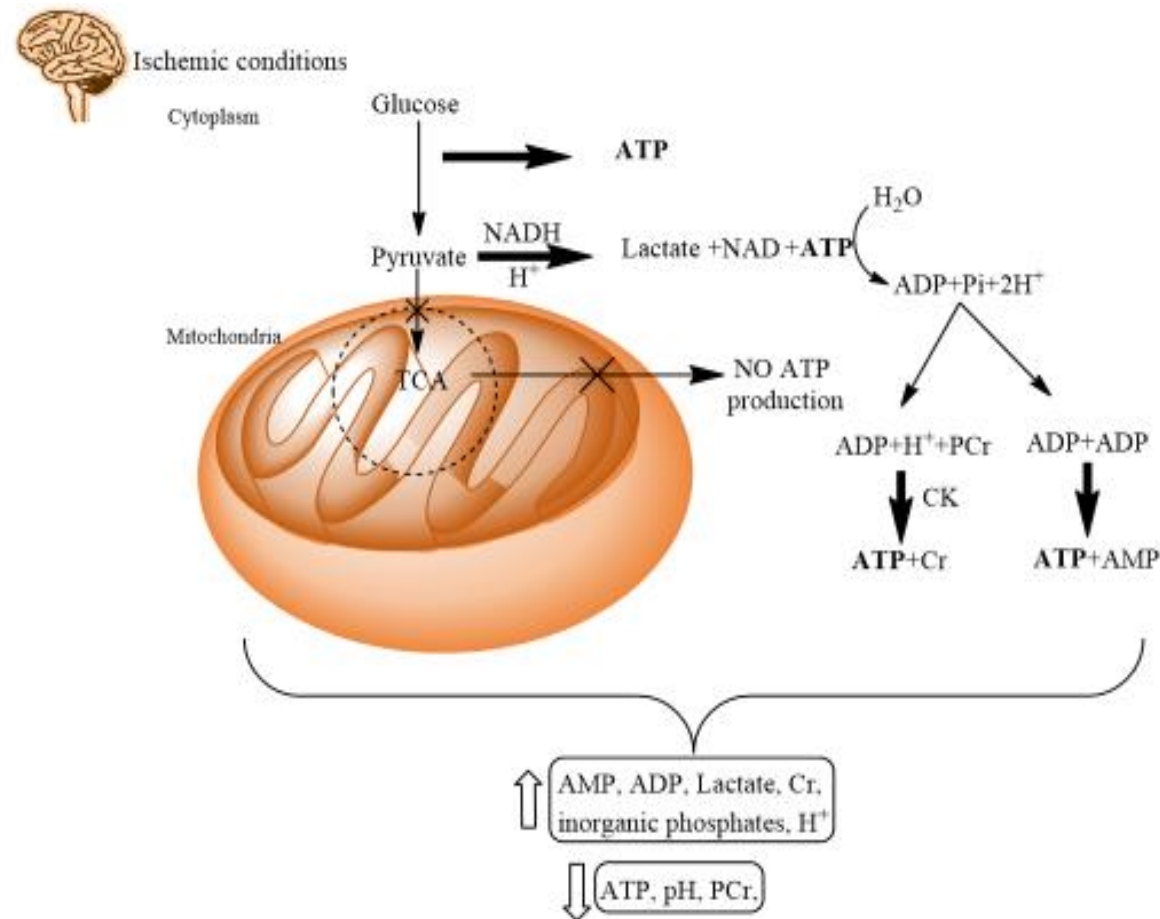
Fig. 1.5.Pictorial representation of bioenergetic failure (compromised ATP production during ischemic conditions).
During ischemic conditions, as pyruvate do not enter TCA cycle due to lack of oxygen, it is being converted to lactate to generate ATP. The brain also maintains ATP balance by utilizing ADP. The ADP formed is converted to ATP by translocation of phosphate from Phospho creatinine. AMP-Adenine Monophosphate, ADP-Adenine Diphosphate, PCr- Phospho Creatinine, CK-Creatinine Kinase, NAD-Nicotinamide Adenine Dinucleotide, FAD-Flavin Adenine Dinucleotide, NADH-Nicotinamide Adenine Dinucleotide Hydride, FADH-Flavin Adenine Dinucleotide Hydride, TCA-Tricarboxylic acid cycle.

1.7.2 Excitotoxicity

The term excitotoxicity refers to neuronal damage induced by over stimulation of excitatory amino acid glutamate and its subsequent action on excitatory amino acid receptors inotropic receptors like N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic receptors present on presynaptic and post synaptic neurons. Excitotoxicity triggered by neuronal injury will lead to neuronal death within minutes in various neuronal pathologies including traumatic brain injury, Huntington's disease, Alzheimer's disease (AD), Parkinson's Disorder (PD) and Stroke (Anglada-Huguet et al., 2017; Prentice et al., 2015).

Under physiological conditions (Fig.1.7), excess glutamate is cleared from synaptic cleft, in an energy and Na^+ dependent manner into neurons and glial cells. However, during pathological conditions like ischemia, traumatic stress and neurodegeneration, neuronal cells undergo anoxic depolarization (Fig.1.6) which not only in results in presynaptic glutamate outburst but also compromise glutamate reuptake through glutamate transporters in astrocytes and neurons. Typically, post synaptic neurons subjected to toxic levels of extracellular glutamate leads to excessive or prolonged stimulation of inotropic (NMDA [*N*-methyl d-aspartate], AMPA [α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid] and kainite) and metabotropic glutamate receptors. Fundamentally, due to high calcium permeability of NMDA receptors, NMDA receptor activation may lead to excitotoxic cell death through disruption of cellular ionic homeostasis (Szydlowska and Tymianski, 2010). Attempts to restore cellular ionic balance and membrane potential may further deplete ATP and NAD resources. Moreover, during ischemia induced excitotoxicity insults (Fig.1.8), ionic imbalance release excess calcium from calcium stores into cytosol and mitochondria that results in altered mitochondrial permeability and impaired oxidative phosphorylation (Lai et al., 2014; Prentice et al., 2015). Further, excess calcium activates various calcium dependent enzymes like proteases, endonucleases, phospholipases and NOS synthases resulting in oxidative and nitrosative damage.

1.7.2.1 *Specific role of NR2B and its downstream modulators*

Neuronal survival during development is supported by multiple signaling pathways and process which includes peptides, neurotrophic factors and neurotransmitters. In the central nervous system (CNS), glutamate is the most prevalent neurotransmitter and regulates an important pro-survival signaling pathway and majority of excitatory signaling. Cellular signaling occurs primarily via inotropic receptors and G-protein coupled receptors mediated via scaffolding proteins signal transduction pathways involving protein phosphorylation cascades. Out of inotropic receptors, NMDA receptors mediate most of the glutamate trophic activity (Habas et al., 2006). The survival role of NMDA receptors has been demonstrated in earlier in vitro studies. However, only basal activity of NMDA-R activity is essential for cell survival, while excessive activation leads to excitotoxic cell death (Bhave et al., 1999; Zhang et al., 1998). An earlier study proposed the recruitment of diverse NMDA receptor subtypes in moderate vs excess glutamate levels. This fact was further strengthened with differential role of NR2A and NR2B subunits of NMDA-R during the physiological and various pathological conditions. For instance, neurons with NR2A were able to rescue neurons from staurosporine-induced apoptosis, while neurons with NR2B subunit resulted in excitotoxic cell death (Hardingham et al., 2002; Mattson, 2003; Takadera et al., 1999). The relative contribution of NR2A and NR2B in the pathological cascade of ischemic injury remains to be elucidated. NMDA-R mediate its pro-survival signaling majorly through post translational modifications that typically include phosphorylation and dephosphorylation of various protein kinases such as Calcium/calmodulin-dependent protein kinase II (CAMKII), Protein Kinase C (PKC), Protein Kinase A (PKA), phosphatidylinositol-3 kinase (PI3K)/Akt and calcium-dependent phosphatase calcineurin (PP2B) (Qiu et al., 2011; Szapiro et al., 2003; Tang et al., 2008). The molecular pathway of PI3K-Akt is the critical transducer for majority of survival signaling pathways. Earlier reports suggest an inhibition of Akt with excessive activation of NMDA, however, it is unclear whether PI3K-Akt contribute to specific NMDA subtype neuroprotection. Further, PI3K-Akt mediates its actions through a group of kinases including Glycogen synthase kinase 3β (GSK3β), an important regulator of neuronal functions ranging from development to apoptosis (Franke et al., 2003; Seira and Del R'\io, 2014; Vara et al., 2004). However, the role of GSK-3β inhibition on the pro-

survival role of PI3K-Akt remains unclear in the context of global cerebral I/R injury. Furthermore, hemeoxygenase-1 (HO-1) an inducible stress protein implicated in the defense mechanism of oxidative injury. Transcriptional regulation of HO-1 is linked to the transcription factor, NF-E2- related factor 2 (Nrf2). Recent results have demonstrated that Nrf2 nuclear translocation requires the activation of several signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), and protein kinase C (PKC) (Min et al., 2011; Yang et al., 2009). Growing evidence suggest the role of HO-1 in cytoprotection, however, the pro-survival role of PI3K-Akt induced HO-1 expression in the cerebral I/R injury remains unclear.
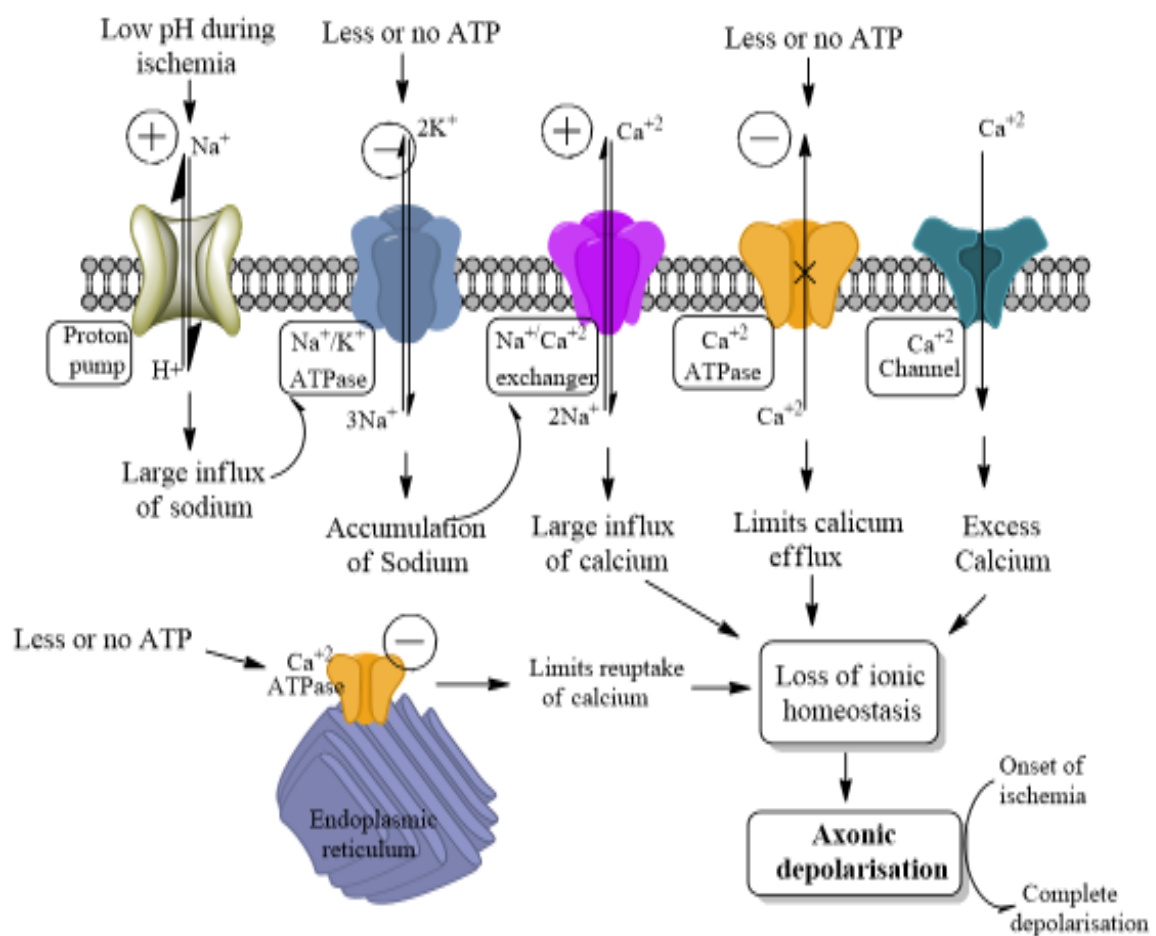
Fig. 1.6. Pictorial representation of ionic imbalance leading to axonic depolarisation during ischemia
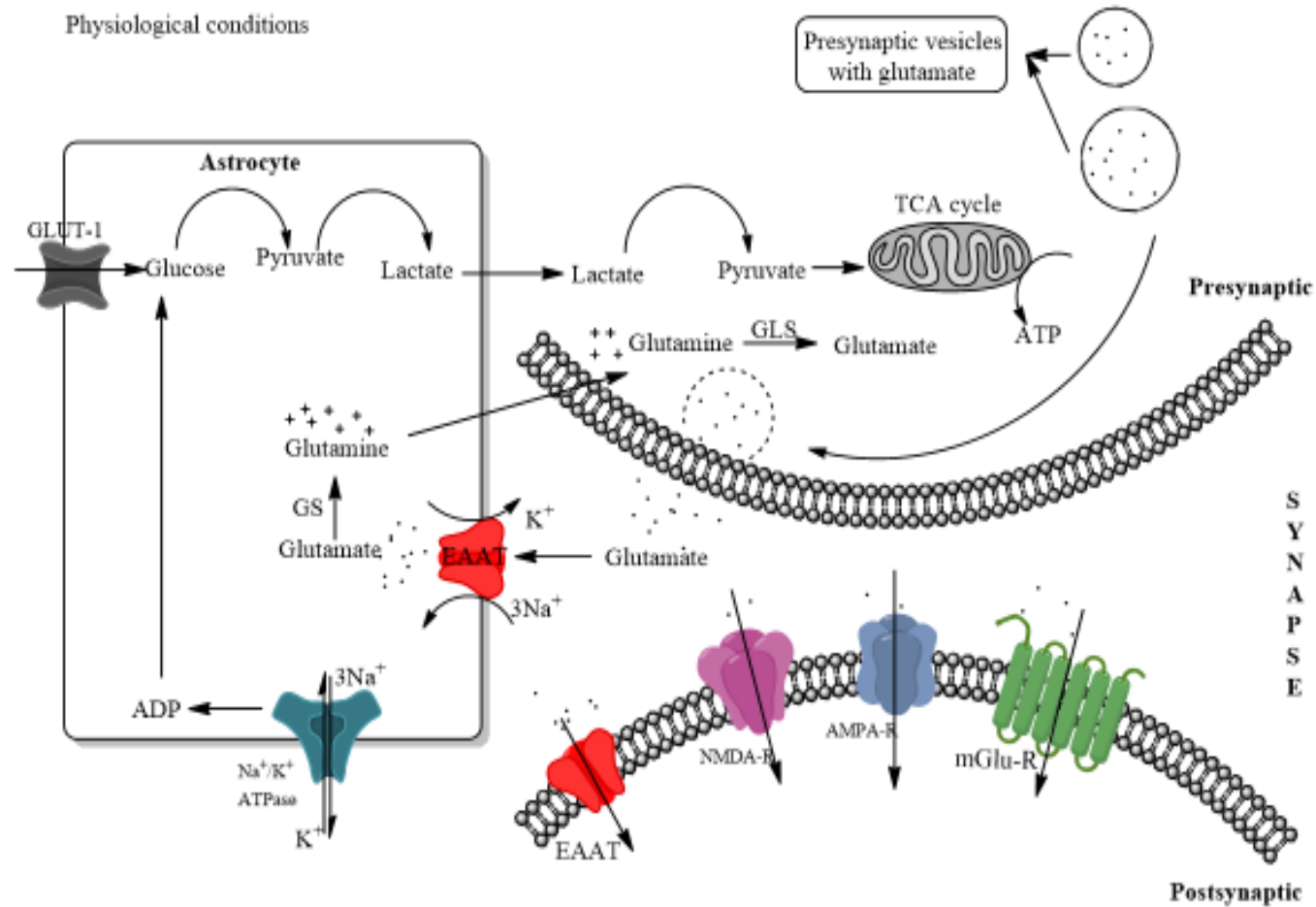
Fig. 1.7. Pictorial representation of astrocyte neuron metabolic interactions during physiological conditions.
EAAT-Excitatory Amino Acid Transporter, GS-Glutamaine Synthetase, GLS-Glutaminases, GLUT1-Glucose Transporter, NMDA-N-methyl-D-aspartate, AMPA-Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, mGlu-metabotrophic glutamate.
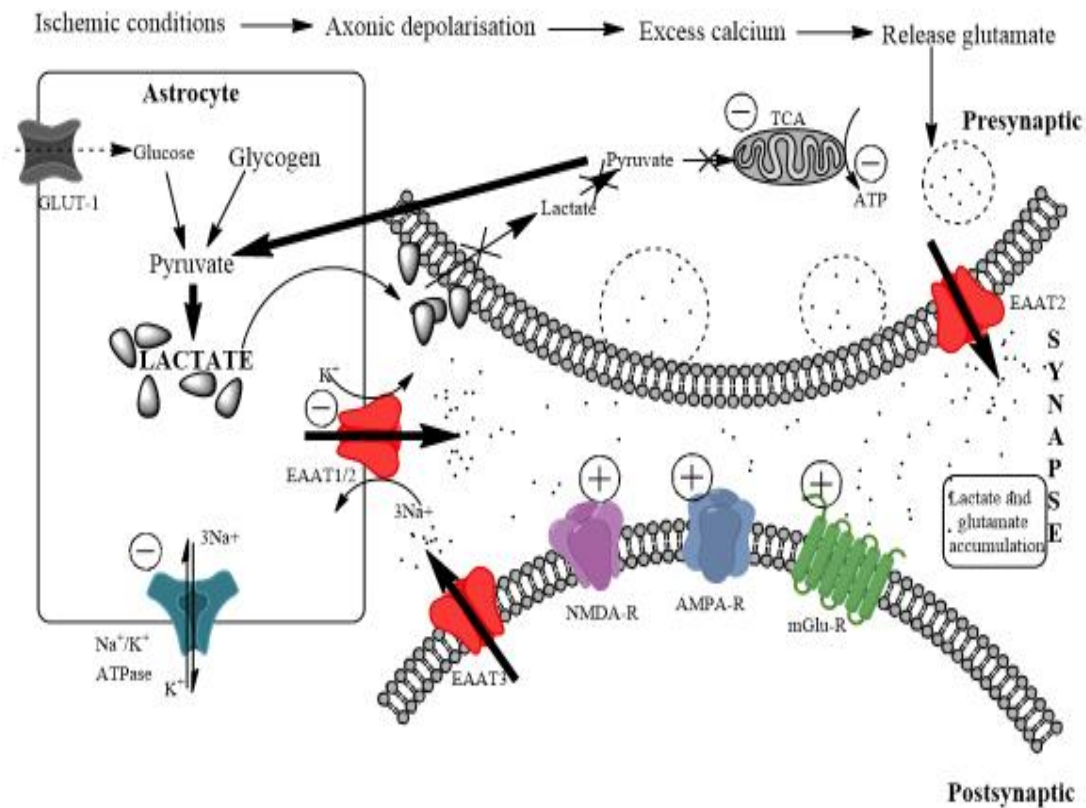
Fig. 1.8. Pictorial representation of excitotoxicity during ischemic injury.
During ischemic injury the calcium toxicity and axonic depolarisation release excess glutamate from the pre synaptic vesicles and activate various glutamate receptors leading to excitotoxicity.

1.7.3   Intracellular calcium toxicity:

During ischemic event (Fig.1.9), accumulation of the intracellular calcium by multiple pathways predominantly by NMDA receptor activation is one of the distinctive highlights in excitotoxicity. NMDA receptor toxicity is mainly dependent on extracellular Ca^{2+}, and reflects a large amount of Ca^{2+} influx directly through the NMDA ion channels. Moreover, NR2B receptors that is regulated by CAMKII, Protein Kinase C (PKC), and other Src family proteins like Phosphatidylinositol 3-Kinase (PI3K) is majorly associated with mitochondrial calcium accumulation, swelling and cell death (Tu et al., 2010). However, AMPA have poor Ca^{2+} permeability that result indirect calcium entry through voltage gated calcium. The influx of calcium activates an array of calcium dependent catabolic enzymes like phospholipases and endonucleases, resulting in breakdown of cellular proteins and cytoskeleton. Generation of inositol-1, 4, 5- triphosphate by the activation of phospholipase A2, further release the calcium from the endoplasmic reticulum (ER) (Doyle et al., 2008; Yao and Haddad, 2004). Excessive calcium triggers various neurotoxic cascades including calcium release from mitochondria, initiation of molecular events for apoptosis and increase free radicals leading to oxidative stress.

1.7.4   Oxidative Stress and ischemic reperfusion injury:

During an ischemic event (Fig.1.10), the brain becomes highly vulnerable to oxidative stress, as it has limited energy resources, less antioxidant capacity and low repair mechanism. Following 15-20 min of ischemic phase, there will be a marked elevation in the glutamate and succinate levels, depletion of glycogen pools and high energy phosphate source along with alteration in NADH/NAD ratio. Moreover, succinate respiration during reperfusion may cause back leak of electrons in Iron-Sulphur (Fe-S) clusters from complex II to I that may react with molecular oxygen resulting in the formation of reactive oxygen species (ROS) (Kristián, 2004; Turrens, 1997). Further, harmful effects like loss of mitochondrial membrane functional integrity upon - excitotoxic stress along with low anti-oxidant capacity, lipid peroxidation and elevated nitrite levels were noticed in reperfusion phase following ischemic injury as seen in Fig.1.11 (Guo et al.,   2016;   Lewen   et   al.,   2000).
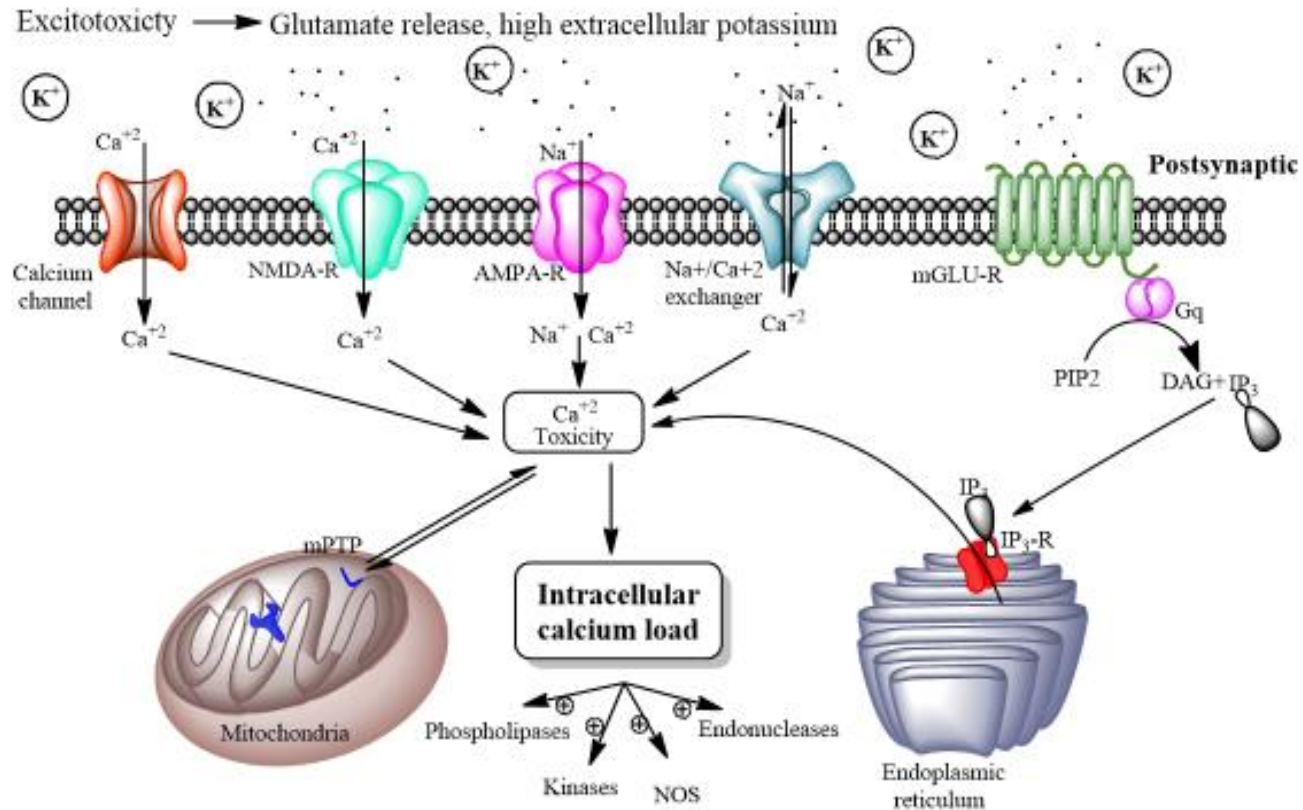
Fig. 1.9. Diagrammatic representation of calcium toxicity.
Excitotoxicity rises levels of glutamate and extracellular potassium concentrations. High levels of glutamate trigger the glutamate receptors like NMDA, AMPA, metabotropic glutamate (mglu) receptors. This leads to influx of calcium not only from the glutamate receptors but also from the calcium ion channels. AMPA initially influx sodium, followed by delayed calcium influx. Mglu receptors upon conversion of PIP2 to IP3 activate IP3 receptors on endoplasmic reticulum which rises the intracellular calcium concentrations. This leads to release of calcium from the mitochondria and opening of mitochondrial permeability transition pore (mPTP). IP3-Inositol triphosphate, NOS-Nitric Oxide Synthase, PIP2-Phosphatidylinositol 4,5-bisphosphate, DAG-Diacyl Glycerol
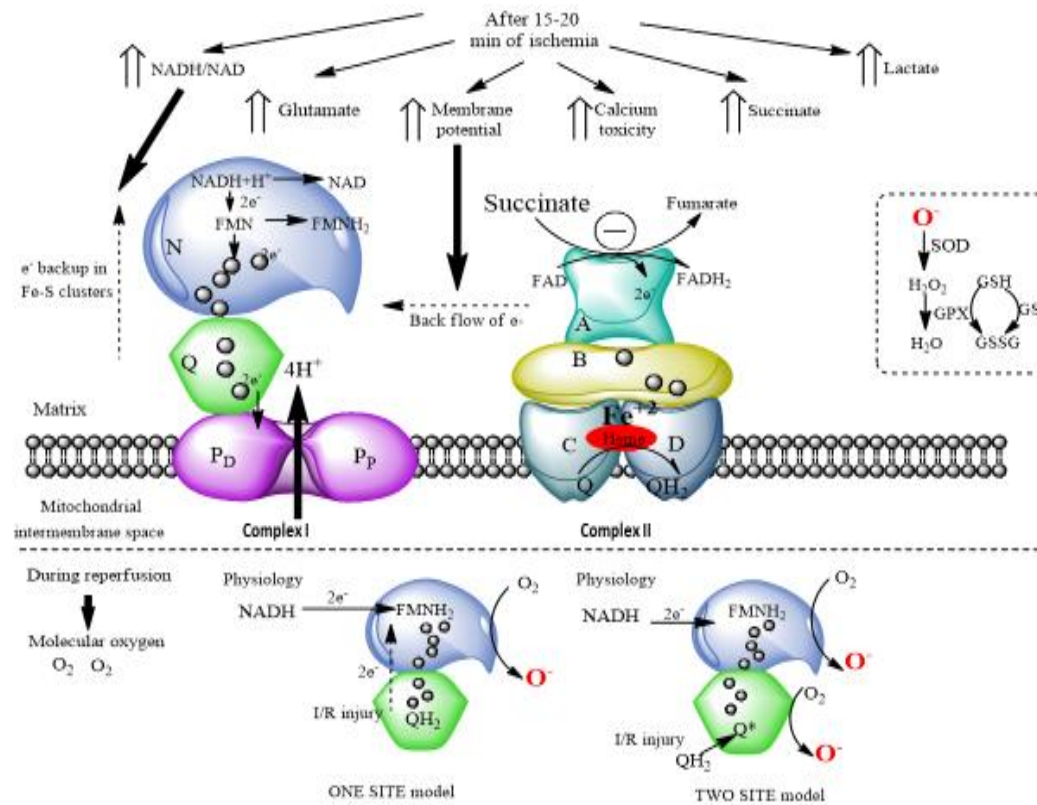
Fig. 1.10. The ischemic cascade for the generation of reactive oxygen species.
After 15-20 min of ischemia, the levels of glutamate, lactate, calcium, succinate and NADH will be high. This causes a back flow of electrons from the complex II to complex I because succinate levels are high due to inhibition of complex II or backup of electrons within the complex I because levels of NADH are high. In one site model FMNH2 produces reactive oxygen species (ROS) both in normal mode when received electrons from NADH and in reverse mode when receives electrons from ubiquinone. In two site model FMNH2 produces ROS only in the normal mode, in reverse mode ROS is produced from the semi-ubiquinone in the Q-site. Black spheres-Fe-S clusters, N-NADH binding site, Q-Ubiquinone binding site, P-Proton pumping sites (distal and proximal), SOD-Superoxide Dismutase, GS-Glutathione reductase, GSH-Glutathione, GPX-glutathione peroxidases, GSSG- glutathione disulfide
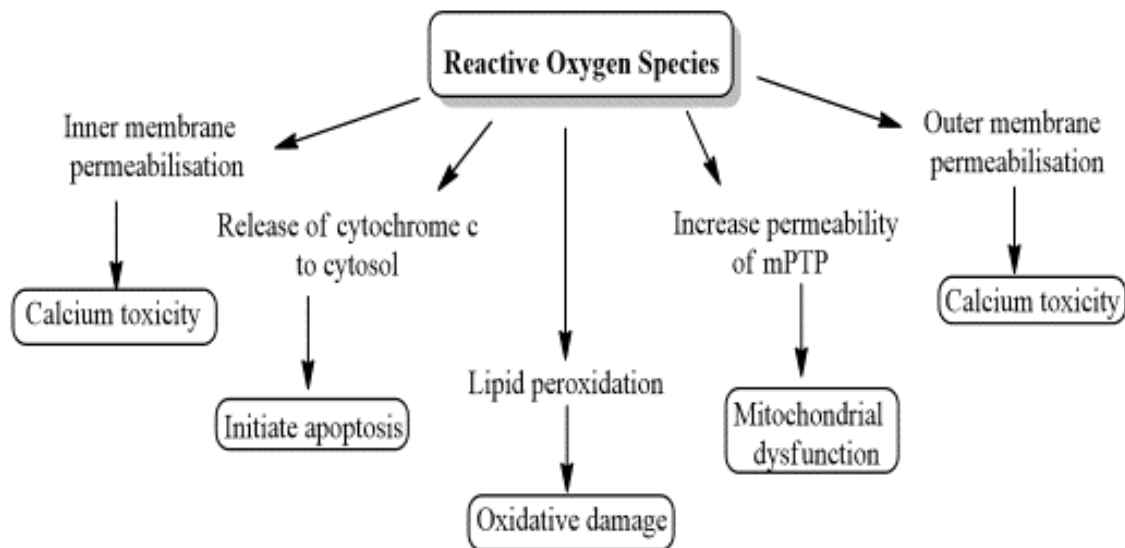
Fig. 1.11. Devastating outcomes of reactive oxygen species during ischemic reperfusion injury

1.7.5   Neuroinflammation:

Compromised cerebral blood flow leads to depletion of energy, rise in intracellular calcium and excitotoxicity resulting in oxidative stress. The generation of ROS either by ischemia or reperfusion can stimulate strong inflammatory reactions (Fig.1.12) that can exacerbate tissue damage and involves inflammatory cell infiltration along with subsequent activation of microglia, macrophage and neutrophils (Kleinig and Vink, 2009; Wang et al., 2007a). Upon activation, inflammatory cells release inflammatory cytokines, ROS, NO and adhesion molecules in the ischemic area, which further intensify the secondary damage. Brief ischemia may also release pro-inflammatory cytokines like Tumor Necrosis factor (TNF-α), interleukin (IL-β) from endothelial cells and macrophages (ARVIN et al., 1996; Lakhan et al., 2009). This may up-regulate intracellular adhesion molecule (ICAM) expression (ICAM 1 and 2, selectins P and selectin E) and promote neutrophil activation that adhere to endothelium of cerebral blood vessels and transmigrate to cerebral parenchyma (Frijns and Kappelle, 2002; Okada et al., 1994; Won et al., 2015). The neutrophil recruitment to cerebral blood vessel may obstruct the cerebral blood flow by accumulation of neutrophils, platelets and red blood cells, prevent restoration of blood flow thus may lead to additional tissue damage (by release of free radicals

and proteolytic enzymes) referred to as "no-reflow" phenomenon (Crack and Wong, 2008). Neutrophil reaction is reported to be followed by activation of monocytes and lymphocytes.

Microglial cells, resident macrophages of the brain are the key modulators of innate immune response. Upon activation of microglia, they transform morphologically from ramified to amoeboid shaped macrophages. They release free radicals, cytotoxic mediators and pro-inflammatory cytokines in the process of clearing cellular debris. The most studied inflammatory cytokines in stroke are TNF-α, IL-6 (ARVIN et al., 1996). They may also guide other inflammatory cells to the ischemic site by releasing chemokines including cytokine-induced neutrophil chemoattractant (CINC) and monocyte chemoattractant protein-1 (MCP-1) (Kreutzberg, 1996; Nedergaard and Dirnagl, 2005). Astrocytes, another glial cells when triggered by physiological and pathological stimuli exhibit various morphological and biochemical alterations (De Keyser et al., 2008; Sofroniew and Vinters, 2010). During acute ischemic phase, astrocytes uptake excess glutamate in the synapse to prevent excitotoxicity, that may act as glycogen store to provide carbon source for anaerobic metabolism in penumbral neurons (Rossi et al., 2007; Swanson et al., 2004). Due to high concentration of intracellular anti-oxidants than neurons, astrocytes may prevent oxidative stress by early detoxification of free radicals (Fernandez-Fernandez et al., 2012; Wilson, 1997). In contrast to their supportive role in ischemia mediated neuronal injury, astrocytes may result in neuronal damage upon excessive activation (Sofroniew and Vinters, 2010; Swanson et al., 2004). Reactive astrocytes are reported to exacerbate excitotoxicity by excess release of glutamate through reversal of ion channels, rise in extracellular glutamate, release ROS, pro-inflammatory cytokines and other molecular events that contribute neuronal cell death (Gomes-Leal, 2012; Pekny and Nilsson, 2005; Rossi et al., 2007; Swanson et al., 2004). Reactive astrogliosis with altered expression of many genes like Glial Fibrillary Acidic protein (GFAP) is a characteristic hallmark of many neurological disorders including cerebral stroke, that may affect disease progression (Nakase et al., 2003; Nawashiro et al., 2000).
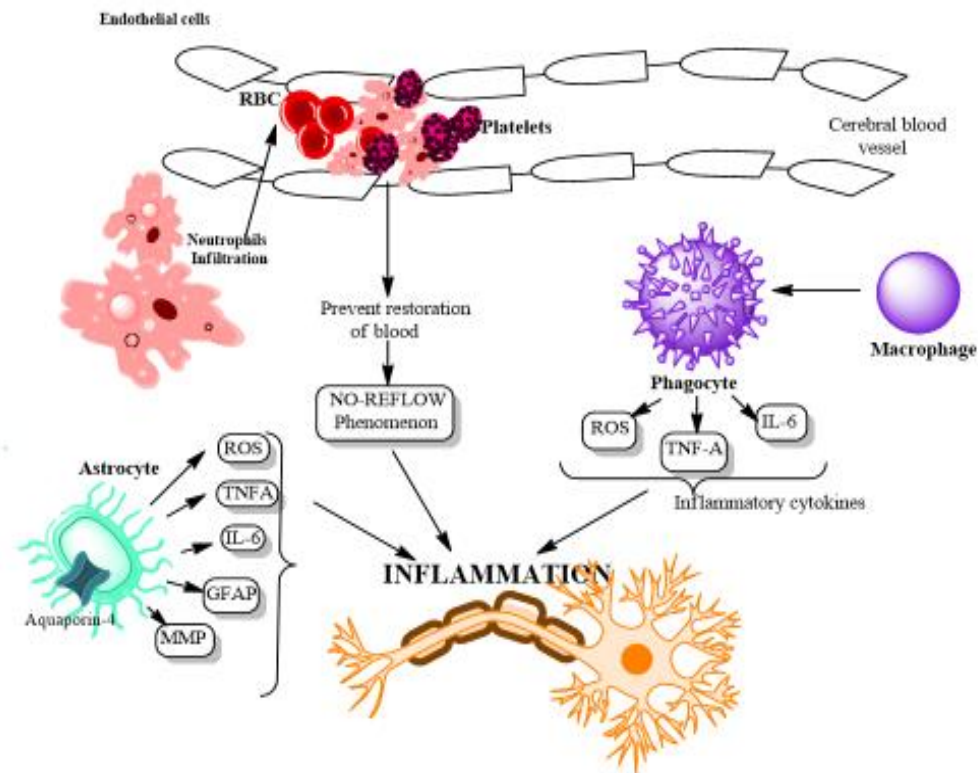
Fig. 1.12. Schematic representation of neuroinflammation during ischemic cascade.
Oxidative damage during ischemia activates inflammatory cascade leading to secondary tissue damage. No reflow phenomenon develops due to accumulation of activation and recruitment of neutrophils into cerebral blood vessels, leading to accumulation of neutrophils, blood cells and platelets in the cerebral blood vessel. Subsequently microglia get activated to phagocytes and astrocytes release reactive oxygen species and inflammatory cytokines. GFAP-Glial Fibrillary Acidic protein, IL-6- Interleukin-6, TNF- Tumor Necrosis Factor, MMP-Matrix Metallo Proteinases.

1.7.6   Mitochondrial permeability transition pore (mPTP) and apoptosis:

Experimental and clinical evidence has demonstrated apoptotic cell death in animal models of cerebral ischemic stroke, hypoxic injury and other neurodegenerative disorders (Elmore, 2007; Mattson, 2000; Raghupathi, 2004; Thompson, 1995). Apoptotic cells are mainly characterized by DNA fragmentation, chromatin condensation, along with a shrunken plasma membrane (Broughton et al., 2009). As mitochondria is source of pro-apoptotic, anti-apoptotic factors and family of intracellular cysteine-aspartate proteases (caspases) it plays an important role in apoptosis and necrosis (Green and Reed, 1998; Wang, 2001). During ischemic injury, excessive glutamate release may result in ionic imbalance and calcium overload that may contribute to mitochondrial swelling leading to opening of mitochondrial permeability transition pore (mPTP) (Fig.1.13). This complex series may lead to release of pro-apoptotic factors from the intermembrane space to cytosol. Initial release of cytochrome-c as seen in Fig.1.14 (Pérez-Pinzón et al., 1999) from the mitochondria activates caspase-9 via pro-caspase-9 and Apa-1. Activation of caspase-9 may initiate a series of downstream mediators like caspase-3, 2, 6, 10 followed by caspase-11 (Kang et al., 2000; Yakovlev and Faden, 2001) which is a crucial initiator for activating caspase 1 and 3 (Doyle et al., 2008; Nakka et al., 2008).   Caspase-3 is the most important end-stream protein in the series of caspases as it cleaves cell signaling, homeostasis, and cytoskeletal proteins that are essential for cell survival as seen in Fig.1.15 (Cregan et al., 1999; Ni et al., 1998).
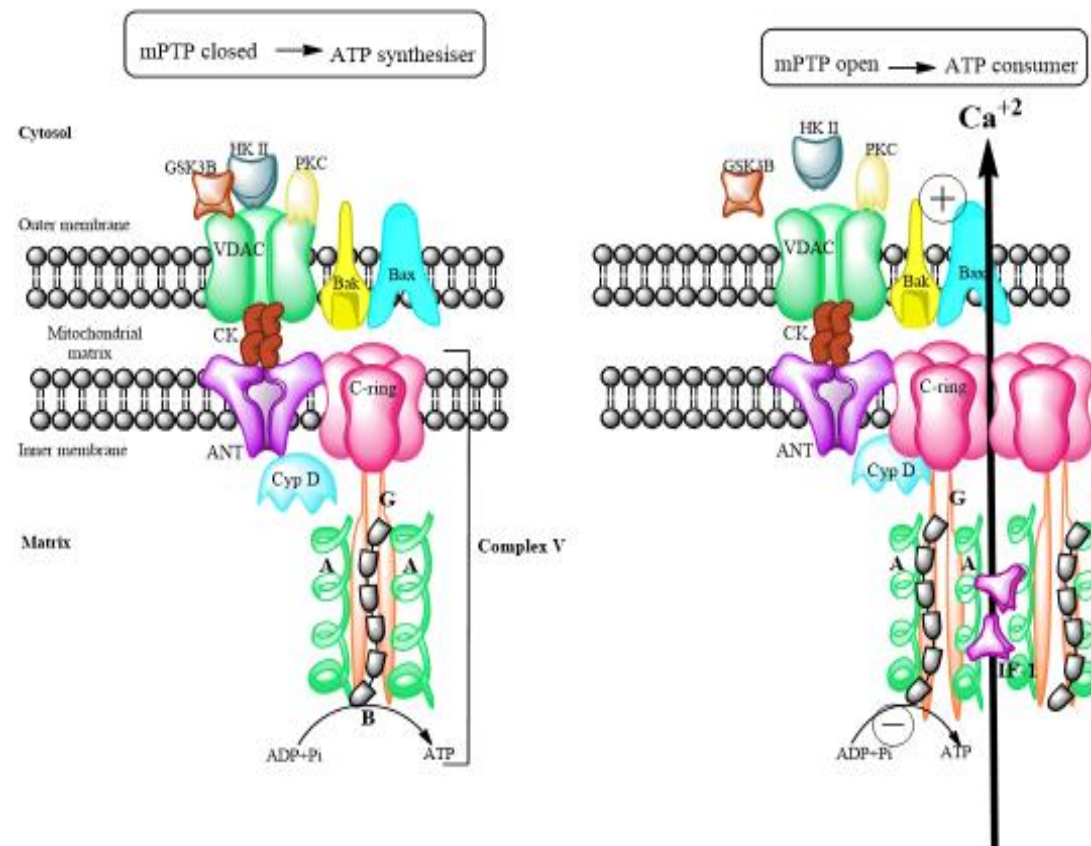
Fig. 1.13. Diagrammatic representation of alterations in mPTP during ischemic injury.
Voltage dependent Anion Channel (VDAC) and Adenosine Nucleotide Transporter (ANT) were located on outer membrane and inner membrane respectively. Other components of the mPTP include creatinine kinase (CK), hexokinase II (HK II), protein kinase C (PKC), Bcl2 family proteins Bax and Bak, the important regulator of mPTP i.e., cyclophilin D (Cyp D) and F1/F0 ATP synthase with alpha (A), beta (B), gamma (G) subunits. During ischemic injury, the c-ring of the ATP synthase may act as pore of mPTP and mitochondrial inhibitory factor 1 (IF-1) dimers the interface of alpha and beta sub-units of ATP synthase resulting in formation of pore. This leads to release of calcium, toxic intramembrane proteins into cytosol apart from activating apoptotic proteins (Bak and Bax).
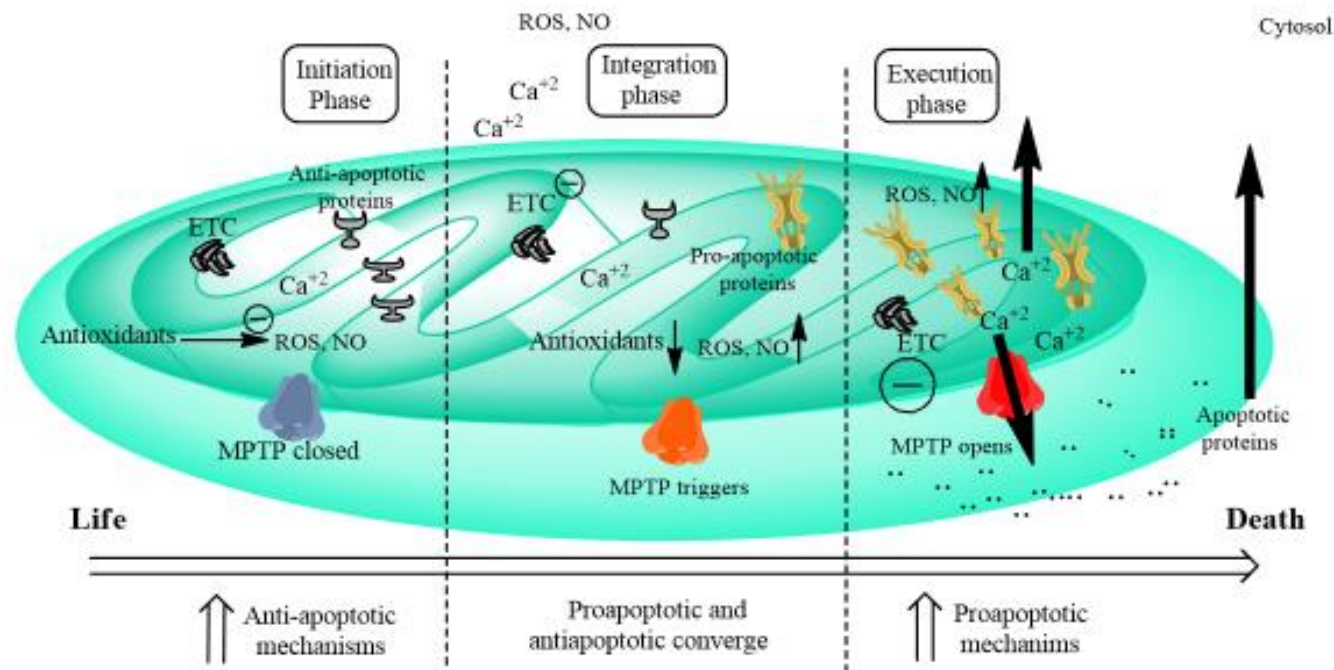
Fig. 1.14. Diagrammatic representation of mitochondrial events leading to opening of mPTP.
During initiation phase, survival mechanisms predominate followed by integration phase where survival and death mechanisms converge. In the final execution phase, the dominance of apoptotic mechanisms opens the mPTP leading to bioenergetic crisis and cell death
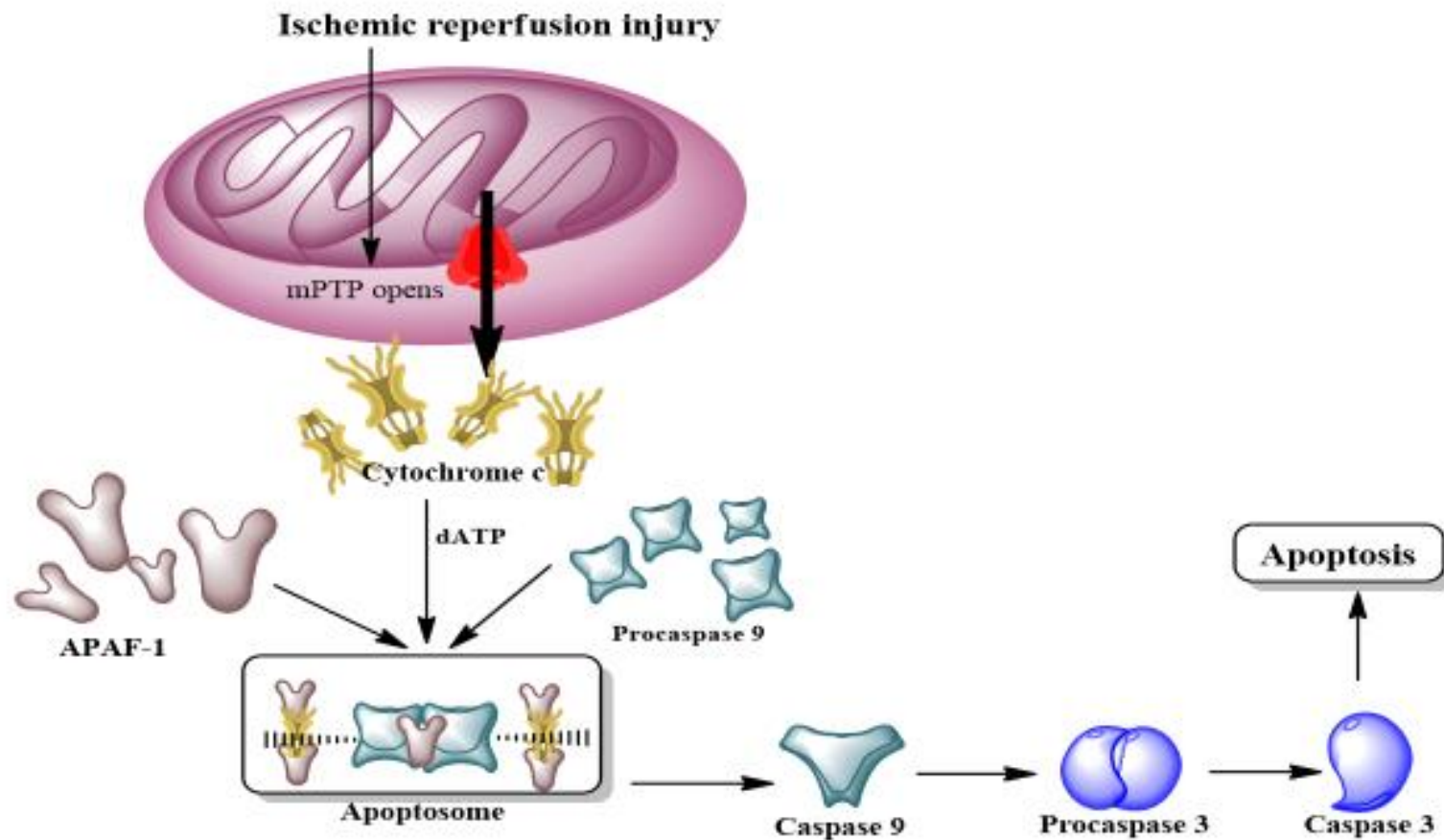
Fig. 1.15. Schematic representation of caspase dependent cell death during ischemic injury.
During ischemic injury, mPTP opening results in release of several cytosolic proteins including cytochrome c. Cytochrome C recruit's apoptotic peptidase activating factor (APAF-1), deoxy adenosine triphosphate and procaspe-9 to form apoptosome, that triggers caspase cascade resulting in activation of caspase 3 leading to apoptosis.

1.7.7   Epigenetic modifications:

In the pathobiology of cerebral I/R injury, reduction of blood flow with low oxygen and glucose supply initiate a highly complex series of events like energy failure, excitotoxicity, oxidative stress leading to secondary phenomenon like neuro-inflammation and neuronal cell death. However, the above mentioned pathological events go hand in hand with great changes in gene expression leading to epigenetic modifications following cerebral I/R injury. Accumulating evidence suggest that epigenetic modifications regulate both neuro-physiological and neuro-pathological process (Schweizer et al., 2013). Epigenetic modifications are not only important for neuronal survival but are also involved in complex process from synaptic plasticity to learning and memory (Levenson and Sweatt, 2005; Zovkic et al., 2013). Modification of epigenetic machinery in the form of DNA methylation, histone methylation and histone acetylation are implicated in various neurodegenerative, neurodevelopmental as well as neurological disorders (Abel and Zukin, 2008; Urdinguio et al., 2009; Zawia et al., 2009). Further, DNA methylation and histone acetylation are more extensively studied post translational modifications over other modifications like sumoylation, phosphorylation, histone methylation. More specifically, disturbance in the histone acetylation homeostasis is identified as the central event in various neurologic pathologies. Histone acetylation at lysine residues neutralizes their positive charge leading to reduced association with DNA (Konsoula and Barile, 2012; Peterson and Laniel, 2004; Strahl and Allis, 2000). Moreover, this open form of chromatin (euchromatin) allows easy access to specific transcription factors to induce active transcription. Histone acetylation and deacetylation is a dynamic process regulated by a balance of histone acetyl transferases (HAT) and histone deacetylases (HDAC). Further, the role of HDAC as important regulators of transcription have been identified to be critical in the pathology of various neurological and neurodegenerative disorders (Kim et al., 2009; Sinn et al., 2007; Steckert et al., 2013).

1.7.7.1   Specific role of histone acetylation and HDAC III

Epigenetics is the mechanism for stable maintenance of gene expression changes that critically involves physically marking DNA and its associated proteins to differentiate phenotypic cells from genotypic cells. However, neuro-epigenetics differ from traditional epigenetics because of

non-diving nature of mature neurons. Further, in the nervous system epigenetic regulation play an important role in acute regulation of gene expression in response to a wide range of environmental signals such as behavioral experience, stress, drugs of abuse, and many others. In addition, epigenetic mechanisms appear to contribute to both psychiatric and neurological disorders (Holliday, 2006, 1994). The two-basic epigenetic mechanisms widely studied at present are regulation of chromatin structure through histone post-translational modifications, and covalent modification of DNA principally through DNA methylation. The area of DNA methylation still remains an area of active research as much attention is focused on hydroxymethyl-cytosine (hmc) and methyl cytosine (mc), however, the functional significant intermediate steps between hmc and mc are yet to be studied (Day and Sweatt, 2010; Huang et al., 1999). Earlier study demonstrated the protective effect of pharmacological inhibition of DNA methylation to improve the neurological outcome in an animal model of ischemia. Further, reduced DNA methyltransferase1 (DNMT1) was found to be protective, however, complete absence did not affect functional recovery indicating that epigenetic changes occur in a gene and promoter specific level rather than global level (Endres et al., 2001; Huang et al., 1999). However, further studies are required to identify the role of specific genes and promoters in the pathology of cerebral ischemic injury.

Further, histone modifications gained much focus in the recent past in the regulation of CNS function. Histones are highly basic proteins whose function is to organize DNA within the nucleus. In the nucleus, DNA is tightly packaged into chromatin, a DNA-protein complex that consists of DNA in a double helix, histone proteins, and many associated regulatory proteins. The basic unit of chromatin is the *nucleosome*, which is composed of an octamer of histone proteins (containing two copies each of histones 2A, 2B, 3, and 4) around the DNA double helix. The degree to which nucleosomes are packed is a critical determinant of the transcriptional activity DNA and this is mediated in part by chemical modifications of the N-terminal tails of histone proteins (Peterson and Laniel, 2004; Strahl and Allis, 2000). Currently, four distinct post-translational modifications of histone tails have been well characterized: acetylation, methylation, ubiquitination and phosphorylation, particularly histone acetylation and phosphorylation may directly influence the chromatin structure. Histone acetylation at lysine

residues neutralizes its positive charge resulting in the open form of chromatin by reducing its association to the negatively charged DNA. During ischemia, there is vast scope for deacetylation, however, based on the dynamic nature of epigenetic modifications this state of repression can be reversed by diverse group of pharmacological modulators (Schweizer et al., 2013).

Acetylation and deacetylation occur in a dynamic process. While the acetylation of lysine residues is carried out by histone acetyl transferases (HATs) using an acetyl group of Acetyl-Co-A, the removal of acetyl groups is performed by histone deacetylases (HDACs). The family of HDACs consists of four classes such as Class I (HDAC 1 to 3 and 8), class II (HDAC 4 to 7, 9, and 10), and class IV (HDAC 11) HDACs are zinc dependent, while the so-called 'sirtuins' of class III act in a NAD dependent manner (Schweizer et al., 2013). In addition, not all members of sirtuins are lysine deacetylases specifically SIRT1, SIRT2, SIRT3 show the maximum histone deacetylase activity, out of which SIRT1 is most extensively studied (Anderson et al., 2014; Khoury et al., 2017). In response to changes in metabolic state, sirtuins coordinate the regulation of various metabolic processes such as glycolysis, fatty acid synthesis, gluconeogenesis, the cell cycle, DNA repair, cell survival, and mitochondrial biogenesis. This is achieved through epigenetic targeting of histones to globally modulate gene expression and targeting specific protein substrates within the mitochondria and cytoplasm. Further, SIRT1 plays a critical role in the regulation of neurodevelopment, learning and memory and metabolic regulation (Morris et al., 2011; Satoh et al., 2013; R.-H. Wang et al., 2008; Feng Zhang et al., 2011). However, the role of SIRT1 and its regulators in the context of cerebral ischemia is still not clear.

1.8 *Selective vulnerability of hippocampal region following global I/R injury:*

Cerebral ischemia can be categorized as focal and global ischemia reperfusion (I/R). Focal ischemia was well explored, while little is known about the global ischemia and mechanisms of cell death following this injury. In clinical conditions, brain injury followed by cardiac arrest is a significant contributor of global I/R injury (Crumrine et al., 1997; Kawai et al., 1992; Nichol et al., 2008). During global I/R injury induced excitotoxicity, specific neuronal population with greater extent of excitatory synapses gets overstimulated and become more susceptible to neuronal damage. Hippocampal *cornu ammonis* (CA1) pyramidal neurons are the most

explored subtype of selectively vulnerable neurons (Pulsinelli, 1985; Wang and Michaelis, 2010). Selective neuronal damage in the hippocampal region with marked neurobehavioral and cognitive impairments are the characteristic features of global I/R injury (Block, 1999; Nunn and Hodges, 1994). The major reason for the selective vulnerability of hippocampus may be its anatomical location, as it lies on watershed between carotid arteries and vertebro-basilar territories (Coceani and Gloor, 1966; Kirino et al., 1985). The hippocampus has two major functional regions, CA1 (corresponds to Sommer's sector) and CA3. The mossy fibers from the dentate gyrus project to CA3 subfield, then Schaffer collaterals are projected to CA1 region. The CA1 region is connected to subiculum in the hippocampus and to entorhinal cortex of the hippocampus. This complete circuit of hippocampus allows for excitatory input that may contribute for over excitation during stress conditions like ischemia (Deshpande et al., 1987). In physiological conditions, neurons possess multiple strategies to buffer large amounts of intracellular calcium induced by NMDA receptor activation during excitotoxic stress (Berridge et al., 2003; Dong et al., 2006). In contrast, a polarized cell uptake large amounts of calcium by Na^+/Ca^{+2} exchanger, Ca^{+2} ATPase, endoplasmic reticulum and mitochondria (Berridge et al., 2003; Hajnóczky et al., 2006; Santo-Domingo and Demaurex, 2010; Verkhratsky and Toescu, 2003). A multitude of factors may play a critical role in the intracellular calcium induced calcium toxicity. In global I/R injury, vast type of neuronal population with stand the brief calcium toxicity during ischemia. However, after 24-48 hours ischemia selectively vulnerable neurons like hippocampal CA1 neurons may receive a secondary pulse of calcium after leading to further excitotoxic damage (Neumar et al., 2001; Szydlowska and Tymianski, 2010). Upon reperfusion, mitochondrial spikes of calcium may lead to mitochondrial membrane depolarisation, impaired oxidative phosphorylation and ATP production contributing to ROS production. In addition, free mitochondrial calcium causes swelling of the matrix that contribute to mPTP opening and release of pro-apoptotic factors as discussed above (Dux et al., 1987; Votyakova and Reynolds, 2005). Above evidences demonstrated the possible role of oxidative stress, inflammatory responses and apoptosis in the hippocampus post I/R injury.

1.9 *Global I/R injury and cognitive deficits:*

Among 15 million people suffering from stroke worldwide, about 30% of survivors after ischemic injury experience physical, neurological and cognitive deficits that affects their social, economic and emotional performance in their daily life and even cause complete dependence (Kalaria et al., 2016). The duration and location of the brain damage strongly impact the intensity and the type of disorders faced by the survivors. The possible interactions among post injury disorders are often complex and remain enigmatic. Motor and sensory deficits are rare to determine a minor portion of post global ischemia. Cognitive deficits are most common among survivors following global I/R injury (Hartman et al., 2005; Stradecki-Cohan et al., 2017). In addition, pre-injury risk factors (modifiable and non-modifiable) differentially contribute to the degree of cognitive impairment. Traditional risk factors like hypertension, smoking, hyperlipidemia and hyperhomocysteinemia were shown to increase cognitive impairment (Leys et al., 2005; Ridker et al., 2008; Rowan et al., 2007; Tamura et al., 2008). The underlying pathological mechanisms are not well known; however, pathogenesis of cognitive impairment may involve vascular cognitive impairment or neurodegeneration type of pathology i.e. Alzheimer's type of pathology.

Cognitive impairment is an umbrella term that can be evaluated in terms of learning and memory, language and praxis and executive function (Cumming et al., 2013). Cerebral performance category test to evaluate gross neurological deficits and modified Rankin scale test to assess overall functional disability which were not able to differentiate the patients with different degree of cognitive deficits that result in underestimation of cognitive deficits (Banks and Marotta, 2007; Stradecki-Cohan et al., 2017). Diagnostics and Statistical Manual of Mental Disorders IV or Mini Mental State Examination (MMSE) is sensitive enough to detect moderate to severe cognitive deficits. Further, 'Montreal Cognitive Assessment' with improved efficiency and sensitivity was developed (Bour et al., 2010; Y. Dong et al., 2010; Stradecki-Cohan et al., 2017) to achieve better evaluation of cognition, to simplify cognitive testing in clinical situations and to provide sensitive and reliable testing computer assessments (Sachdev et al., 2004; Torgersen et al., 2010). Upon validation and proper implementation, above tests would allow for early detection of cognitive deficits. So far, there is no effective therapeutic intervention to

treat cognitive post-stroke cognitive impairment but clinically approved drugs for AD such as donepezil, rivastigmine, galantamine and memantine shown to be efficacious for post-stroke cognitive impairment. Earlier studies demonstrated that these drugs significantly improved certain cognitive domain like executive function (Ballard et al., 2008; Dichgans et al., 2008; J.-H. Sun et al., 2014). However, the benefit of the drug on the overall global function and daily activities involving cognition still remains uncertain.

1.10 *Unsuccessful treatment options:*

Numerous chemical compounds targeting different molecular mechanisms such as excitotoxicity, oxidative stress, calcium toxicity, neuroinflammation that are involved in the pathology of I/R injury with potential efficacy in the animal models have been translated to clinical trials as seen in table1.1. The failure of translation from preclinical to clinical may involve several factors which includes therapeutic time window, gender, age, ischemia duration, identification of stroke type and morphological differences in brain structure (Sena et al., 2007; Tymianski, 2010). Despite international recommendations by STAIR (Stroke Therapy Academic Industry Roundtable) criteria followed for preclinical research to maintain quality of animal studies, there is limited success in clinical trials (Fisher et al., 2009; Sutherland et al., 2012). Therefore, there is a pressing need to address the drawback of the present therapy and to investigate novel therapeutic interventions to treat cerebral I/R injury and its associated co-morbidities.

Abbreviations for table1.1.

ASTIN- (Acute Stroke Therapy by Inhibition of Neutrophils), **PAIS**-Paracetamol (Acetaminophen) In Stroke, **RANTTAS**-A randomized trial of tirilazad mesylate in patients with acute stroke, **FAST-MAG-** Field Administration of Stroke Therapy-Magnesium, **CLASS-** Clomethiazole acute stroke study in ischemic stroke, **AXIS-** Atrial in acute stroke patients, **SCAST-** The angiotensin-receptor blocker candesartan for treatment of acute stroke, **FIST-** Flunarizine in stroke treatment, **TRUST-** Randomised, double-blind, placebo-controlled trial of nimodipine in acute stroke, **NICE-** Rationale and design of a double-blind, placebo-controlled, randomized trial to evaluate the safety and efficacy of nimodipine in preventing cognitive impairment in ischemic cerebrovascular event, **VENUS-** Very Early Nimodipine Use in Stroke, **AbseTT II-** Abciximab in Emergency Treatment of Stroke Trial, **MATCH-** Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients, **SPS3-** Secondary Prevention ofSmall Subcortical Stroke, **HAEST-** Heparin in Acute Embolic Stroke Trial, **IST-** international Stroke Trial, **SaTIS-** Safety of Tirofiban in acute Ischemic Stroke, **TAPRISS-**Triflusal versus Aspirin for Prevention of Infarction: a Randomized Stroke Study, **ICTUS-** Citicoline in the treatment of acute ischaemic stroke, **WEST-** Women Estradiol for Stroke Trial, **mRECT-** Modified Randomized Exposure Controlled Trial, **NEST-3-**NeuroThera® Efficacy and Safety Trial, **MAST-** Multi-center Acute Stroke Trial, **NMDA-** N-Methyl-D-aspartic acids, **AMPA**-2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid, **COX-** Cyclooxygenase, **ICAM-**Intercellular Adhesion Molecule 1.

S. No	Drug	Mechanism of action	Result	Phase	Reference
1.	Licostinel (ACEA 1021)	Competitive antagonist of glycine at NMDA	Safe and tolerable	III	(Albers et al., 1999)
2.	Selfotel	NMDA antagonist	Neurotoxic	III	(Davis et al., 2000)
3.	Aptiganel	NMDA antagonist	May have detrimental effects	III	(Albers et al., 2001)
4.	Gavestinel	Selective antagonist of glycine at NMDA receptor	Lack of efficacy	III	(Lees et al., 2000)
5.	Eliprodil	NMDA Polyamine antagonist	Lack of efficacy	II	(Ikonomidou and Turski, 2002)
6.	Magnesium	NMDA channel blocker, calcium antagonist (FAST-MAG)	No rapid neurological improvement as soon as 45 min after stroke	III	(Balucani et al., 2016)
7.	ZK200775	AMPA antagonists	Worsen neurological condition	II	(Elting et al., 2002)
8.	Lifarizine	Sodium channel blocker	Well tolerated, Lack of efficacy	Pilot	(Squire et al., 1996)
9.	Lubeluzole	Sodium channel blocker	Lack of efficacy	III	(Diener et al., 2000)
10.	Sipatrigine (BW619C89)	Sodium channel antagonist	No improvement in neurological recovery	II	(Muir et al., 2000)
11.	Baclofen	GABA agonists	To be determined	Ongoing	(Maneyapanda et al., 2017)
12.	Clomethiazole	GABA receptor mimetic (CLASS-1)	No improvement in neurological recovery	III	(Lyden et al., 2002)
13.	Nalmefene	Kappa opoid antagonist	Safe and well tolerated	III	(Clark et al., 2000)
14.	Naloxone	Opoid antagonist	No improvement in neurological	III	(Fallis et al., 1984)

S. No	Drug	Mechanism of action	Result	Phase	Reference
			recovery		
15.	BMS-204352	Potassium channel opener (POST)	Failed to show efficacy	III	(Jensen, 2002)
16.	Cyclosporine A	Immunosuppressant	No infarct size reduction (alone)	II	(Osman et al., 2011)
17.	FK 506	Immunosuppressant	Adverse events reported	II	
18.	Enlimomab	Anti-ICAM1 antibody (EAST)	Lack of efficacy Worsen stroke outcome	III	(Investigators and others, 2001)
19.	UK-279,276	Neutrophil inhibitory factor (ASTIN)	No improvement in neurological recovery in futility analysis	II	(Krams et al., 2003)
20.	Paracetamol	COX inhibitor	Might be beneficial on functional outcome. Do not support use of high dose	III	(den Hertog et al., 2009)
21.	Minocycline	Tetracycline antibiotic	Significant neurological improvement only in males	III	(Amiri-Nikpour et al., 2015)
22.	Tirilazad	Lipid peroxidation inhibitor (RANTTAS)	No improvement in neurological recovery	III	(Investigators and others, 1996)
23.	Ebselen	Lipid peroxidation inhibitor	No improvement in neurological recovery	II	
24.	Granulocytecolony stimulating factor (G-CSF)	Stem cell regeneration (AXIS)	No improvement in neurological recovery	IIb	(Floel et al., 2011)

S. No	Drug	Mechanism of action	Result	Phase	Reference
25.	Atenolol	Selective β-1 receptor antagonist	No difference in stroke outcome		
26.	Candesartan	AT1 receptor antagonist (SCAST)	Harmful effects reported	III	(Sandset et al., 2011)
27.	Flunarizine	Calcium channel blocker (FIST)	No improvement in neurological recovery	III	(Franke et al., 1996)
28.	Nimodipine	Calcium channel blocker (TRUST, NIMPAS, NEST, VENUS and NICE)	No improvement in neurological recovery	III	(Horn et al., 2001; Horn and Limburg, 2001)
29.	PY 108-068	Calcium antagonist	Lack of efficacy but safe	NA	(Vorstrup et al., 1986)
30.	Abciximab	Platelet glycoprotein IIb/IIIa inhibitor (AbseTT)	Lack of efficacy and safe	III	(Taqi et al., 2012)
31.	Clopidogrel	Anti-platelet (MATCH, SPS3)	Lack of efficacy in preventing secondary attack	III	(Diener et al., 2005, 2004)
32.	Dalteparin	Anti-coagulant (HAEST)	No improvement in neurological recovery	III	(Thon and Gurol, 2016)
33.	Heparin	Anti-coagulant (IST)	No improvement in neurological recovery	III	(Group, 1997)
34.	Tirofiban	GP-IIb/IIIa inhibitor (SaTIS)	Safety, No improvement in neurological recovery	III	(Ciccone et al., 2014; Siebler et al., 2011)
35.	Pentoxifylline	Blood viscosity reducer	No improvement in neurological recovery	III	(Hsu et al., 1988)
36.	Triflusal	Anti-platelet (TAPRISS)	No improvement in neurological recovery	NA	(Culebras et al., 2004)
37.	Mannitol	Hyper osmotic agent	Harmful effects reported	NA	(Bereczki et al., 2001)
38.	Hyperbaric oxygen delivery	Oxygen supply	May be harmful for acute ischemic stroke	NA	(Anderson et al., 1991; Rusyniak et al., 2003)
39.	Normobaric oxygen delivery	Oxygen supply	Transient improvement of clinical deficits. Further studies required	Pilot	(Lo et al., 2003; Singhal et al., 2005)

S. No	Drug	Mechanism of action	Result	Phase	Reference
40.	Hypothermia	Hypoperfusion	Risk of infection and cardiovascular complications	III	(Linares and Mayer, 2009)
41.	Citicoline (CDP choline)	Cell membrane stabilizer (ICTUS)	Lack of efficacy	III	(Dávalos et al., 2012)
42.	Estradiol	Anti-oxidant, anti-inflammatory (WEST)	Worsen neurological recovery	III	(Horowitz and Brass, 1994; Viscoli et al., 2005)
43.	ONO-2506	Astrocyte Modulating Factor	Lack of efficacy in futility analysis	II	(Lakhan et al., 2009; Pettigrew et al., 2006)
44.	Repinotan	Serotonin agonist (mRECT)	No improvement in neurological recovery	IIb	(Teal et al., 2009)
45.	Transcranial laser therapy	Neuron repair by various mechanisms (NEST-3)	Futile	III	(Teal et al., 2009)
46.	Streptokinase	Thrombolytic (MAST)	No improvement in neurological recovery	III	(Teal et al., 2009)

Table 1.1. Representing the failed clinical trials categorized based on mechanism of action.

1.11 *Reason for exploration of novel targets:*

From the above facts, an ischemic event is critically damaging to the brain and needs rapid treatment. It is estimated that millions of neurons die and billions of synapses are lost in the human brain per minute following a prolonged ischemic event. The potential loss of neurons may escalate to billions if the treatment is delayed. Therefore, research on stroke has emphasized the significance of time in the treatment of ischemic injured brain. Stroke is currently treated with the classical therapy, rt-PA with a therapeutic window of 3-4.5 hours and recently by endovascular procedures with an extended window of 6 hours within stroke. Despite extensive research in the identification and development of neuroprotective compounds targeting various mechanisms in the pathology of ischemic injury only few strategies were translated into clinical trials. However, most of the neuroprotective compounds that are translated to clinical trials have shown lack of efficacy, primarily because of overrated efficacy of the compounds in the preclinical studies. Ischemic injury involves a complex array of molecular changes in the pathological cascade, therefore, future studies should focus on multi-target approach rather than single target.

Interestingly, from an evolutionary instinct of adaptive responses brain initiates spontaneous recovery mechanism after an ischemic event which includes alteration in bioenergetics, survival pathways, synaptic plasticity and neurogenesis to a limited extent. During initial phases of ischemic injury, the identification of endogenous protective mechanisms may provide therapeutic options to target a wide range of patients. Therefore, development of novel therapeutic interventions to trigger endogenous molecular targets may reveal specific targets to augment brain repair following cerebral I/R injury.

2. Review of literature

2.1 *Endogenous mechanisms of brain*

The brain itself has an immense capacity for self-preservation during conditions of stress or ischemia (Albers et al., 2011; Lo, 2008). The ischemic brain activates certain endogenous molecular pathways to produce an "ischemic tolerant phase" (Dirnagl et al., 2003). Extensive research is being conducted to understand the underlying molecular mechanisms involved in endogenous self-protective pathways/tolerance following ischemia. However, the intricate mechanisms behind the endogenous recovery are not well understood.

The term "tolerance" and "preconditioning" are often interchanged. The concept of pre-conditioning has been known since ancient times. The king of Pontus, Mithridates IV (c 132-63 B.C.), ingested daily small doses of poison and toxin to protect himself against poisoning (Dirnagl et al., 2003). Further, Paracelsus father of toxicology, introduced the concept of "dose makes the poison" (Dirnagl et al., 2003). In the field of literature and philosophy, preconditioning is termed as "what does not kill you makes you stronger" by Nietzsche. Preconditioning can be in many form including pharmacological agents or stimuli like ischemia or hypoxia. For example, a daily routine of exercise may also be considered as a form of preconditioning (Kavazis, 2009; F Zhang et al., 2011).
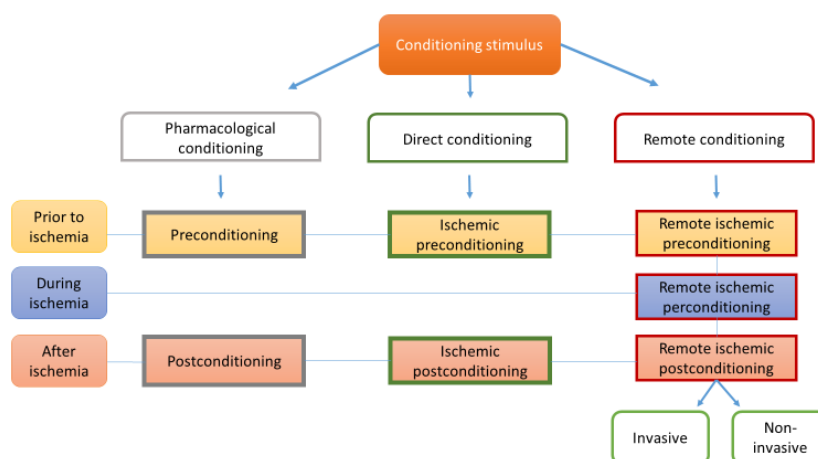
Fig. 2.1. Pictorial representation of timing of conditioning stimulus with respect to an ischemic event

S.NO	TERMS	DEFINITION
1.	Pharmacological preconditioning	Administration or treatment with pharmacological agent before a prolonged/fatal ischemic event
2.	Pharmacological preconditioning	Administration or treatment with pharmacological agent after a prolonged/fatal ischemic event
3.	Ischemic preconditioning (IPC)	Application of brief periods of cycles of ischemia and reperfusion to arteries supplying blood to vital organs (brain or heart) before a prolonged ischemic phase
4.	Ischemic postconditioning (IPOC)	Application of brief periods of cycles of ischemia and reperfusion to arteries supplying blood to vital organs (brain or heart) after a prolonged ischemic phase
5.	Remote ischemic preconditioning (RIPC)	Application of brief periods of cycles of ischemia and reperfusion to non-vital arteries (renal, abdominal, femoral) to provide protection to vital organs before a prolonged ischemic phase
6.	Remote ischemic perconditioning (RIPEC)	Application of brief periods of cycles of ischemia and reperfusion to non-vital arteries (renal, abdominal, femoral) to provide protection to vital organs during a prolonged ischemic phase
7.	Remote ischemic postconditioning (RIPOC)	Application of brief periods of cycles of ischemia and reperfusion to non-vital arteries (renal, abdominal, femoral) to provide protection to vital organs after a prolonged ischemic phase
8.	Invasive remote conditioning	Renal and abdominal arteries can be assessed only through invasive remote conditioning. Invasive remote conditioning is performed on femoral artery as well.
9.	Non-invasive remote conditioning	This technique is suitable for femoral artery. Non-invasive remote conditioning can be performed by placing a thin elastic tourniquet around the upper third of the limb in a tight position to occlude the arterial blood supply. The ischemia to hind limb will be confirmed by cyanosis and hypothermia of limb; after reperfusion, the skin color returned to normal pink colour.

Table 2.1. Introduction to the terms of types of conditioning stimulus
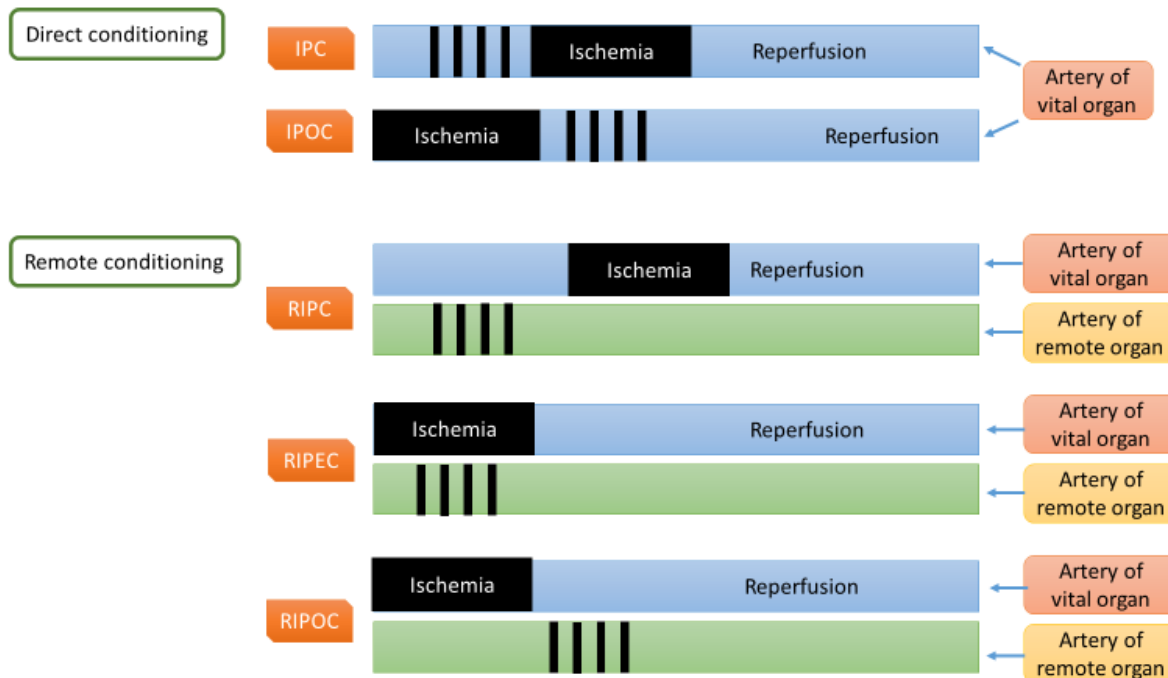
Fig. 2.2. Treatment algorithm of direct and remote ischemic conditioning.
IPC-ischemic preconditioning; IPOC-Ischemic Post conditioning; RIPC-Remote Ischemic preconditioning; RIPEC-Remote ischemic preconditioning; RIPOC-Remote Ischemic Post conditioning.

2.2 *Ischemic preconditioning and ischemic postconditioning:*

The revolutionary concept of ischemic preconditioning (IPC) was initially demonstrated by Murray *et al* in 1986 in an animal model of myocardial injury. Briefly, four alternating cycles of ischemia and reperfusion of 5 min each was performed on vital artery (supplying heart) before a prolonged ischemic event i.e., 40 min circumflex coronary artery occlusion in dogs. They observed that there was 25% reduction in infarct size when compared to injury group which indicated IPC induced cardio protection. They demonstrated that high energy phosphates were preserved in preconditioned heart (Murry et al., 1986). Further, the efficacy of IPC in rats was first demonstrated by Li and his colleagues in 1992. They found that preconditioning induced by 3 cycles of ischemia and reperfusion 3 min each, protected the rat heart as evidenced by reduced infarct size in an animal model of low collateral blood flow (Li et al., 1992). Another group showed that single cycle of ischemic and reperfusion for 5 min each appears to be sufficient to protect the rabbit heart against myocardial injury (Cohen et al., 1991). Further, protection against coronary artery by-pass grafting was demonstrated by Yellon and his colleagues with two cycles of ischemia (3 min) and reperfusion (2 min) (Yellon et al., 1993).

Later studies by Murray *et al* and various research groups suggested that episodic circumflex occlusion and left anterior descending artery (LAD) occlusion could induce cardio protection in circumflex distribution and LAD distribution respectively. Using a canine model, Przyklenk and colleagues demonstrated that brief episodes of ischemia and reperfusion in circumflex artery provided protection against 60 min occlusion of LAD. However, due to unpredictability of ischemic injury there is a need to develop therapeutic options that are protective after I/R injury.

Therefore, in the recent past considerable effort has been made to discover therapeutic interventions that are effective post ischemic injury. The concept of ischemic postconditioning (IPOC) was clearly demonstrated by Zhao *et al* in a canine model of myocardial injury while IPOC was first demonstrated and coined by Na *et al* (Na et al., 1996). IPOC can be defined as brief alternate cycles of ischemia and reperfusion to the artery supplying vital organ that is applied immediately after the onset of reperfusion or delayed after reperfusion following an ischemic event to provide protection to the target organ. Briefly, they showed that IPOC achieved by 3 alternative cycles of 30 sec ischemia and 30 sec reperfusion during early onset of reperfusion attenuated infarct size, endothelial dysfunction and tissue edema as effectively as IPC in a canine model of 60 min LAD occlusion and 3 h of reperfusion (Zhao et al., 2003).

Like myocardial ischemia, the treatment goal of cerebral I/R injury is to revascularise the occluded cerebral blood vessels to allow early reperfusion (Fig2.3). Moreover, the mechanisms of I/R injury share similar concept like myocardial I/R injury. The translation of postconditioning concept from cardiology to neurology was first demonstrated in 2006 simultaneously by two research groups. Burda *et al* demonstrated that IPOC performed 2 days following lethal ischemic phase (delayed IPOC) was protective against transient ischemia. In this study, IPOC at right time and optimal intensity provides significant protection against 15 min of transient ischemia (Burda et al., 2006). In the same year Zhao *et al* demonstrated that IPOC induced by 3 cycles of 10 sec occlusion and 30 sec reperfusion attenuates the infarct size, reduced free radical generation and apoptosis in a model of focal ischemia (Zhao et al., 2006).
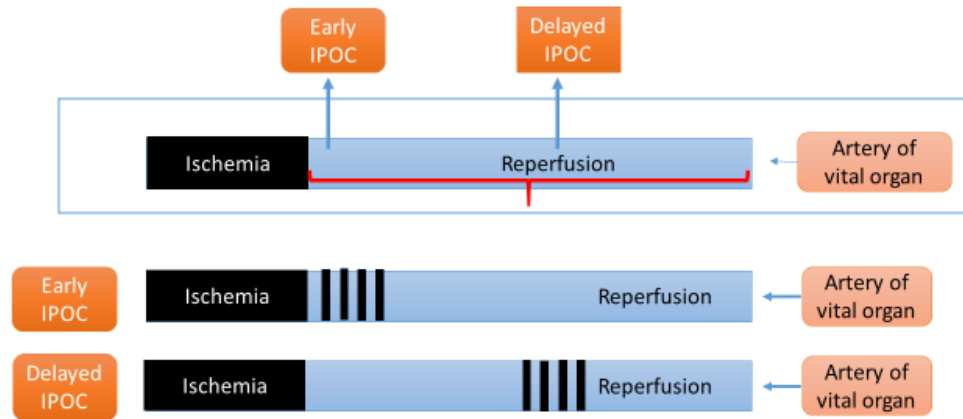
Fig. 2.3. Diagrammatic representation of early and delayed types of ischemic post conditioning

Meanwhile the first evidence for the protective effects of IPOC against global ischemia was provided by Rehni and Singh *et al* (Rehni and Singh, 2007). They found that three 10 sec episodes of common carotid artery (CCA) ameliorates short term memory, motor coordination and lateral push response. Another study demonstrated the protective effect of IPOC using various algorithms. They found that three cycles of 15 sec or 30 sec conditioning stimulus and three cycles of 15 sec conditioning stimulus 45 min after lethal ischemia were effective against global ischemia (J. Wang et al., 2008). Further, various invitro and invivo studies demonstrated that the potential neuroprotective role of IPOC against cerebral I/R injury(Burda et al., 2006; Gao et al., 2008; Loukogeorgakis et al., 2007; J. Wang et al., 2008; Xing et al., 2008).

Despite of numerous reports on IPOC neuroprotection, there are certain factors to be considered to translate IPOC into clinical trials. Translation of IPOC into clinical studies is not just science, rather it involves psychological, social and most importantly medical ethics as IPC and IPOC involves mechanical interruption of vital arteries. The major principles of medical ethics include *Non-maleficence* and *beneficence* which refers to "no harm to patients" and "any benefit to patient". For any treatment or intervention there should be a balance between the above two principles. In clinical trials, whether IPC and IPOC is detrimental or patients may benefit from the damaging effects of stroke is to be considered. The factors to be taken into consideration for the translation of IPC and IPOC into clinical studies includes the patient and medical doctor's concerns regarding the risk of additional injury and fear for the acceptance of

the procedure. Therefore, development of least hazardous, yet beneficial therapeutic interventions are crucial for successful clinical translation (Hess et al., 2015).

2.3 *Remote ischemic preconditioning*

To avoid the mechanical interruption of vital arteries and to preserve the beneficial effects of conditioning stimulus, a novel technique termed as "regional preconditioning" or "preconditioning at a distance" or "intra-organ preconditioning" where preconditioning at different region provide protection to the nearby region in the same organ was introduced by (Przyklenk et al., 1993). This research paved the way for further research in remote preconditioning where stimulus to one remote organ protect another distant organ. In 1977, Birnbaum and colleagues for the first time demonstrated that brief episodes of ischemia and reperfusion to rabbit hind limb by electrical stimulation was protective against heart ischemia (Birnbaum and Kloner, 1997). Further, in the same year Oxman and his colleagues introduced hind limb ischemia as preconditioning stimulus, a minimally invasive or non-invasive technique of remote ischemic preconditioning (RIPC) that was achieved by 10 min limb ischemia using a thin elastic tourniquet. They demonstrated that brief ischemia to the remote organ, hind limb protects heart against tachyarrhythmia in rats (Oxman et al., 1997). Later this was extended to humans by Kharbanda and colleagues in 2002. For the first time they demonstrated that clinically feasible transient limb ischemia prevents endothelial ischemic reperfusion injury in humans (Kharbanda et al., 2002). Consequent studies demonstrated the protective effects of renal and abdominal preconditioning on myocardial infarction (Dickson et al., 2002; Lang et al., 2006). This preconditioning at a distance or RIPC applied to wide range of remote tissue induces multi-organ protection including brain against subsequent ischemic reperfusion injury. In subsequent studies on cerebral ischemia, RIPC was found to be protective against cerebral injury (Malhotra et al., 2011; Rehni et al., 2007). In a mouse model of embolic ischemic stroke, RIPC was shown to be protective alone or in combination with tissue plasminogen activator (Hoda et al., 2012). In another study, RIPC improved the functional outcome, attenuated the infarct size in animal models of focal and global ischemic reperfusion injury (Ren et al., 2008; Wei et al., 2012). The support from the preclinical evidences on effectiveness of RIPC in multi-organ protection (heart and brain), paved the way for rapid translation of RIPC to clinical trials.

Moreover, RIPC is economical and easy to perform in human subjects using simple blood pressure cuff. Various clinical studies like Effect of Remote Ischemic preconditioning on Clinical outcomes in patients undergoing Coronary Artery bypass graft surgery (ERICCA), Cardiac Remote Ischemic Preconditioning in Coronary Stenting (CRISP Stent) and the Remote Ischemic Preconditioning for Heart Surgery study (RIP Heart- Study) evaluated the protective effect of RIPC in CABG and coronary stenting (Hausenloy et al., 2012; Hoole et al., 2009). Further, randomized clinical trials in patients demonstrated effectiveness of RIPC in pediatric congenital cardiac defect surgery, coronary valve surgery, cardiopulmonary bypass surgery (Cheung et al., 2006; Kharbanda et al., 2006; Xie et al., 2012). The probable reason for clinical applicability of RIPC in cardiac research rather than on cerebral research may be due to the fact that most of the cardiac surgeries are elective. Moreover, the initial studies were performed in myocardial salvage that leads to dominance of RIPC in cardiac studies. The most important reason could be the myocardial ischemia, which is an important adverse outcome of most of the cardiac surgeries; this makes RIPC an attractive cardio-protective strategy. However, there are few studies that supported the neuroprotective role of RIPC in humans. In the carotid endarterectomy study, RIPC non-significantly preserved the saccadic latency, a measure of neurologic function (Walsh et al., 2010). RIPC attenuated the neurological injury biomarkers in patients undergoing cervical decompression procedure (Hu et al., 2010).

Though earlier preclinical and clinical studies have evidenced the cardio-protective and some neuroprotective effect of RIPC, there are still some gaps majorly on practical application that researchers must overcome. There are certain queries regarding the methods in RIPC research like site of RIPC, duration and cycles of conditioning stimulus. Several experimental and human studies stated that remote limb ischemia is the most attractive strategy of RIPC (Faries et al., 2008; Hoda et al., 2012; Kharbanda et al., 2006; Walsh et al., 2010; Xie et al., 2012). As cerebral ischemic injury is an emergency condition with minimal signs and symptoms during the progression of the disease, the clinical applicability of RIPC is uncertain. Moreover, it is difficult to outweigh the advantages of RIPC when compared to its lethal effects during prolonged ischemia. Further, neurovascular conditions are often associated with co-morbid conditions, the effect of RIPC on age, gender, co-existing disease; current therapies are not well

understood. However, large scale multicenter studies will be required for successful clinical translation of RIPC.

Despite extensive research in the area of RIPC, the underlying moleular mechanisms are yet to be explored. It seems to work by activation of several complementary molecular pathways including intracellular survival kinases and susbsequent modification of mitochondrial function (Hausenloy and Yellon, 2008; J. Li et al., 2011). However, the exact nature of signal transduction from RIPC to target tissue is unknown. Out of several communication theories proposed for RIPC, neural, humoral and systemic anti-inflammatory responses were known to be investigated in the earlier studies (Fig.2.4). The neural link in the transfer of RIPC stimulus was supported in a study when ganglionic blocker hexamethonium attenauted the protective effects of RIPC (H.-L. Dong et al., 2010; Gho et al., 1996). The humoral theory suggests that certain ciculating factors are released from the site of RIPC to act on target organ (Kharbanda et al., 2009; Lim et al., 2010; Patel et al., 2002). In addition to the release of bloodborne and neurogenic factors, RIPC was shown to suppress the pro-inflammatory cytokine expression and neutrophil adhesion (Konstantinov et al., 2004; Shimizu et al., 2010).

The window of protection offered by RIPC may be divided into two distinct phases (Fig.2.5). The primary window of protection induced by RIPC will lasts for about few hours (2-3 hours) and then secondory window of protection repappears after 24-72 hours, and can persists upto 72-96 hours. The first window of protection occurs within 2-3 hours of RIPC, therefore, to maximise the protective effect of RIPC in patients stimulus should be applied no more than three hours before the ischemic event. The second window of protection provides sustained effect. However, the magnitude of protection is less than first window. The initial window of protection may arise due to activation of certain existing intracellular kinases whereas increased expression of signalling molecules may be the putative reason for the delayed form of protection by RIPC (Dezfulian et al., 2013; Heusch, 2013). Evidence for this distinct window of protection by RIPC was provided by Loukogeorgakis in a series of experiments on healthy individuals. Breifly, they estimated the RIPC ability to attenuate endothelial I/R injury at different time course like immediate, 4 hour, 24 hour and 48 hour before I/R injury. Ultrasound assessment of endothelial injury showed that RIPC was effective at immediate, 24 and 48 hour

following reperfuion but not when applied at 4 hour before injury (Loukogeorgakis et al., 2005). In a pediatric study, children with complex congenital heart disease undergoing RIPC 24 hours before displayed low levels of natriuretic peptide post surgery. However, no beneficial effect was observed in inflammatory response and cardiac clinical parameters, providing the evidence that late phase of RIPC was not clinically effective against I/R injury assocaited with cardiopulmonary bypass in children (Pavione et al., 2012).
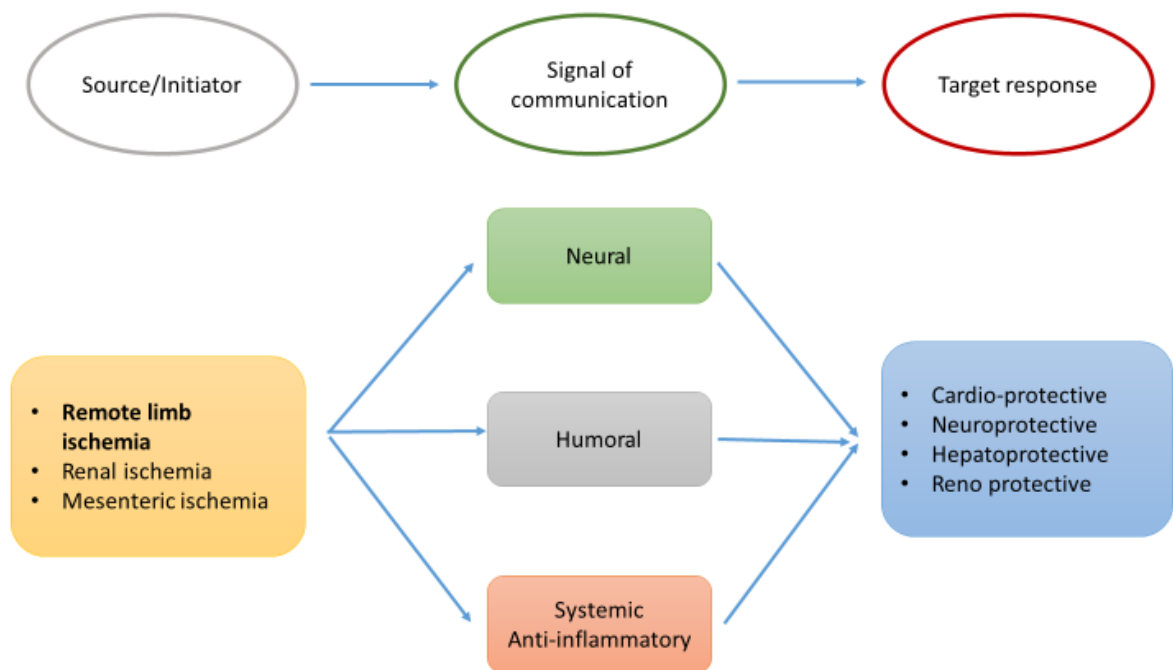
Fig. 2.4. Diagrammatic illustration of RIPC signal initiation in remote organ, communication to target organ and effect on target organ
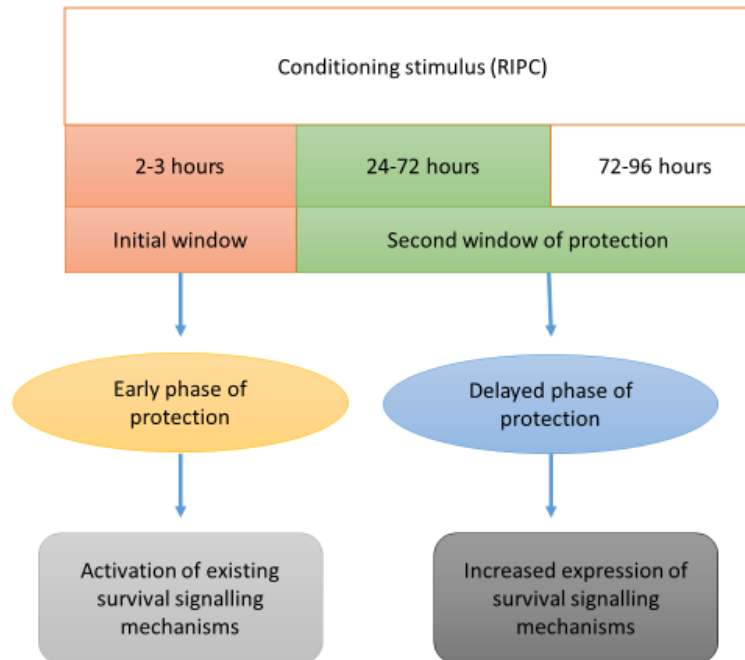
Fig. 2.5. Pictorial representation of window of protection with remote preconditioning (RIPC)

Studies to evalute the combined effects of early and late phase should be performed in future to maximise the protective effects of RIPC. In the field of cardiac research, RIPC has been clinically applied and explored more than a decade now, still there are no conclusive evidences for the safety and efficacy of RIPC intervention. Current theraputic guidelines for heart treatment still did not include RIPC as an intervention, indicating there is a need of further pre-clinical and clinical studies to identify the time course and molecular mechanisms of RIPC. Aditionally, RIPC in experimental cerebral I/R injury may help us discriminate between the protective and delerious effects following I/R injury. Identification of endogenous molecular signalling cascades may provide promising startegies for the treatment of cerebral I/R injury. Moreover, the identified effectors and transducers of RIPC can be used as preventive measures for patients who are risk of brain I/R injury in near future (based on modifiable and non-modifiable risk factors).

2.4 *Remote ischemic perconditioning:*

Brief interupted cycles of ischemia and reperfusion to the remote organs during the time of prolonged ischemia is termed as remote ischemic perconditioning (Schmidt et al., 2007) and as

remote ischemic periconditioning (Rentoukas et al., 2010; Zhao et al., 2009). The meaning of peri means "around something in time" while per means "during the course of something" which is more appropiate. Moreover, Schmidt *et al*, who first proposed the technique in 2007, proposed the term as "remote perconditioning", as it can be applied after ischemia and before reperfusion (Schmidt et al., 2007). Breifly, in a model of LAD occlusion, 4 cycles of non-invasive RIPEC (5 min each) was performed in pigs. The protective effects of RIPEC were evidenced by improved cardiac parameters and reduced myocardial infarction through KATP dependent mechanism (Schmidt et al., 2007). In a previous study RIPEC in combination with IPOC was found to increase the expression of neuoglobin that contribute to the neuroprotection of conditioning stimulus (Ren et al., 2015). The major advantage of this technique in clinical situations would be that it can applied during the ischemia of emergency or elective surgeries during the transit of the patient in the ambulance.

Based on the timing on the conditioning stimulus there may be differences in the activation of biochemical and signal transduction pathways by various types of remote conditioning. Due to limited research in RIPEC, the molecular mechanism of this relatively novel concept is still not clear. However, there may be some overlap in the molecualr mechanisms of RIPC and RIPEC, as it is practically difficult to discriminate RIPC and RIPEC in clinical settings. In addition, RIPEC is applied during the ischemic phase, therefore, the fundamental biology of RIPEC protection to target organ may slightly vary from RIPC. Future studies may provide a scope on signalling mechanisms of RIPEC. According to a hypotheis, the protective pathways of RIPEC were classified as connective mechanisms that include the conventional neural, humoral and systemic mechanisms and the signal and effector mechanisms (Szijártó et al., 2012).

Due to limited research, there is less evidence to support the role of humoral and neural factors in RIPEC. The possible connective mechanism in RIPEC technique may be through systemic inflammatory response. Data from earlier suggests that RIPEC is able to alter the gene expression of anti-inflammatory genes. In a rat model of LAD occlusion, 4 cycles of 5 min of ischemia and reperfusion to hind limb during ischemic phase attenuated the inflammatory responses (Wei et al., 2011).In another study, RIPEC reduces the renal dysfunction by causing a down-regulation in the expression of pro-inflammatory cytokines (Sedaghat et al., 2016). A

significant and favorable alteration in the expression of tumor necrosis factor –alpha (TNF-α) by RIPEC was reported in a model of liver I/R injury (Czigány et al., 2013). Additionally, non-invasive RIPEC was found reduce neutrophil accumulation and exert anti-inflammatory activity to reduce I/R injury in liver transplantation (Jia et al., 2015). In a transient middle cerebral artery occlusion (MCAO) model of stroke RIPEC was shown exert superior neuroprotection when compared to RIPC (Hahn et al., 2011). Another study showed the protective effects of RIPEC in an model of embolic stroke in ovariectomized rats with or without treatment of tissue plasminogen activator (Hoda et al., 2014).

Considering the promising results of RIPEC against cerebral, hepatic, renal and cardiac I/R injury in pre-clinical evidences, RIPEC protective effect has been explored in certain clinical conditions. Moreover, RIPEC can be applied in emergency and elective surgeries by simple blood pressure cuffs is more acceptable in clinical settings with minimal or no side-effects. In a randomized controlled clinical trial RIPEC attenuated the myocardial injury in patients undergoing valve replacement (Li et al., 2010). RIPEC as an adjuvant to angioplasty improved the myocardial salvage during hospital transfer in acute myocardial infarction patients (Bøtker et al., 2010). The neuroprotective effect of RIPE against acute stroke was demonstrated in an earlier study (Hougaard et al., 2010). However, future studies are required to demonstrate the neural and humoral mechanisms as well as signaling mechanisms of RIPEC. Multicenter large randomized clinical studies are required to confirm the efficiency of this technique and that will allow RIPEC as a routine clinical practice to protect wide range of target organs.

2.5   *Remote postconditioning*

The preclinical and clinical data of RIPC and RIPEC against cardiac, hepatic and renal ischemic injury have been satisfactory while unsatisfactory for emergency conditions like cerebral ischemic injury. The clinically feasible manifestations targeting unpredictable damage of cerebral I/R injury must be developed with a similar magnitude of protection like RIPC and RIPEC. RIPOC is a novel technique induced by brief interrupted cycles of ischemia and reperfusion to remote or distal organ to induce protection in vital organs (heart or brain). The conditioning stimulus in RIPOC may be applied during early onset of reperfusion or delayed onset of reperfusion depending upon the clinical setting as it can be applied in elective and

unpredictable/emergency conditions. RIPOC phenomenon was first demonstrated by Kerendi *et al* in 2005. They showed that renal artery occlusion immediately before reperfusion of a coronary artery occlusion, reduced the myocardial infarction (Kerendi et al., 2005). Later in 2007, Andreka *et al* demonstrated the protective effects of RIPOC in a porcine model of myocardial injury. In that study, they induced myocardial infarction with balloon inflations of LAD artery and RIPOC was achieved by four 5 min cycles of ischemia and reperfusion to lower limb in pigs. They showed that RIPOC applied during early reperfusion was able to reduce infarct size in myocardial injury without any potential adverse effects (Andreka et al., 2007). Ren *et al* for the first time expanded the concept of remote postconditioning (RIPOC) against cerebral I/R injury in 2009. They found that early RIPOC performed immediately after onset and delayed RIPOC (Fig.2.6) conducted 3h after reperfusion in the hind limb was protective against focal cerebral I/R injury (Ren et al., 2009).
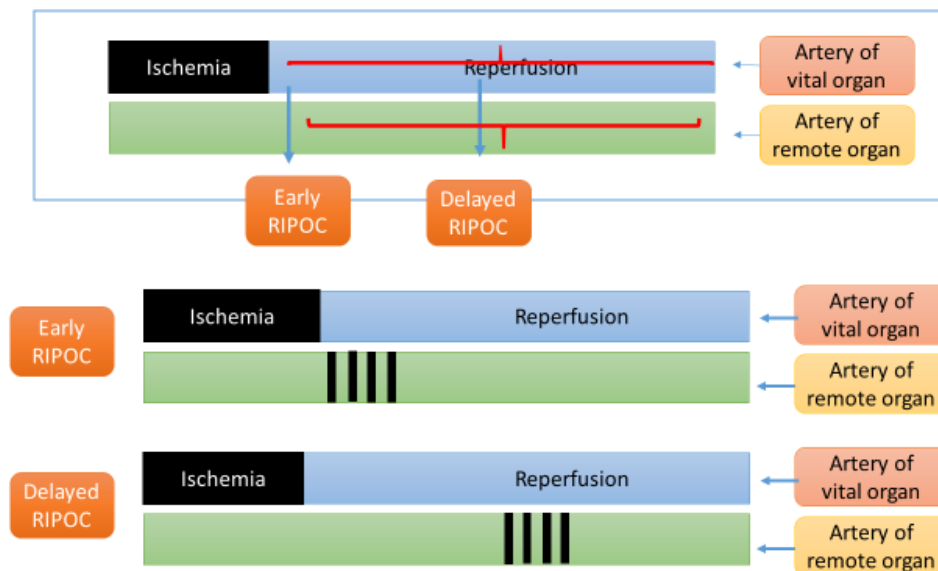
Fig. 2.6. Diagrammatic representation of early and delayed remote post conditioning (RIPOC)

Due to limited research in the field of RIPOC, the fundamental biology of neuroprotection with brief episodes of ischemia and reperfusion to hind limb remains to be elucidated. Moreover, the signaling pathway involved in the communication of the protective signals from remote organ to target organ are yet to be discovered. Strong evidences from the cardiac research on RIPC and RIPEC suggests that the existence of neural, humoral and systemic mechanisms in RIPOC protection for transmission of the signal to target organ.

The neural hypothesis suggested that RIPOC induction to the hind limb generates an endogenous substance that activates local afferent neural pathway which stimulates efferent pathway that terminates at the target organ. Ren and his colleagues found that RIPOC protection against infarction was abolished in the presence of afferent nerve blocker, capsaicin. This suggests that afferent nerve pathways serves in connection between the remote and target organ (Ren et al., 2009). The neural pathway existence in the neuroprotection was further supported by other two earlier studies. Pignataro *et al* demonstrated the presence of neural pathway communication in RIPOC neuroprotection. They found that hexamethonium, selective ganglioplegic blocker of the autonomic nervous system partially prevented the RIPOC neuroprotection, suggesting the neuronal pathway activation by RIPOC (Pignataro et al., 2013). They demonstrated that peripheral nerve induction by electrical stimulation and RIPOC share similar protective mechanisms and RIPOC protection might involves neural pathway modulation (Xiao et al., 2015).

The humoral mechanism involves the transmission of signal from the remote organ to the target organ via humoral or blood borne factors. Data from cardiovascular research in RIPC identified interleukin-10, plasma nitrite, micro RNA 144, stromal derived factor 1 a (SDF-1) as the humoral mediators for transmission of protective stimulus for remote conditioning (Hess et al., 2015). However, the humoral transmission in RIPOC is yet to be discovered. There are evidences from the previous studies for the immune cells and inflammatory response to be part of remote conditioning protective systemic response (Konstantinov et al., 2004). However, RIPOC effect on expression of inflammatory genes still remains to be explored.

Further, few studies suggested the involvement of mediator signaling pathways which includes various molecular pathways like MAPK, KATP, STAT3 and apoptosis in the focal model of cerebral I/R injury (Cheng et al., 2014; Peng et al., 2012; Sun et al., 2012b). However, the exact molecular mechanism of RIPOC neuroprotection involving the trigger, mediator and its end effector still remains uncertain. In addition, the fundamental biology involved in RIPOC protection against functional and cognitive dysfunction is yet to be explored. Moreover, most importantly effective non-vital artery for application of RIPOC intervention, the algorithm of RIPOC i.e. the number of cycles of ischemia and reperfusion and duration of the ischemia and

reperfusion to be applied are yet to be clarified. However, few earlier studies were involved in standardisation of RIPOC algorithm as seen in Table.2.1. the effective algorithm for RIPOC intervention was not clear.

S. No	Algorithm				Model of I/R injury		SP	Parameters evaluated	References
	Onset	Isc (m)	Rep (m)	Cycles	Isc (m)	Rep (h)			
1	Onset/3h/6h	15/15/15	15/15/15	03-03-2003	pMCAO+CCAO (both) 30min		R	Vibrissae forelimb testing; Infarct size	(Ren et al., 2009)
	Invasive left femoral artery; Early and delayed								
2	Onset	10	10	3	120	24/72	R	NSS, infarct volume 72h; TUNEL 24h	(Wang et al., 2011)
	Invasive right femoral artery; early				Right MCAO				
3	Onset	10	10	3	90	24	R	Infarct size; BBB integrity	(Ren et al., 2011)
	Invasive bilateral femoral artery; early				Right MCAO				
4	3/6h	5	5	3	90	72	R	NSS; Infarct volume; TUNEL	(Sun et al., 2012)
	Invasive bilateral femoral artery; Delayed				Right MCAO				
5	Onset	15	15	3	8	24/48/7days	R	MWM task; Nissil and TUNEL staining	(Peng et al., 2012)
	Invasive bilateral femoral artery; Early				4VO				
6	Onset/10/30min	10-10-2010	10-10-2010	03-03-2003	120	24	R	NSS; Infarct size; IHC and assessment of autophagy	(Qi et al., 2012)
	Invasive bilateral femoral artery; Early and delayed				Right MCAO				
6	All onset	05-05-2005 10-10-2010 15/15/15	05-05-2005 10-10-2010 15/15/15	½/3 ½/3 ½/3	90	22	R	NSS; infarct volume; BBB integrity; TUNEL; mPTP opening	(Xu et al., 2012)
	Invasive bilateral femoral artery; Early				Right MCAO				

S. No	Algorithm				Model of I/R injury		SP	Parameters evaluated	References
	Onset	Isc (m)	Rep (m)	Cycles	Isc (m)	Rep (h)			
7	0/0/0/10/10/10/5	10/5/5/10/20/10/5	10/5/5/NA/NA/10/NA	10/2/3/1/1/2/1	100	24/7day	R	NSS; Infarct size	(Pignataro et al., 2013)
	10/20/30/40 min	20/20/20/20	NA for all	1/1/1/1	100	24			
	Invasive; Early and delayed				Right tMCAO				
8	Onset	15	15	3	5/15/30/60	24	R	NSS; Infarct size	(Hasseldam et al., 2013)
	Non-invasive right femoral artery; Early				MCAO or 2VO (10min)				
9	Onset	10	10	3	120	4/24/72	R	NSS, infarct size, TUNEL, Immunofluorescence	(X. Liu et al., 2014)
	Invasive bilateral femoral artery; Early				Right MCAO				
10	Onset	5	5	3	90	24	R	NSS, infarct size, TUNEL, H and E	(Cheng et al., 2014)
	Non-invasive right femoral artery; Early				Right MCAO				
11	8 and 24	10	10	3	120	Aug-24	R	NSS, infarct size, edema	(Q. Liu et al., 2014)
	Invasive bilateral femoral artery; Delayed				Right MCAO				
12	2 days	20	NA	1	10	7days	R	Fluoro Jade b staining, IHC, MWM	(Burda et al., 2014)
	Non-invasive bilateral femoral artery; Delayed				Transient forebrain				

Table 2.2.Representing algorithms of RIPOC in specific animal model

3. Hypothesis and objectives

3.1 *Hypothesis*

Cerebral I/R injury associated with stroke is the leading cause of mortality and morbidity worldwide according to WHO. Ischemic stroke is currently treated with Food and Drug Administration (FDA) approved thrombolytic, tissue Plasminogen Activator (tPA) as a standard therapy with therapeutic window of 4.5 hours. Recently, ischemic stroke is treated with mechanical endovascular procedures with or without thrombolysis and is considered to be effective technique, but is applied to less than 5% of stroke patients. Despite extensive efforts on identification and development of diverse neuroprotective compounds, the treatment options are limited. Therefore, exploration and activation of endogenous mechanisms that arise from an evolutionary basic instinct of adaptation gives impetus to the exploration of novel protective strategies for neuroprotection. In a similar line, the potential of RIPOC may be great as it can be applied in emergency clinical situations; however, there are many barriers that researchers must overcome. There is uncertainty regarding the option of non-vital artery (femoral, renal and abdominal), optimal duration and number of ischemia and reperfusion cycles to be applied. However, the molecular mechanisms of RIPOC in trigger, mediator and end-effector pathway during recovery of I/R injury and associated cognitive deficits are also yet to be explored.

During cerebral I/R injury, reduced oxygen and glucose supply due to compromised CBF may lead to ionic imbalance that may deplete nicotinamide adenine dinucleotide (NAD). Further, impaired energy metabolism may prevent glutamate reuptake by neurons and astrocytes that result in prolonged stimulation of NR2B receptors and modulation of its downstream regulators leading to excitotoxicity. The imbalance in bioenergetics and excitotoxicity may act as triggers to mediate disease progression through oxidative stress, neuroinflammation, inhibition of survival kinases that may finally converge at mitochondrial dysfunction by mPTP opening as end-effector. The above mentioned pathological cascade of cerebral I/R injury subsequently may also result in altered gene expression leading to histone modifications particularly histone acetylation, due to alteration in balance of HAT and HDAC. In addition, energy failure/deficit

induced NAD depletion during cerebral I/R injury may modify the levels of NAD dependent HDAC III (SIRT1). Further, NAMPT is the rate limiting enzyme in NAD biosynthesis, therefore, may regulate the activities of NAD and NAD dependent SIRT1. Therefore, in this study, we hypothesize to investigate the role of RIPOC in modulation of trigger, mediator and end-effector pathway involved in pathological cascade of cerebral I/R injury and associated cognitive deficits.
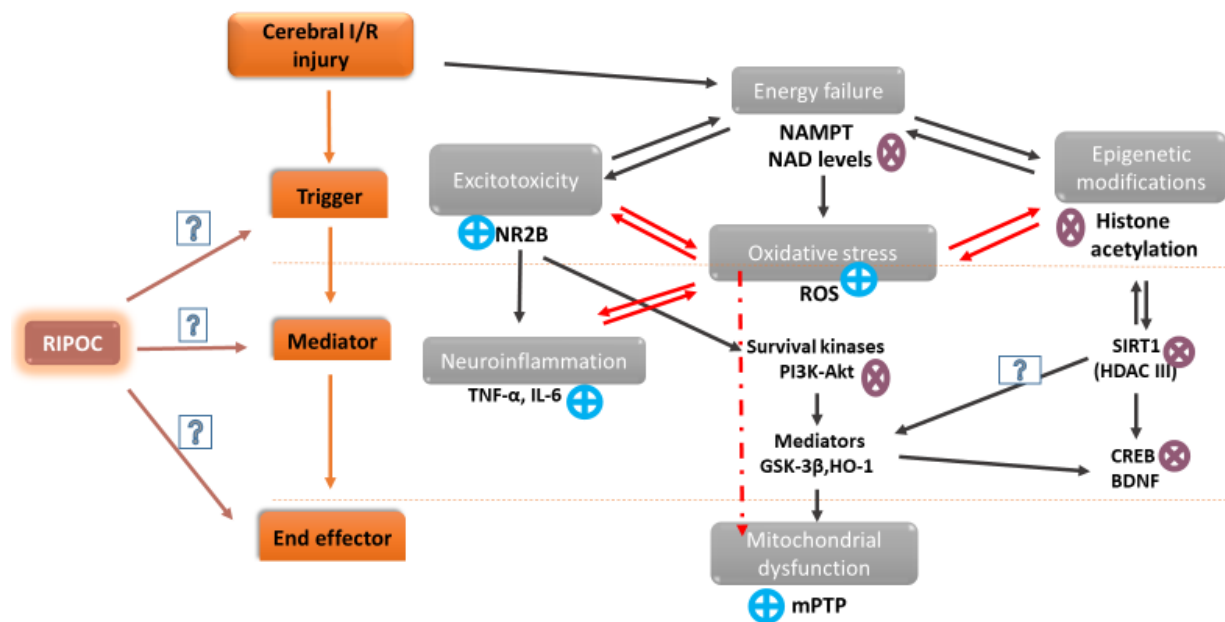
Fig. 3.1. Proposed hypothetical representation for the various molecular targets in the ischemic pathological cascade

3.2   *Objectives*

- To standardise the animal model for cerebral I/R injury associated with co-morbid conditions like cognitive dysfunction.

- To standardise effective algorithm for neuroprotective potential of RIPOC

- To study the role of RIPOC in trigger, mediator and effector pathways in the pathological cascade of cerebral I/R injury

- To suggest newer strategies and molecular mechanisms for better outcome of cerebral I/R injury associated with co-morbid conditions like cognitive dysfunction based on analysis of result obtained

4. Materials and Methods

4.1 *Animals and ethical approval:*

Male Wistar rats (weighing 220-250g) were obtained from central animal facility, Birla Institute of Technology and Science (BITS) Pilani, Pilani campus, India. All rats were housed in a group of three per cage in established laboratory conditions of adequate temperature (22 ± 2°C) and relative humidity (60%) with a 12 hrs light/dark reverse cycles. Rats were allowed for free access to food in the form of dry pellets and water. All the behavioral experiments were carried out between 9.00 and 17.00 Indian Standard Time. All protocols and laboratory work were approved by Institutional Animal Ethics Committee of BITS Pilani (IAEC/RES/19/11/Rev-1/21/10/Rev-2/23/14). The number of animals in each group were 4-8 depending upon the parameter analysed.

4.2 *Drugs and chemicals:*

TNF-α kit (Wuhan Fine Biotechnology, Wuhan, China), IL-6 kit (Wuhan Fine Biotechnology, Wuhan, China) BDNF kit (Boster Biological Technology, CA, USA) and GSK-3β kit and CREB kit (YH Bio search Laboratory, Shanghai, China),HO-1 kit (StressMarq biosciences, USA), Cytochrome C kit (Universal Biologicals, Cambridge, UK), TUNEL kit (Calbiochem, Darmstadt, Germany),SIRT 1 kit (Everon life sciences), NAD kit (Cusabio), Hemin (Himedia), Global histone H3 acetylation kit (EpiQuik™), Lithium chloride (Central Drug House (P) Ltd), resveratrol (TCI), P7C3-A20 (Biotechno labs) , FK866 (Sigma Aldrich, St. Louis, Missouri, United States), Atractyloside (Sigma), LY294002 and Ifenprodil (Cayman Chemicals, Michigan, USA), Quinolinic acid (Fischer Scientific, New Hampshire, USA) were procured and other biological reagents of analytical grade were obtained from Hi-media and CDH. All the biological solvents for the study were prepared just before use.

Quinolinic acid (QA) was dissolved in phosphate buffer saline (PBS) and was injected I.C.V at the dose of 100nmol/1 µl according to the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to the sagittal midline and 3.8 mm in depth 15 min before surgery (Block et al., 1993; Jamwal et al., 2015; Santamar'\ia and R'\ios, 1993). Ifenprodil was dissolved in normal saline and administered i.p. at the dose of 20mg/kg (Mishra et al., 2011; Picconi et al., 2006).

LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one] was dissolved in DMSO and diluted with PBSand was injected 5µl at dose of 25µg according to the following co-ordinates from bregma: anteroposterior, 0.8 mm; mediolateral, 1.5 mm; dorsoventral, 3.5 mm) 20 min before surgery(Zhao et al., 2005; Zhu et al., 2013). Atractyloside (Atr) was administered 5mg/kg intravenously (i.v) 30 min prior to surgery (Fang et al., 2009; Wang et al., 2015; Yang et al., 2015). P7C3-A20 was dissolved in DMSO and 5% dextrose and was administered intraperitoneally (i.p) at the dose of 10mg/kg (Walker et al., 2015; Yin et al., 2014). Resveratrol was dissolved in 50% ethanol and PBS and administered at the sub-therapeutic dose of 5mg/kg, i.p. based on previous studies and laboratory pilot studies (W. Li et al., 2015; Lu et al., 2006). Hemin was prepared by dissolving in 0.1 N NaOH and diluted with phosphate-buffered saline (pH-7.4) and was administered at a sub-therapeutic dose of 2mg/kg i.p. based on previous studies and laboratory pilot studies (Gupta et al., 2016; Yamauchi et al., 2004). Lithium chloride was dissolved in sodium chloride and was administered i.p at the sub-effective dose of 0.5mmol/kg based on laboratory pilot studies and previous studies (Boyko et al., 2015; H. Li et al., 2011).

4.3 *Equipments:*

- ✓ Stereotaxic Instrument: Inco Ambala, India
- ✓ Hamilton syringe
- ✓ Actophotometer: Inco Ambala
- ✓ Rotarod: Inco Ambala
- ✓ Passive Avoidance Task: Inco Ambala
- ✓ Centrifuge: Eppendorf refrigerated centrifuge, 5702-R, Eppendorf AG, German
- ✓ Elisa Plate reader: Ark Diagnostic, India
- ✓ Tissue Homogenizer: KinematicaTM PolytronTM Homogenizers, Germany
- ✓ Spectrophotometer: UV-1800 Shimadzu, Japan
- ✓ Digital Microscope: Optika, Microscopes, Italy
- ✓ Deep freeze (-80°C): OPR-DFC-300CE, Operon Co. Ltd., Korea.

4.4   *Surgical protocol and post-operative care*

4.4.1   *Middle cerebral artery occlusion (MCAO)*

Rats were anesthetized using ketamine (80mg/kg) and xylazine (10mg/kg). Rectal [core] temperature was recorded and maintained at 37 °C throughout the surgical procedure and up to 90mins after reperfusion. A midline incision was made and the right common carotid artery, external carotid artery and internal carotid artery were exposed. A 4.0 monofilament nylon thread [Ethicon, Johnson and Johnson] with its tip rounded by heating quickly near a flame, was advanced from the external carotid artery into the lumen of the internal carotid artery until the resistance was felt which ensures the occlusion of the origin of middle cerebral artery. The nylon filament was allowed to remain in the place for 90 min. After ischemia, the filament was retracted so as to allow the reperfusion. The incision was sutured back in layers, cleaned with antiseptic solution and neosporin powder was applied in the end. For volume replenishment, 0.5 ml of saline was administered intraperitoneally for all rats at the conclusion of the surgical procedure. The rats were maintained in a well-ventilated room at 25 ± 2°C until they gained full consciousness, and were housed together in a group of two rats per cage until they recovered. Throughout the surgical protocol, regional cerebral blood flow and body temperature (36.9°C to 37.6°C) was monitored using a flexible optical fiber probe attached to the skull over the ipsilateral parietal cortex at one point (1mm posterior and 5 mm lateral to bregma) using Laser-Doppler Perfusion and Temperature Monitor (moor VMF – LDF2, UK)(Sun et al., 2012b).
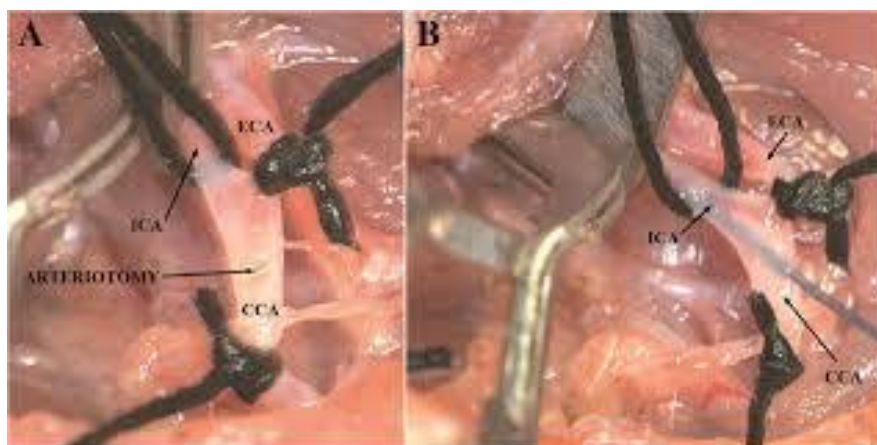
Fig. 4.1. Pictorial representation of middle cerebral artery occlusion (MCAO)

4.4.2   Bilateral common carotid artery occlusion (BCCAO)

All rats were allowed free access to food and water, but were fasted 12 hrs before surgery. The rats were anesthetized using ketamine (80 mg/kg) and xylazine (10mg/kg). A midline incision was made in between neck and sternum region, to expose trachea. Both the common carotid arteries (CCA) located parallel to sternocleidomastoid muscle, were freed from surrounding tissue and vagus nerve with utmost care. Global cerebral ischemia was standardised by 20 minutes of BCCAO and followed by reperfusion for 72 hrs. The incision was sutured back in layers, cleaned with antiseptic solution and neosporin powder was applied in the end. For volume replenishment, 0.5 ml of saline was administered intraperitoneally for all rats after the surgical procedure. The rats were maintained in a well-ventilated room at 25 ± 2°C until they gained full consciousness, and were housed together in a group of two rats per cage until they recovered.

Fig. 4.2. Representative image of Bilateral Common Carotid Artery Occlusion (BCCAO)

S. No	Animal used	Ischemia duration	Reperfusion duration	Outcome of the study	References
1.	Wistar rats M	30 min	60 min	Oxidative stress and microvascular damage	(Lapi et al., 2012)
2.	Wistar rats M	15 min	Continuous Reperfusion	Infarction and cognitive abnormalities	(Singh and Chopra, 2014)
3.	Wistar rats	30 min	4 hrs	Elevation of oxidative damage	(Mansoorali et al., 2012)

S. No	Animal used	Ischemia duration	Reperfusion duration	Outcome of the study	References
4.	Wistar rats	30 min	60 min	Modulation of endocannabinoid system	(Quartu et al., 2017)
5.	Wistar rats M	10 min	Continuous Reperfusion	Oxidative stress and cognitive abnormality	(Kaur et al., 2016)
6.	Wistar rats M	20 min	30 min	Oxidative stress and neuroinflammation	(Quartu et al., 2012)
7.	Wistar rats M	15 min	Continuous Reperfusion	Oxidative stress and cognitive abnormality	(Singh and Chopra, 2013)
8.	Wistar rats M	30 min	24 hrs	Mitochondrial enzyme dysfunction and hippocampal damage	(Gaur et al., 2009)
9.	Wistar rats M	10 min	72 hrs	Oxidative damage	(Kakkar et al., 2013)
10.	Wistar rats M	20 min	24, 96, 168 hrs	Oxidative damage and apoptosis	(Akinrinmade et al., 2017)
11.	Wistar rats M	20 min	96 hrs	Hippocampal apoptosis	(Erfani et al., 2015)

Table 4.1. Algorithms for BCCAO used in previous studies

4.5   *EXPERIMENTAL DESIGN*

4.5.1   *Development of focal model*

Group	Group name	Description
1	Sham	Surgical control
2	Ischemia-reperfusion injury (I/R)	Ischemia of 90minutes followed by 24 hrs reperfusion

Fig. 4.3. Representative image of focal and global model of cerebral I/R injury

4.5.2   Development of global model

Group	Group name	Description
1	Sham	Surgical control
2	I/R 5min	5mins of ischemia followed by 72hours of reperfusion
3	I/R 10min	10mins of ischemia followed by 72hours of reperfusion
4	I/R 15min	15mins of ischemia followed by 72hours of reperfusion
5	I/R 20min	20mins of ischemia followed by 72hours of reperfusion

4.5.3   Development of Remote Ischemic Post Conditioning (RIPOC) model

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	2/15 RIPOC	After induction of I/R injury, RIPOC was induced by 2cycles of 15min ischemia and 15min reperfusion during the onset of reperfusion
4	3/10 RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
5	4/5 RIPOC	After induction of I/R injury, RIPOC was induced by 4cycles of 5min ischemia and 5min reperfusion during the onset of reperfusion
6	1/20 RIPOC	After induction of I/R injury, RIPOC was induced by 1cycle of 20min ischemia followed by reperfusion during the onset of reperfusion

4.5.4   Development of early and delayed RIPOC

Group	Group name	Description
1	I/R	20mins of ischemia followed by 72hours of reperfusion
2	Onset RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
3	10min RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion after 10mins of reperfusion
4	30min RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion after 30min of reperfusion
5	1hr RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion after 1hr of reperfusion
6	3hr RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion after 3hr of reperfusion
7	6hr RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion after 6hr of reperfusion

4.5.5   Effect of RIPOC and NR2B agonist, quinolinic acid (QA) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	QA	Administered ICV 15 min prior to surgery
4	RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
5	RIPOC + QA	QA administered 20min prior to I/R, RIPOC intervention was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion

4.5.6   Effect of NR2B antagonist-Ifenprodil (IFN) and PI3-Akt inhibitor-LY294002 (LY) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	IFN	Administered during onset of reperfusion followed till end of reperfusion with 24hr interval
4	LY + IFN	LY was administered ICV 20 min prior to I/R, IFN was administered during the onset of reperfusion followed till end of reperfusion with 24hr interval

4.5.7   Effect of RIPOC and PI3-Akt inhibitor-LY294002 (LY) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	LY294002	Administered ICV 20min before surgery
4	RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
5	RIPOC + LY294002	LY294002 was administered 30min before induction of ischemia, after induction of I/R injury RIPOC intervention was performed during onset of reperfusion.

4.5.8   Effect of RIPOC and mPTP opener atractyloside (Atr) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion

Group	Group name	Description
3	Atr	Administered 30 min prior to surgery
4	RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
5	Atr + RIPOC	Administered 30 min prior to surgery, after induction of I/R injury RIPOC intervention was performed during onset of reperfusion.

4.5.9   Effect of NAMPT activator (A20) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	A-20	Administered during reperfusion

4.5.10   Effect of RIPOC and NAMPT inhibitor (FK866) on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by 72hours of reperfusion
3	FK866	Administered ICV 15 min prior to surgery
4	RIPOC	After induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion
5	RIPOC + FK866	Administered ICV 15 min prior to surgery, after induction of I/R injury, RIPOC was induced by 3cycles of 10min ischemia and 10min reperfusion during the onset of reperfusion

4.5.11   Effect of sub-effective combination of resveratrol and lithium chloride on cerebral I/R injury and associated cognitive deficits

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by reperfusion of 7 days
3	Resveratrol	Administered during reperfusion and continued till 7 days
4	Lithium chloride	Administered during reperfusion and continued till 7 days
5	Resveratrol + lithium chloride	Administered during reperfusion and continued till 7 days

4.5.12 *Effect of sub-effective combination of resveratrol and hemin cerebral I/R injury and associated cognitive deficits*

Group	Group name	Description
1	Sham	Surgical control
2	I/R	20mins of ischemia followed by reperfusion of 7 days
3	Resveratrol	Administered during reperfusion and continued till 7 days
4	Hemin	Administered during reperfusion and continued till 7 days
5	Resveratrol + Hemin	Administered during reperfusion and continued till 7 days

4.6 *Behavioral Methodology:*

4.6.1 *Neurological Scoring:*

Neurological behavior was scored based on below mentioned six tests: 1. spontaneous activity; 2. symmetry in the movement of forelimbs; 3. forepaw outstretching; 4. climbing; 5. body proprioception; 6. Vibrissae touch response. The first three tests were scored from 0 to 3 points, whereas the later three tests were scored from 1 to 3 points which finally gives a minimum score of 3 and 18 as maximum using Garcia scoring system(Garcia et al., 1995).

4.6.2 *Evaluation of spontaneous locomotor activity:*

Each rat was tested for spontaneous locomotor activity after reperfusion phase. Rats were observed over a period of 7 minutes in a square closed arena (30×30 cm2) equipped with infrared light-sensitive photocells using a digital actophotometer in Fig.4.4 (INCO, India). The results were expressed as locomotor activity (Kumar et al., 2007; Mahesh et al., 2012).

Fig. 4.4. Pictorial representation of Actophotometer

4.6.3   Evaluation of anxiety using elevated plus maze (EPM):

EPM consisted of two open (50×10 cm) crossed with two enclosed arms with similar dimensions and 40m wall height (Fig.4.5). The open and closed arms are connected to a central platform (10×10cm) and adjusted to a light intensity of 80-100 lux. The apparatus was adjusted to a height of 25 cm above the floor to avoid jumping of rats. Each rat was individually placed at the end of open arm facing away from central platform of the maze. The time taken by the rat to completely (with both forelimbs and hind limbs) enter enclosed arm from open arm was considered as the latency time (LT). All rats were given acquisition trial before induction of cerebral I/R injury to record the initial trial latency (ITL). Rats were allowed to explore the maze for 30 secs after recording ITL. After the reperfusion phase of 48 hrs, transfer trial latency (TLT) was recorded. Utmost care was taken regarding the relative location of plus maze with respect to any object serving as visual clue laboratory (Gaur and Kumar, 2012).

Fig. 4.5. Pictorial representation of Elevated plus maze

4.6.4   Assessment of associative learning by passive avoidance task:

In the passive avoidance task (Fig.4.6), the rat learns to suppress a motor response to avoid exposure to the aversive event, such as electric shock in a dark compartment. The ability to avert electric shock by preferring brightly illuminated compartment, allows the assessment of learning memory in rodents. Data was expressed as retention trial latency, shorter retention period indicated poor learning. The apparatus consisted of two compartments, one brightly Illuminated and one dark and were separated by a guillotine door. During the acquisition trial on day 2, each rat was placed in the illuminated compartment. After 60 s of habituation, a

guillotine door was opened and the initial latency to enter the dark chamber was recorded. As rat entered the dark compartment, the door was closed and an electric foot shock (50 V, 0.2mA, 50Hz) was delivered through floor grids for 2 s. The rat was then removed from the dark chamber 5 s later and returned to its home cage. Retention latency was measured 24 h later in the same way as in the acquisition trial, but foot shock was not delivered. The latency time to reach dark chamber was recorded to a maximum of 300 s(Foley et al., 2004; Park et al., 2000).

Fig. 4.6. Pictorial representation of passive avoidance task

4.6.5   Evaluation of spatial working memory by Y-maze spontaneous alteration:

Y-Maze spontaneous alteration is a behavioral test for measuring the willingness of rodents to explore new environments and estimate its spatial working memory. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. Y-maze used in the study consisted of three arms with 120° angles between each arm (30×20×10cm) and an equilateral triangular central area (Fig.4.7). The Y-maze test consisted of 3 arms named as A, B, C. After reperfusion phase, rats were placed in any one of the arm and allowed to move freely in all the arms for duration of 5 min. An entry into arm was recorded when four limbs are within the arm. Data was expressed as percentage of spontaneous alterations (Dong et al., 2011; Li et al., 2008).

Fig. 4.7. Pictorial representation of Y-maze

4.7 Biochemical parameters:

4.7.1 Brain Homogenate Preparation:

Rat was sacrificed by decapitation; brain was carefully removed and rinsed with ice-cold (0.9 % w/v) isotonic saline. The brain was then homogenized with ice-cold 0.1 M phosphate buffer (pH 7.4) 4.5ml. The homogenate was centrifuged (Remi Cooling compufuge CPR 24) at 10,000 rpm for 15 minutes (4°C), and aliquots of supernatant were separated and used for biochemical estimations.

4.7.2 Estimation of malondialdehyde (MDA) levels:

The major product of lipid peroxidation, MDA, was assayed quantitatively in the form of thiobarbituric acid reactive substances (TBARS). 0.1 ml of the brain homogenate was heated at 95°C for 1 hour in a mixture of 0.1 ml of 8.1% sodium dodecyl sulphate (SDS), 0.75 ml 20% glacial acetic acid, 0.75 ml of 0.8% thiobarbituric acid (TBA) and 0.3 ml of distilled water. The supernatant obtained was separated by centrifugation at 10,000 rpm for 10 minutes. The amount of MDA in the supernatant was measured spectrophotometrically at a wavelength of 532 nm using Tetramethoxypropane was used as standard (Epoch microplate spectrophotometer, Biotek, Winooski, US). The values were expressed as nanomole of MDA per milligram of protein (Wills, 1966).

4.7.3   Estimation of nitrite levels:

The levels of nitrite, an indicator of nitric oxide (NO) production were determined by a colorimetric assay using Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid). Equal volumes of supernatant and Griess reagent were mixed, the mixture was incubated for 10 minutes at room temperature and the absorbance was determined at 540 nm (Epoch microplate spectrophotometer, Biotek, Winooski, US). The concentration of nitrite in the brain homogenate was calculated using a sodium nitrite standard curve. The values were expressed as micromole per milligram of protein (Green et al., 1982)*.*

4.7.4   Estimation of reduced glutathione (GSH) levels:

Reduced glutathione in the brain was estimated using Ellman method. Equal volumes of homogenate and 5% sulphosalicylic acid were mixed and kept cold digested at 4°C for 1 hour. The supernatant was separated by centrifugation at 10,000 rpm for 10 minutes at 4°C. 50 µl of the clear supernatant was mixed with 450 µl of phosphate buffer and 1.5 ml of 5,5-dithiobis-(2-nitrobenzoic acid) in 0.1M phosphate buffer, (pH 8.0). Reaction mixture was incubated for 10 minutes at 37°C and colour developed was measured at 412 nm (Epoch microplate spectrophotometer, Biotek, Winooski, US). Results were expressed as micromole per milligram protein(Sedlak and Lindsay, 1968).

4.7.5   Estimation of SOD levels:

Autoxidation of epinephrine at pH 10.4 was spectrophotometrically measured. In this method, the supernatant of the tissue was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4 and the reaction was started by the addition of 0.02 ml (−)-epinephrine. After 5 min, the absorbance was measured at 480 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). The activity of SOD was expressed as U/mg of protein(Crapo et al., 1978; Jindal et al., 2015).

4.7.6   Protein Determination:

Protein content in the brain samples was measured by the method of Lowry et al., using bovine serum albumin (BSA) (1 mg/ml) as a standard(Jindal et al., 2013).

4.8   *Estimation of neuroinflammation – TNF-α and IL-6:*

The levels of TNF-α and IL-6 were determined according to manufacturer's instructions. The cytokine standards and brain homogenates were added to the wells of coated ELISA plate. Initially the plates were coated using capture antibody. The plate was sealed and incubated at 37°C for 90 min. Following the incubation period, wells were aspirated and washed 2 times with wash buffer. Further, 0.1ml of biotin labeled antibody was added and incubated for 1 h at 37°C, followed by washing for 3 times. 100 µl HRP-Streptavidin Conjugate was added to each well and incubated for 30 min at 37°C. The contents of the wells were aspirated and wells were washed for 5 times. Finally, 90 µl of Tetramethylbenzidine (TMB) was added as a substrate and incubated at 37°C in dark for 15-30min and reaction was terminated by adding 50 µl stop solution. The plate was read at 450 nm and concentrations of TNF-α and IL-6 were obtained from their respective standard curve. The values were expressed as pg/mg protein.

4.9   *Estimation of Nicotinamide (NAD) level*

NAD level was determined by using a commercially available ELISA kit as per the manufactures instructions. The samples, standards and reagents were prepared fresh as instructed in the protocol. 100µl of standard or sample was added to each well and incubated for 2 hours at 37°C. After aspiration of wells, 100µl of biotin-antibody was added to each well and incubated for one hour at 37°C. After this step, the wells were aspirated and washed for 3 times with wash buffer, 100µl of HRP-avidin was added to each well and incubated for one hour at 37°C. The wells were aspirated and washed for 5 times, 90µl of TMB substrate was added to each well and incubated at 37°C for 15-30 min away from light. The reaction was terminated by stop solution and absorbance was read at 450nm.The data was expressed as µMol/mg protein.

4.10   *Estimation of global histone H3 acetylation levels*

The level of Global Histone H3 acetylation was estimated by using Global Histone H3 Acetylation Assay Kit as per manufacturer's instruction. The protocol consists of three parts: Nucleic Extraction Preparation, Histone Extraction and Histone H3 Acetylation Detection. First, the nucleic extraction was performed by homogenizing the tissue with lysis buffer. Then, the histone proteins were extracted using the extraction buffer. At the end of histone extraction

procedure, the protein concentration was measured using Lowry's method. After this, the histone proteins were stably spotted on the strip wells. The acetylated histone H3 was recognized with a high affinity antibody. The amount of acetylated histone H3 was quantified through HRP conjugated secondary antibody-color development system and was proportional to the intensity of color development.

4.11   *Estimation of Sirtuins (SIRT1) levels*

The levels of SIRT1 were determined according to manufacturer's instructions. Initially, 100µl of standard or sample was added to each well. It was then incubated for 90minutes at 37°C. Following aspiration, 100µl of biotinylated detection Ab was added and incubated for 1 hour at 37°C. It was then followed by aspiration and washing for 3 times. 100µl of HRP conjugate was added and incubated for 30 minutes at 37°C. It was aspirated and washed for 5 times. After that, 90µl of substrate reagent was added and incubated for 15 minutes at 37°C. Then, 50µl of stop solution was added and absorbance was read at 450nm immediately. The results were calculated and expressed as ng/mg protein.

4.12   *Estimation of hemeoxygenase (HO-1) levels:*

Ho-1 level was determined by using a commercially available ELISA kit as per the manufactures instructions. The samples and standard were prepared in standard and sample diluents provided in the ELISA kit. Initially, the plates were treated with 50 µl of pre-treated buffer, followed by addition of samples and standards to appropriate wells. The plate was sealed and incubated at 25°C for 2 hours. After the incubation period, the plate was washed four times with wash buffer. 100 µl of biotinylated antibody working solution was added to each well, sealed and incubated at room temperature for one hour. The plate was washed again following the incubation period as described above. 100 µl of Streptavidin-HRP working solution was added into each well, sealed and incubated at room temperature for 30min. Then, 100 µl of TMB substrate was added and plate was developed in dark at room temperature for 30 min. The assay reaction was ceased by adding 50 µl of stop solution and absorbance was measured at 450 nm. The values were expressed as ng/mg protein.

4.13 *Estimation of Brain Derived Neurotrophic factor (BDNF) levels:*

BDNF level was determined by using a commercially available ELISA kit as per the manufactures instructions. In brief, 100 µl of BDNF standards and samples were added onto pre-coated plates and incubated at 37°C for 90 min. In the next step, 100 µl of antibody working solution was added and incubated at 37°C for 60 min. The plate was washed for three times with PBS, later 100 µl of streptavidin HRP working solution was added, the plate was incubated at 37°C for 30 min. The plate was developed at 37°C in dark followed by addition of TMB substrate. 100 µl of stop solution was used to terminate the assay and the absorbance was recorded at 450nm. The values were expressed as ng/mg protein.

4.14 *Estimation of Cyclic AMP Response Element Binding (CREB) levels:*

The levels of CREB were determined by using a commercially available ELISA kit as per the manufactures instructions. In this protocol, 40 µl of sample/standard, 10 µl of CREB antibody working solution, 50 µl of streptavidin HRP working solution were incubated at 37°C for 60 min. After incubation period, the contents of the plate were aspirated and wells were washed for 5 times with wash buffer. Later, the plate was developed in dark at 37°C for 10 min with 50 µl of chromogen A and 50 µl of chromogen B. The reaction was terminated using 50 µl of stop solution and absorbance was calculated at 450nm. The values were expressed as pg/mg protein.

4.15 *Estimation of Glycogen Synthase Kinase (GSK-3β) activity:*

GSK-3βlevel was determined by using a commercially available ELISA kit as per the manufactures instructions. Briefly, 40 µl of sample/standard, 10 µl of GSK-3β antibody working solution, 50 µl of streptavidin HRP working solution were incubated at 37°C for 60 min. After incubation period, the contents of the plate were aspirated and wells were washed for 5 times with wash buffer. Later, the plate was developed in dark at 37°C for 10 min with 50 µl of chromogen A and 50 µl of chromogen B. The reaction was terminated using 50 µl of stop solution and absorbance was calculated at 450nm. The values were expressed as pg/mg protein.

4.16 *Estimation of apoptosis by cytochrome-c levels:*

Cytochrome-c levels were determined by using a commercially available ELISA kit as per the manufactures instructions. Equal volume of (100 µl) of sample and standards were added to the wells. The plate was sealed and incubated at 37°C for 90 min. Following the incubation period, wells were aspirated and washed 2 times with wash buffer. Further, 0.1ml of biotin labeled antibody was added and incubated for 1 h at 37°C, followed by washing for 3 times. 100 µl HRP-Streptavidin Conjugate was added to each well and incubated for 30 min at 37°C. The contents of the wells were aspirated and wells were washed for 5 times. Finally, 90 µl of Tetramethylbenzidine (TMB) was added as a substrate and incubated at 37°C in dark for 15-30min and reaction was terminated by adding 50 µl stop solution. The cytochrome-c concentration of the samples can be interpolated from the standard curve. The values were expressed as ng/mg protein.

4.17 *Histological evaluation by Triphenyl tetrazolium Chloride staining (TTC)*

The brain was sliced into 2 mm thick coronal sections in a brain matrix and stained with 2% (w/v) TTC (Sigma-Aldrich) for 30 min at 37ºC followed by immersion in 4% (w/v) paraformaldehyde in phosphate buffer overnight for colour fixation. The non-infarct region turns red, whereas infarct region remained unstained (white). The infarct area was demarcated and analysed using image J software. The data expressed as % infarction (Bederson *et al.*, 1986).

4.18 *Histological evaluation by Hematoxylin and eosin (H and E) staining*

The brains were rapidly removed after 72 hrs of reperfusion and fixed by immersion in 10% formalin. Subsequently they were embedded in paraffin wax, cut into 5 µm thick sections coronally and stained with H and E stain. Hippocampal CA1 and dentate gyrus region of brain were examined under bright field illumination using ''Optika TCB5'' microscope (Optika Research Microscope, Italy) at total of 100x and 400x. Degenerating neurons were counted using the ImageJ cell counter tool and expressed as the percentage of degenerating neurons (Taliyan and Ramagiri, 2016). Neurons in hippocampal CA1 regions, which were round and robust shape, visible cytoplasm, spherical or slightly oval nucleus with single large nucleolus are

considered as healthy neurons and indicated by yellow arrows. The H and E stained sections which showed shrinkage in cell size or pyramidal shaped with darkly stained nuclei were considered as pyknotic or degenerating neurons and indicated by red arrows(Fischer et al., 2008). The images were analysed using image J software. The data expressed as % of damaged neurons in hippocampal CA1 region.

4.19 *Estimation of DNA fragmentation by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay:*

The evaluation of apoptosis in hippocampal (dentate gyrus and CA1) regions was performed on paraffin-embedded tissue slides by TUNEL assay kit as per manufacturer's instructions. The slides were initially deparaffinised and rehydrated using xylene and decreasing concentrations of ethanol. Permeabilization of the specimen was achieved by covering the specimen with 100 μl of 20 μg/ml proteinase K and incubating at room temperature for 20 min. For the inactivation of endogenous peroxidases, the specimens were covered with 100 μl of 3%H_2O_2 and incubated at room temperature for 5 min. The specimens were carefully blotted with equilibration mixture followed by application of 60 μl of TdT labelling reaction mixture and incubated in a humidified chamber at 37°C for 1.5 hours. The labelling reaction was terminated by 50 μl of stop solution. For detection, the specimens were covered with 100 μl of blocking buffer and incubated at RT for 10 min. After washing with 1X TBS, the specimens were covered with 100 μl of DAB solution and incubated at RT for 15 min. For counterstaining, the specimens were covered with 100 μl of methyl green counterstain, followed by dehydration using xylene and absolute ethanol. Hippocampal CA1 region of brain were examined under bright field illumination using ''Optika TCB5'' microscope (Optika Research Microscope, Italy) at a total of 40X.

4.20 *Statistical Analysis*

All data were expressed as mean ± SEM, except for neurological scores which were expressed as median. The data obtained from different groups in behavioral and other biochemical tests was statistically analysed using t-test (only for two groups) one-way analysis of variance (ANOVA) (for more than two groups) followed by the post hoc Tukey's test in graph pad prism

software (version 5.0, La Jolla, CA, USA), except for neurological scores. Neurological deficit scores were estimated by Kruskal -Wallis test followed post hoc by Dunn's test. The value of *P <0.05* was considered as statistically significant.

5. Results

5.1 *Development of focal cerebral Ischemic Reperfusion (I/R) injury*

Focal I/R injury was induced by middle cerebral artery occlusion (MCAO) model and monitoring of regional cerebral blood flow (CBF) was done by laser doppler to ensure successful MCAO as represented in Fig.5.1.
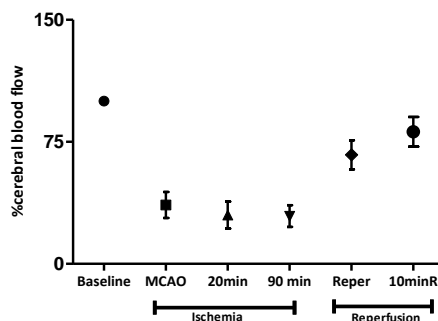

Fig. 5.1. Graphical representation of changes in cerebral blood flow validated by laser Doppler.
Rats were subjected to 90 min of ischemia by middle cerebral artery occlusion (MCAO) followed by reperfusion.

5.1.1 *Effect of focal I/R injury on neurological scores:*

Rats induced with focal I/R injury exhibited significant (P<0.05) neurological deficits parameters such as spontaneous activity, symmetry in four limb movement, forepaw outstretching, climbing, body proprioception and vibrissae touch response when compared to sham as evidenced in Fig.5.2.
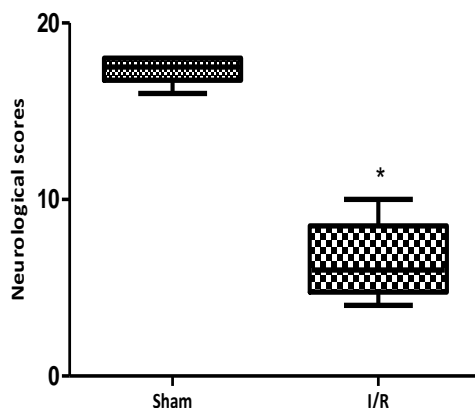

Fig. 5.2. Effect of focal I/R injury on neurological scores (NSS)
NSS were evaluated after reperfusion using Garcia scoring system. Data represented as median and analysed by non-parametric test (Mann-Whitney) with stastical significance of P< 0.05.

5.1.2   Effect of focal I/R injury on biochemical parameters

Oxidative markers like MDA ($P<0.001$) and nitrite ($P<0.01$) levels were found to be significantly elevated after MCAO induction in comparison to sham control rats. Concurrently, I/R injury induced a marked decrease in contents of anti-oxidants like SOD ($P<0.01$) and GSH ($P<0.001$) when compared to sham control rats as described in table.5.1.

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.55±0.31	5.9±2.82	2.38±0.51	17.5±3.87
I/R	2.05±0.56***	20.5±7.25**	0.58±0.35***	4.77±2.36**

Table 5.1. Effect of focal I/R injury on oxidative markers (MDA, nitrite) and anti-oxidants (GSH and SOD). Values were expressed as mean±S.D. ***$P<0.001$ Sham vs I/R (MDA and GSH), **$P<0.01$ Sham vs I/R (nitrite and SOD).

5.1.3   Effect of focal I/R injury on neuroinflammatory parameters

MCAO induction significantly up-regulated the expression of pro-inflammatory cytokines as evidenced by increased levels of TNF-α ($P<0.01$) and IL-6 ($P<0.001$) as seen in Fig.5.3, when compared to sham control rats.
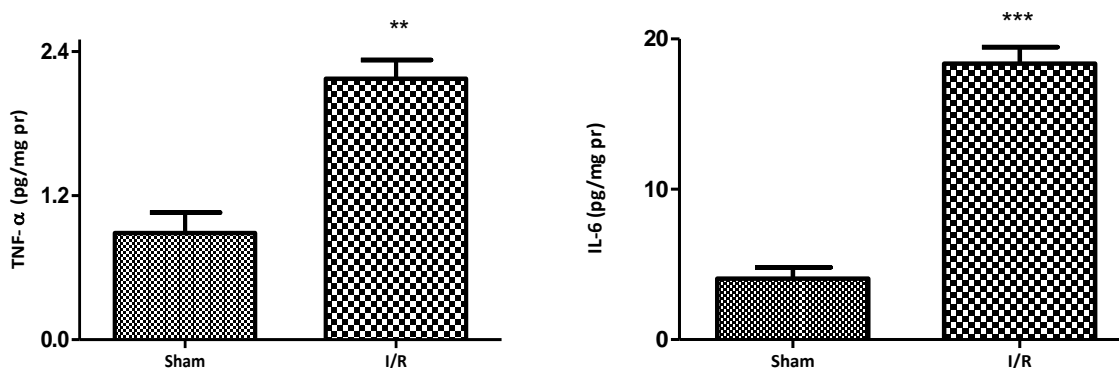

Fig. 5.3. Effect of focal I/R injury on inflammatory parameters (TNF-α and IL-6). Values were expressed as mean±S.E.M. **$P<0.01$ Sham vs I/R (TNF- α), ***$P<0.001$ Sham vs I/R (IL-6).

5.1.4   Effect of focal I/R injury on infarct volume and cortical neurons

MCAO induced an increase in percentage infarction as evidenced in TTC staining (indicated in Fig.5.4). In hematoxylin and eosin (HE) staining, we observed neurons with intact nucleus and cytoplasm along with intact shape and size in sham control rats. In contrast, neurons with shrinkage of nucleus and cytoplasm were noticed in MCAO treated rat brains when compared to sham control group as depicted in Fig.5.5.
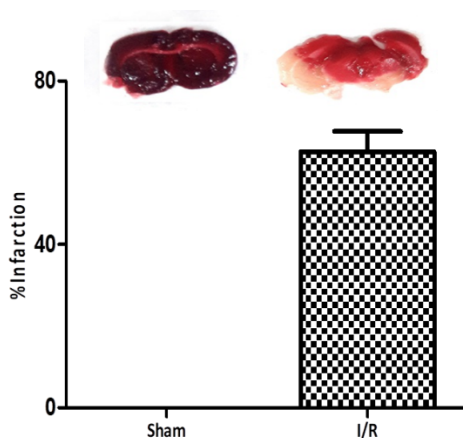

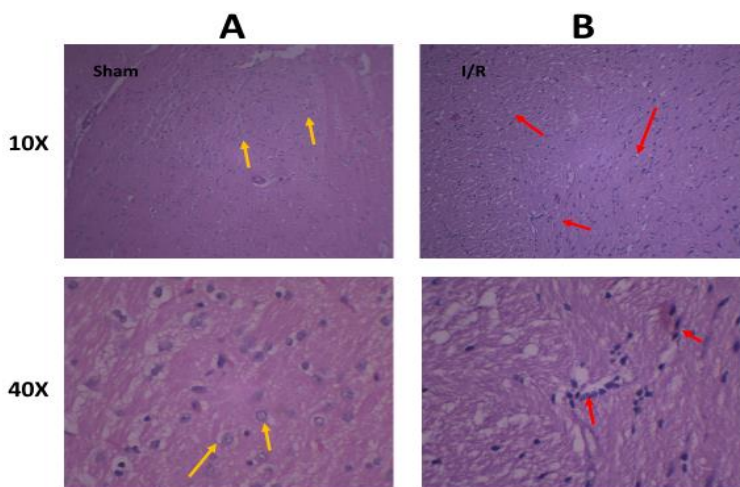**Fig. 5.4. Effect of focal I/R injury on %infarction. Values were expressed as mean±S.E.M.**

Fig. 5.5. Effect of focal I/R injury on neuronal alterations assessed by Hematoxylin and Eosin staining.

5.2 Development of global cerebral I/R injury

All animals in the study were subjected to habituation trial of passive avoidance, Y-maze, elevated plus maze and actophotometer before the induction of global I/R injury. For validating the model of global I/R injury (considered as cerebral I/R injury or I/R injury in the thesis), rats were divided into five groups 1. Sham, 2. Ischemia 5 min and reperfusion 72 hours, 3. Ischemia 10 min and reperfusion 72 hours, 4. Ischemia 15 min and 72 hours of reperfusion, 5. Ischemia 20 min and 72 hours' reperfusion. After reperfusion phase a battery of tests were performed to assess the effect of I/R injury on various behavioral, biochemical, neuro-inflammatory and histological parameters.

5.2.1 Effect of cerebral I/R injury on behavioral parameters

5.2.1.1 Effect of cerebral I/R injury on memory in passive avoidance task

Passive avoidance test was performed on day 2 and day 3 following I/R injury to assess the cognitive deficits induced by cerebral I/R injury. Acquisition trial of the passive avoidance task was performed on day 2 and we did not observe any significant difference in the initial trial latency among different groups in the study as seen in Fig.5.6.
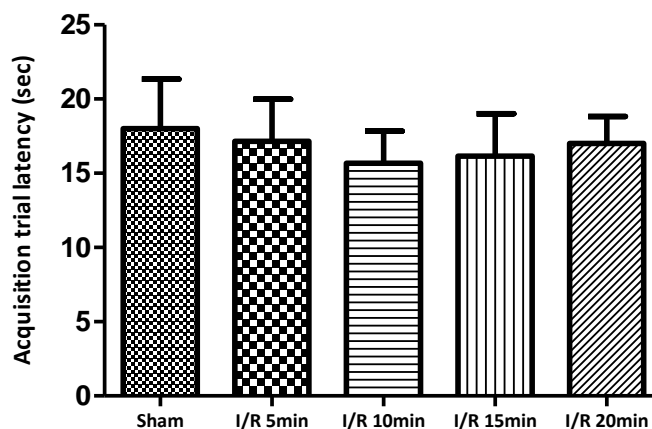

Fig. 5.6. Effect of cerebral I/R injury on memory in acquisition trial of passive avoidance task. Values were expressed as mean±S.E.M. No significant difference was observed among the groups.

Sham control rats were reluctant to enter the dark chamber and showed high retention trial latency among different groups of the study. Cerebral I/R injury groups with 5, 10, 15 min of ischemia and 72 hrs of reperfusion showed a decrease in trend of retention trial latency when compared to sham rats. However, I/R injury with 20 min of ischemia and 72 hrs of reperfusion

showed a significant (P<0.01) reduction in the retention trial latency when compared to sham group as represented in Fig.5.7.
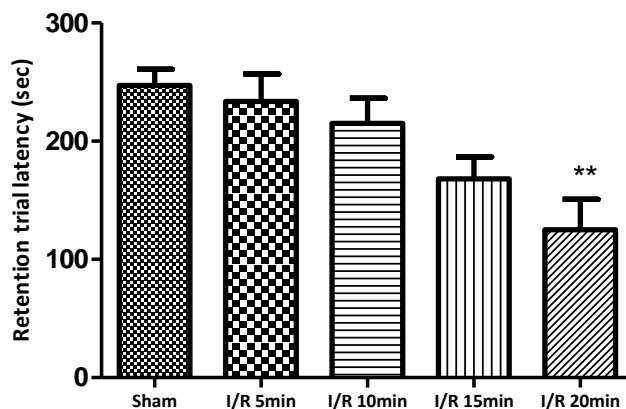

Fig. 5.7. Effect of cerebral I/R injury on memory in retention trial of passive avoidance task. Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R 20min.

5.2.1.2   Effect of cerebral I/R injury on memory in Y-maze task

We estimated the spatial working memory and the willingness of the rodents to explore the novel environment by Y-maze task. During the Y-maze task (Fig.5.8) sham group showed maximum spontaneous alterations when compared to other groups in the study. Induction of cerebral I/R injury with 5 and 10 min of ischemia followed by 72 hours of reperfusion reduced the percentage spontaneous alterations in a non-significant manner. However, I/R injury of 15 min (P<0.05) and 20 min (P<0.01) ischemia followed by 72 hr of reperfusion induced significant reduction in percent spontaneous alterations.
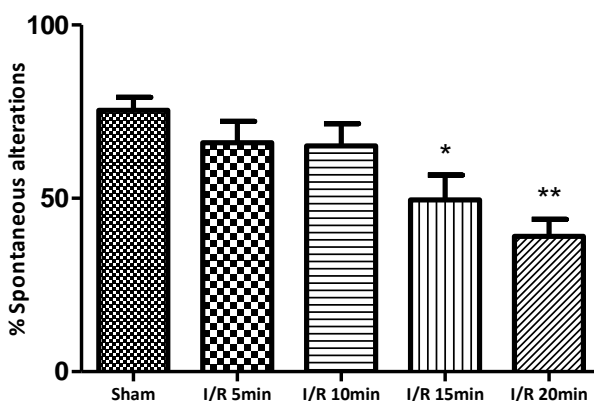

Fig. 5.8. Effect of cerebral I/R injury on memory in Y-maze task. Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 15min and **P<0.01 sham vs I/R 20min.

5.2.1.3   Effect of cerebral I/R injury on memory in Elevated Plus maze (EPM)

To assess the spatial working memory, we have estimated the transfer trial latency taken by the rat to reach the enclosed arm in EPM. The acquisition trial of the EPM was performed just before ischemia and the retention trial was performed at 48 hrs of reperfusion phase. During the acquisition trial of the elevated plus maze task the transfer trial latency was invariable among the different groups in the study (Fig.5.9).
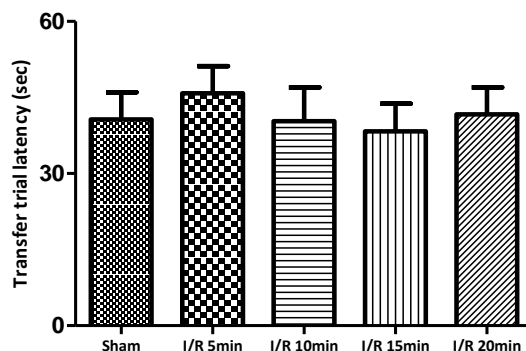

Fig. 5.9. Effect of cerebral I/R injury on memory in elevated plus maze task acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups in the study.

Cerebral I/R injury induced by 5 min and 10 min of ischemia followed by 72 hours of reperfusion increased the transfer trial latency to reach enclosed arm in EPM (Fig.5.10) when compared to sham rats but it was not statistically significant. However, I/R injury induced by ischemia of 15 min (P<0.05) and 20 min (P<0.01) followed by 72 hrs reperfusion significantly increased the time taken to reach the enclosed arm, indicating poor retention of memory.
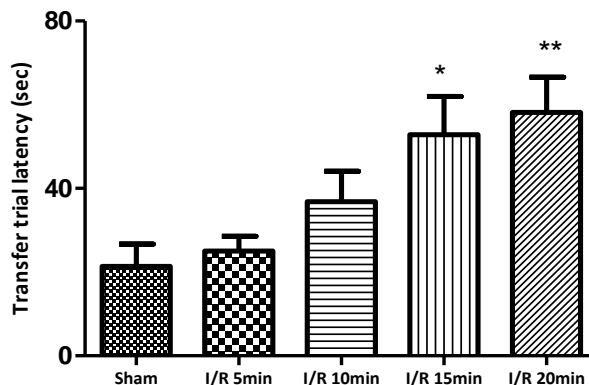

Fig. 5.10. Effect of cerebral I/R injury on memory in elevated plus maze task acquisition trial.
Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 15min and **P<0.01 sham vs I/R 20min.

5.2.1.4   Effect of cerebral I/R injury on locomotor activity

To rule out the effect of motor abnormality in cognitive behavior we have performed spontaneous activity test. No significant difference in locomotor activity was observed among different groups of the study, indicating that there may not be any possible interference of motor abnormality in the cognitive deficits elicited by cerebral I/R injury as evidenced in Fig.5.11.
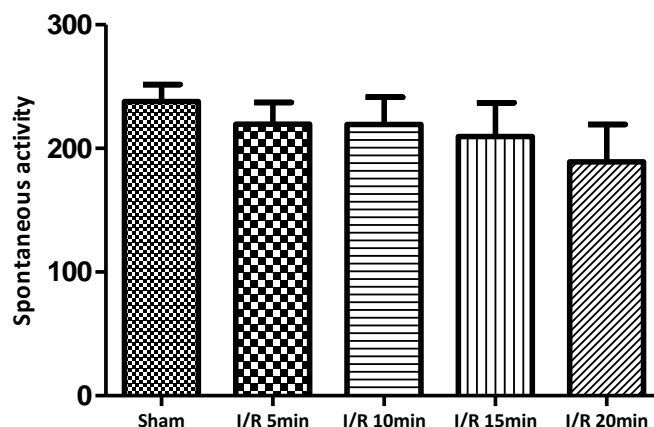

Fig. 5.11. Effect of cerebral I/R injury on spontaneous locomotor activity.
Values were expressed as mean±S.E.M. No significant difference was observed among different groups of the study.

5.2.2   Effect of cerebral I/R injury on biochemical parameters

5.2.2.1   Oxidative markers – Malondialdehyde (MDA) and nitrite

Cerebral I/R injury elevated the oxidative stress as indicated by increased levels of oxidative markers such as MDA (Fig.5.12) and nitrite when compared to sham control rats. Cerebral I/R injury of 5 min and 10 min with 72 hours reperfusion induced oxidative stress but it was not significant when compared to sham group. However, I/R injury induced by ischemia of 15 min (P<0.01) and 20 min (P<0.001) followed by 72 hours reperfusion significantly increased the oxidative stress as indicated by elevated MDA and nitrite (Fig.5.13) levels when compared to sham control rats.
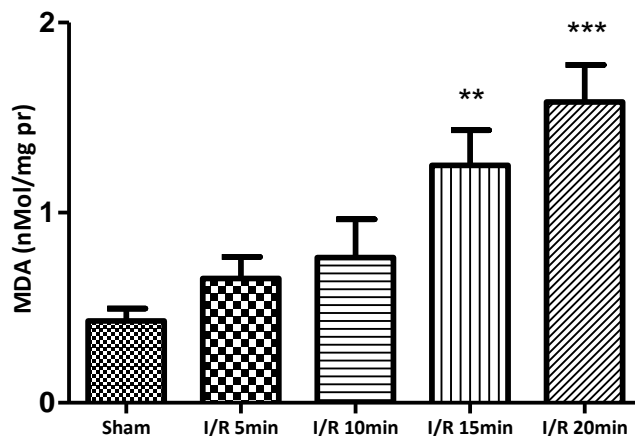
Fig. 5.12. Effect of cerebral I/R injury on MDA levels.
Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R 15min and ***P<0.001 sham vs I/R 20min.
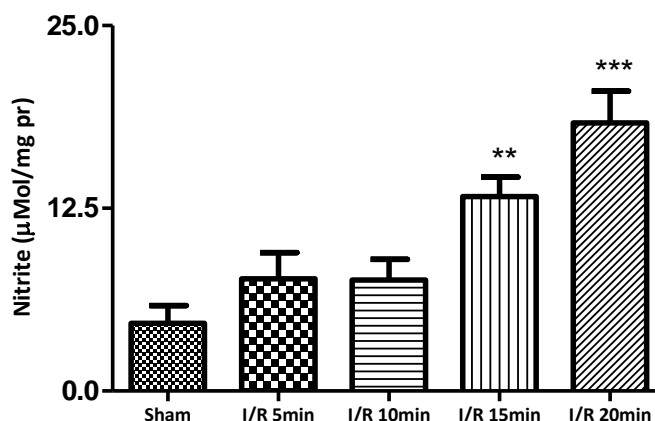

Fig. 5.13. Effect of cerebral I/R injury on nitrite levels.
Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R 15min and ***P<0.001 sham vs I/R 20min.

5.2.2.2   *Anti-oxidants- Glutathione (GSH) and Superoxide Dismutase (SOD)*

During model validation, I/R injury of 5 min, 10 min and 15min with 72 hours of reperfusion reduced the anti-oxidant content, but it was not statistically significant. However, I/R injury induced by ischemia of 20 min followed by 72 hours of reperfusion significantly reduced the anti-oxidants like GSH ($P<0.05$) and SOD ($P<0.001$) (Fig.5.14 and 5.15) when compared to sham control rats. In addition, cerebral I/R injury induced by 15 min of ischemia followed by 72 hours of reperfusion significantly reduced the levels of SOD when compared to sham group.
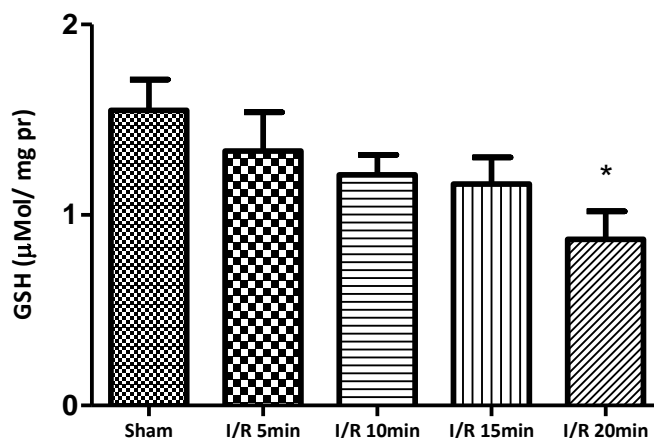
Fig. 5.14. Effect of cerebral I/R injury on GSH levels.
Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 20min

Fig. 5.15. Effect of cerebral I/R injury on SOD levels.
Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 15min and ***P<0.001 sham vs I/R 20min

5.2.3   *Effect of cerebral I/R injury on neuroinflammatory parameters*

5.2.3.1   *Tumor Necrosis factor-α (TNF-α)*

Cerebral I/R injury induced by 15 min (P<0.01) and 20 min (P<0.001) of ischemia followed by 72 hours of reperfusion significantly increased the neuroinflammation as evidenced by elevated levels of TNF-α (Fig.5.16) when compared to sham control rats.
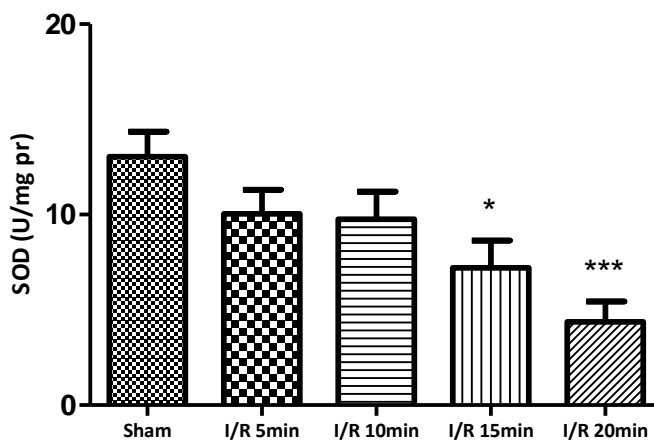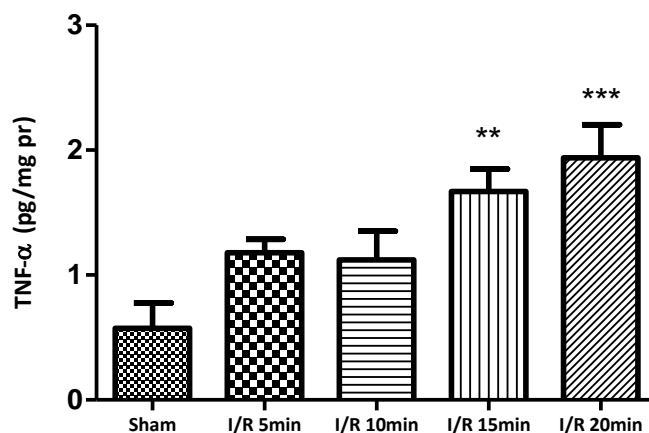
Fig. 5.16. Effect of cerebral I/R injury on TNF-α levels.
Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R 15min and ***P<0.001 sham vs I/R 20min

5.2.3.2   Interleukin-6 (IL-6)

In our study, cerebral I/R injury increased the expression of IL-6 and we found that I/R injury in 15 min (P<0.05) and 20 min (P<0.001) groups significantly up-regulated IL-6 levels when compared to sham control rats as depicted in Fig.5.17.
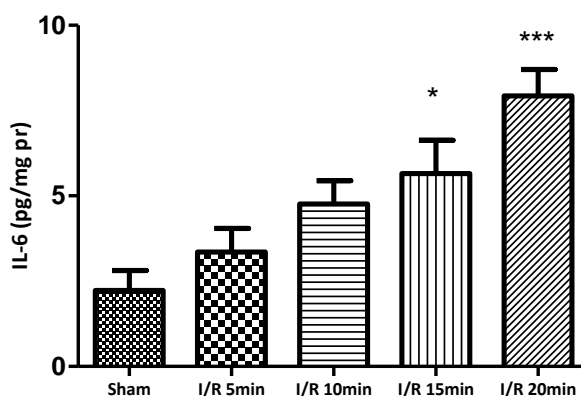
Fig. 5.17. Effect of cerebral I/R injury on IL-6 levels.
Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 15min and ***P<0.001 sham vs I/R -20min

5.2.4   Effect on cerebral I/R injury on morphological characteristics of hippocampal CA1 neurons

Along with memory impairment observed in behavioral parameters, I/R injury resulted in significant morphological alterations in the hippocampal CA1 region as assessed by HE staining. In HE stained brain sections of sham rats we observed neurons with robust shape, clear nucleus along with visible cytoplasm. However, cerebral I/R injury with 10 min ischemia followed by 72

hrs reperfusion did not change the integrity of the hippocampal CA1 neurons. In addition, cerebral I/R injury with 15 min of ischemia and 72 hrs reperfusion resulted in CA1 regions visualized ($P<0.05$) in the form of gradual cell loss and disorganization. In contrast, cerebral I/R injury with 20 min of ischemia and 72 hrs reperfusion resulted in significant ($P<0.01$) neuronal degeneration with increased pyknotic neurons in hippocampal CA1 region. The pyknotic neurons were shrunken in size reflecting almost sickle shape, darkly stained with no visible clear cell contents and indicated by red arrows in Fig. 5.18 (a). Further, the percentage of damaged neurons in the hippocampal CA1 region were calculated as represented in Fig. 5.18 (b).

Fig. 5.18. (a) Effect of cerebral I/R injury on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R 10 min, (Panel C) I/R 15 min, (Panel D) I/R 20 min. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
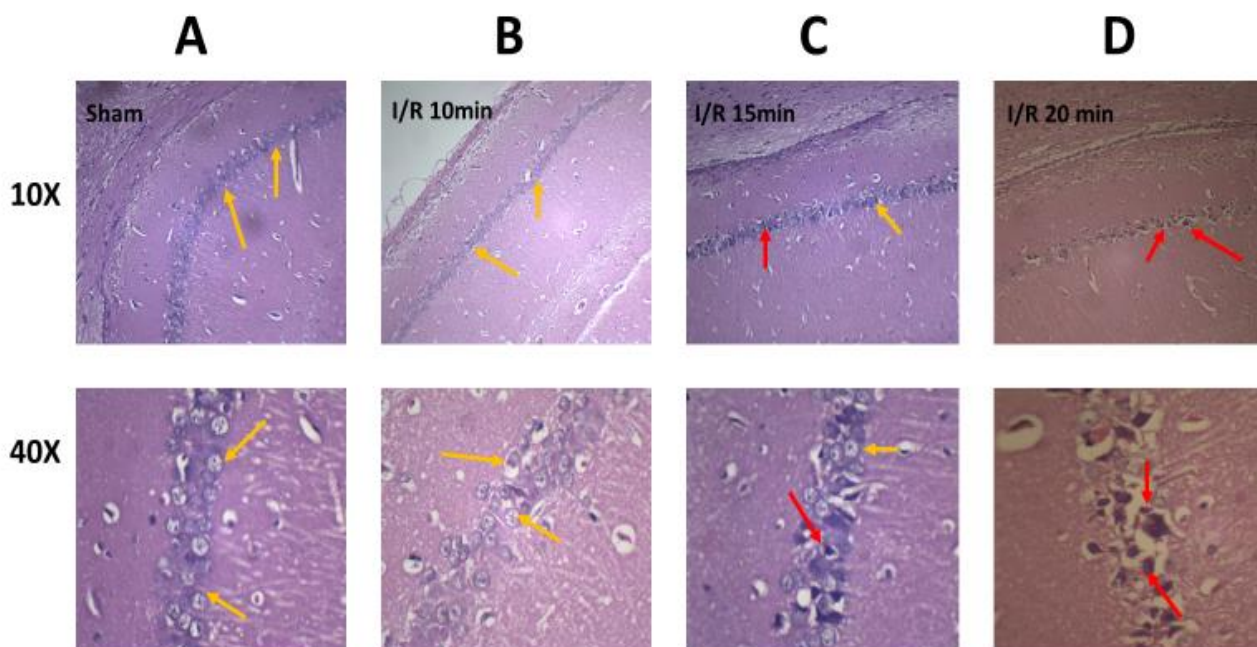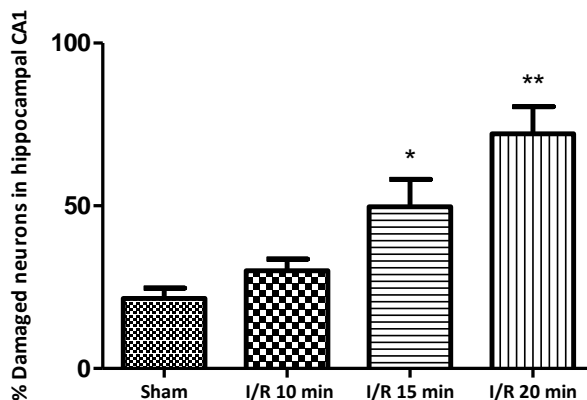
Fig. 5.18 (b). Effect of cerebral I/R injury on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. *P<0.05 sham vs I/R 15min and **P<0.01 sham vs I/R 20min.

5.3   *Development of Remote limb ischemic post conditioning (RIPOC)*

From the earlier study, it is evident that ischemia of 20 min followed by reperfusion of 72 hrs could induce significant cerebral I/R injury. Therefore, I/R of 20 min ischemia and 72 hrs reperfusion was considered as I/R group in the rest of the studies in this thesis. Further, to validate RIPOC we have performed RIPOC on femoral artery, renal artery and abdominal artery. Pilot studies were performed in renal and abdominal arteries, however due to high mortality rates, invasive surgical procedure, the above-mentioned arteries were not considered. However, RIPOC intervention at femoral artery reduced the mortality and post-surgical complications when compared to renal and abdominal arteries as there is no direct contact of vital organs. Therefore, we have considered femoral artery as the target artery to perform RIPOC. Moreover, we performed non-invasive RIPOC which is clinically beneficial and it is being mentioned as RIPOC only rather than NRIPOC in this thesis.

Further, to validate the remote limb ischemic post conditioning (RIPOC) we have tested various duration of ischemia and reperfusion and different number of cycles of ischemia and reperfusion. Based on earlier studies and laboratory pilot studies, we have induced RIPOC for a total duration of 40-60 min to femoral artery. During the standardisation protocol, we have induced RIPOC on femoral artery using 4 cycles of 5 min ischemia and 5 min reperfusion (mentioned as 4/5 in the study), 3 cycles of 10 min ischemia and 10 min reperfusion (indicated as 3/10 in the study), 2 cycles of 15 min ischemia and 15 min reperfusion (named as 2/15 in the study) and one cycle of 20 min ischemia followed by reperfusion (referred as 1/20 in the study).

5.3.1 Effect of RIPOC on behavioral parameters

5.3.1.1 Effect of RIPOC on memory in passive avoidance

We have not observed any significant change in the acquisition trial of passive avoidance task in Fig.5.19.
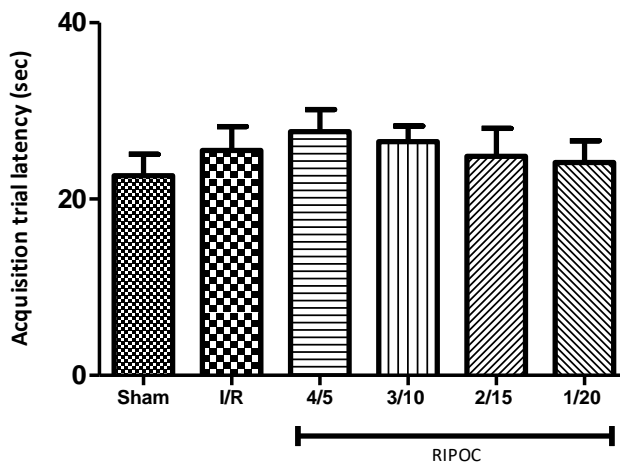
Fig. 5.19. Effect of RIPOC on acquisition trial latency of passive avoidance task.
Values were expressed as mean±S.E.M. No significant differences were observed among the groups.

RIPOC in various duration of ischemia and reperfusion, and different number of cycles was shown to be protective when compared to I/R injury as indicated by improved retention in light chamber as shown in Fig.5.20. However, 3/10 RIPOC could produce significant (P<0.05) effect as evidenced by increased retention trial latency when compared to I/R injury.
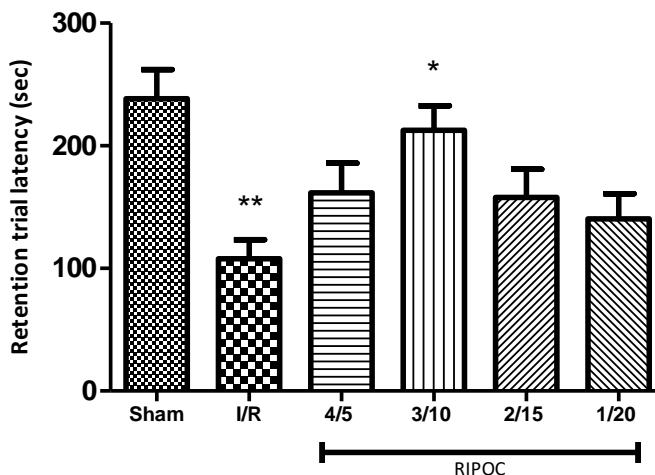

Fig. 5.20. Effect of RIPOC on retention trial latency of passive avoidance task.
Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R and *P<0.05 I/R vs 3/10 RIPOC.

5.3.1.2   Effect of RIPOC on memory in Y-maze test:

To assess the memory function and willingness of the rodents to explore the novel environment we have performed Y-maze task (Fig.5.21). We noticed that RIPOC intervention induced by different algorithms improved the spontaneous alterations. However, 3/10 RIPOC has shown significant spontaneous alterations (P<0.05) when compared to I/R injury.
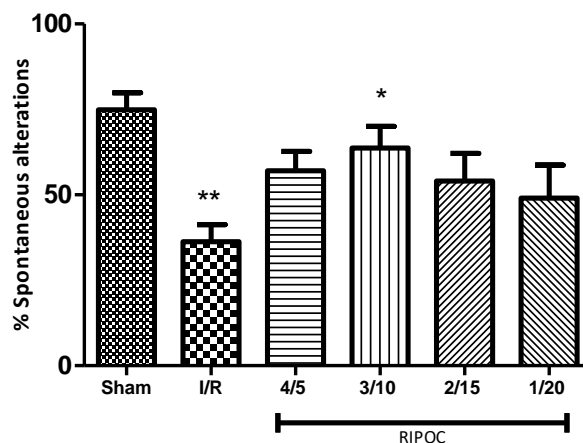
Fig. 5.21. Effect of RIPOC on percent spontaneous alterations in Y-maze task.
Values were expressed as mean±S.E.M. **P<0.01 sham vs I/R and *P<0.05 I/R vs 3/10 RIPOC

5.3.1.3   Effect of RIPOC on memory in elevated plus maze (EPM)

Cognitive dysfunction induced by I/R injury can be assessed by EPM task as seen in Fig.5.22 & 5.23. No significant difference in transfer trial latency was noticed during acquisition trial of EPM task. During retention trial, RIPOC in various combinations of I/R duration and cycles was shown to be protective. However, 4/5 (P<0.05) and 3/10 RIPOC (P<0.01) groups were shown to be markedly protective as indicated by reduced transfer trial latency in EPM task.
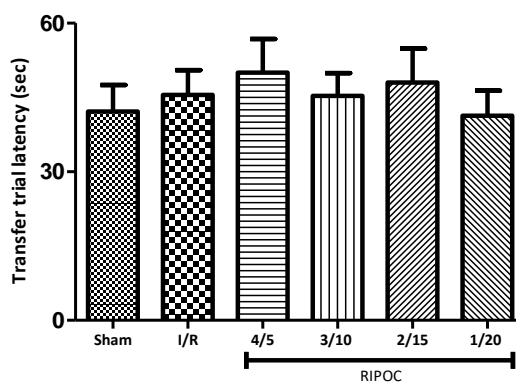
Fig. 5.22. Effect of RIPOC on transfer trial latency in elevated plus maze task.
Values were expressed as mean±S.E.M. No significant difference was observed among the groups during acquisition trial.
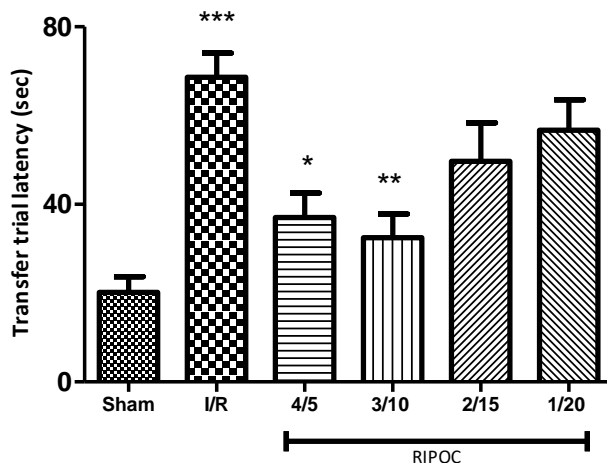
Fig. 5.23. Effect of RIPOC on retention transfer trial latency in elevated plus maze task.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, *P<0.05 I/R vs 4/5 RIPOC and **P<0.01 I/R vs 3/10 RIPOC.

5.3.1.4 *Effect of RIPOC on locomotor activity*

Locomotor activity was assessed after the reperfusion phase to eliminate the possibility of any motor abnormality in the cognitive deficits. We observed that there is no significant difference in the locomotor activity among different groups in the study indicating that motor abnormality may not be the reason for the poor performance in memory parameters elicited by I/R injury as seen in Fig.5.24.
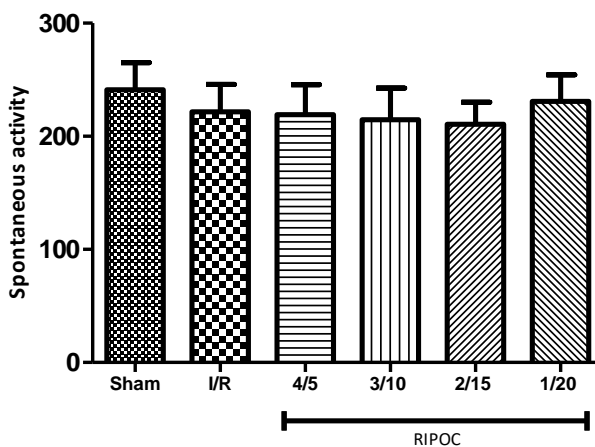
Fig. 5.24. Effect of RIPOC on locomotor activity.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups in the study.

5.3.2   Effect of RIPOC on biochemical parameters

5.3.2.1   Oxidative markers-MDA and nitrite

In comparison to cerebral I/R injury group, RIPOC intervention in different algorithms reduced the levels of oxidative markers such as MDA and nitrite. However, 3/10 RIPOC was shown to significantly ($P<0.05$) reduce the levels of oxidative markers such as MDA (Fig.5.25) and nitrite (Fig.5.26) when compared to I/R injury group. Moreover, 2/15 RIPOC markedly ($P<0.05$) reduced the nitrite levels when compared to I/R injured rats.
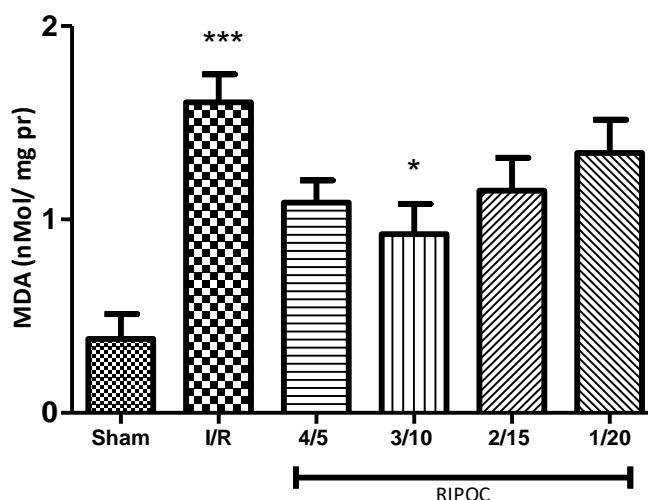
Fig. 5.25. Effect of RIPOC on MDA levels.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R and *P<0.05 I/R vs 3/10 RIPOC

Fig. 5.26. Effect of RIPOC on nitrite levels.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R and *P<0.05 I/R vs 3/10 RIPOC and 2/15 RIPOC.

5.3.2.2   Anti-oxidants-GSH and SOD

We observed that 3/10 RIPOC significantly increased the level of GSH (P<0.01) and SOD (P<0.001) when compared to I/R injury as seen in Fig.5.27 and 5.28 respectively. Moreover, 4/5 RIPOC significantly (P<0.05) improved the levels of SOD when compared to I/R injury group.
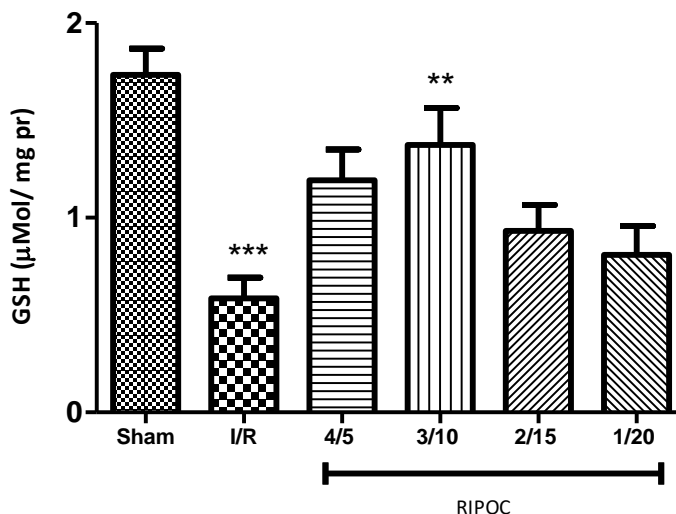
Fig. 5.27. Effect of RIPOC on GSH levels.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R and **P<0.01 I/R vs 3/10 RIPOC
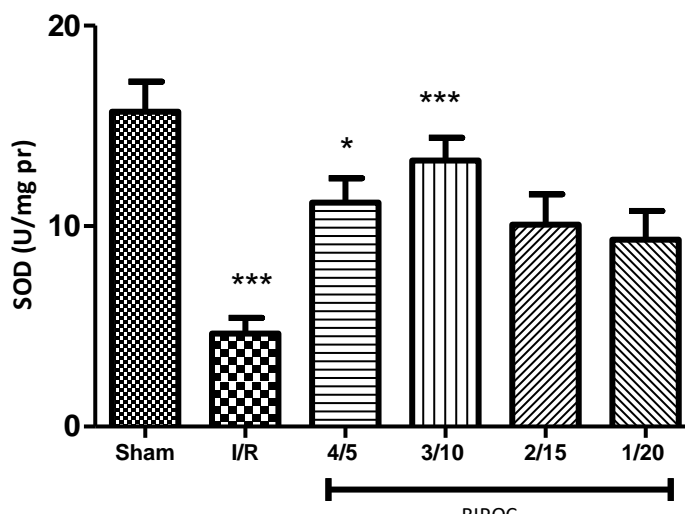
Fig. 5.28. Effect of RIPOC on SOD levels.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, *P<0.05 I/R vs 4/5 RIPOC and ***P<0.001 I/R vs 3/10 RIPOC

5.3.3 Effect of RIPOC on neuroinflammatory parameters

5.3.3.1 TNF-αlevels

RIPOC induced by different algorithms reduced the neuroinflammation as evidenced by reduced TNF-α levels as depicted in Fig.5.29. However, 4/5 RIPOC (P<0.05) and 3/10 RIPOC (P<0.01) significantly reduced the neuroinflammation as evidenced by low TNF-α levels when compared to I/R injury.
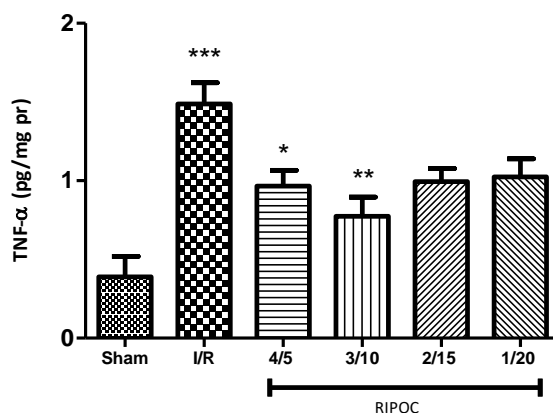
Fig. 5.29. Effect of RIPOC on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.01 sham vs I/R, *P<0.05 I/R vs 4/5 RIPOC and **P<0.01 I/R vs 3/10 RIPOC

5.3.3.2 IL-6

We found that 4/5, 2/15 and 1/20 RIPOC were able to reduce IL-6 levels but it was not significant when compared to I/R injury (Fig.5.30). However, 3/10 RIPOC was found to significantly (P<0.01) reduce the neuroinflammation as evidenced by reduced levels of IL-6 when compared to I/R injury.
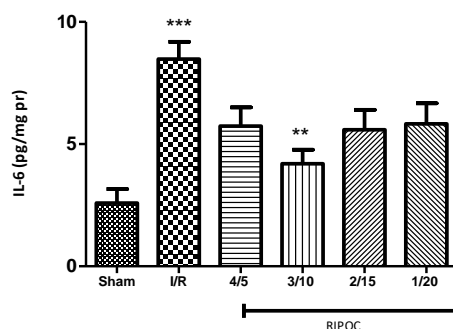
Fig. 5.30. Effect of RIPOC on IL-6 levels.
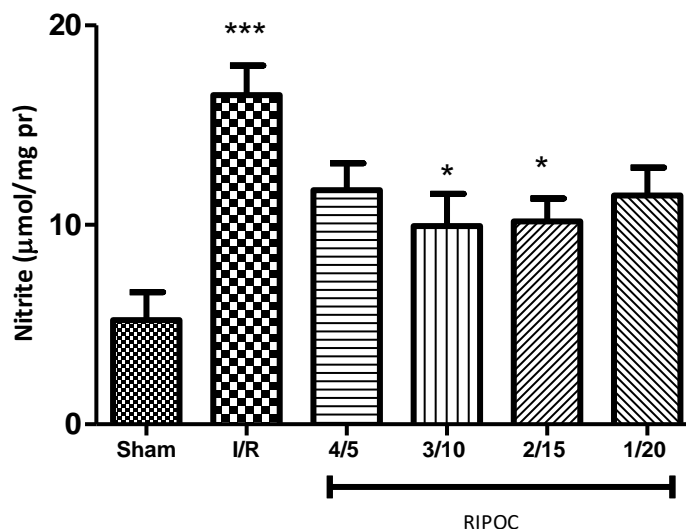**Values were expressed as mean±S.E.M. ***P<0.01 sham vs I/R and **P<0.01 I/R vs 3/10 RIPOC**

5.3.4   Effect of RIPOC on hippocampal CA1 neurons

The microscopic images from hippocampal CA1 region of cerebral I/R injured rats showed decreased neuronal density and increased pyknotic neuronal count. In addition, neurons represented shrunken and sickle shape with no visible nucleus and cytoplasm. However, in 4/5 RIPOC and 2/15 RIPOC groups, we found improved healthy neuronal count while some neurons with degenerating morphology were also noticed. In contrast 3/10 RIPOC significantly (P<0.05) improved the neuronal count where we visualized neurons with robust shaped along with clear nucleus and visible cytoplasm as indicated in Fig. 5.31 (a & b).
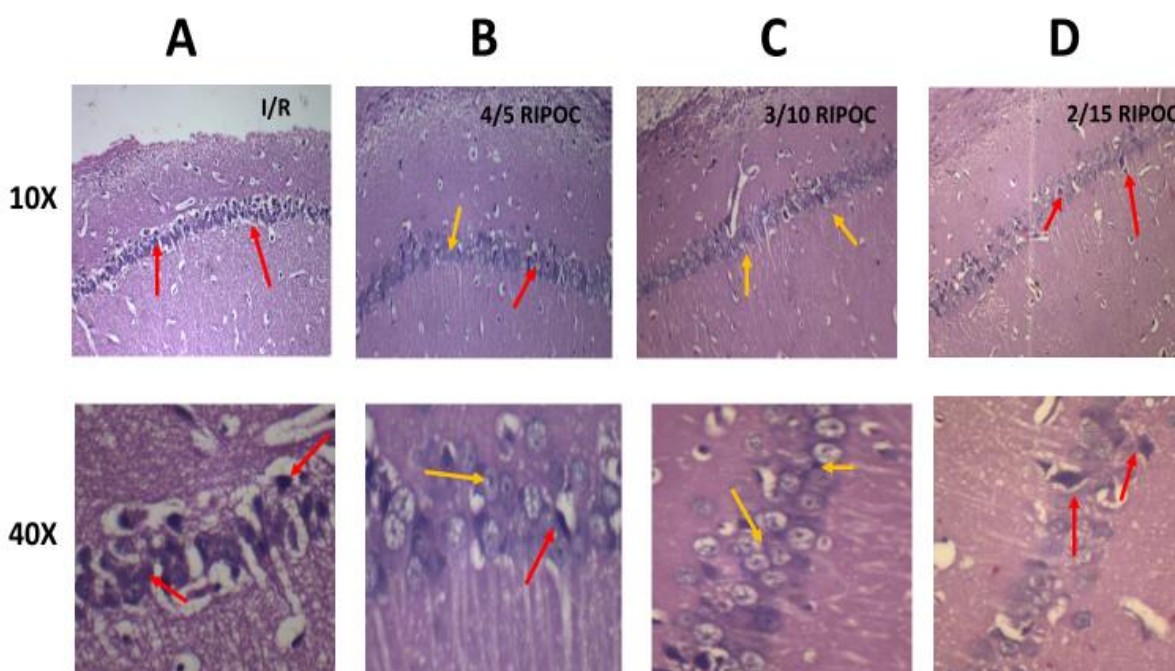
Fig. 5.31 (a). Effect of RIPOC on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) I/R injury, (Panel B) 4/5 RIPOC, (Panel C) 3/10 RIPOC, (Panel D) 2/15 RIPOC. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
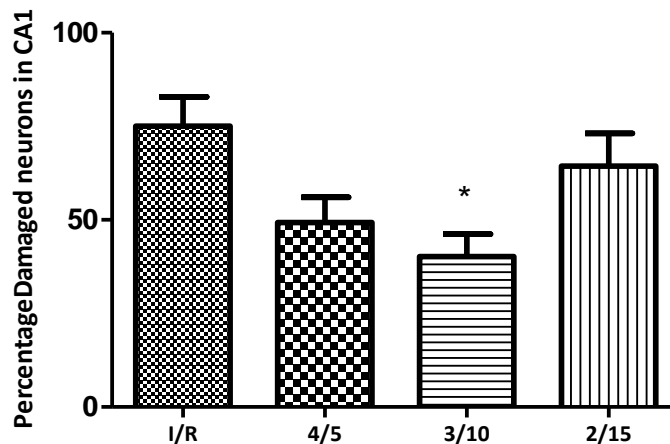
Fig. 5.31 (b). Effect of RIPOC on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. *P<0.05 I/R vs 3/10 RIPOC

5.4 Development of early and delayed RIPOC

In the earlier study, 4/5, 2/15, 3/10, 1/20 RIPOC were applied during the onset of reperfusion to enhance the potential of RIPOC. Based on the results of the earlier study, it is evident that RIPOC in different algorithms significantly reduced I/R injury induced behavioral, biochemical and inflammatory parameters. Therefore, based on analysis of results from previous study i.e. model validation of RIPOC it was evident that 3 cycles of 10 min of ischemia and 10 min of reperfusion was effective to reduce I/R injury induced cerebral damage. Therefore, we explored the role of 3/10 RIPOC when applied during the onset, 10min, 30 min, 1 hour, 3 hours, 6 hours after the reperfusion i.e., the protective effect of early and delayed RIPOC (3/10 RIPOC was considered as RIPOC in the thesis).

5.4.1 Effect of early and delayed RIPOC on behavioral parameters

5.4.1.1 Effect of early and delayed RIPOC on memory in passive avoidance task

During the acquisition trial of passive avoidance task, there was no significant difference in the initial trial latency among different groups of the study as seen in Fig. 5.32. However, during the retention trial of the passive avoidance task, RIPOC performed at onset and 10 min significantly (P<0.05) improved the retention trial latency. In addition, RIPOC induced at 1 hour, 3 hours and at 6 hours after reperfusion was able to improve the retention trial latency, but it was not significant when compared to I/R injury group (Fig. 5.33).
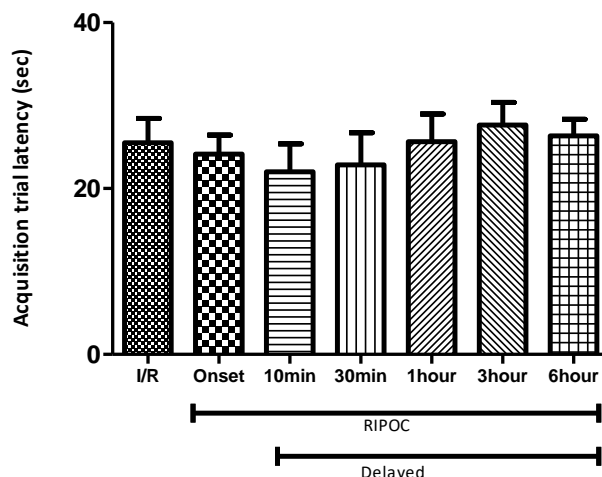
Fig. 5.32. Effect of early and delayed RIPOC on initial trial latency in passive avoidance task.
Values were expressed as mean±S.E.M. No significant differences were observed among the rats in different groups.
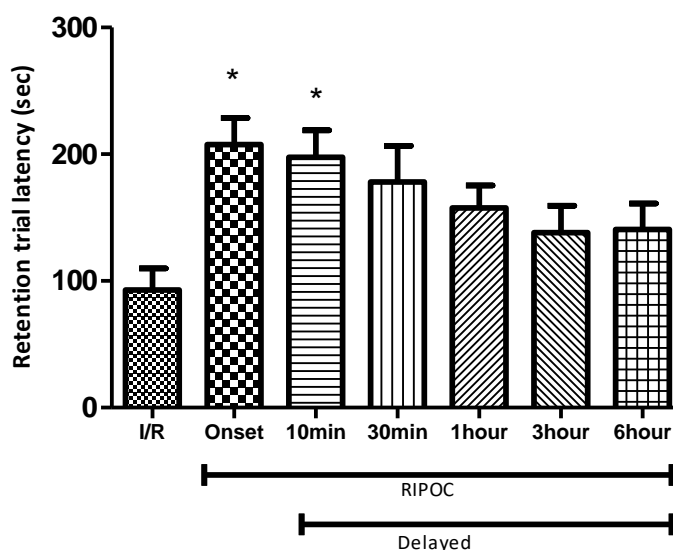
Fig. 5.33. Effect of early and delayed RIPOC on retention trial of passive avoidance task.
Values were expressed as mean±S.E.M. *P<0.05 I/R vs onset RIPOC and 10 min RIPOC

5.4.1.2   *Effect of early and delayed RIPOC on memory in Y-maze test*

Delayed RIPOC was able to induce the spontaneous alteration as evidenced by improved percent spontaneous alterations in a non-significant manner when compared to I/R injured rats as seen in Fig. 5.34. However, RIPOC intervention applied during the onset of reperfusion significantly (P<0.05) improved the percent spontaneous alterations when compared to I/R injury.

Fig. 5.34. Effect of early and delayed RIPOC on percent spontaneous alterations.
Values were expressed as mean±S.E.M. *P<0.05 I/R vs onset RIPOC

5.4.1.3   Effect of early and delayed RIPOC on memory in EPM task

During the acquisition trial of the EPM task (Fig.5.35), there was no significant difference in the transfer trial latency. However, during the retention trial 3/10 RIPOC applied during the onset (P<0.01) and a delay of 10 min (P<0.05) was able to markedly reduce the transfer trial latency in the EPM task when compared to I/R injury as observed in Fig.5.36.
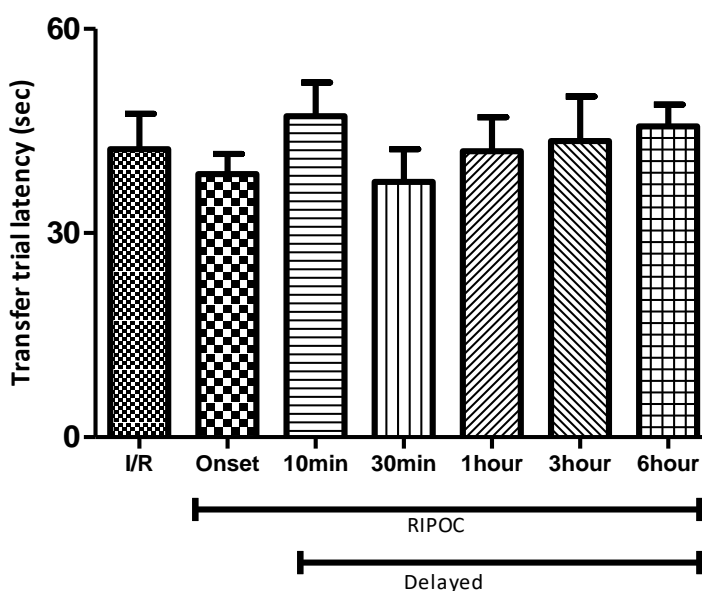
Fig. 5.35. Effect of early and delayed RIPOC on transfer trial latency during acquisition trial of EPM task.
Values were expressed as mean±S.E.M. No significant task was observed among different groups in the study.
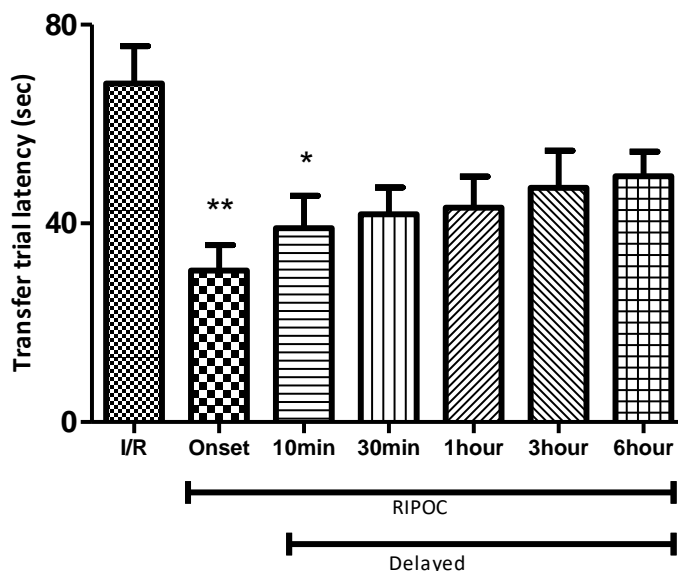
Fig. 5.36. Effect of early and delayed RIPOC on transfer trial latency during retention trial of EPM task. Values were expressed as mean±S.E.M. **P<0.01 I/R vs onset and *P<0.05 I/R vs 10 min

5.4.1.4   Effect of early and delayed RIPOC on spontaneous activity

We did not observe any significant difference in the locomotor activity among various groups in the study indicating that the motor abnormalities may be eliminated as a reason for poor cognitive performance (Fig. 5.37).
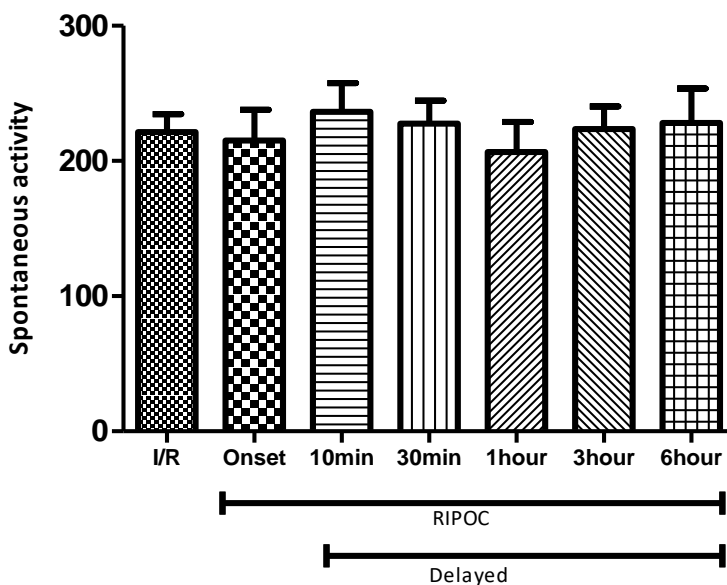
Fig. 5.37. Effect of early and delayed RIPOC on locomotor activity. Values were expressed as mean±S.E.M. No significant differences were observed among the groups.

5.4.2   Effect of early and delayed RIPOC on biochemical parameters

5.4.2.1   Effect of early and delayed RIPOC on oxidative markers- MDA and nitrite

Rats subjected to cerebral I/R injury exhibited significant increase in the MDA and nitrite levels. However, significant ($P<0.05$) reduction of MDA and nitrite levels were observed with RIPOC intervention applied during onset of reperfusion when compared to I/R injury group as seen in Fig. 5.38 and 5.39. In contrast, delayed RIPOC was able to reduce oxidative stress as evidenced by reduced by MDA and nitrite but it was not significant when compared to I/R injury group.
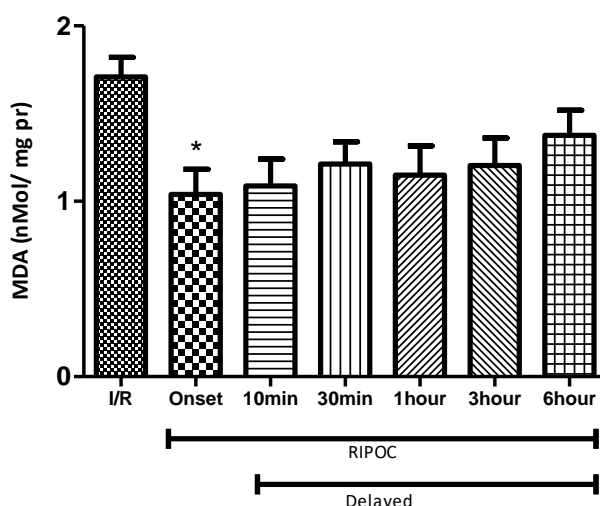
Fig. 5.38. Effect of early and delayed RIPOC on MDA levels.
Values were expressed as mean±S.E.M. *P<0.05 I/R vs onset RIPOC

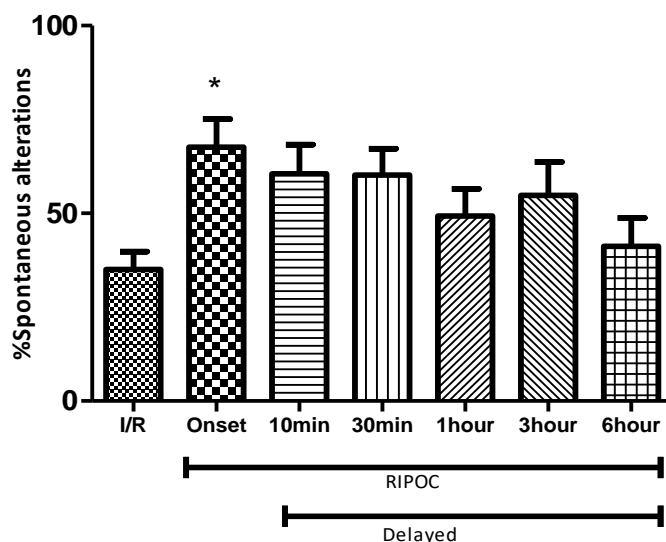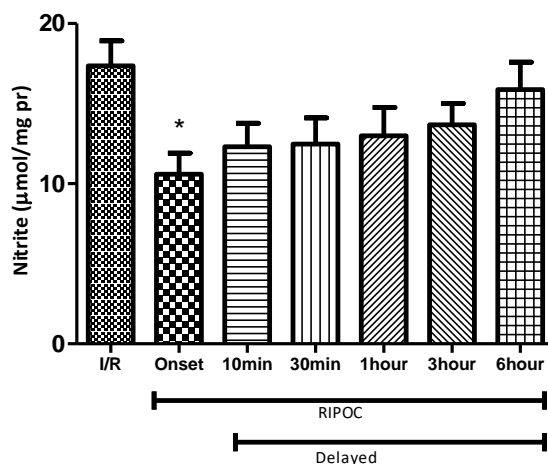**Fig. 5.39. Effect of early and delayed RIPOC on nitrite levels.**
Values were expressed as mean±S.E.M. *P<0.05 I/R vs onset RIPOC

5.4.2.2   Effect of early and delayed RIPOC on anti-oxidants- GSH and SOD

A significant reduction in the anti-oxidant levels such as GSH and SOD was observed in I/R injury group. However, RIPOC applied during onset (P<0.01) or at a delay of 10 (P<0.05) and 30 min (P<0.05) was able to improve GSH levels (Fig.5.40) when compared to I/R injury group. In comparison to I/R group, RIPOC induced at onset and a delay of 10 min of reperfusion significantly (P<0.01) improved SOD levels as noticed in Fig. 5.41.
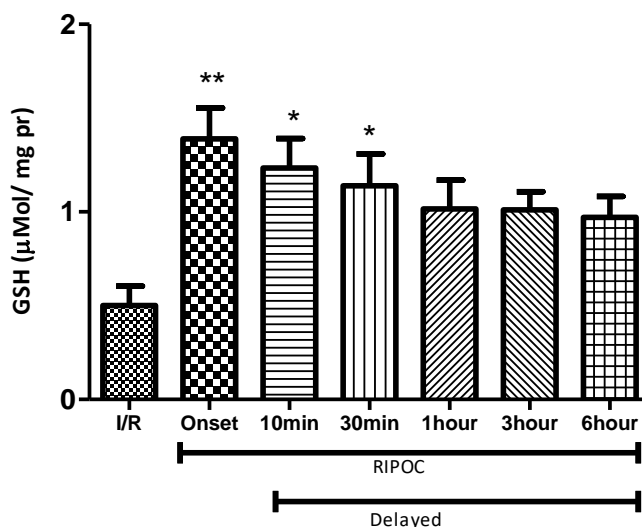
Fig. 5.40. Effect of early and delayed RIPOC on GSH levels.
Values were expressed as mean±S.E.M. **P<0.01 I/R vs onset RIPOC, *P<0.05 I/R vs 10 min and 30 min RIPOC
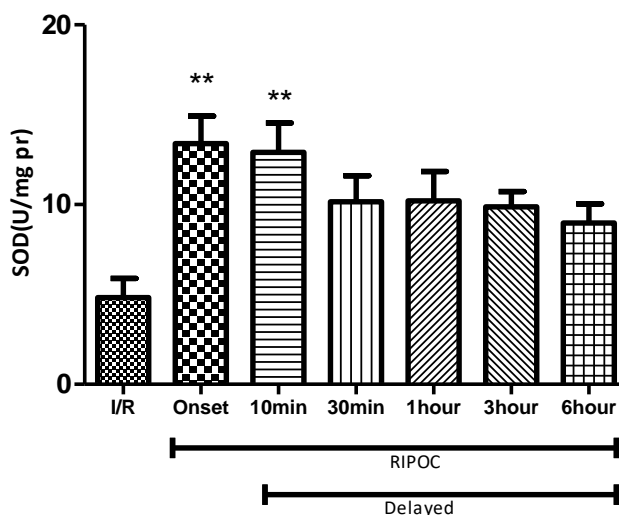
Fig. 5.41. Effect of early and delayed RIPOC on SOD levels.
Values were expressed as mean±S.E.M. **P<0.01 I/R vs onset RIPOC, **P<0.01 I/R vs 10 min RIPOC

5.4.3   Effect of early and delayed RIPOC on neuroinflammatory parameters

5.4.3.1   Effect of early and delayed RIPOC on TNF-α and IL-6

Cerebral I/R injury produced neuroinflammation as evidenced by increased levels of TNF-α and IL-6. However, early and delayed RIPOC intervention prevented the up-regulation of pro-inflammatory cytokines including TNF-α (Fig.5.42) and IL-6 (Fig.5.43). RIPOC performed after a delay of 30min, 1 hour, 3 hours, 6 hours after reperfusion was able to reduce the pro-inflammatory cytokine levels but it was not significant. In contrast, RIPOC intervention applied at onset (P<0.001) and 10 min (P<0.01) delay of reperfusion significantly reduced the levels of TNF-α and IL-6.
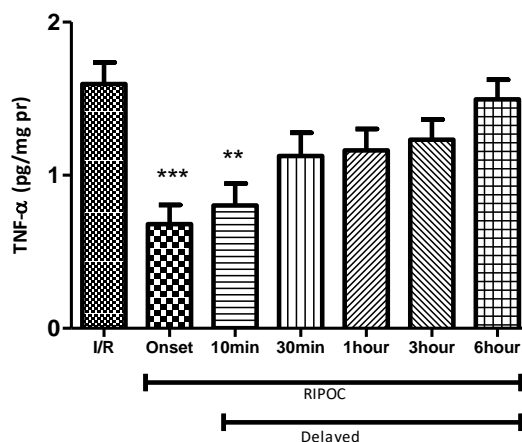
Fig. 5.42. Effect of early and delayed RIPOC on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 I/R vs onset RIPOC and **P<0.01 I/R vs 10 min RIPOC
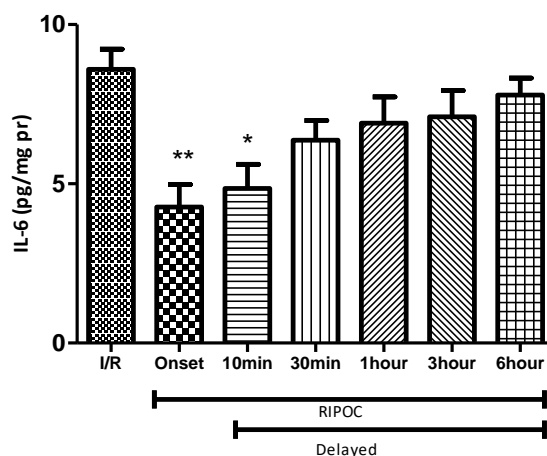
Fig. 5.43. Effect of early and delayed RIPOC on IL-6 levels.
Values were expressed as mean±S.E.M. **P<0.01 I/R vs onset RIPOC and *P<0.05 I/R vs 10 min RIPOC

5.4.4 Effect of early and delayed RIPOC on hippocampal CA1 neurons

Microscopic analysis of HE sections of I/R injured rats revealed significant neuronal damage as evidenced by pyknotic neurons that were characterized by sickle shape, dark stain and indicated by red arrows Fig.5.44 (a). However, early RIPOC showed a significant (P<0.05) reduction in the percentage of damaged neurons in the hippocampal CA1 region as depicted in Fig.5.44 (b). The neurons in early RIPOC group were robust in shape, with clear large nucleus and visible cytoplasm and indicated by yellow arrows, while in delayed RIPOC groups we noticed an increase in pyknotic neuronal count with delay in RIPOC intervention.
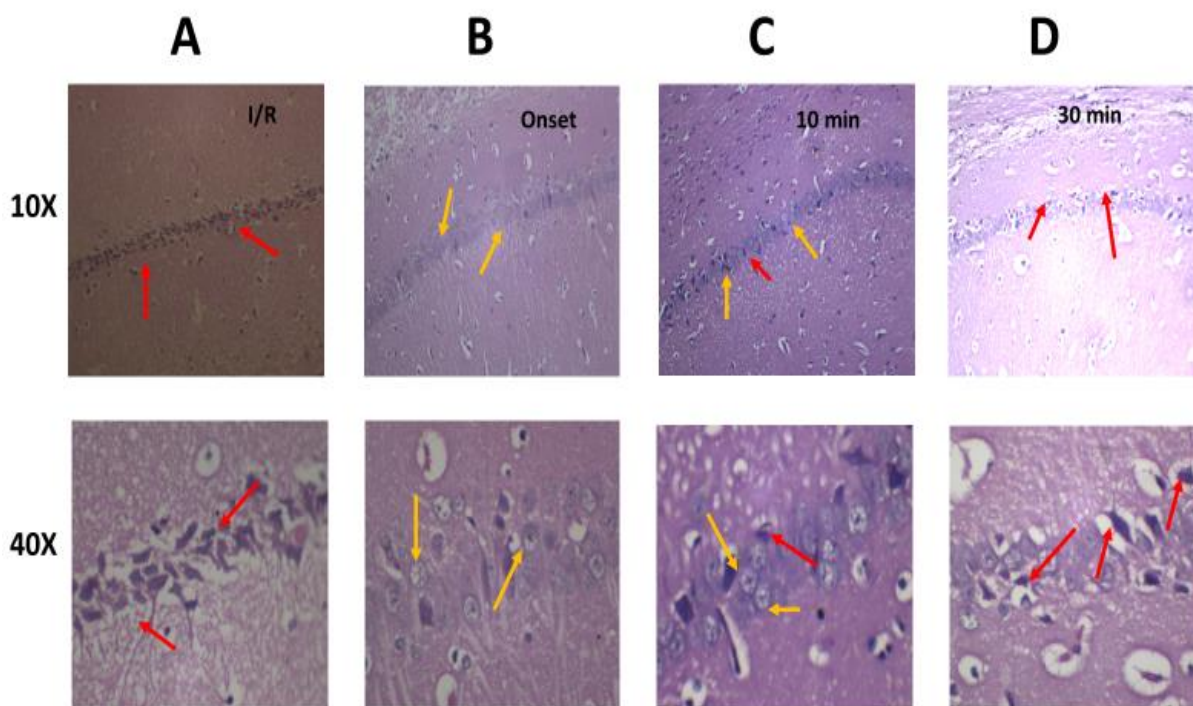
Fig. 5.44(a). Effect of early and delayed RIPOC on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) I/R injury, (Panel B) onset RIPOC, (Panel C) 10 min RIPOC, (Panel D) 30 min RIPOC. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
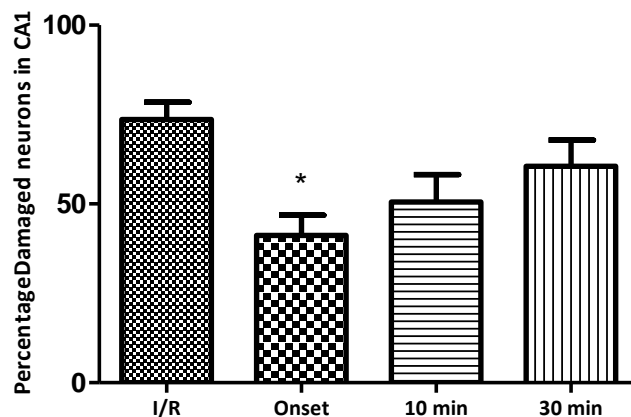
Fig. 5.44 (b). Effect of early and delayed RIPOC on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. *P<0.05 I/R vs onset RIPOC.

5.5   *Effect of RIPOC and NR2B agonist on cerebral I/R injury and associated cognitive deficits*

5.5.1   Effect of RIPOC and NR2B agonist (Quinolinic acid [QA] 100nmol/1 μl) on behavioral parameters

5.5.1.1   Effect of RIPOC and QA on memory in passive avoidance task

During the acquisition trial of the passive avoidance task, there was no significance in acquisition trial latency among different groups of the study (Fig.5.45). In comparison to I/R injured group, RIPOC intervention significantly (P<0.01) improved the retention trial latency. However, the protective effect of RIPOC was significantly (P<0.05) abolished in presence of QA as evidenced by reduced retention latency as depicted in Fig.5.46.
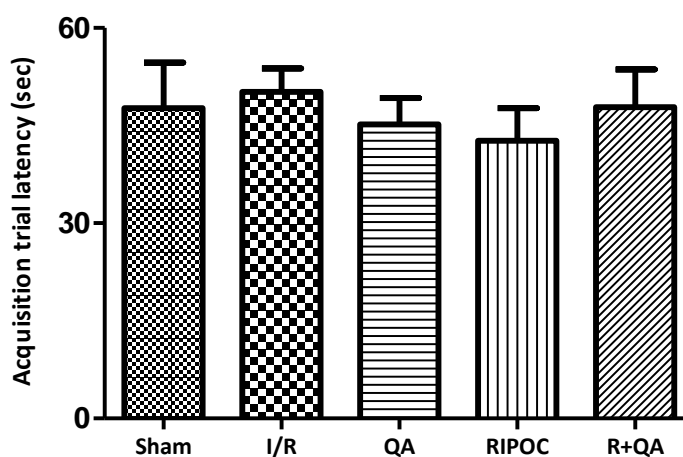
Fig. 5.45. Effect of RIPOC and NR2B agonist-quinolinic acid on acquisition trial during passive avoidance task. Values were expressed as mean±S.E.M. No significant differences were observed among the different groups of the study
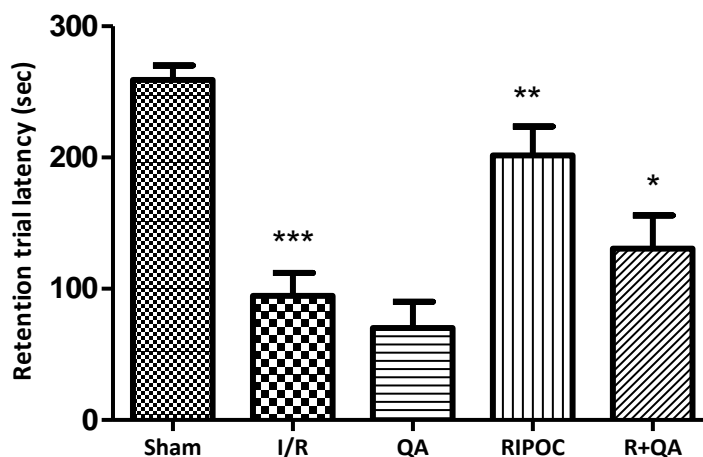
Fig. 5.46. Effect of RIPOC and NR2B agonist-quinolinic acid on retention trial during passive avoidance task. Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC, *P<0.05 RIPOC vs QA

5.5.1.2   Effect of RIPOC and QA on memory in Y-maze task

Cerebral I/R significantly (P<0.01) reduced the percentage spontaneous alterations when compared to sham rats. In comparison to I/R injury, RIPOC intervention significantly (P<0.05) improved the percentage spontaneous alterations, while the protective effects of RIPOC were markedly (P<0.05) abolished by QA treatment, as indicated by less spontaneous alterations as seen in Fig.5.47.
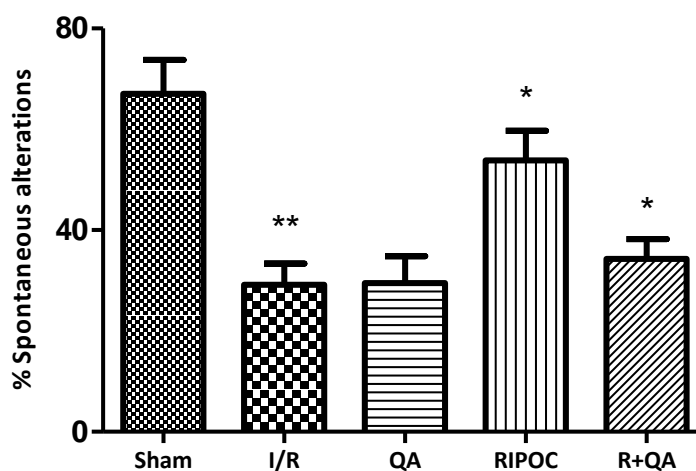
Fig. 5.47. Effect of RIPOC and NR2B agonist-quinolinic acid on spontaneous alterations during Y-maze task. Values were expressed as mean±S.E.M. **P<0.01 Sham vs I/R, *P<0.05 I/R vs RIPOC and *P<0.05 RIPOC vs QA

5.5.1.3   Effect of RIPOC and QA on memory in elevated plus maze test

During the acquisition trial of the EPM test, no significant difference in the transfer trial latency to enclosed arm was observed among different groups in the study (Fig.5.48). In the retention trial of the elevated plus maze task, I/R injured rats took more time to enter the enclosed arm as evidenced by marked (P<0.001) increased transfer trial latency when correlated to sham group. When correlated to I/R injured rats, RIPOC intervention significantly (P<0.01) reduced transfer trial latency to reach the enclosed arm, however, the protective effects of RIPOC were markedly (P<0.05) abolished in presence of QA (Fig.5.49).
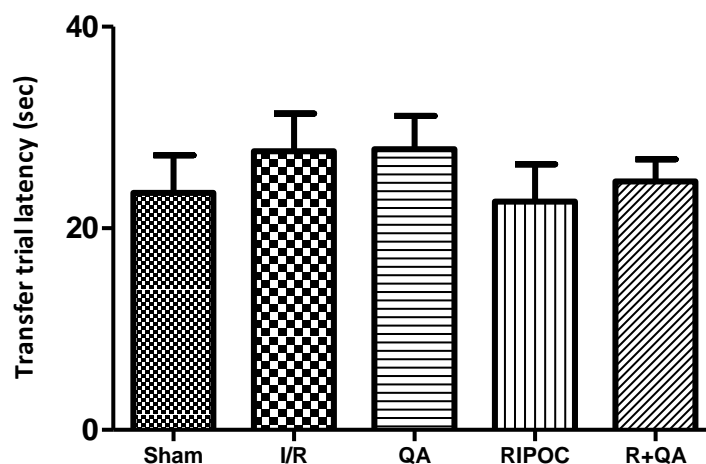
Fig. 5.48. Effect of RIPOC and NR2B agonist-quinolinic acid on transfer trial latency in acquisition trial of EPM. Values were expressed as mean±S.E.M. No significant differences were observed among different groups in the study.
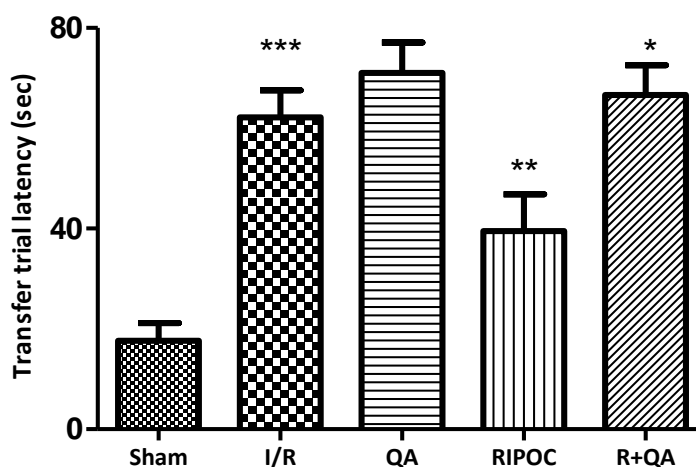
Fig. 5.49. Effect of RIPOC and NR2B agonist-quinolinic acid on transfer trial latency in retention trial of EPM. Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs QA

5.5.1.4   Effect of RIPOC and QA on locomotor activity

We did not observe any significant difference in locomotor activity among different groups in the study, this indicates that there no interference of motor abnormality in cognitive dysfunction (Fig.5.50).
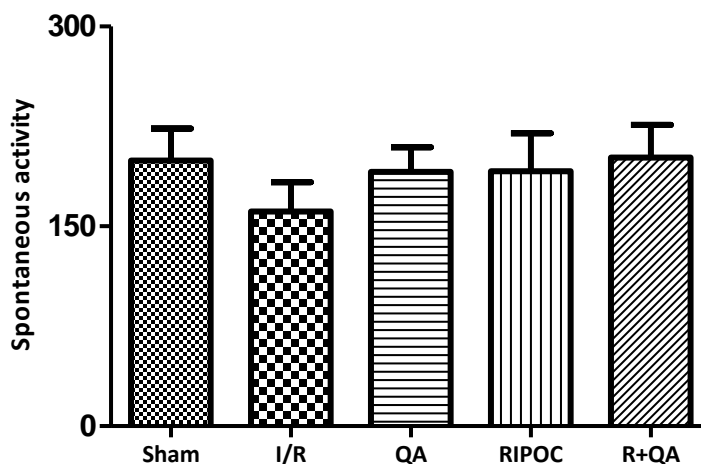
Fig. 5.50. Effect of RIPOC and NR2B agonist-quinolinic acid on locomotor activity.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups in the study.

5.5.2   Effect of RIPOC and QA on biochemical parameters

5.5.2.1   Effect of RIPOC and QA on oxidative markers and anti-oxidants

Induction of IR injury resulted in significant rise in oxidative markers such as MDA ($P<0.001$) and nitrite ($P<0.01$) along with reduction in anti-oxidants like GSH ($P<0.001$) and SOD ($P<0.001$). RIPOC intervention reduced the oxidative stress as evidenced by significantly reduced levels of MDA ($P<0.05$) and nitrite ($P<0.01$) along with increased levels of GSH ($P<0.01$) and SOD ($P<0.01$). However, treatment with QA before RIPOC intervention abolished the protective effects as evidenced by increased levels of oxidative markers and significantly ($P<0.01$) reduced levels of anti-oxidants (Table.5.2.).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.63±0.34	8.2±3.18	1.625±0.44	16.7±3.68
I/R	1.63±0.24***	23.42±5.73**	0.54±0.27***	6.88±2.96***
QA	1.85±0.36	26.80±6.08	0.49±0.12	5.27±1.03
RIPOC	0.93±0.39*	11.3±5.07**	1.43±0.47**	14.84±3.89**
R+QA	1.39±0.32	19.81±6.01	0.72±0.38*	8.19±1.65**

Table 5.2. Effect of RIPOC and NR2B agonist-quinolinic acid on biochemical parameters.
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R (MDA and SOD), *P<0.05 Sham vs I/R (nitrite and GSH), **P<0.01 I/R vs RIPOC (SOD), *P<0.05 I/R vs RIPOC (MDA, nitrite and GSH), *P<0.05 RIPOC vs QA (MDA and GSH) and **P<0.01 RIPOC vs QA.

5.5.3 Effect of RIPOC and QA on neuroinflammatory parameters

5.5.3.1 Effect of RIPOC and QA on TNF-α and IL-6

We found that I/R injury resulted in significant (P<0.001) neuroinflammation when compared to sham rats as evidenced by increased levels of TNF-α and IL-6 (Fig. 5.51 and 5.52). Early intervention of RIPOC during onset of reperfusion markedly (P<0.01) reduced the levels of TNF-α and IL-6. However, RIPOC protective effect was significantly (P<0.05) abolished in presence of QA, as observed by increased levels of TNF-α and IL-6.
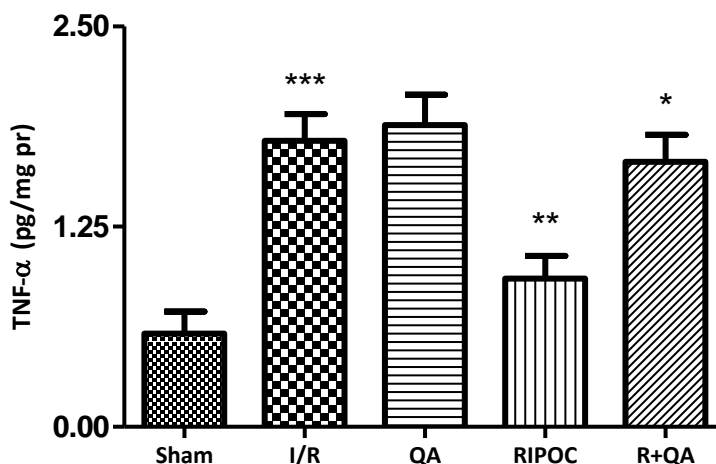
Fig. 5.51. Effect of RIPOC and NR2B agonist-quinolinic acid on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs QA
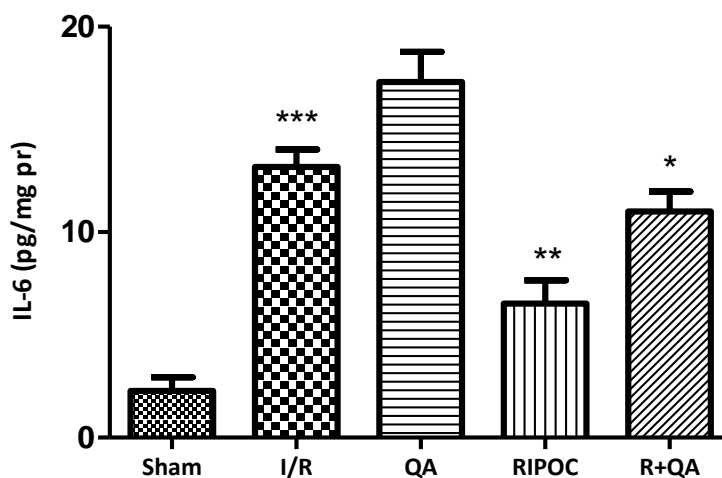
Fig. 5.52. Effect of RIPOC and NR2B agonist-quinolinic acid on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs QA

5.5.4 Effect of RIPOC and QA on hippocampal neurons

Microscopic analysis of HE stained sections of sham rats showed healthy neurons. The healthy neurons were robust in shape, had a nearly spherical nucleus with visible and clear cytoplasm as indicated by yellow arrows as observed in Fig.5.53 (a & b). However, in I/R injury and QA groups neurons were darkly stained, shrunken in size with darkened nuclei and indicated by red arrows. RIPOC intervention resulted in significant improvement in preservation of cell size, shape and content. In addition, there was marked improvement in neuronal density and subsequent reduction in the percentage of pyknotic neurons following RIPOC intervention, while this effect was abolished with prior treatment with QA before RIPOC intervention.
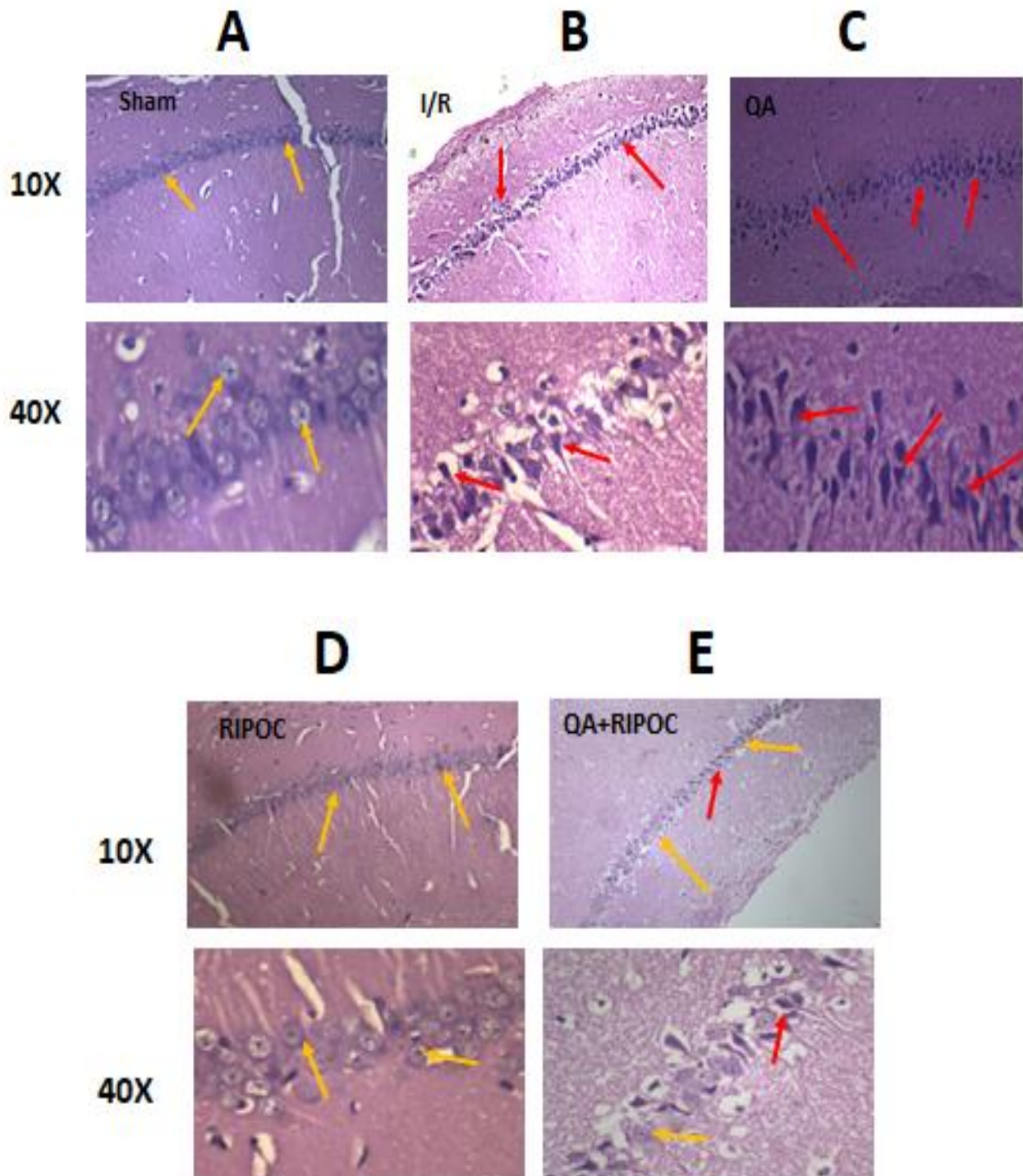
Fig. 5.53 (a). Effect of RIPOC and NR2B agonist-quinolinic acid on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) Quinolinic acid (QA) (Panel D) RIPOC, (Panel E) QA+RIPOC. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
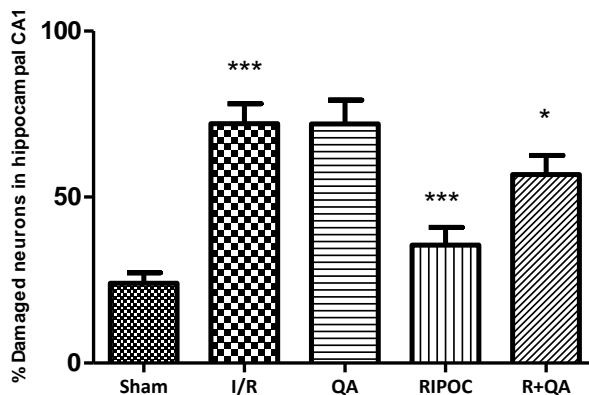
Fig. 5.53 (b). Effect of early and delayed RIPOC on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, ***P<0.001 I/R vs RIPOC and *P<0.05 RIPOC vs R+QA.

5.6 *Effect of NR2B antagonist-Ifenprodil and PI3K-Akt inhibitor-LY294002 on cerebral I/R injury and associated cognitive deficits*

5.6.1 Effect of Ifenprodil (IFN, 20mg/kg) and LY294002 (25µg) on behavioral parameters

5.6.1.1 Effect of Ifenprodil (IFN) and LY294002 on memory in passive avoidance acquisition trial

No significant difference in the acquisition trial latency was observed among different groups of the study during acquisition trial of passive avoidance task (Fig.5.54). However, during retention trial cerebral I/R injured rats markedly (P<0.001) reduced the retention trial latency when compared to sham group, while treatment with NR2B antagonist, Ifenprodil (IFN) significantly (P<0.01) improved the retention trial latency. However, IFN protective effects were significantly (P<0.05) abolished with PI3K-Akt inhibitor, LY294002 (Fig.5.55).
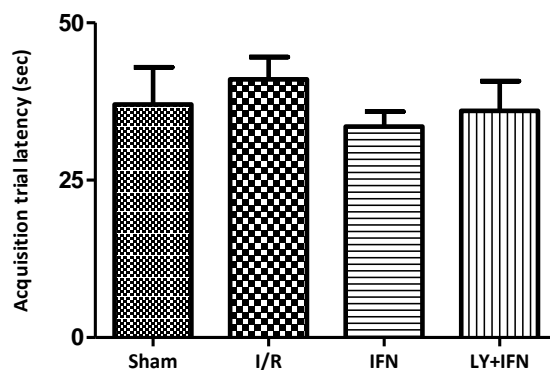
Fig. 5.54. Effect of IFN and LY294002 on memory in passive avoidance acquisition trial. Values were expressed as mean±S.E.M. No significant difference among different groups of the study.
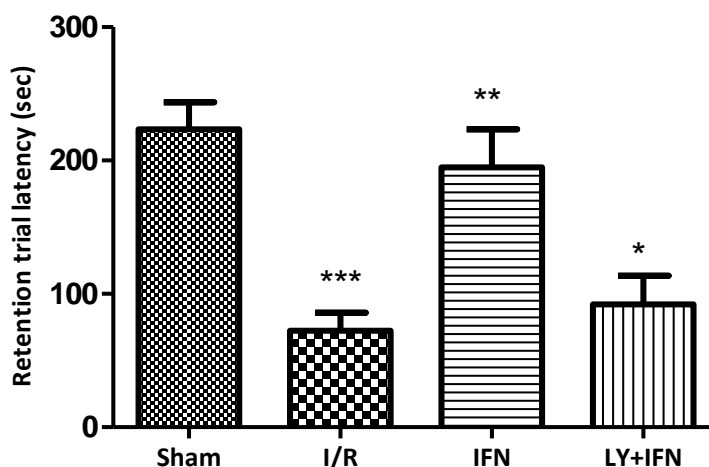
**Fig. 5.55. Effect of IFN and LY294002 on memory in passive avoidance acquisition trial.**
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs QA

5.6.1.2   Effect of Ifenprodil (IFN) and LY294002 on memory in Y-maze

Induction of cerebral I/R injury led to a significant (P<0.001) decrease in percentage spontaneous alterations when correlated to sham rats. However, IFN markedly (P<0.01) ameliorated the percentage spontaneous alterations while the protective effects of IFN were significantly (P<0.05) abolished with LY294002 pretreatment (Fig.5.56).

Fig. 5.56. Effect of IFN and LY294002 on memory in Y-maze task.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC, *P<0.05 RIPOC vs QA

5.6.1.3   Effect of Ifenprodil (IFN) and LY294002 on memory in EPM task

No significant difference was observed in the transfer trial latency during acquisition trial of the EPM task (Fig.5.57). In contrast, during retention trial induction of cerebral I/R injury

significantly (P<0.001) increased the transfer trial latency when compared to sham rats. However, a marked (P<0.001) reduction in transfer trial latency was noticed with IFN administration, while pre-treatment with LY294002 significantly (P<0.01) prevented the IFN induced protective effects (Fig.5.58).
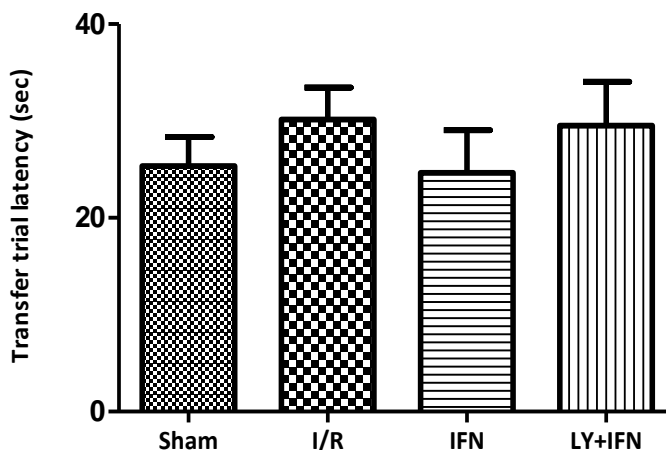
Fig. 5.57. Effect of IFN and LY294002 on memory in EPM acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups
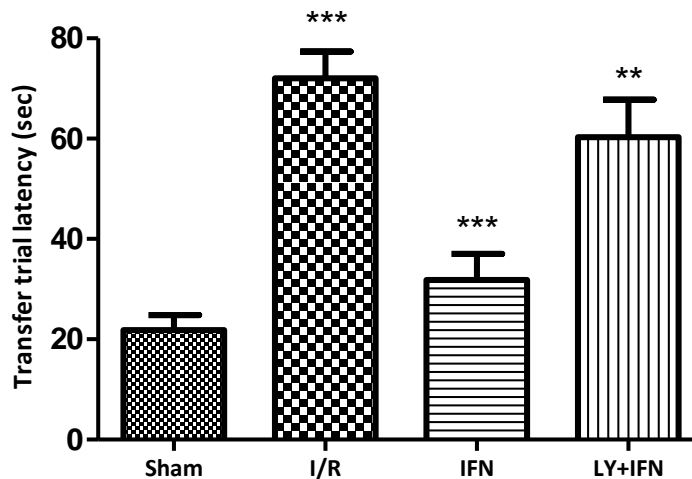
Fig. 5.58. Effect of IFN and LY294002 on memory in EPM retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC, **P<0.01 RIPOC vs QA

5.6.1.4   *Effect of Ifenprodil (IFN) and LY294002 on locomotor activity*

No significant differences in locomotor activity were observed among different groups of the study, indicating that there is no effect of motor abnormality on cognitive deficits (Fig.5.59).
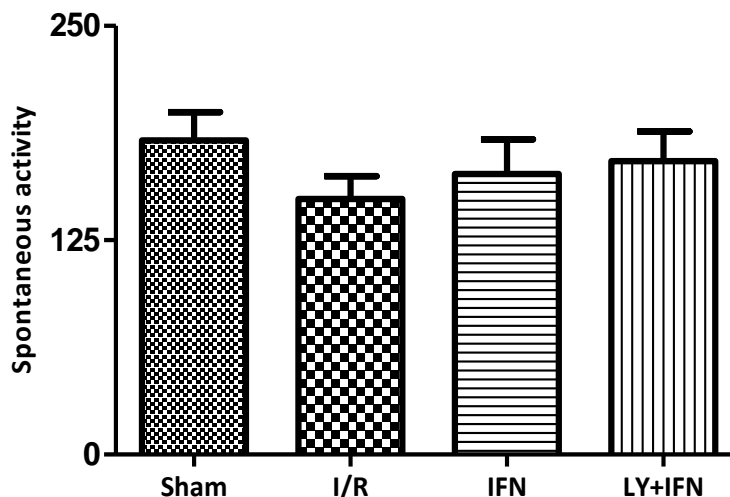
Fig. 5.59. Effect of IFN and LY294002 on locomotor activity.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups of the study.

5.6.2   Effect of Ifenprodil (IFN) and LY294002 on biochemical parameters

5.6.2.1   Effect of Ifenprodil (IFN) and LY294002 on oxidative markers and anti-oxidants

A significant (P<0.001) rise in oxidative marker level such as MDA and nitrite along with attenuation of anti-oxidants such as GSH and SOD was noticed in ischemic rats when compared to sham control rats. In contrast, IFN treatment attenuated levels of MDA (P<0.01) and nitrite (P<0.05), and ameliorated GSH (P<0.01) and SOD (P<0.05) levels. However, IFN induced protective effects on oxidative stress were significantly (P<0.01) abolished in presence of LY294002 (Table.5.3).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.5±0.3	6.35±2.34	1.8±0.39	15.9±4.00
I/R	1.79±0.26***	19.3±5.16***	0.6±0.28***	5.94±2.99***
IFN	1.02±0.34**	10.1±4.17*	1.46±0.38**	12.22±3.91*
IFN+LY	1.73±0.43**	20.0±5.93**	0.66±0.22**	6.75±2.22*

Table 5.3. Effect of IFN and LY294002 on biochemical parameters.
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs IFN (MDA and GSH), *P<0.05 I/R vs IFN (Nitrite and SOD), *P<0.05 IFN vs IFN+LY (SOD) and **P<0.01 IFN vs IFN+LY (MDA, nitrite and GSH).

5.6.3   Effect of Ifenprodil (IFN) and LY294002 on neuroinflammatory parameters

5.6.3.1   Effect of Ifenprodil (IFN) and LY294002 on TNF-α and IL-6

I/R injury resulted in significant (P<0.001) rise of inflammatory cytokine levels like TNF-α (Fig.5.60) and IL-6 (Fig.5.61) when compared to sham rats. However, administration of IFN significantly (P<0.01) reduced the neuroinflammation as evidenced by low levels of TNF-α and IL-6 when compared to I/R injury group, while pre-treatment with LY294002 markedly (P<0.01) abolished the protective effects of Ifenprodil.
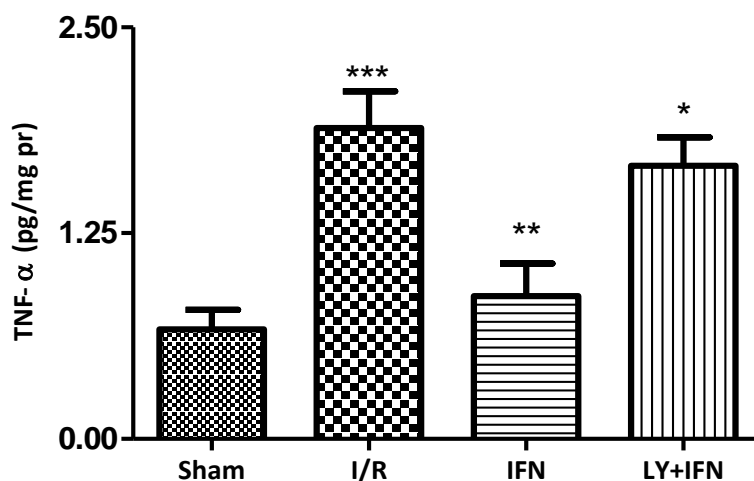
Fig. 5.60. Effect of IFN and LY294002 on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs QA
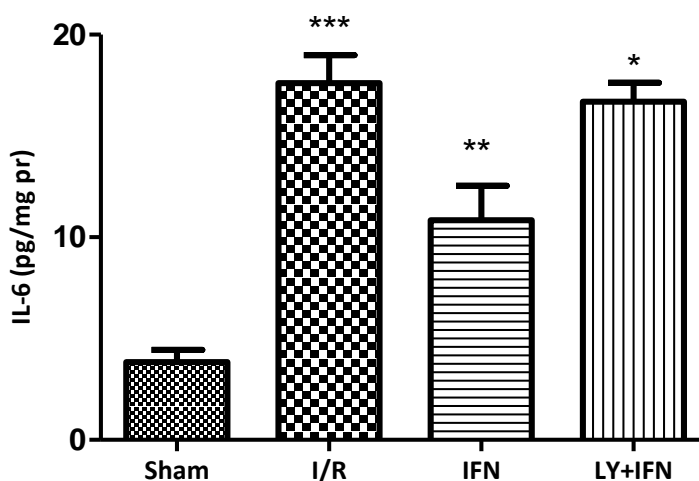
Fig. 5.61. Effect of IFN and LY294002 on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC, *P<0.05 RIPOC vs QA

5.6.4   Effect of Ifenprodil (IFN) and LY294002 on molecular estimations

5.6.4.1   Effect of Ifenprodil (IFN) and LY294002 on CREB and BDNF levels

The levels of CREB (Fig.5.62) and neurotrophic factor such as BDNF (Fig.5.63) are significantly (P<0.001) reduced by induction of I/R injury when compared to sham rats. However, IFN treatment significantly improved the levels of CREB (P<0.001) and BDNF (P<0.05), while LY294002 pretreatment significantly abolished this protective effect of IFN as evidenced by reduced CREB (P<0.001) and BDNF (P<0.05) levels.
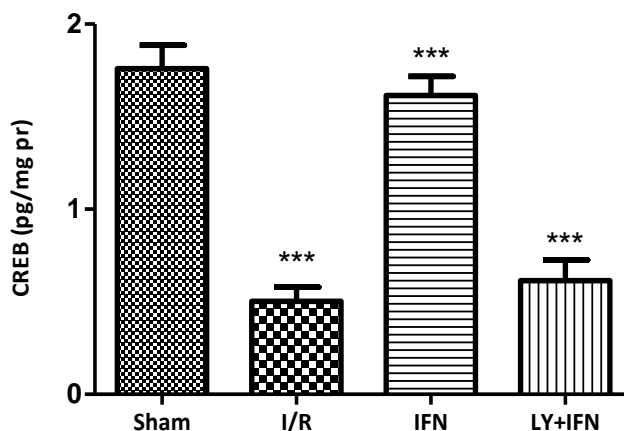
Fig. 5.62. Effect of IFN and LY294002 on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC, ***P<0.001 RIPOC vs QA
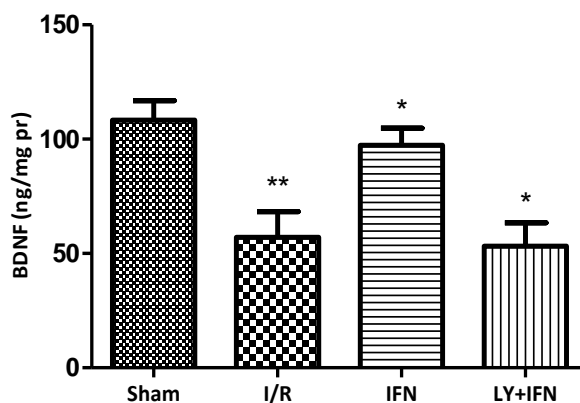
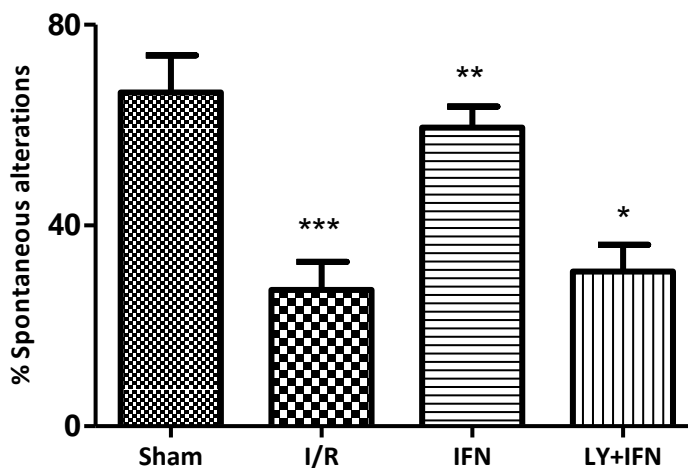**Fig. 5.63. Effect of IFN and LY294002 on BDNF levels.**
Values were expressed as mean±S.E.M. **P<0.001 Sham vs I/R, *P<0.05 I/R vs RIPOC and *P<0.05 RIPOC vs QA

5.6.5   Effect of Ifenprodil (IFN) and LY294002 on hippocampal CA1 neurons

Light microscopic results of HE stained sections showed healthy neurons in the CA1 region of sham control rats. Healthy neurons appeared robust in shape, had a spherical or slightly oval

nucleus and a single large nucleolus with clear visible cytoplasm as indicated by yellow arrows in Fig.5.64 (a). However, induction of cerebral I/R injury showed marked (P<0.001) changes in the disorganization, severe cell loss and darkened nuclei along with a marked shrinkage in the pyramidal cell size. Treatment with IFN caused significant (P<0.01) preservation of pyramidal cells, while this protective effect was significantly (P<0.05) abolished with LY294002 pre-treatment (Fig.5.64 (b).
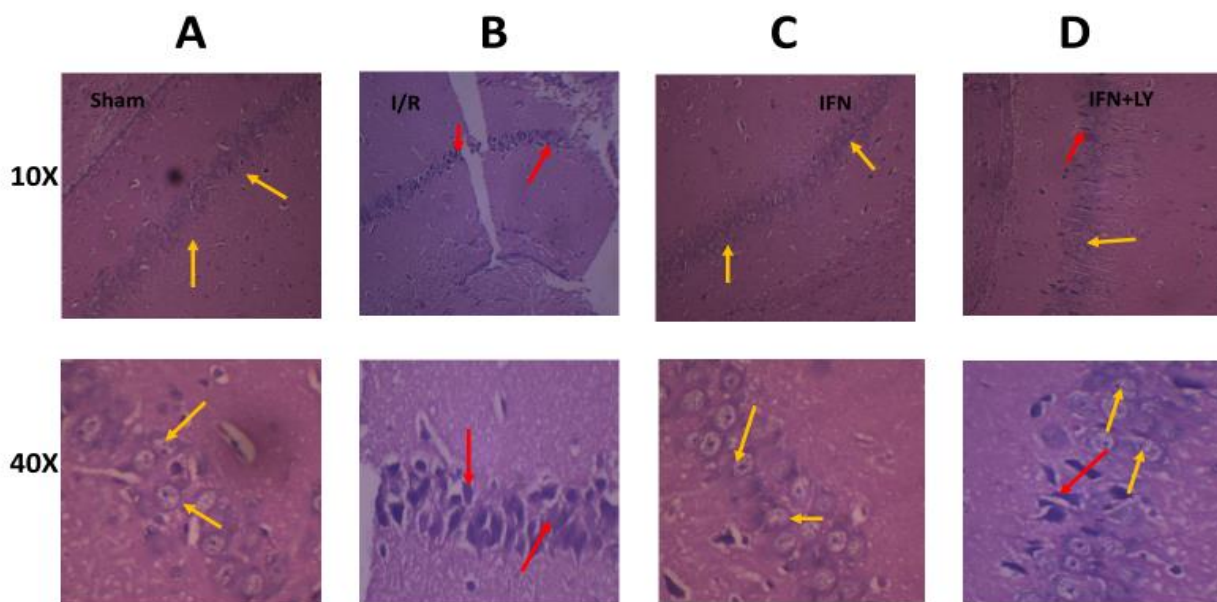
Fig. 5.64 (a). Effect of IFN and LY294002 on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) IFN-Ifenprodil (Panel D) IFN+LY-Ifenprodil+LY294002. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
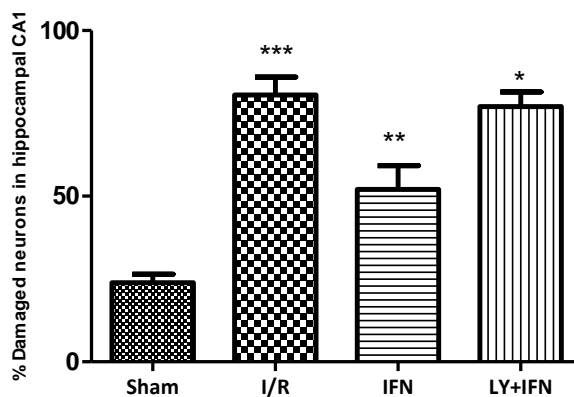
Fig. 5.64 (b). Effect of IFN and LY294002 on percentage damaged neurons in hippocampus CA1 region.
Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, **P<0.01 I/R vs IFN and *P<0.05 IFN vs LY+IFN

5.7 *Effect of RIPOC and LY294002 on cerebral I/R injury and associated cognitive deficits*

5.7.1 *Effect of RIPOC and LY294002 on behavioral parameters*

5.7.1.1 *Effect of RIPOC and LY294002 on memory in passive avoidance acquisition trial*

In the acquisition trial of the passive avoidance task, no significant differences in the acquisition trial latency were observed among different groups in the study (Fig.5.65). In the retention trial, in comparison to sham control group I/R injured rats significantly (P<0.001) reduced retention trial latency. In contrast, a marked (P<0.05) improved retention latency was observed with RIPOC intervention, while LY294002 pretreatment significantly (P<0.05) abolished the protective effect of RIPOC (Fig.5.66).
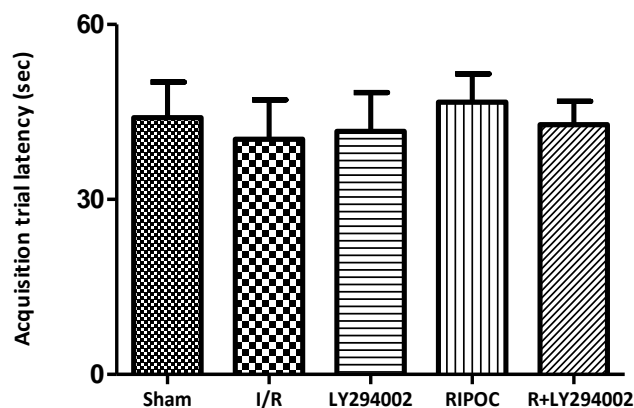
Fig. 5.65. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on memory in passive avoidance acquisition trial. Values were expressed as mean±S.E.M. No significant differences were observed among the various groups
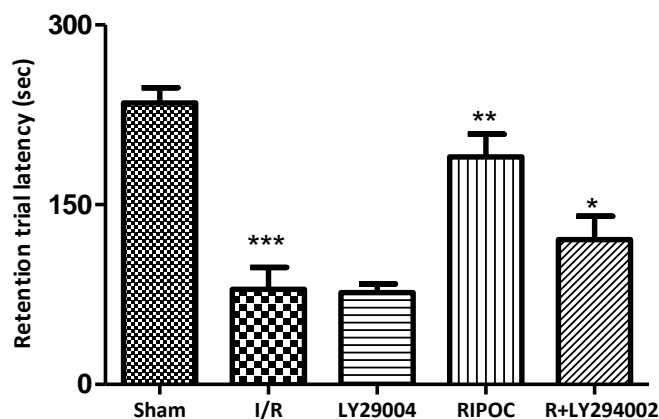

Fig. 5.66. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on memory in passive avoidance retention trial. Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs LY294002

5.7.1.2   Effect of RIPOC and LY294002 on memory in Y-maze task

Cerebral I/R injury significantly (P<0.001) reduced the percentage spontaneous alterations when compared to sham (Fig. 67). However, RIPOC intervention significantly (P<0.01) improved the percentage spontaneous alterations when correlated I/R group, while the protective effect of RIPOC was markedly (P<0.05) abolished by LY294002 pre-treatment.

Fig. 5.67. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on memory in Y-maze task.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs LY294002

5.7.1.3   Effect of RIPOC and LY294002 on memory in EPM task

In elevated plus maze task, during acquisition trial there was no significant differences in transfer trial latency among the different groups of the study (Fig. 5.68). In the retention trial (Fig.5.69), cerebral I/R injury significantly (P<0.001) increased the transfer trial latency when compared to sham rats. However, transfer trial latency was markedly (P<0.05) reduced by RIPOC treatment when compared to cerebral I/R injury group, while pre-treatment with LY294002 significantly (P<0.05) abolished the protective effects of RIPOC.
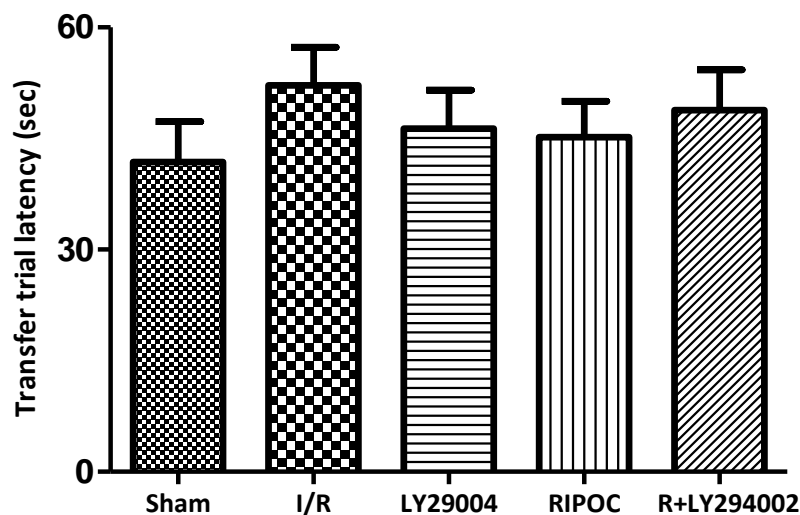
Fig. 5.68. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on memory in EPM acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups of the study.
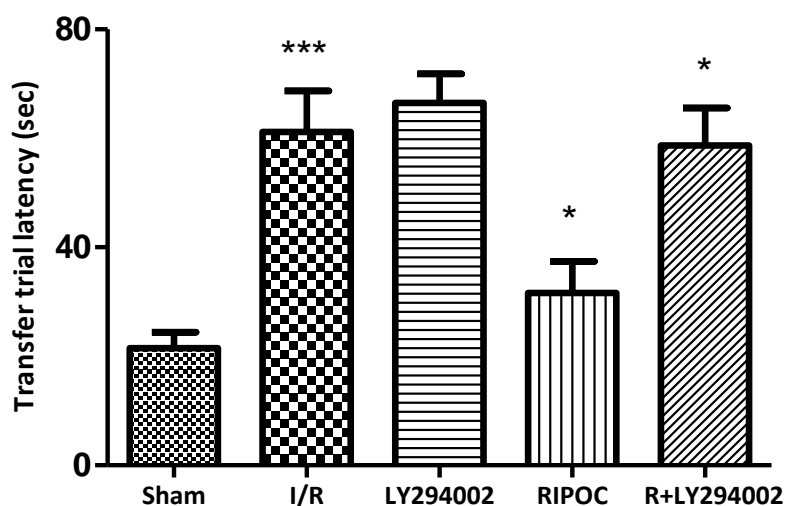
Fig. 5.69. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on memory in EPM retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, *P<0.05 I/R vs RIPOC and*P<0.05 RIPOC vs LY294002

5.7.1.4   *Effect of RIPOC and LY294002 on locomotor activity*

To rule out the interference of motor abnormalities in cognitive dysfunction, we performed locomotor activity task. No significant difference in the locomotor activity was observed among different groups in the study (Fig.5.70).
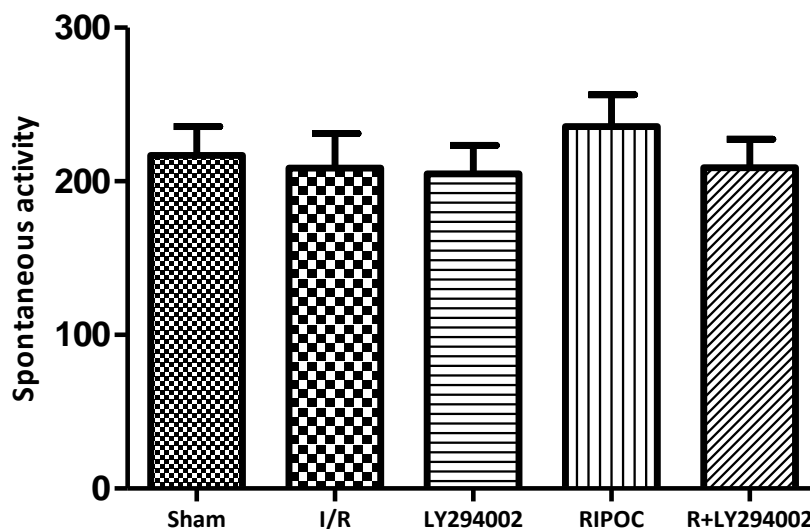
Fig. 5.70. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on locomotor activity.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups

5.7.2   Effect of RIPOC and LY294002 on biochemical parameters

5.7.2.1   Effect of RIPOC and LY294002 on oxidative markers and anti-oxidants

Oxidative markers such as MDA and nitrite were significantly ($P<0.001$) increased while anti-oxidants like GSH and SOD were significantly ($P<0.001$) reduced with induction of cerebral I/R injury when compared to sham. However, RIPOC intervention significantly ($P<0.001$) increased the anti-oxidants and reduced the oxidative marker levels when compared to I/R injury group, while LY294002 pre-treatment markedly ($P<0.05$) abolished the protective effect of RIPOC (Table.5.4).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.45±0.23	5.06±2.05	1.78±0.48	15.64±3.8
I/R	1.72±0.45***	18.91±4.27***	0.48±0.25***	5.37±2.91***
LY294002	1.90±0.38	21.04±4.05	0.46±0.21	3.60±1.12
RIPOC	0.77±0.28***	9.17±2.12**	1.47±0.29***	12.77±3.77**
R+ LY294002	1.43±0.31*	15.71±4.88*	0.84±0.31*	6.61±2.39*

Table 5.4. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on biochemical parameters.
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC (MDA and GSH),
**P<0.01 I/R vs RIPOC (Nitrite and SOD), *P<0.05 RIPOC vs R+LY294002

5.7.3   Effect of RIPOC and LY294002 on neuroinflammatory parameters

5.7.3.1   Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on TNF-α and IL-6

Cerebral I/R injury significantly (P<0.001) increased the levels of neuroinflammatory cytokines like TNF-α (Fig.5.71) and IL-6 (Fig.5.72) when compared to sham rats. However, RIPOC intervention significantly reduced the neuroinflammation as evidenced by reduced levels of TNF-α (P<0.001) and IL-6 (P<0.01), while RIPOC induced protection was markedly (P<0.01) abolished by LY294002 pre-treatment.
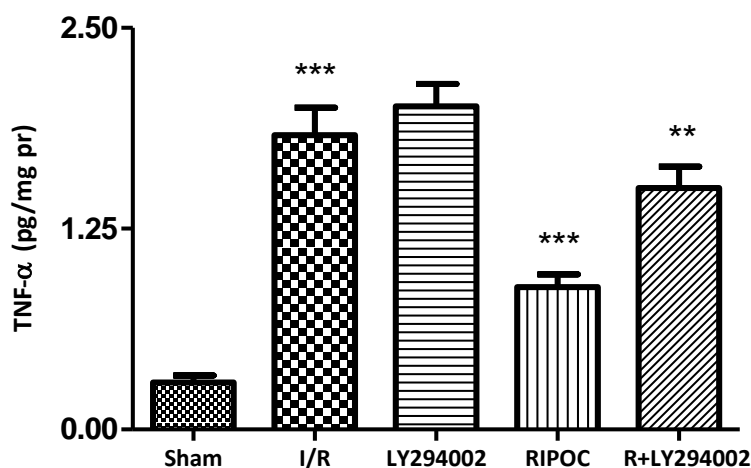
Fig. 5.71. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC and **P<0.01 RIPOC vs LY294002
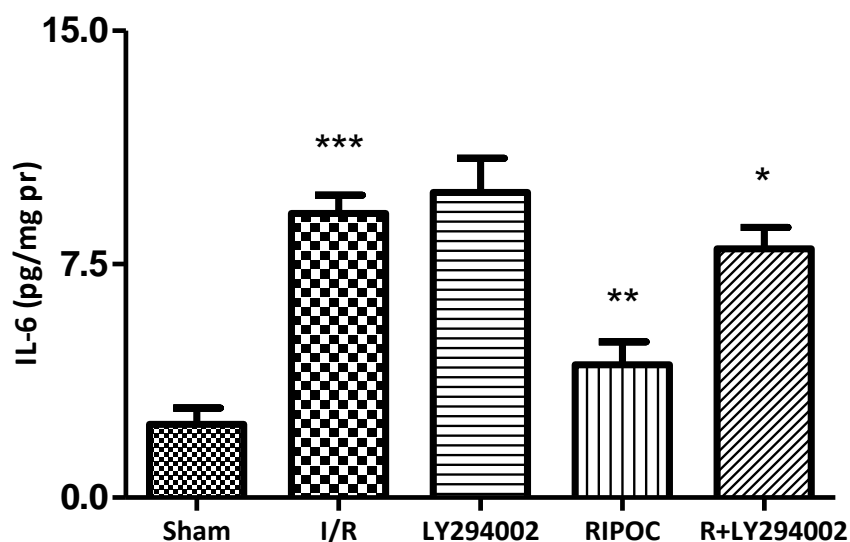
Fig. 5.72. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs LY294002

5.7.4 Effect of RIPOC and LY294002 on molecular estimations

5.7.4.1 Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on CREB and BDNF

Cerebral I/R injury resulted in significant (P<0.001) reduction in the CREB (Fig.5.73) and BDNF (Fig.5.74) levels when compared to sham control rats. RIPOC intervention markedly improved the levels of CREB (P<0.001) and BDNF (P<0.01) when compared to the ischemic group. However, pretreatment with LY294002 significantly abolished the protective effect of RIPOC intervention as evidenced by reduced levels of CREB (P<0.05) and BDNF (P<0.01).
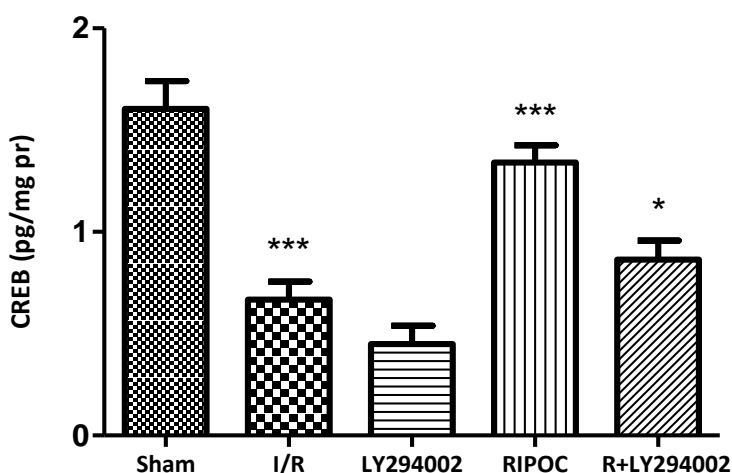
Fig. 5.73. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC and *P<0.05 RIPOC vs LY294002
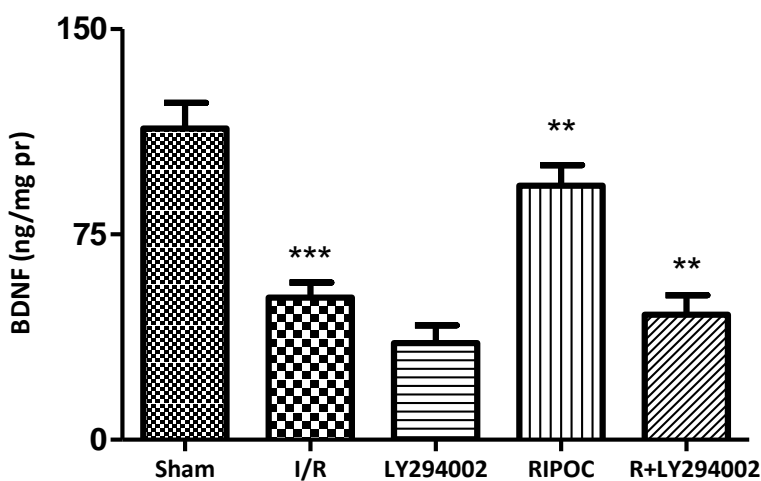
Fig. 5.74. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on BDNF levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and **P<0.01 RIPOC vs LY294002

5.7.4.2   Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on GSK-3β and HO-1

Cerebral I/R injury resulted in significant (P<0.001) increase in the levels of GSK-3β (Fig.5.75) and reduced HO-1 (Fig.5.76) when compared to sham. However, RIPOC intervention showed significant (P<0.01) attenuation of GSK-3β and up-regulated HO-1 expression, while the protective effect was significantly (P<0.05) reduced by LY294002.
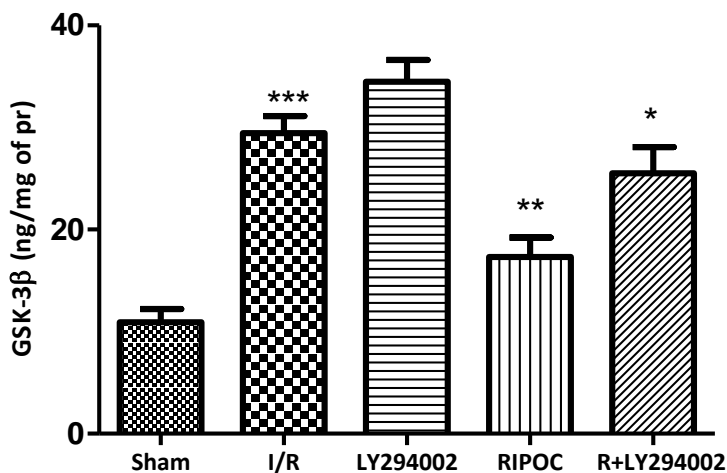
Fig. 5.75. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on GSK-3β levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs LY294002

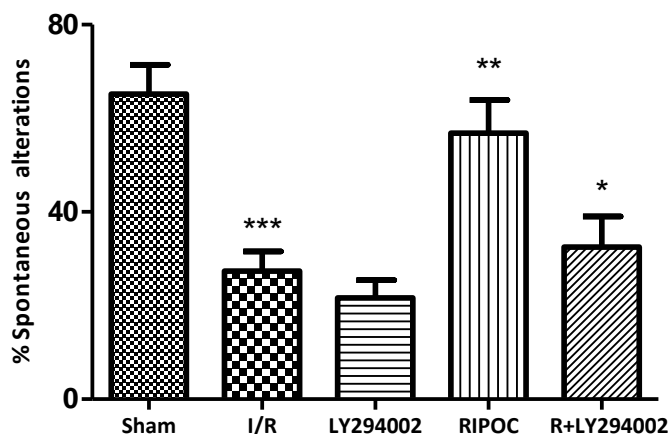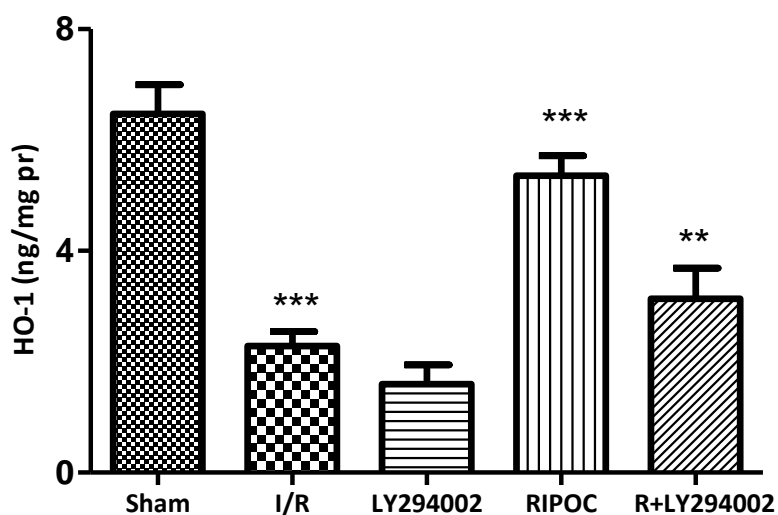**Fig. 5.76. Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on HO-1 levels.**
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC and **P<0.01 RIPOC vs LY294002

5.7.5   Effect of RIPOC and LY294002 on hippocampal CA1 neurons

The induction of cerebral I/R injury induced significant morphological alterations and neuronal loss in the CA1 hippocampal region. The HE stained sections of cerebral I/R injury showed increased pyknotic neurons that are visualized as dark stained cells with disorganized cell mass. In contrast, healthy neurons with robust shape, slightly spherical or oval nucleus with visible clear cytoplasm were visualized in sham rat brain sections. RIPOC intervention markedly reduced the pyknotic neurons and improved the neuronal density, while this effect was abolished in presence of LY294002 (Fig.5.77 [a & b]).
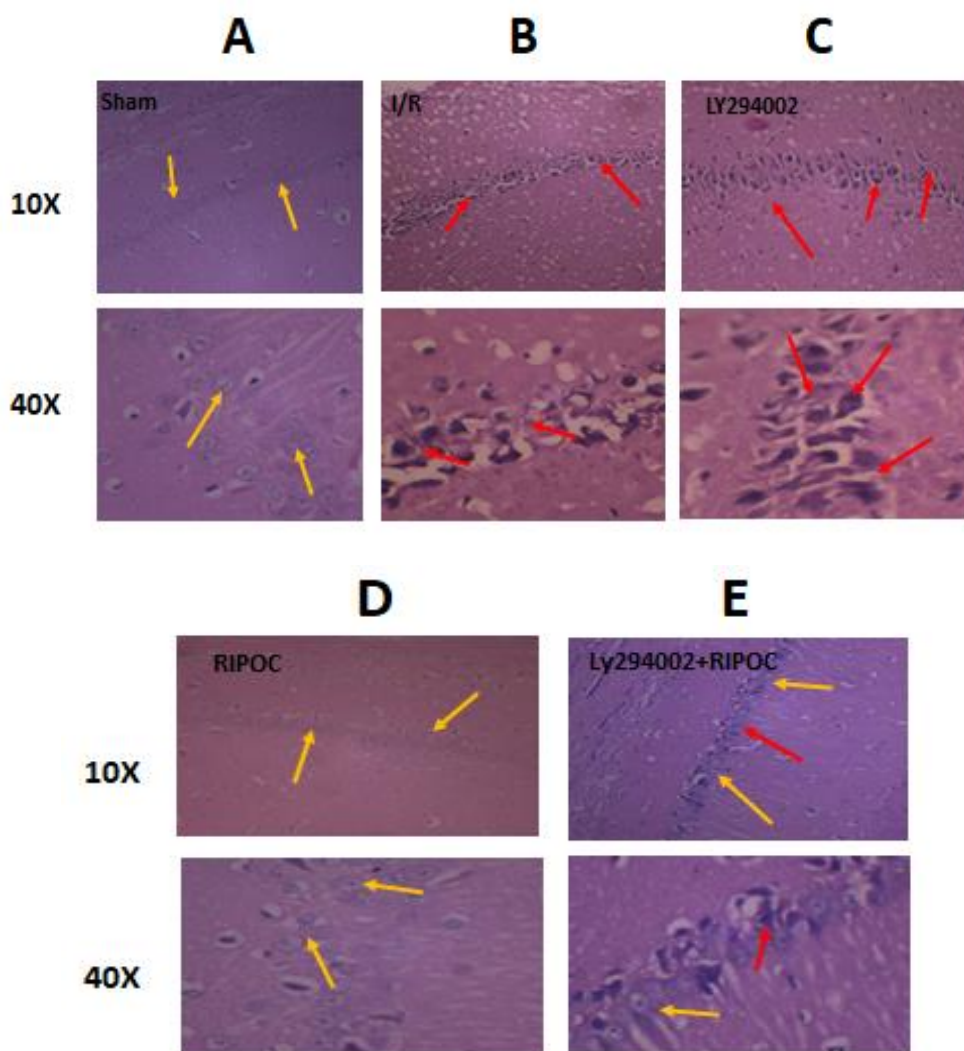
Fig. 5.77 (a). Effect of RIPOC and PI3K-Akt inhibitor – LY294002 on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) LY294002 (Panel D) RIPOC, (Panel E) LY294002+RIPOC. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
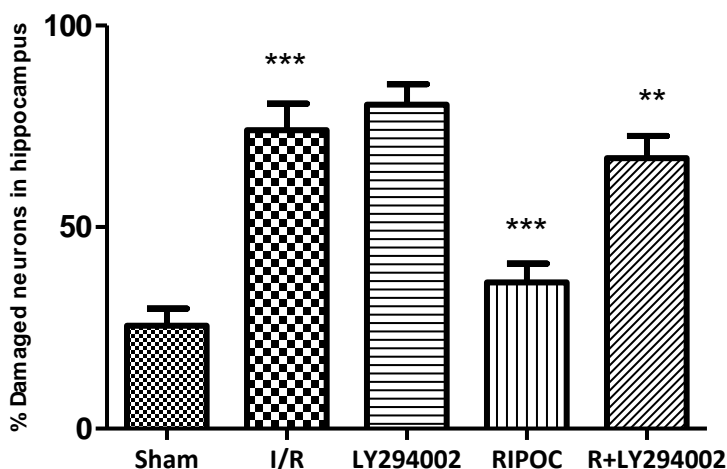
Fig. 5.77 (b). Effect of RIPOC and LY294002 on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, ***P<0.001 I/R vs RIPOC and **P<0.01 RIPOC vs R+LY294002

5.8   *Effect of RIPOC and mPTP opener on cerebral I/R injury and associated cognitive deficits*

5.8.1   *Effect of RIPOC and mPTP opener- atractyloside (5mg/kg) on biochemical parameters*

5.8.1.1   *Effect of RIPOC and mPTP opener- atractyloside on biochemical parameters*

Cerebral ischemic injury markedly (P<0.001) increased the oxido-nitrosative stress as evidenced by increased MDA and nitrite levels when compared to sham rats. However, RIPOC intervention markedly (P<0.001) reduced the levels of MDA and nitrite, and improved the anti-oxidant levels such as GSH (P<0.001) and SOD (P<0.01) while RIPOC protective effect against oxidative damage was significantly (P<0.05) abolished in presence of mPTP opener, atractyloside (Table.5.5).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.53±0.2	5.23±1.84	1.87±0.81	18.39±5.12
I/R	1.7±0.28***	17.8±3.51***	0.54±0.26***	5.4±2.3***
Atr	1.99±0.34	22.4±7.29	0.37±0.14	4.5±2.08
RIPOC	0.98±0.14***	8.23±2.45**	1.54±0.23***	13.0±2.96**
Atr+RIPOC	1.42±0.22*	14.56±3.68*	1.07±0.31	7.1±2.68*

Table 5.5. Effect of RIPOC and mPTP opener-atractyloside on biochemical parameters. Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, **P<0.001 I/R vs RIPOC (MDA and GSH), **P<0.01 I/R vs RIPOC (Nitrite and SOD), *P<0.05RIPOC vs Atr+RIPOC (MDA, nitrite and SOD)

5.8.2   Effect of RIPOC and mPTP opener- atractyloside on neuroinflammatory parameters

5.8.2.1   Effect of RIPOC and mPTP opener- atractyloside on TNF-α and IL-6

A significant (P<0.001) rise in neuroinflammatory cytokines including TNF-α (Fig.5.78) and IL-6 (Fig.5.79) were observed in cerebral I/R injury group when compared to sham rats. However, treatment with RIPOC significantly attenuated the levels of neuroinflammatory markers like TNF-α (P<0.001) and IL-6 (P<0.01), while pre-treatment with atractyloside markedly (P<0.01) abolished the protective effect of RIPOC.
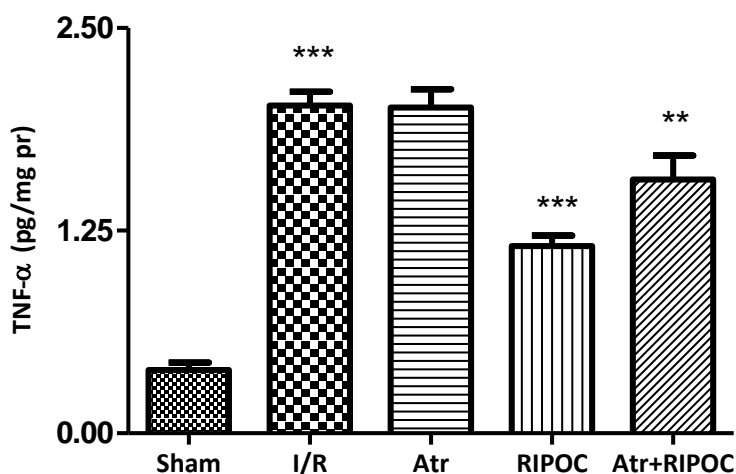
Fig. 5.78. Effect of RIPOC and atractyloside on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC and **P<0.01 RIPOC vs Atr+RIPOC
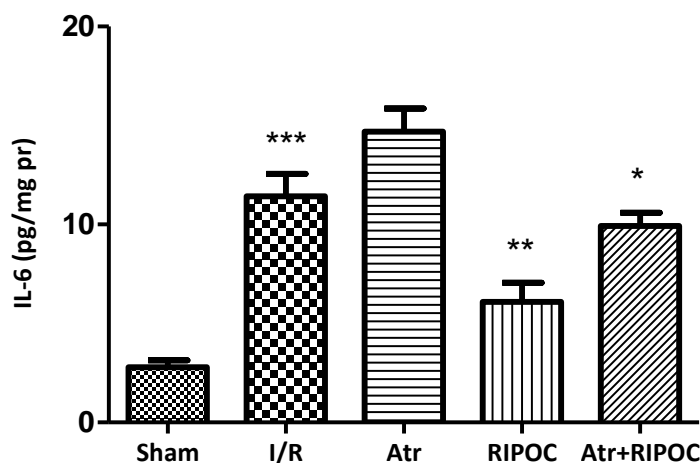
Fig. 5.79. Effect of RIPOC and atractyloside on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs Atr+RIPOC

5.8.3   Effect of RIPOC and mPTP opener- atractyloside on cytochrome c (cyt c) estimation

When correlated to sham control group, induction of cerebral I/R injury significantly (P<0.001) increased the levels of cyt c. However, RIPOC intervention significantly (P<0.01) reduced the levels of cyt c, while this protective effect was significantly abolished (P<0.05) in presence of atractyloside (Fig.5.80).
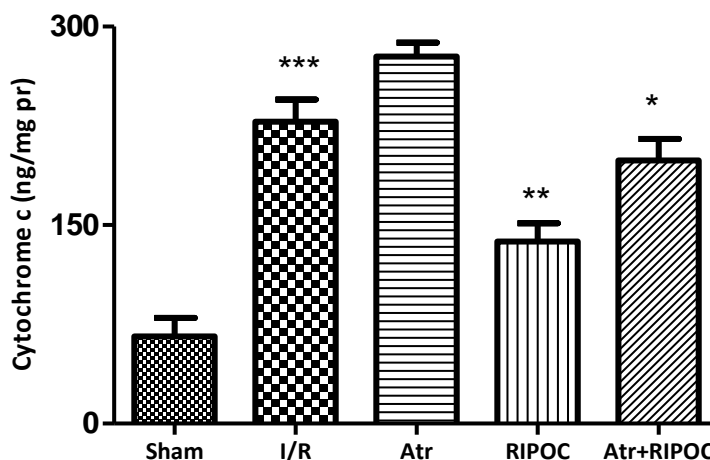
Fig. 5.80. Effect of RIPOC and atractyloside on cyt c levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC and *P<0.05 RIPOC vs Atr+RIPOC

5.8.4   Effect of RIPOC and mPTP opener- atractyloside on apoptotic cell death in hippocampus

DNA fragmentation study revealed an increased apoptotic cell death in hippocampal CA1 of cerebral I/R injured rats when compared to sham control rats. However, RIPOC intervention results in significant attenuation of apoptotic cell death in hippocampal CA1 region (Fig.5.81).
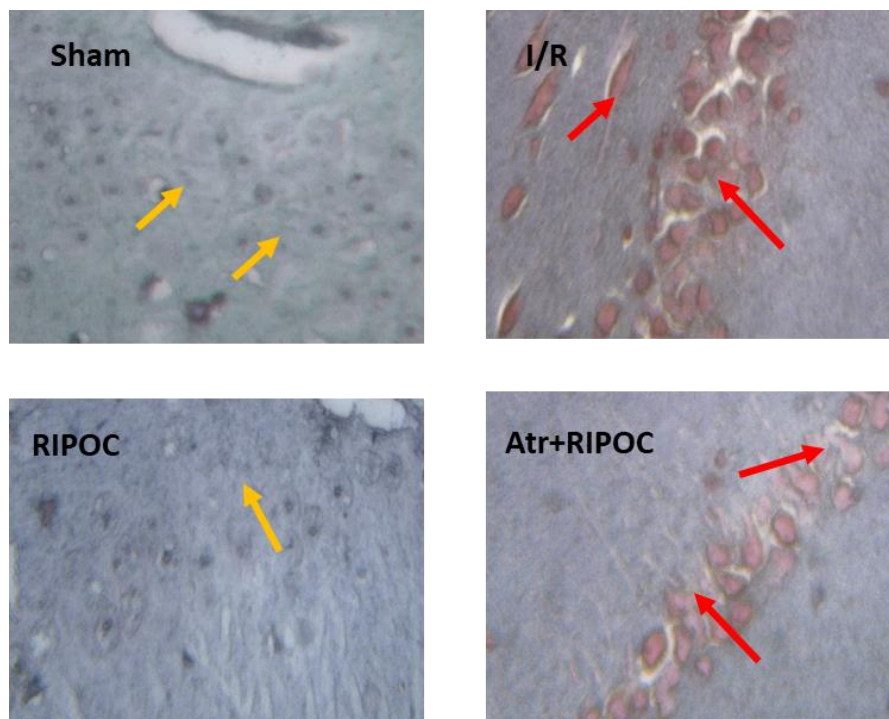
Fig. 5.81. Effect of RIPOC and atractyloside on hippocampal CA1 neurons: DNA Fragmentation (TUNEL) assay. Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) RIPOC, (Panel D) Atr+RIPOC. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.

5.9 *Effect of cerebral I/R injury on histone H3 acetylation levels*

Induction of cerebral I/R injury caused transcriptional repression as evidenced by reduced global histoneH3 acetylation when compared to sham control rats, however, RIPOC intervention improved the histoneH3 acetylation levels (Fig.5.82).
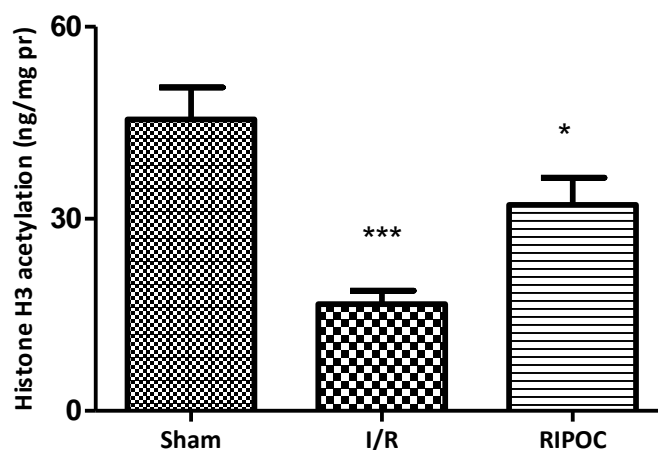
Fig. 5.82. Effect of cerebral I/R injury on global H3 acetylation levels
Values were expressed as mean±S.E.M. ***$P<0.001$ Sham vs I/R and *$P<0.05$ I/R vs RIPOC

5.10 *Effect of NAMPT activator on cerebral I/R injury and associated cognitive deficits*

5.10.1 Effect of NAMPT activator (A-20, 10mg/kg) on behavioral parameters

5.10.1.1 Effect of NAMPT activator (A-20) on memory in passive avoidance task

The acquisition trial latency during the acquisition trial of passive avoidance task did not differ among different groups in the study (Fig.5.83). However, during the retention trial I/R injury significantly (P<0.001) reduced retention latency when compared to sham group. In contrast, treatment with NAMPT activator, A-20 markedly (P<0.01) improved the retention trial latency when compared to I/R injured rats (Fig.5.84).
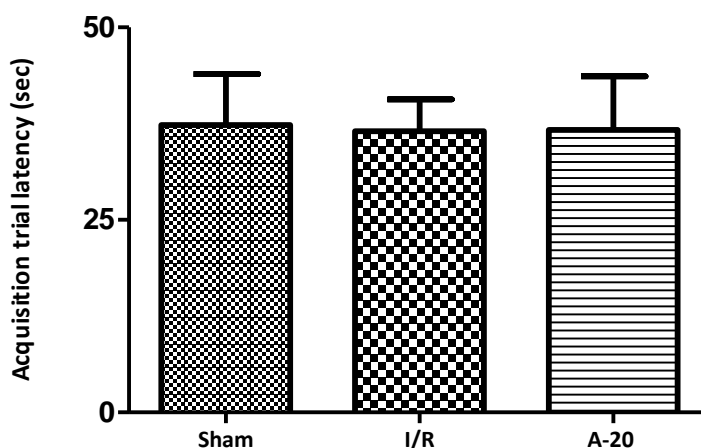
Fig. 5.83. Effect of NAMPT activator (A-20) on memory in passive avoidance acquisition trial. Values were expressed as mean±S.E.M. No significant difference was observed among different groups of the study in acquisition trial.
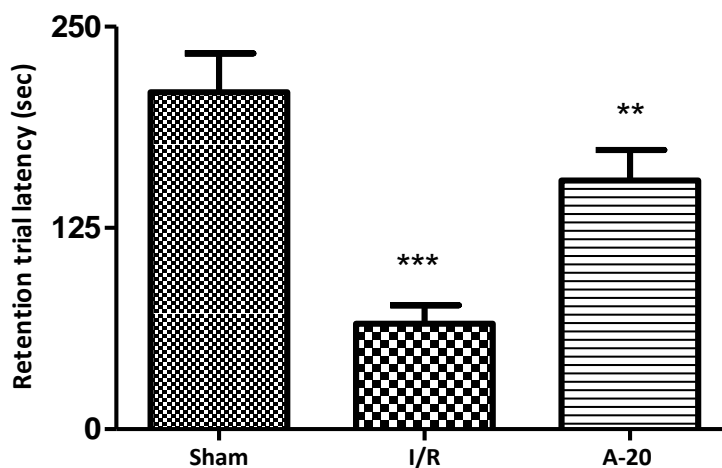
Fig. 5.84. Effect of NAMPT activator (A-20) on memory in passive avoidance retention trial. Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01I/R vs A-20

5.10.1.2 Effect of NAMPT activator (P7C3-A20) on memory in Y-maze test

In correlation to sham, I/R injury group resulted in significantly (P<0.01) less number of spontaneous alterations. However, treatment with A-20 significantly (P<0.05) improved the percentage spontaneous alterations when correlated to I/R injury group (Fig.5.85).
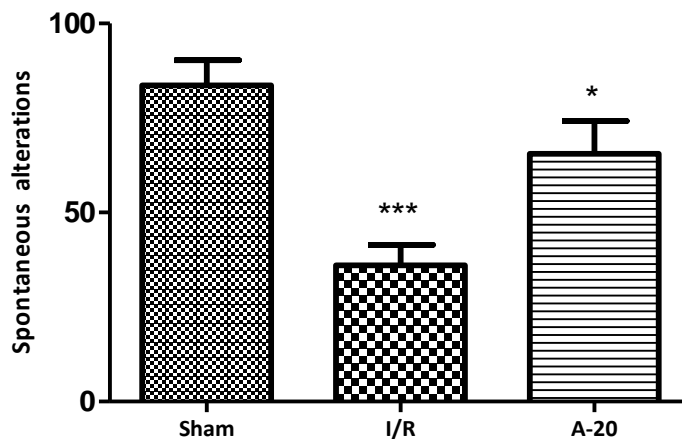
Fig. 5.85. Effect of NAMPT activator (A-20) on memory in Y-maze task.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and *P<0.05 I/R vs A-20

5.10.1.3 Effect of NAMPT activator (P7C3-A20) on memory in EPM task

During the acquisition trial, no significant difference in the transfer trial latency was observed among different groups in the study (Fig.5.86). However, induction of I/R injury significantly (P<0.001) increased the retention trial latency while treatment with A-20 markedly (P<0.01) reduced the transfer trial latency to enter the enclosed arm (Fig.5.87).
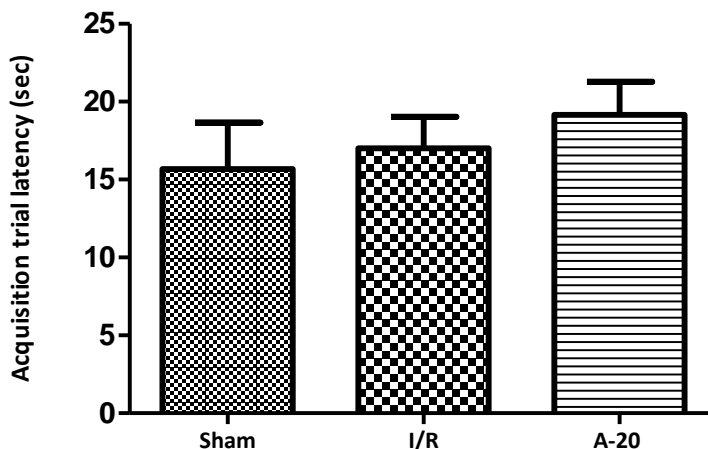
Fig. 5.86. Effect of NAMPT activator (A-20) on memory in EPM acquisition trial.
Values were expressed as mean±S.E.M. No significant difference was observed in acquisition trial latency.
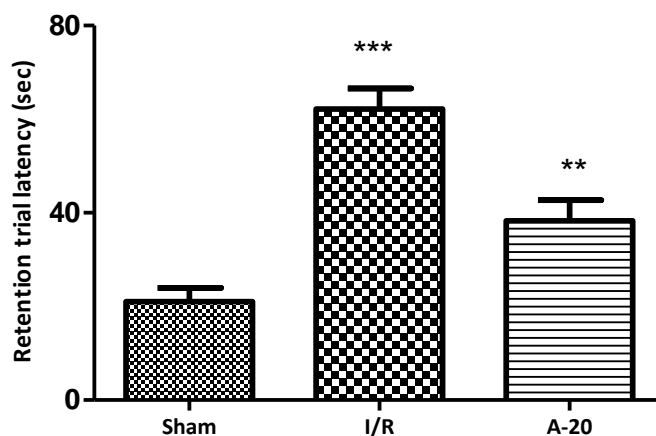
Fig. 5.87. Effect of NAMPT activator (A-20) on memory in EPM retention trial
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs A-20

5.10.1.4 *Effect of NAMPT activator (P7C3-A20) on locomotor activity*

We did not observe any significant difference in the locomotor activity among different groups of the study, which suggests that there is no interference of motor dysfunction in the cognitive abnormality (Fig.5.88).
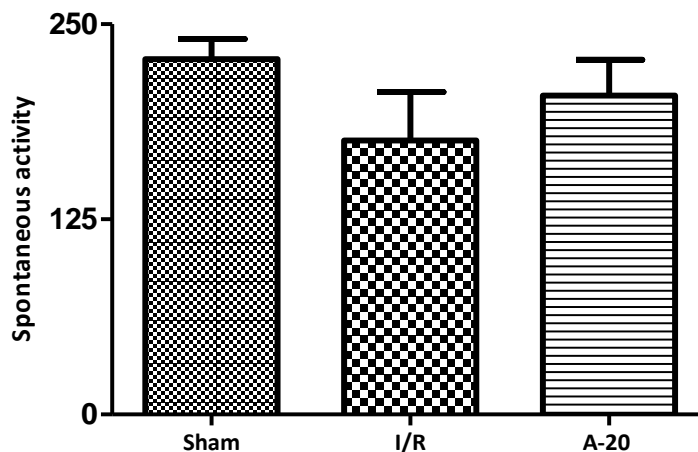
Fig. 5.88. Effect of NAMPT activator (A-20) on locomotor activity.
Values were expressed as mean±S.E.M. No significant difference was observed in locomotor activity among different groups of the study.

5.10.2 *Effect of NAMPT activator (A-20) on biochemical parameters*

5.10.2.1 *Effect of NAMPT activator (P7C3-A20) on oxidative markers and anti-oxidants*

The oxidative stress induced by cerebral I/R injury was estimated in terms of oxidative markers like MDA and nitrite and anti-oxidants including GSH and SOD. I/R injury induced significant

(P<0.001) elevation in oxidative markers along with reduction in anti-oxidants when compared to sham. However, treatment with A-20 reduced the oxidative markers such as MDA (P<0.001) and nitrite (P<0.05) concurrently improved the anti-oxidant levels like GSH (P<0.01) and SOD (P<0.01) when compared to cerebral I/R injury group (Table.5.6).

Groups	MDA (nMol/mg pr)	Nitrite µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.42±0.17	4.33±1.36	1.56±0.22	17.83±5.26
I/R	2.03±0.39***	18.47±4.55***	0.30±0.11***	4.36±2.07***
A-20	1.02±0.22***	12.85±3.27*	0.78±0.29**	12.73±3.05**

Table 5.6. Effect of NAMPT activator (A-20) on biochemical parameters
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, ***P<0.01 I/R vs A-20 (MDA), *P<0.05 I/R vs A-20 (nitrite) and **P<0.01 I/R vs A-20 (GSH and SOD)

5.10.3   Effect of NAMPT activator (A-20) on molecular markers

5.10.3.1   Effect of NAMPT activator (P7C3-A20) on Nicotinamide Adenine Dinucleotide (NAD) and sirtuins (SIRT1)

We found that cerebral I/R injury significantly (P<0.001) reduced the levels of NAD (Fig.5.89) and SIRT1 (Fig.5.90) when compared to sham control group. However, treatment with A-20 significantly (P<0.01) improved the levels of NAD and SIRT1 when correlated to cerebral I/R injury group.
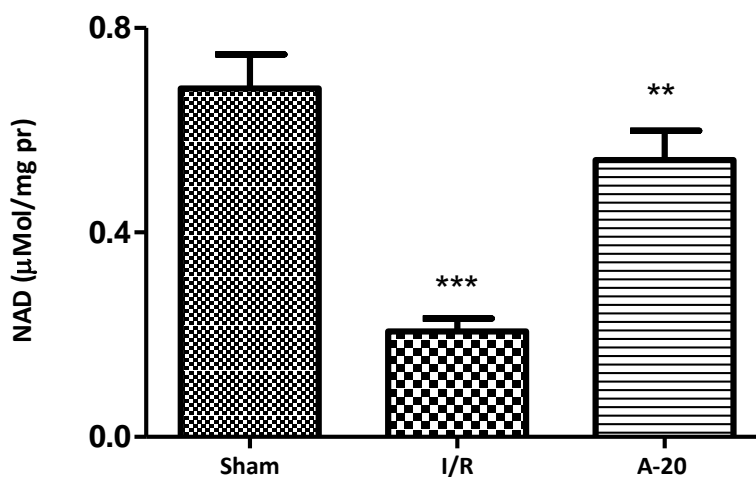
Fig. 5.89. Effect of NAMPT activator (A-20) on NAD levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs A-20
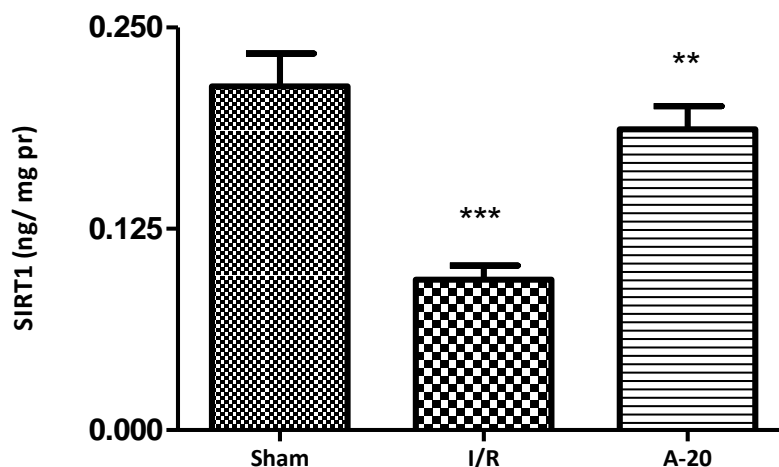
Fig. 5.90. Effect of NAMPT activator (A-20) on SIRT1 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs A-20

5.10.3.2 Effect of NAMPT activator (P7C3-A20) on CREB and BDNF

When correlated to sham group, I/R injured rats showed marked (P<0.001) reduction in CREB (Fig.5.91) and neurotrophic factors like BDNF (Fig.5.92). However, treatment with A-20 significantly improved the levels of CREB (P<0.001) and BDNF (P<0.05) in correlation to I/R injured rats.
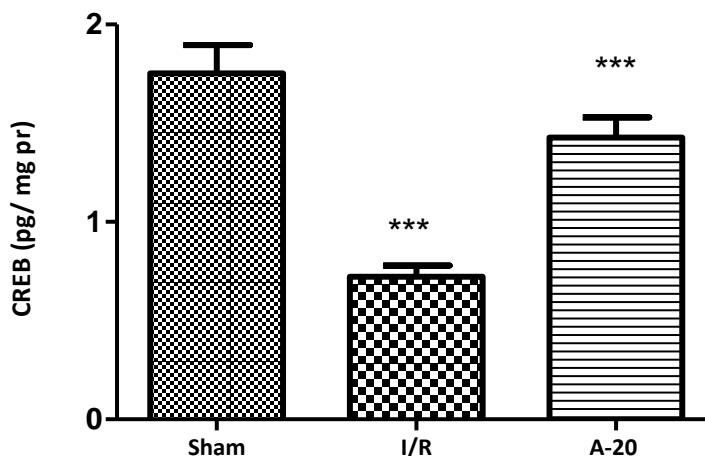
Fig. 5.91. Effect of NAMPT activator (A-20) on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and ***P<0.001 I/R vs A-20
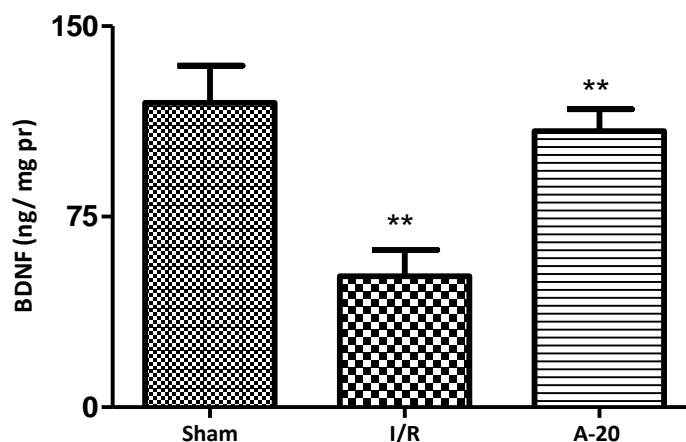
Fig. 5.92. Effect of cerebral I/R injury NAMPT activator (A-20) on BDNF levels.
Values were expressed as mean±S.E.M. **P<0.01 Sham vs I/R and **P<0.01 I/R vs A-20

5.10.4 *Effect of NAMPT activator (A-20) on hippocampal CA1 neurons*

The HE brains sections of sham control rats showed healthy neurons in the hippocampal CA1 region. Healthy neurons were robust in shape and had a pale and spherical or slightly oval nucleus and a single large nucleolus with clear visible cytoplasm. In this study, pyknotic neurons with darkly stained nucleus with shrunken and sickle shaped were visualized in brain sections of I/R injured rats. The microscopic pictures of HE sectioned rat brains showed a marked (P<0.001) cell loss with decreased neuronal density and increased pyknotic cell count when compared to sham rats. However, NAMPT activator A-20 significantly (P<0.01) improved the neuronal density and reduced the pyknotic neuronal count (Fig.5.93 [a&b]) as correlated to cerebral I/R injury group.

Fig. 5.93(a). Effect of NAMPT activator (A-20) on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) NAMPT activator-A20. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.

Fig. 5.93 (b). Effect of NAMPT activator (A-20) on percentage damaged neurons in hippocampus CA1 region.
Values were expressed as mean±S.E.M. ***$P<0.001$ sham vs I/R, ** $P<0.01$ I/R vs A-20

5.11 *Effect of RIPOC and NAMPT inhibitor-FK866 on cerebral I/R injury and associated cognitive deficits*

5.11.1 *Effect of RIPOC and NAMPT inhibitor-FK866 on behavioral parameters*

5.11.1.1 *Effect of RIPOC and NAMPT inhibitor-FK866 on memory in passive avoidance task*

During the acquisition trial of the passive avoidance task, the trial latency did not differ among different groups of the study (Fig.5.94). Cerebral I/R injury significantly ($P<0.001$) reduced

retention latency while RIPOC treatment significantly (P<0.01) improved the retention trial latency. However, the protective effect of RIPOC was abolished in presence of FK866 (Fig.5.95).
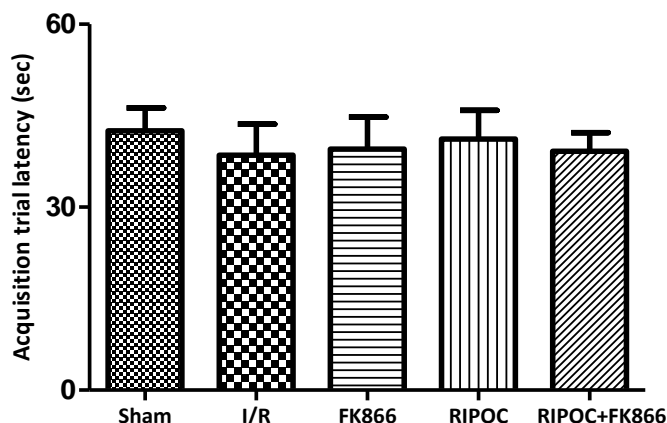
Fig. 5.94. Effect of RIPOC and NAMPT inhibitor FK866 on memory in passive avoidance task.
Values were expressed as mean±S.E.M. No significant difference was observed among different groups of the study.

Fig. 5.95. Effect of RIPOC and NAMPT inhibitor FK866 on memory in passive avoidance retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs RIPOC

5.11.1.2 *Effect of RIPOC and NAMPT inhibitor-FK866 on memory in Y-maze task*

In correlation to sham, induction of cerebral I/R injury resulted in significantly (P<0.001) less number of spontaneous alterations. In contrast, RIPOC intervention marked (P<0.01) improved the percentage spontaneous alterations, while the protective was significantly (P<0.001) abolished in presence of FK866 (Fig.5.96).
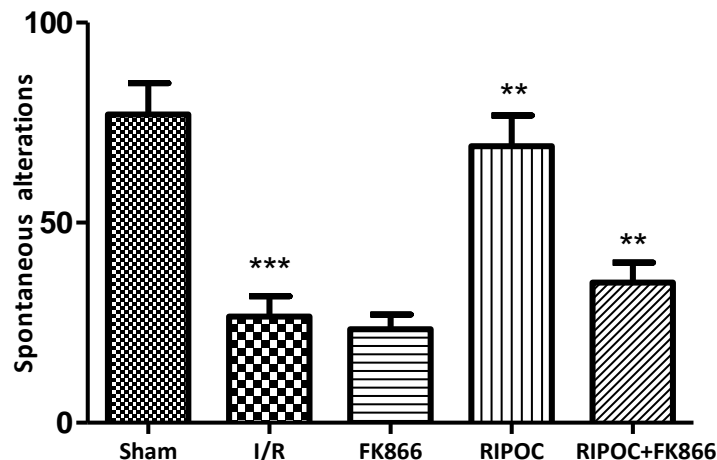
Fig. 5.96. Effect of RIPOC and NAMPT inhibitor FK866 on memory in Y-maze test.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs RIPOC and RIPOC vs
**P<0.01 RIPOC+FK866

5.11.1.3 Effect of RIPOC and NAMPT inhibitor-FK866 on memory in EPM task

In elevated plus maze task during the acquisition trial, there was no significant difference in the transfer trial latency among different groups of the study (Fig.5.97). Cerebral I/R injury significantly (P<0.001) increased the transfer trial latency when compared to sham group. However, RIPOC intervention markedly (P<0.01) reduced the retention trial latency, while pretreatment with FK866 significantly (P<0.05) abolished the protective effect of RIPOC (Fig.5.98).
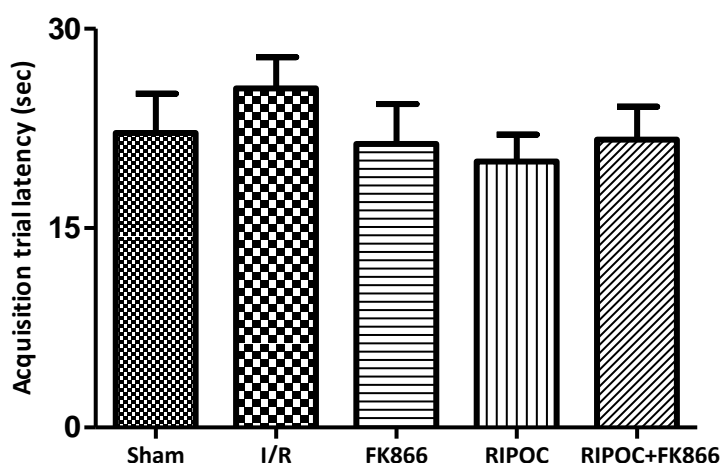
Fig. 5.97. Effect of RIPOC and NAMPT inhibitor FK866 on memory in EPM task.
Values were expressed as mean±S.E.M. No significant difference was observed in acquisition trial latency among different groups of the study.
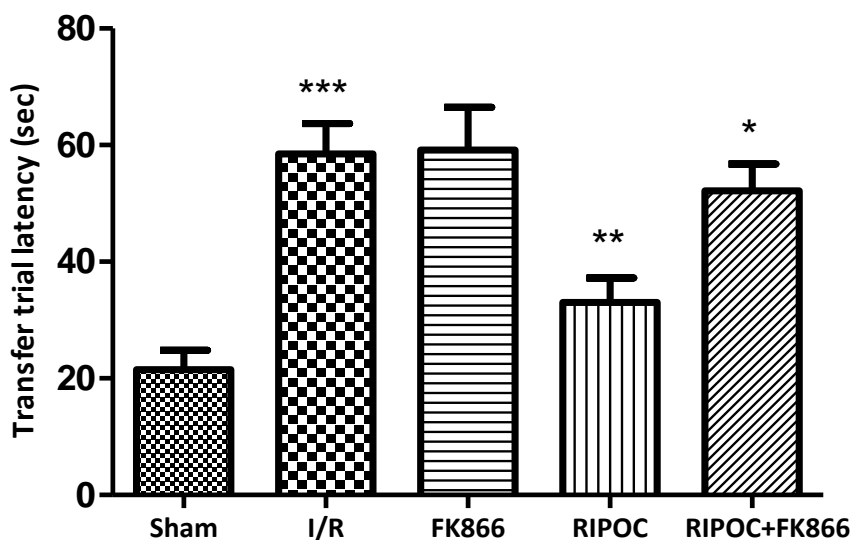
Fig. 5.98. Effect of RIPOC and NAMPT inhibitor FK866 on memory in Y-maze test.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs RIPOC and RIPOC vs *P<0.05 RIPOC+FK866

5.11.1.4 *Effect of RIPOC and NAMPT inhibitor-FK866 on locomotor activity*

We did not observe any significant difference in the locomotor activity among different groups of the study that indicates that there is no interference of motor dysfunction in the cognitive abnormality (Fig.5.99).
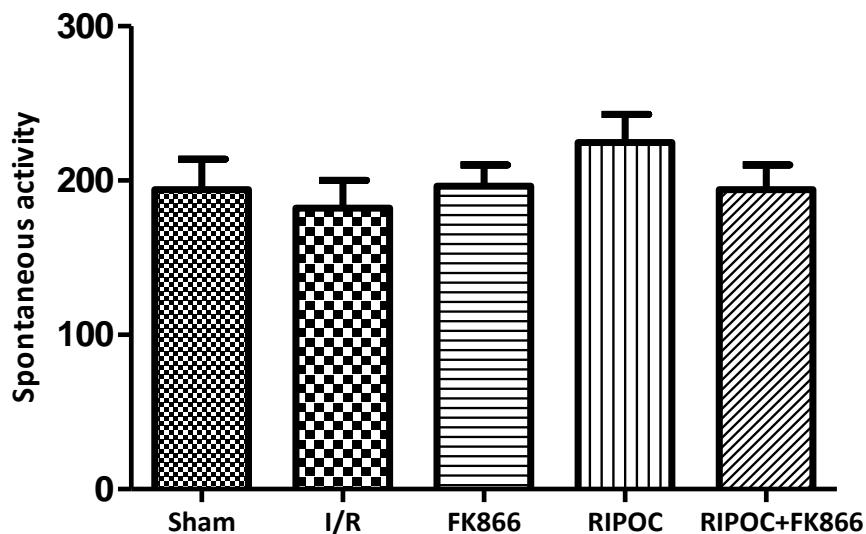
Fig. 5.99. Effect of RIPOC and NAMPT inhibitor FK866 on locomotor activity.
Values were expressed as mean±S.E.M. No significant difference was observed in locomotor activity among different groups of the study.

5.11.2   Effect of RIPOC and NAMPT inhibitor-FK866 on biochemical parameters

5.11.2.1   Effect of RIPOC and NAMPT inhibitor-FK866 on oxidative markers and anti-oxidants

The oxidative stress induced by cerebral I/R injury was estimated in terms of oxidative markers like MDA and nitrite and anti-oxidants including GSH and SOD. A significant (P<0.001) elevation of oxidative markers along with reduction in anti-oxidants were noticed in I/R injured rats and FK866 group. Moreover, RIPOC intervention significantly (P<0.001) improved the antioxidants and markedly (P<0.001) reduced the levels of oxidative markers which was significantly (P<0.01) abolished in presence of FK866 (Table.5.7).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.29±0.17	3.60±1.58	1.58±0.32	17.17±2.77
I/R	1.84±0.31***	16.68±4.10***	0.27±0.12***	4.36±1.30***
FK866	1.80±0.25	17.94±4.36	0.34±0.17	3.47±1.36
RIPOC	1.02±0.22***	9.43±2.12**	1.31±0.18***	14.41±3.03***
FK866+RIPOC	1.56±0.33*	15.16±3.26*	0.76±0.29**	8.67±1.94**

Table 5.7. Effect of RIPOC and NAMPT inhibitor FK866 on biochemical parameters.
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs RIPOC (Nitrite, GSH and SOD)
***P<0.01 I/R vs RIPOC (MDA), *P<0.05 RIPOC vs RIPOC+FK866 (MDA and nitrite) and **P<0.01 RIPOC vs
RIPOC+FK866 (GSH and SOD)

5.11.3   Effect of RIPOC and NAMPT inhibitor-FK866 on molecular estimations

5.11.3.1   Effect of RIPOC and NAMPT inhibitor-FK866 on NAD and SIRT1

Cerebral I/R injury significantly (P<0.001) reduced the levels of NAD and SIRT1 when compared to sham group. However, RIPOC intervention significantly improved the levels of NAD (P<0.001) and SIRT1 (P<0.01) when correlated to cerebral I/R injury group, while the protective effect of RIPOC was markedly (P<0.05) abolished in presence of FK866 (Fig.5.100 and 5.101).
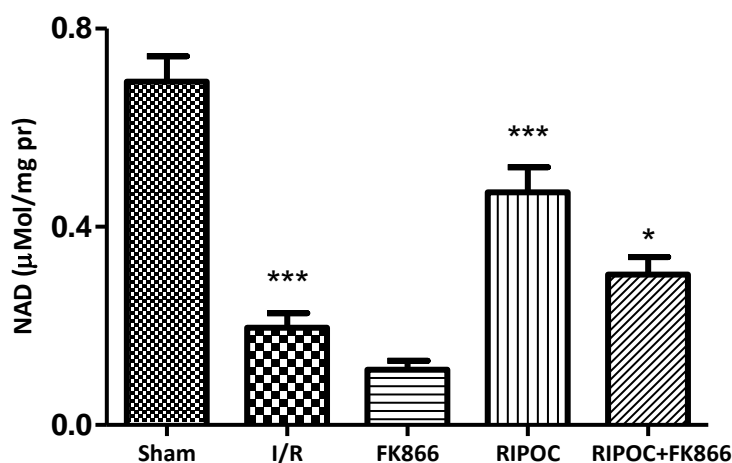
Fig. 5.100. Effect of RIPOC and NAMPT inhibitor FK866 on NAD levels.
Values were expressed as mean±S.E.M. ***$P<0.001$ Sham vs I/R and ***$P<0.001$ I/R vs RIPOC and *$P<0.05$ RIPOC vs RIPOC+FK866
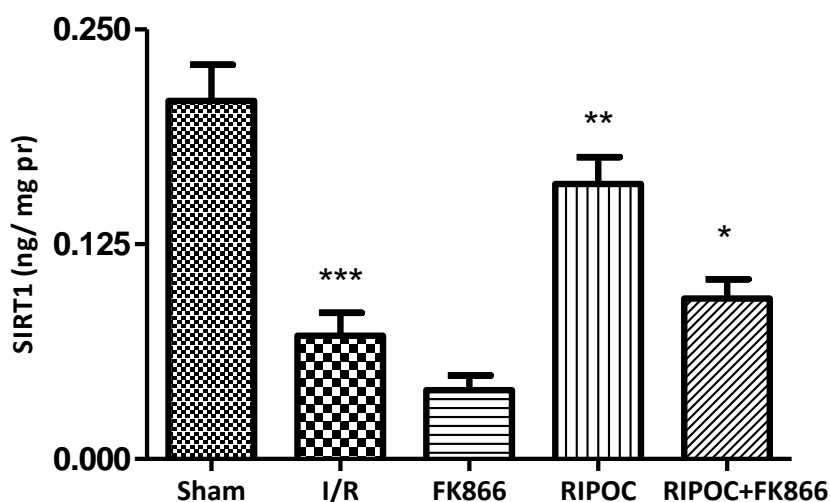
Fig. 5.101. Effect of RIPOC and NAMPT inhibitor FK866 on SIRT1 levels.
Values were expressed as mean±S.E.M. ***$P<0.001$ Sham vs I/R and **$P<0.01$ I/R vs RIPOC and *$P<0.05$ RIPOC vs RIPOC+FK866

5.11.3.2 Effect of RIPOC and NAMPT inhibitor-FK866 on CREB and BDNF

When correlated to sham group, cerebral I/R injured group showed significant ($P<0.001$) reduction in the levels of CREB (Fig.5.102) and BDNF (Fig.5.103). However, RIPOC intervention significantly ($P<0.001$) improved the levels of CREB and BDNF, while FK866 pre-treatment markedly abolished the protective effect of RIPOC as evidenced by reduced levels of CREB ($P<0.01$) and BDNF ($P<0.05$).
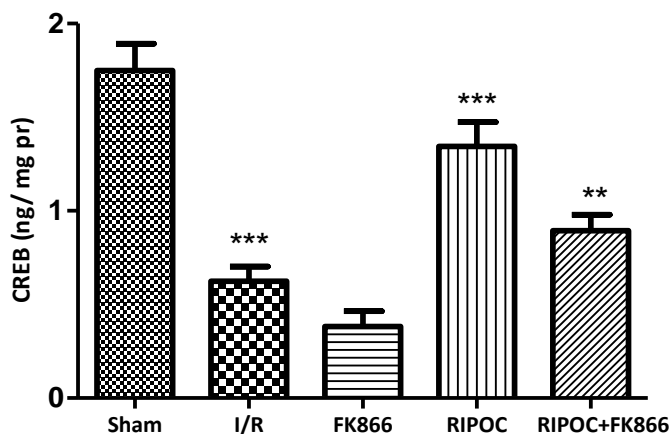
Fig. 5.102. Effect of RIPOC and NAMPT inhibitor FK866 on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and ***P<0.001 I/R vs RIPOC and **P<0.01 RIPOC vs RIPOC+FK866
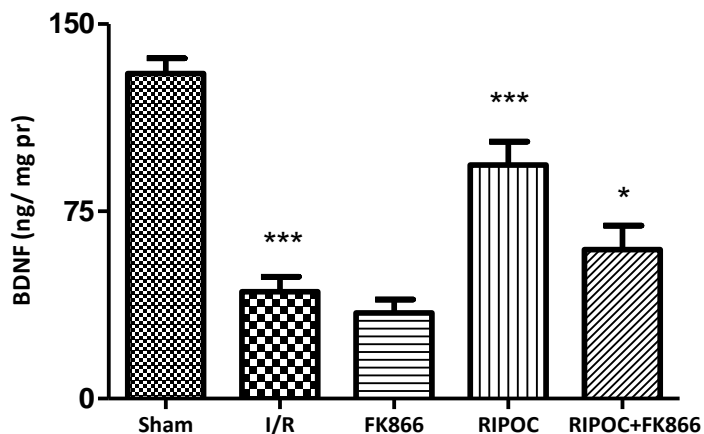
Fig. 5.103. Effect of RIPOC and NAMPT inhibitor FK866 on BDNF levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs RIPOC, *P<0.05 RIPOC vs RIPOC+FK866

5.11.4 *Effect of RIPOC and NAMPT inhibitor-FK866 on hippocampal CA1 neurons*

The HE brains sections showed healthy neurons in the CA1 region of hippocampus of sham group. Healthy neurons were robust in shape, had a spherical or slightly oval nucleus and a single large nucleolus with clear visible cytoplasm as indicated by yellow arrows. The induction of cerebral I/R injury resulted in significant neuronal cell loss and increased pyknotic neuronal count in CA1 region of hippocampus. The pyknotic neurons were darkly stained with no nucleus or visible nucleolus and few cells were shrunken and sickle shaped and indicated by red arrows. However, there was a marked improvement in neuronal density and reduction in pyknotic

neurons with RIPOC intervention, while this protective effect was abolished by NAMPT inhibitor FK866 (Fig.5.104 [a & b]).
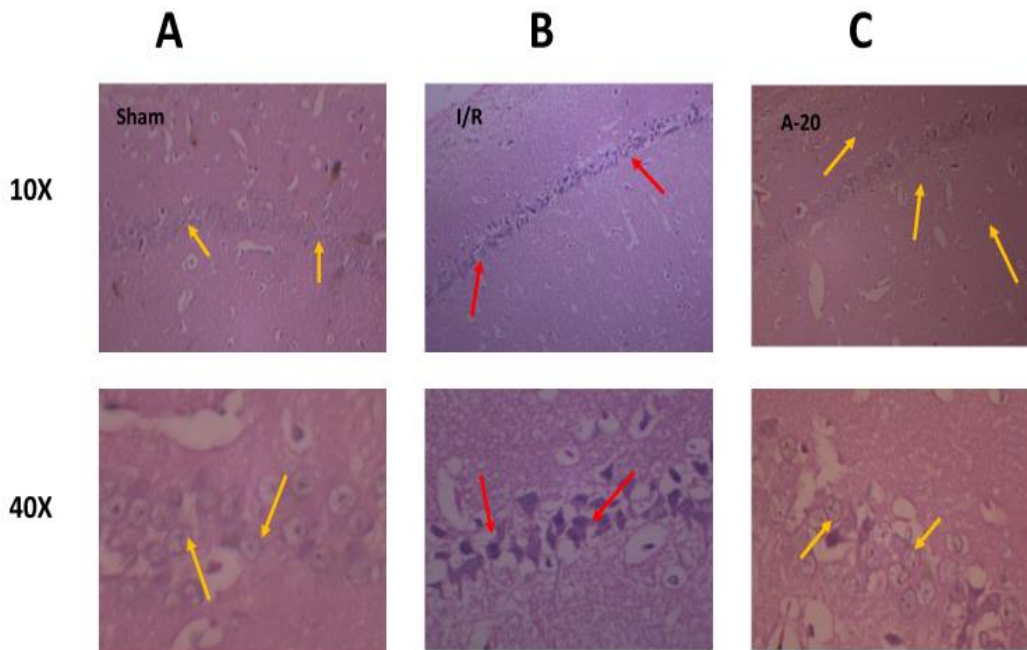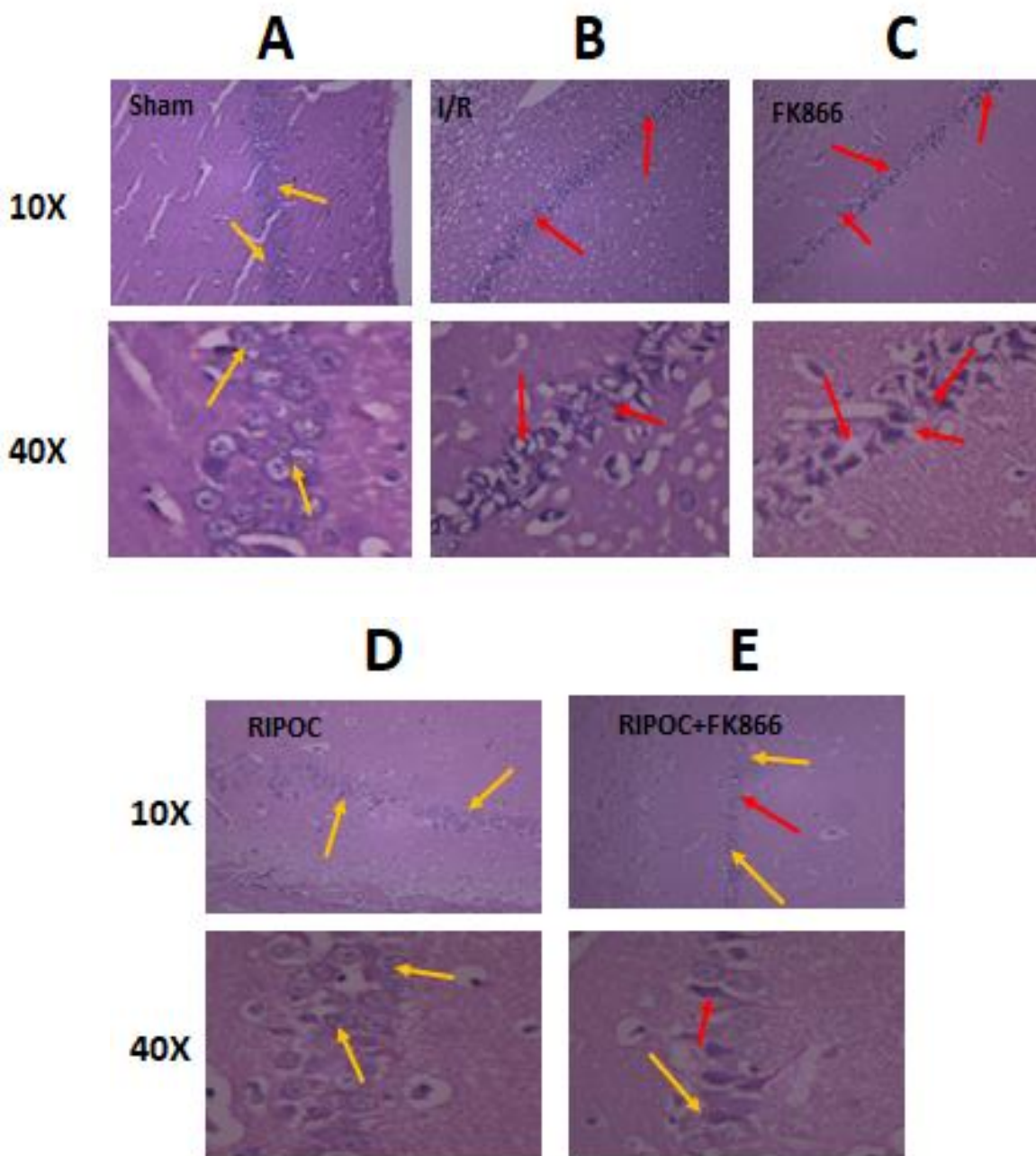

Fig. 5.104 (a). Effect of RIPOC and NAMPT inhibitor FK866 on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) FK866, (Panel D) RIPOC, (panel E) RIPOC+FK866. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
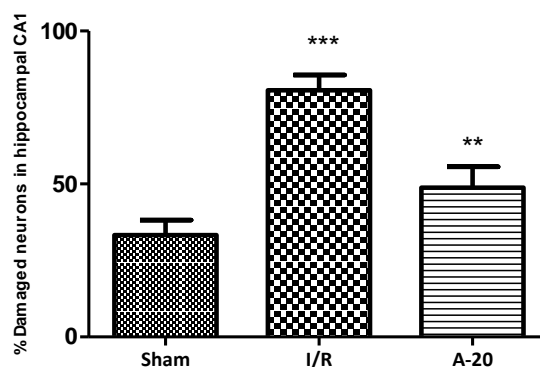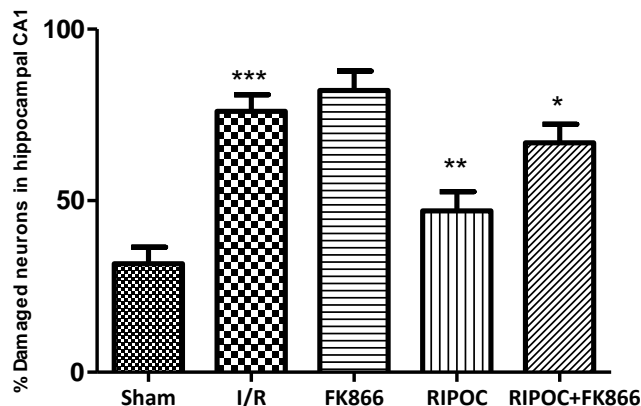
Fig. 5.104 (b). Effect of RIPOC and FK866 on percentage damaged neurons in hippocampus CA1 region. Values were expressed as mean±S.E.M. ***P<0.001 sham vs I/R, **P<0.01 I/R vs RIPOC, *P<0.05 RIPOC vs RIPOC+FK866

5.12 *Combination study (SIRT1 Activator-Resveratrol & GSK-3βInhibitor-Lithium chloride)*

5.12.1 Effect of sub-effective combination of resveratrol and lithium chloride on behavioral parameters

5.12.1.1 Effect of resveratrol and lithium chloride on memory in passive avoidance task

The initial latency in the acquisition trial did not differ significantly among different groups of the study (Fig.5.105). However, during the retention trial cerebral I/R injury significantly (P<0.001) reduced the retention trial latency as compared to sham control rats, while the sub-effective combination of resveratrol and lithium chloride significantly (P<0.01) improved the retention latency when compared to I/R injury group and either drug alone (Fig.5.106).
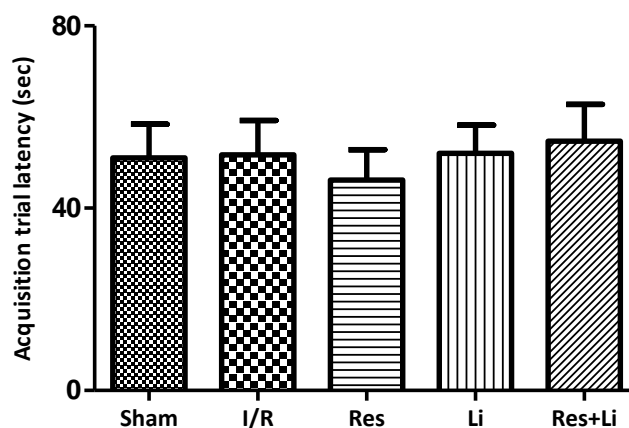
Fig. 5.105. Effect of resveratrol and lithium chloride on memory in passive avoidance task. Values were expressed as mean±S.E.M. No significant differences were observed among various groups of the study
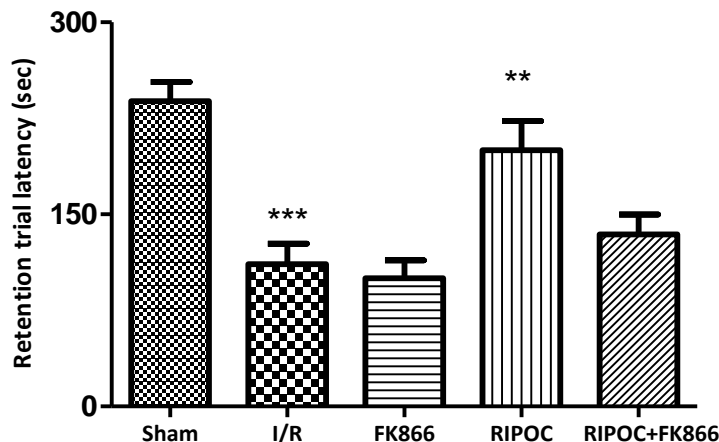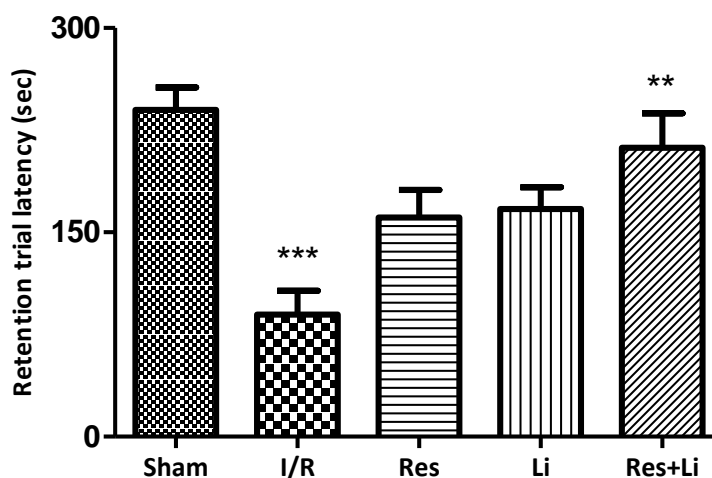
Fig. 5.106. Effect of resveratrol and lithium chloride on memory in passive avoidance task retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li

5.12.1.2 Effect of resveratrol and lithium chloride on memory in Y-maze task

Induction of cerebral I/R injury significantly (P<0.001) reduced the spontaneous alterations as compared to sham group. However, treatment with sub-effective combination of resveratrol and lithium chloride has significantly (P<0.01) improved the percentage spontaneous alterations when correlated to I/R injury group and either drug alone (Fig.5.107).
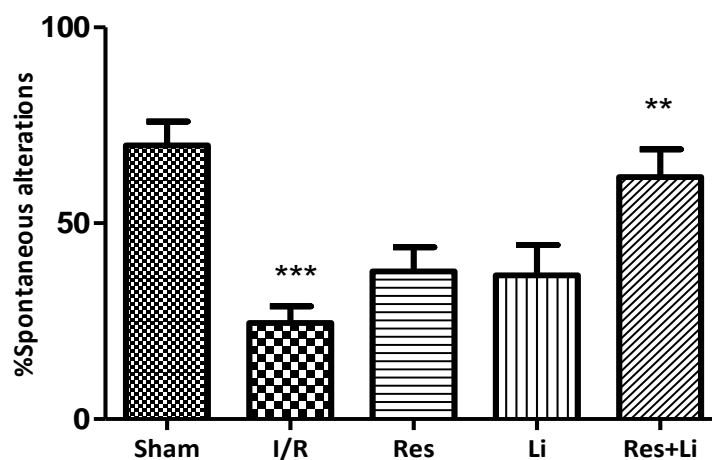
Fig. 5.107. Effect of resveratrol and lithium chloride on memory in Y-maze task
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li

5.12.1.3 Effect of resveratrol and lithium chloride on memory in EPM task

We found no significant difference in the transfer trial latency among different groups in the study during the EPM acquisition trial (Fig.5.108). However, during the retention trial cerebral

I/R injury significantly (P<0.001) increased the transfer latency when compared to sham rats. A sub effective combination of resveratrol and lithium chloride markedly (P<0.001) improved the transfer latency as compared with cerebral I/R injured group or either drug alone (Fig.5.109).
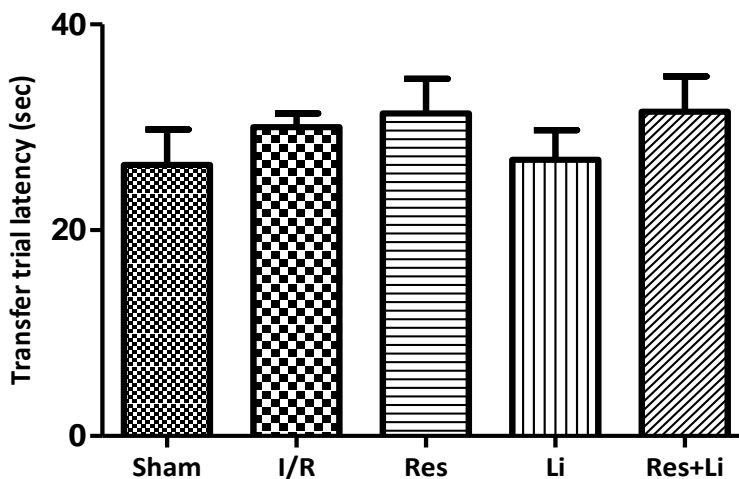
Fig. 5.108. Effect of resveratrol and lithium chloride on memory in EPM acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among various groups of the study.
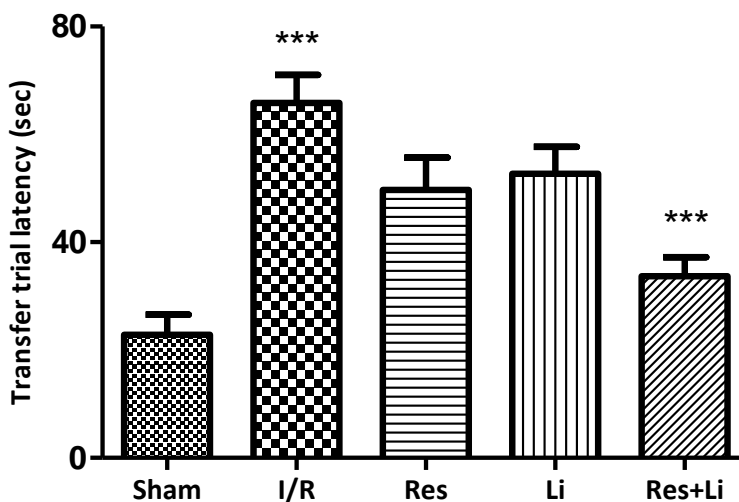
Fig. 5.109. Effect of resveratrol and lithium chloride on EPM retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and ***P<0.001 I/R vs Res+Li

5.12.1.4 *Effect of resveratrol and lithium chloride on locomotor activity*

We did not observe any significant difference in the locomotor activity among different groups of the study (Fig.5.110).
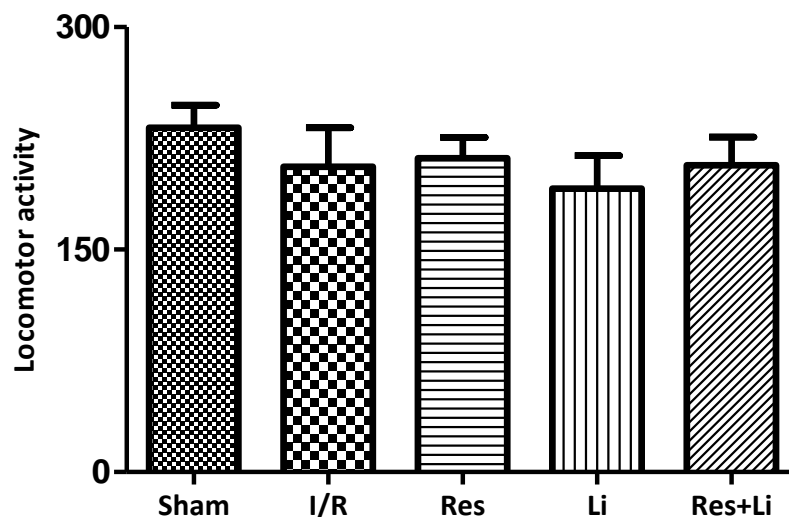
Fig. 5.110. Effect of resveratrol and lithium chloride on locomotor activity.
Values were expressed as mean±S.E.M. No significant differences were observed among various groups of the study.

5.12.2 Effect of combination of sub-effective resveratrol and lithium chloride on biochemical parameters

Cerebral I/R injury intervention resulted in significant (P<0.001) elevation of oxidative markers such as MDA and nitrite and anti-oxidants such as GSH and SOD. In contrast, rats treated with sub-effective combination of resveratrol and lithium chloride showed significant (P<0.001) amelioration of anti-oxidant levels and attenuation of oxidative marker levels as compared to cerebral I/R injury or individual drug treatment (Table.5.8).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.31±0.1	4.9±1.66	1.74±0.32	5.42±1.49
I/R	1.72±0.41***	15.5±2.5***	0.36±0.14***	18.4±2.32***
Res	1.62±0.36	11.9±2.45	0.66±0.17	15.8±2.74
Li	1.55±0.34	13.01±2.57	0.72±0.19	16.1±2.62
Res+Li	0.99±0.24**	7.08±1.70***	1.5±0.23***	11.3±3.00***

Table 5.8. Effect of resveratrol and lithium chloride on biochemical parameters.
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, **P<0.01 I/R vs Res+Li (Nitrite, GSH and SOD)
P<0.01 I/R vs Res+Li (MDA)

5.12.3 Effect of combination of sub-effective resveratrol and lithium chloride on neuroinflammatory parameters

5.12.3.1 Effect of resveratrol and lithium chloride on TNF-α and IL-6

A significant (P<0.001) elevation in neuroinflammatory markers such as TNF-α (Fig.5.111) and IL-6 (Fig.5.112) were noticed with induction of cerebral I/R injury when compared to sham control rats. However, a sub-effective combination of resveratrol and lithium chloride significantly (P<0.01) reduced the levels of TNF-α and IL-6 as compared to cerebral I/R injury group or either drug alone.
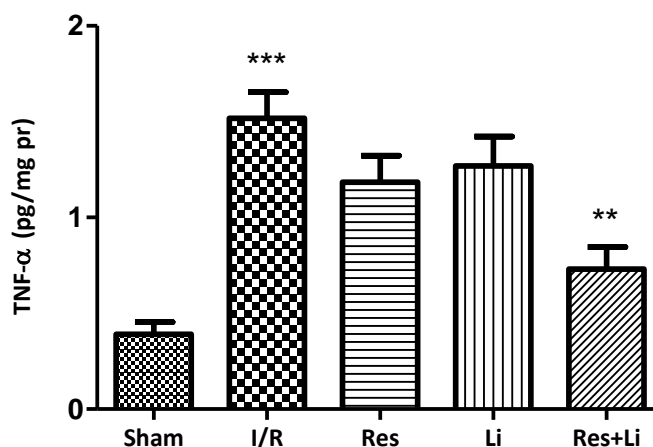
Fig. 5.111. Effect of resveratrol and lithium chloride on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li
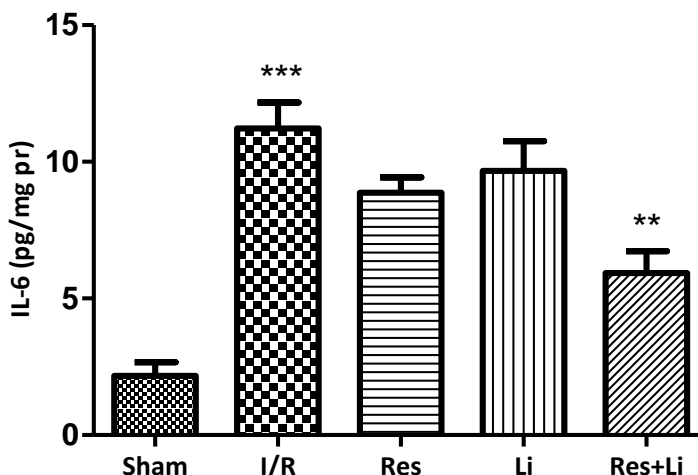
Fig. 5.112. Effect of resveratrol and lithium chloride on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li

5.12.4 Effect of combination of sub-effective resveratrol and lithium chloride on molecular parameters

5.12.4.1 Effect of resveratrol and lithium chloride on CREB and BDNF

Significantly (P<0.001) reduced levels of CREB (Fig. 5.113) and BDNF (Fig.5.114) were noticed in cerebral I/R injury group when compared to sham rats. In contrast, treatment with sub-effective combination of resveratrol and lithium chloride significantly reduced the levels of CREB (P<0.001) and BDNF (P<0.01) when compared to cerebral I/R injured groups or either drug alone.
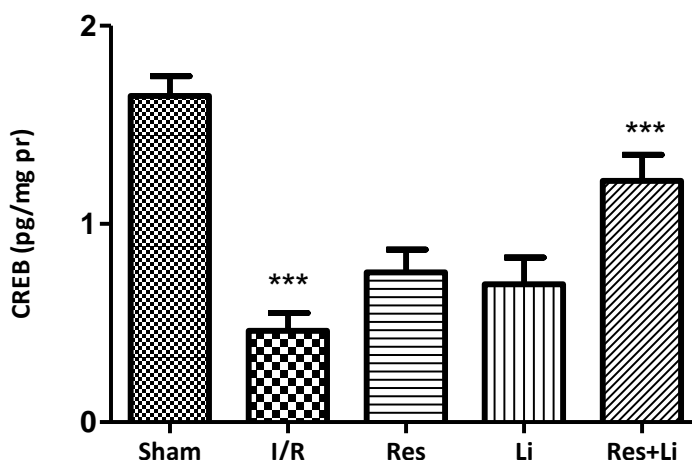
Fig. 5.113. Effect of resveratrol and lithium chloride on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and ***P<0.001 I/R vs Res+Li
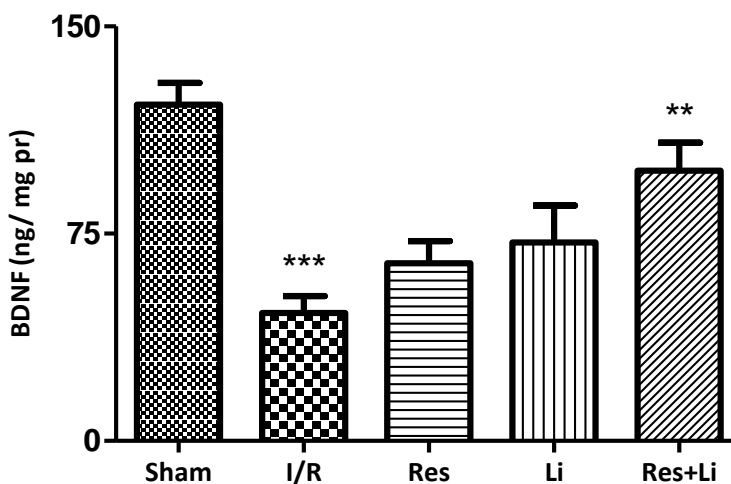
Fig. 5.114. Effect of resveratrol and lithium chloride on BDNF levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li

5.12.4.2   Effect of resveratrol and lithium chloride on GSK-3β

The levels of GSK-3β were found to be significantly elevated in cerebral I/R injured rats when compared to sham group (Fig.5.115). However, either resveratrol or lithium chloride in sub-effective dose did not produce any significant (P<0.001) difference on levels of GSK-3β when compared to I/R injured rats. In addition, sub-effective combination of resveratrol and lithium significantly (P<0.001) attenuated the level of GSK-3β when compared to cerebral I/R injury or either drug alone.
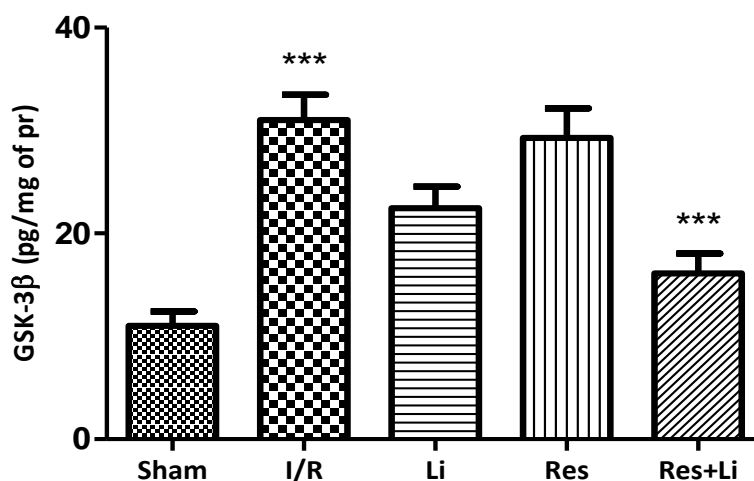
Fig. 5.115. Effect of resveratrol and lithium chloride on GSK-3β levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and ***P<0.001 I/R vs Res+Li

5.12.5   Effect of combination of sub-effective resveratrol and lithium chloride on hippocampal CA1 neurons

The HE sections of sham rats showed healthy neurons in CA1 hippocampal region. The healthy neurons appeared round in shape, robust in shape with large distinct nucleus with a clear visible cytoplasm as indicated by yellow arrows (Fig.5.116 [a&b]). In contrast, induction of cerebral I/R injury resulted in significant morphological alterations as evidenced by increased number of pyknotic neurons. Treatment with sub-effective resveratrol or lithium chloride failed to exert significant effect on alteration of neuronal morphology of hippocampal CA1 region. However, a combination of sub-effective dose of resveratrol and lithium chloride resulted in marked improvement in neuronal density and reduction in pyknotic neurons.
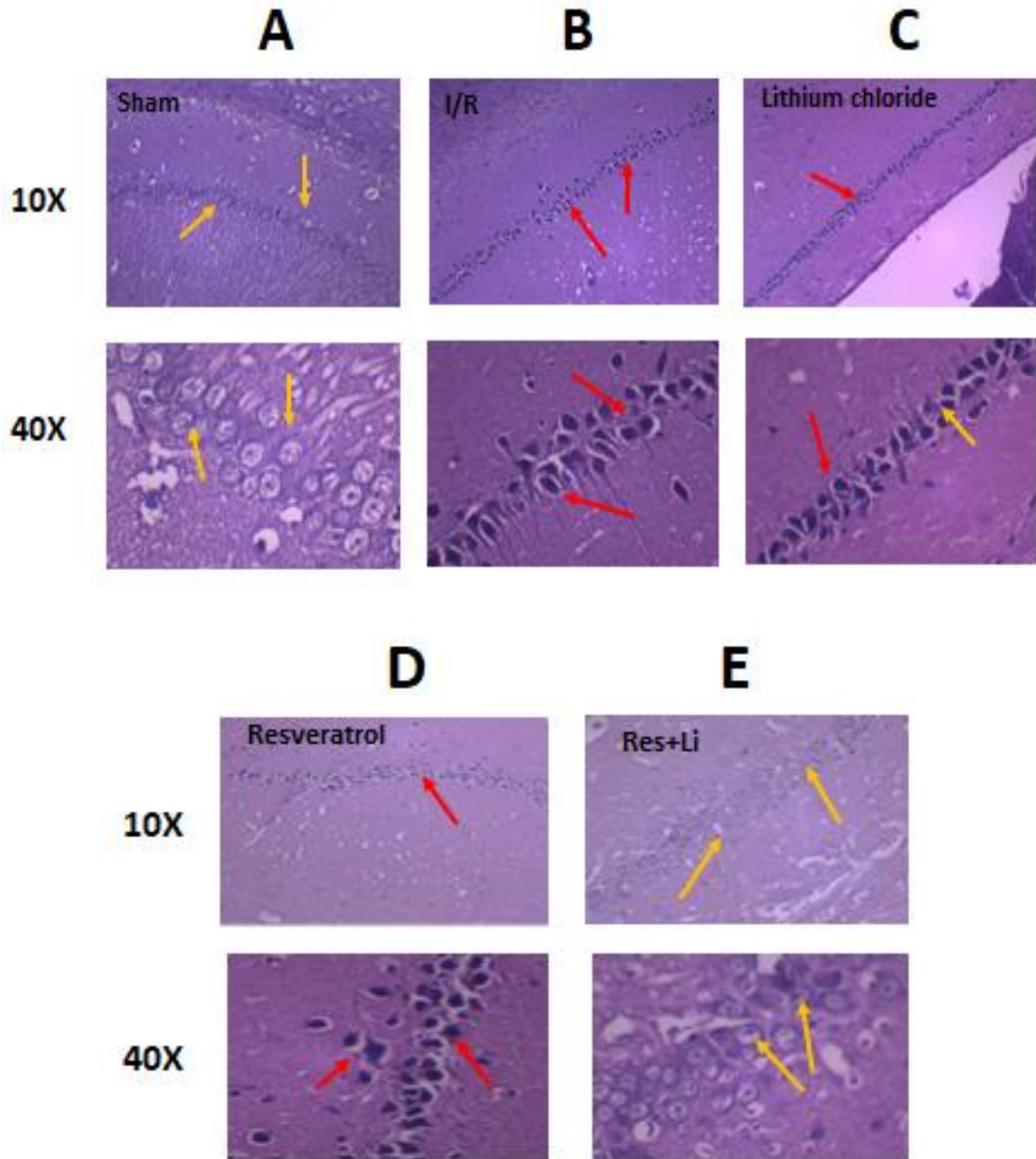
Fig. 5.116 (a). Effect of resveratrol and lithium chloride on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) Lithium chloride, (Panel D) Resveratrol, (panel E) Res+Li. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
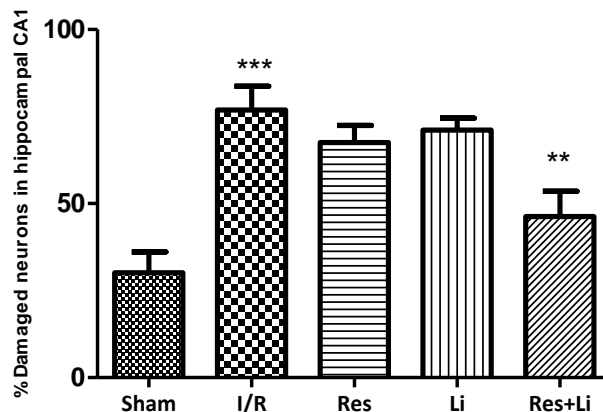
Fig. 5.116 (b). Effect of resveratrol and lithium chloride on hippocampal CA1 neurons
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Li

5.13 Combination study (SIRT1 Activator-Resveratrol & HO-1 Activator-Hemin)

5.13.1 Effect of sub-effective combination of resveratrol and hemin on behavioral parameters

5.13.1.1 Effect of resveratrol and hemin on memory in passive avoidance task

The acquisition trial latency to reach dark chamber did not differ significantly among different groups of the study (Fig.5.117). However, during the retention trial latency we observed a significant (P<0.001) decrease in retention latency with induction of cerebral I/R injury when compared to sham rats. In contrast, sub-effective combination of resveratrol and hemin significantly (P<0.01) improved the retention latency when compared to individual treatment or ischemic group (Fig.5.118).
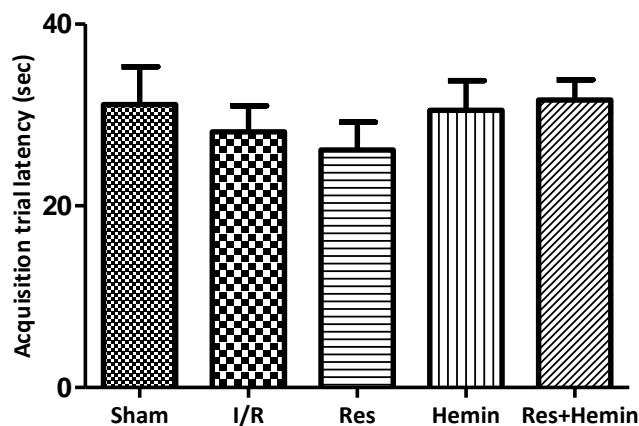
Fig. 5.117. Effect of resveratrol and hemin on memory in passive avoidance acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups of the study
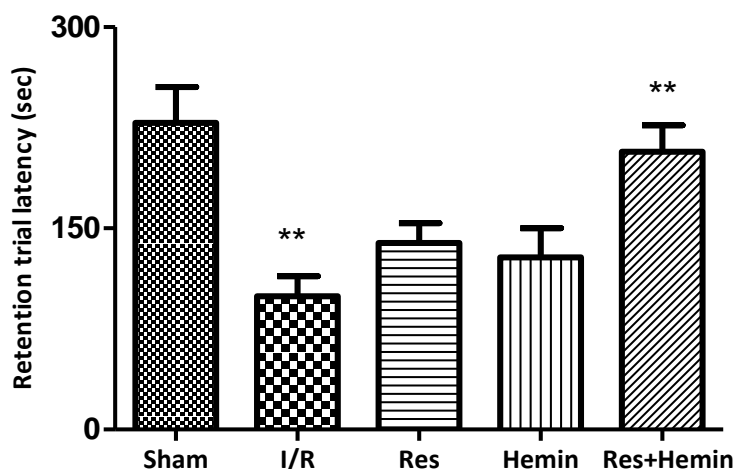
Fig. 5.118. Effect of resveratrol and hemin on memory in passive avoidance retention trial.
Values were expressed as mean±S.E.M. **P<0.01 Sham vs I/R and I/R vs Res+Hemin

5.13.1.2 Effect of resveratrol and hemin on memory in Y-maze test

A significant (P<0.001) decrease in the percentage spontaneous alterations were observed with induction of cerebral I/R injury when compared to sham rats. The sub-effective combination of resveratrol and hemin significantly (P<0.001) improved the spontaneous alterations when compared to cerebral I/R injury group or either drug alone (Fig.5.119).
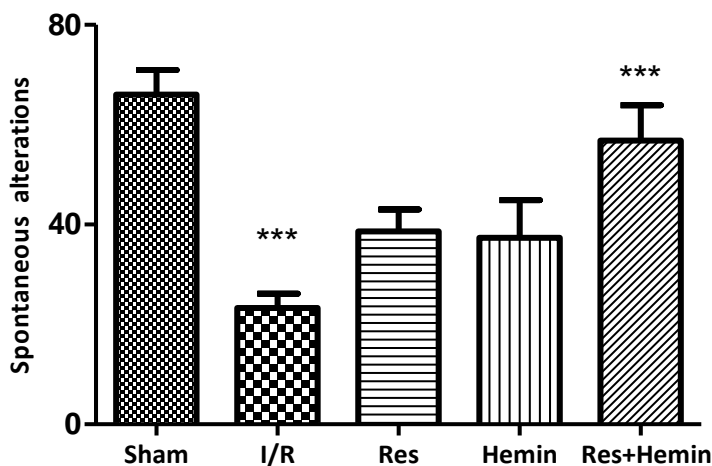
Fig. 5.119. Effect of resveratrol and hemin on memory Y-maze task.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and I/R vs Res+Hemin

5.13.1.3 Effect of resveratrol and hemin on memory in EPM test

In the EPM task, during acquisition trial, no significant difference in the transfer trial latency was noticed among different groups of the study (Fig.5.120). However, during the retention session, I/R injured rats had significantly (P<0.001) higher transfer trial latency when compared to sham rats. Treatment with sub-effective combination of resveratrol and hemin significantly (P<0.001) reduced the transfer latency when compared to ischemic group or either drug alone (Fig.5.121).
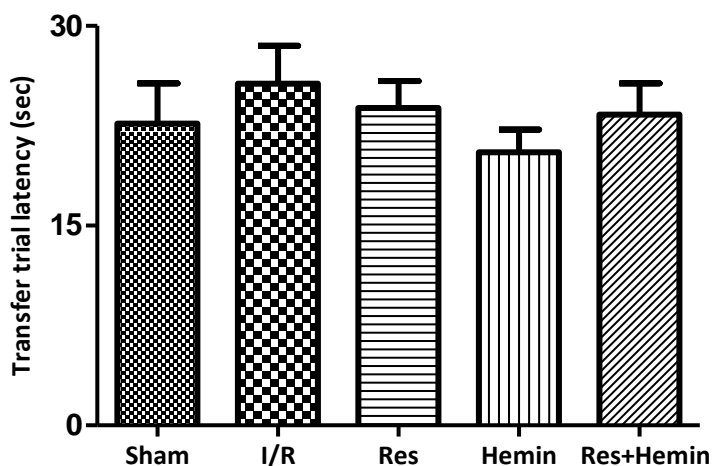
Fig. 5.120. Effect of resveratrol and hemin on memory in EPM acquisition trial.
Values were expressed as mean±S.E.M. No significant differences were observed among different groups of the study
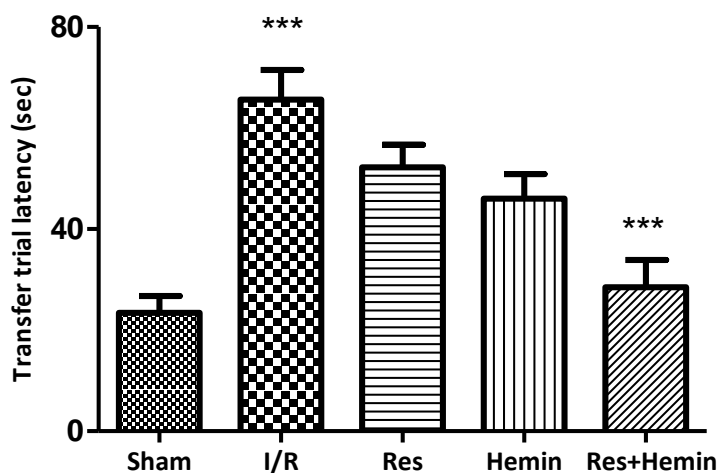
Fig. 5.121. Effect of resveratrol and hemin on memory EPM retention trial.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and I/R vs Res+Hemin

5.13.1.4 Effect of resveratrol and hemin on memory on locomotor activity

The spontaneous locomotor activity was performed to rule out the possibility of effect of impaired locomotion on performance of cognitive parameters. No significant difference was observed among different groups of the study (Fig.5.122).
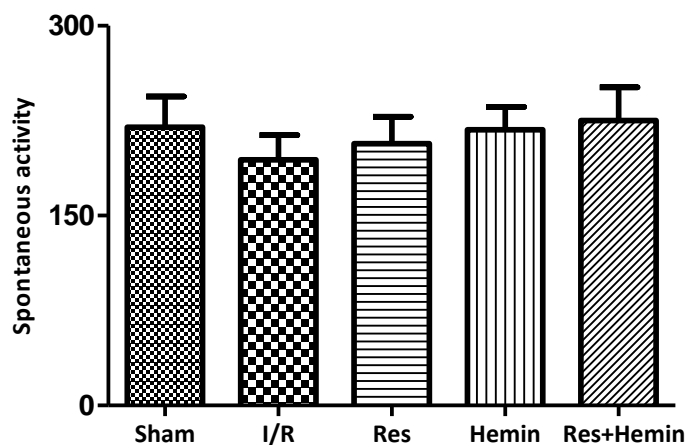
Fig. 5.122. Effect of resveratrol and hemin on locomotor activity.
No significant differences were observed among different groups of the study.

5.13.2 Effect of sub-effective combination of resveratrol and hemin on biochemical parameters

Cerebral I/R injury intervention resulted in significant (P<0.001) increase in oxidative markers such as MDA and nitrite along with reduction in anti-oxidants such as GSH and SOD. However, the rats treated with sub-effective combination of resveratrol and hemin significantly ameliorated the anti-oxidants such as GSH (P<0.01) and SOD (P<0.05) while reduced the levels of oxidative markers like MDA (P<0.001) and nitrite (P<0.05) when compared to I/R injury or either drug alone (Table.5.9).

Groups	MDA (nMol/mg pr)	Nitrite (µMol/mg pr)	GSH (µMol/mg pr)	SOD (U/mg pr)
Sham	0.22±0.08	5.07±1.23	1.93±0.33	6.36±1.84
I/R	2.25±0.36***	13.7±3.01***	0.52±0.36***	16.9±4.4***
Res	1.71±0.56	12.2±1.75	0.87±0.18	15.12±2.91
Hemin	1.92±0.33	12.8±2.66	0.94±0.13	14.79±3.24
Res+Hemin	0.82±0.2***	8.48±1.49*	1.61±0.27**	9.30±2.18*

**Table 5.9. Effect of resveratrol and hemin on biochemical parameters.**
Values were expressed as mean±S.D. ***P<0.001 Sham vs I/R, ***P<0.001 I/R vs Res+Li (MDA) *P<0.05 I/R vs Res+Hemin (Nitrite and SOD) and **P<0.01 I/R vs Res+Hemin (GSH)

5.13.3   Effect of sub-effective combination of resveratrol and hemin on neuroinflammatory parameters

Elevated (P<0.001) levels of TNF-α and IL-6 were noticed with induction of cerebral I/R injury when compared to sham group. Treatment with sub-effective combination of resveratrol and hemin significantly reduced the neuroinflammation as evidenced by low levels of TNF-α (P<0.05) and IL-6 (P<0.01) as correlated to I/R injury group or either drug alone (Fig.5.123 and 5.124).
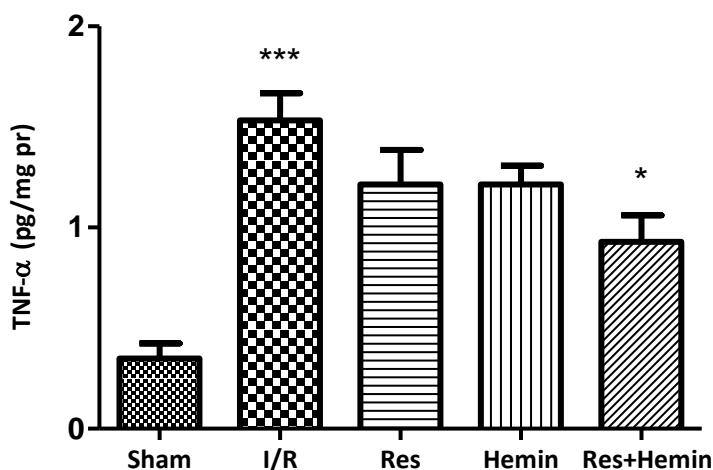
Fig. 5.123. Effect of resveratrol and hemin on TNF-α levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and *P<0.05 I/R vs Res+Hemin
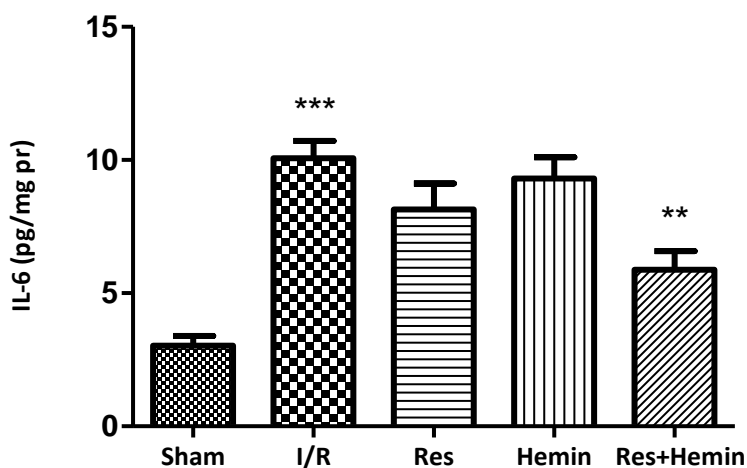
Fig. 5.124. Effect of resveratrol and hemin on IL-6 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Hemin

5.13.4   Effect of sub-effective combination of resveratrol and hemin on molecular parameters

5.13.4.1   Effect of resveratrol and hemin on CREB and BDNF

The levels of CREB (Fig.5.125) and BDNF (Fig.5.126) were found to be markedly (P<0.001) decreased with cerebral I/R injury intervention when compared to sham rats. In contrast, treatment with sub-effective combination of resveratrol and hemin significantly (P<0.01) improved the levels of CREB and BDNF when compared to ischemic group or either drug alone.
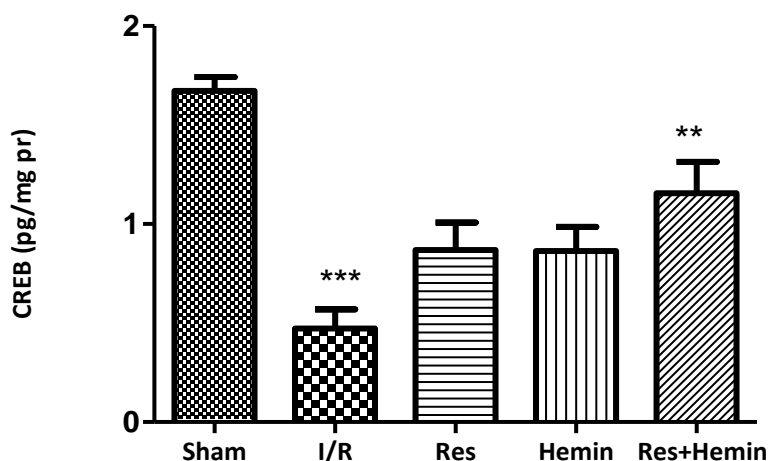
Fig. 5.125. Effect of resveratrol and hemin on CREB levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Hemin
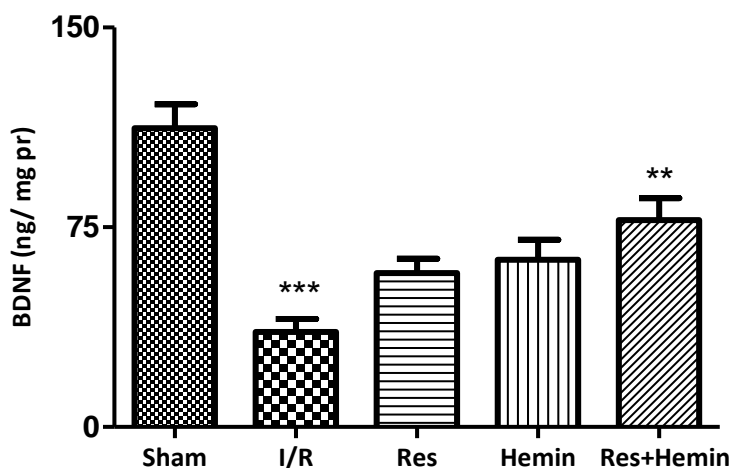
Fig. 5.126. Effect of resveratrol and hemin on BDNF levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Hemin

5.13.4.2 Effect of resveratrol and hemin on HO-1

Cerebral I/R injury significantly (P<0.001) reduced the levels of HO-1 (Fig.5.127) when compared to sham rats, while treatment with resveratrol and hemin combination at sub-effective significantly (P<0.001) improved the HO-1 levels when correlated to ischemic group or either drug alone.
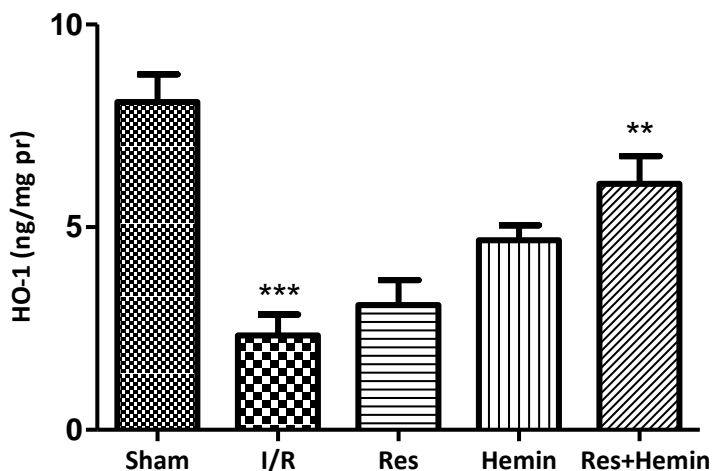
Fig. 5.127. Effect of resveratrol and hemin on HO-1 levels.
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and **P<0.01 I/R vs Res+Hemin

5.13.5 Effect of sub-effective combination of resveratrol and hemin on hippocampal CA1 neurons

Cerebral I/R injury resulted in a range of morphological alterations in hippocampal CA 1 region when compared to sham group. The neurons in I/R group were shrunken in size and shape, darkly stained with no distinct cell mass. In comparison to treatment with sub-effective resveratrol or hemin, its combination significantly improved the neuronal density with healthy neurons, indicating their neuroprotective action when administered together (Fig.5.128 [a&b]).
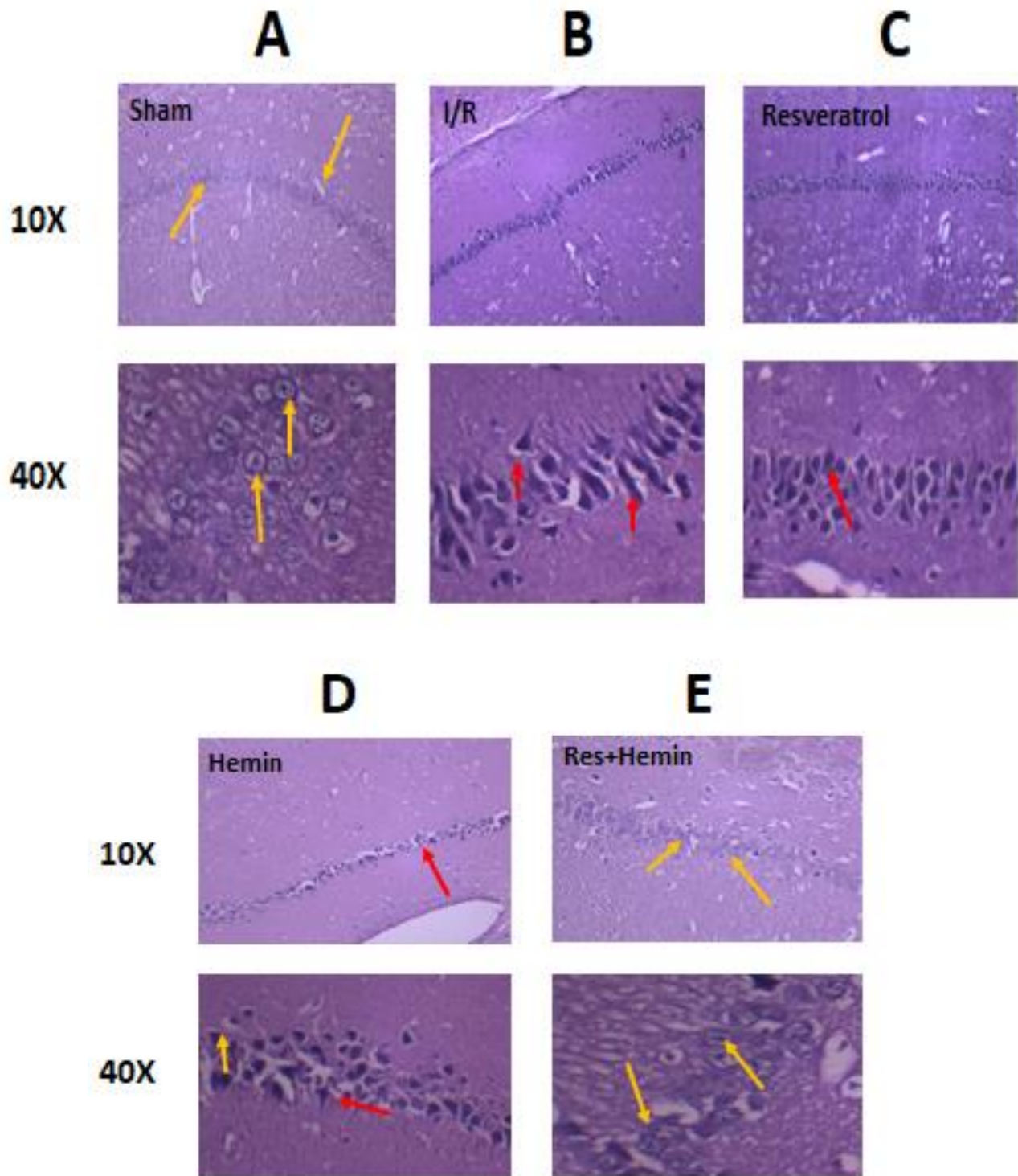
Fig.5.128 (a). Effect of RIPOC and on hippocampal CA1 neurons.
Figure shows photomicrographs of CA1 (Panel A) sham, (Panel B) I/R injury, (Panel C) Resveratrol, (Panel D) Hemin, (panel E) Res+Hemin. Yellow arrows indicate normal healthy neurons; Red arrows indicate damaged or sickle shaped pyknotic neurons.
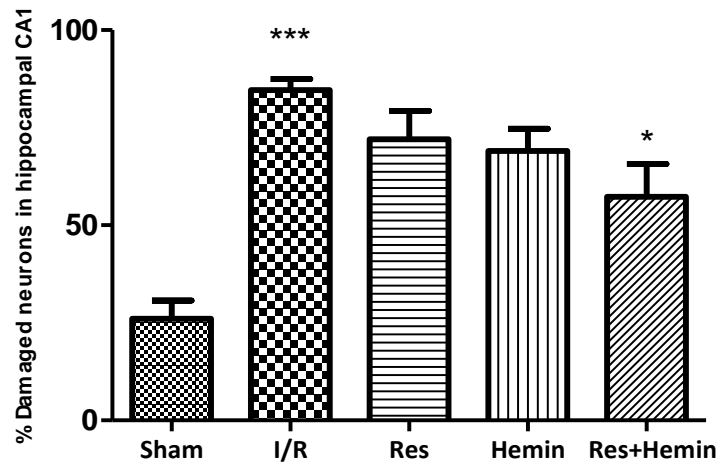
Fig. 5.128 (b). Effect of resveratrol and hemin on hippocampal CA1 neurons
Values were expressed as mean±S.E.M. ***P<0.001 Sham vs I/R and *P<0.05I/R vs Res+Hemin

6. Discussion

In the present study, we observed cerebral I/R injury induced by global model resulted in consistent neuronal damage and cognitive deficits. In addition, we observed beneficial effects of RIPOC and explored its molecular mechanisms against cerebral I/R injury and associated cognitive deficits. We found that RIPOC mediates neuroprotection via inhibition of NR2B, subsequent activation of PI3K/Akt and modulation of its downstream targets GSK-3β and HO-1 along with inhibition of mPTP. Further, we observed that RIPOC exerts neuroprotection by modulation of NAMPT/NAD/SIRT1 pathway.

6.1 *Model validation of cerebral I/R injury*

Cerebral ischemic reperfusion (I/R) injury can be induced by middle cerebral artery occlusion (MCAO) model (MCAO) i.e., focal ischemia and bilateral common carotid artery occlusion (BCCAO) model i.e., global ischemia (Bacigaluppi et al., 2010). Focal ischemia can be developed by mechanical occlusion of middle cerebral artery (proximal or distal), or by thrombotic occlusion or by i.v. injection of Rose Bengal (Bederson et al., 1986; Brint et al., 1988; Gerriets et al., 2003). Proximal MCAO model is the most commonly used experimental technique for induction of stroke because it represents the pathological conditions similar to human stroke and induces a constant infarct size (Bederson et al., 1986; Takano et al., 1997). Moreover, focal ischemia induced by unilateral MCAO results in contralateral neurological deficits due to selective neuronal damage in lesion side striatum and cortex (longer duration occlusions). Motor and sensory deficits including hemiplegia are well known sequalae of focal ischemia, and the severity of neurological deficits was well correlated with the infarct volume in previous studies (Rogers et al., 1997; Yonemori et al., 1998). In line with previous studies, we noticed significant neurological deficits and increased % infarction following induction of cerebral I/R injury using focal model of stroke. Further, we noticed an up-regulation of neuroinflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) along with significant neuronal damage as evidenced by HE staining. However, sensory and motor deficits induced by focal model may interfere with evaluation of cognitive parameters.

Global cerebral ischemia was reported to induce selective forebrain injury with a tailored ischemic duration. Global ischemic can be induced by four-vessel occlusion (4VO) and two vessel occlusion (2VO) models. The 4VO model causes ischemic damage in bilateral forebrain and brain stem, while 2VO model with or without hypotension results in selective CA1 hippocampal neuronal injury, caudate putamen and neocortex damage. Thus, 2VO i.e., by occluding bilateral common carotid occlusion may result in cognitive abnormalities following cerebral I/R injury.

Different algorithms have been suggested for induction of BCCAO to achieve consistent neuronal damage (Table.4.2). Therefore, to standardise the animal model in male wistar rats in our laboratory, we treated rats with different duration of ischemia followed by 72 hours of reperfusion. Data obtained from a battery of behavioral, biochemical and histochemical parameters suggested that cerebral I/R injury induced by 20min of ischemia and 72 hours of reperfusion were able to induce consistent neuronal damage. The molecular mechanisms leading to tissue damage from cerebral I/R injury are complex and multi-factorial. Growing evidence from earlier studies suggest the role of oxidative stress and neuroinflammation in mediating disease progression (Allen and Bayraktutan, 2009; Danton and Dietrich, 2003). Earlier studies revealed that an increased production of oxygen free radicals and pro-inflammatory cytokines like TNF-α and IL-6 is an important underlying cause for neuronal damage following cerebral I/R injury (Collino et al., 2006; Crack and Wong, 2008). In this study, we observed oxido-nitrosative stress as evidenced by increased levels of oxidative markers like MDA and nitrite, and reduced levels of anti-oxidants like GSH and SOD.

Earlier experiments revealed that excessive production of ROS in acute phase of I/R injury, results in secondary injury by amplifying the brain inflammatory mechanisms including resident microglial activation, leukocyte and neutrophil infiltration, leading to neuroinflammation (Danton and Dietrich, 2003; Jin et al., 2010). In addition, microglial activation is the major inflammatory mechanism to exacerbate ischemic injury as they respond early to ROS. Neurotoxicity of the microglia is exerted through production of NADPH oxidase and pro-inflammatory cytokines like IL-6 and TNF-α (Danton and Dietrich, 2003; Jin et al., 2010). In the

present study, an elevation in neuroinflammation was evidenced by increased levels of pro-inflammatory cytokines like TNF-α and IL-6.

Cerebral I/R injury induced oxidative stress and neuroinflammation may affect a vast type of neuronal population that is more susceptible to ischemic damage. Hippocampal CA1 region is the most explored subtype of selectively vulnerable neuronal population following cerebral I/R injury (Pulsinelli, 1985). The putative reason for the selective hippocampal damage by ischemia or anoxic depolarisation may be the difference in the chemical characteristics and complex circuitry of the hippocampal neurons (Kirino et al., 1985). It is well known that selective hippocampal damage lead to learning and memory impairments(Davis et al., 1986; Deshpande et al., 1987; Gehrmann et al., 1992; Saad et al., 2015).Therefore, we designed to explore the relation between hippocampal damage following cerebral I/R injury and the cognitive deficits. To achieve this, initially we have screened the effect of cerebral I/R injury on locomotor activity and found no significant effect of cerebral I/R injury on spontaneous locomotor activity. This indicates that motor abnormality may not be a reason for poor cognitive performance. Then, we used passive avoidance task, Y-maze test and elevated plus maze to evaluate the effect of cerebral I/R injury on cognitive impairment. Passive avoidance task is based on rat ability to avert electric shock and is useful to assess learning and memory in rodents (Foley et al., 2004; Park et al., 2000). We found that, cerebral I/R injured rats showed less retention latency to enter dark chamber in comparison to sham rats. Further, cerebral I/R injured rats showed increased transfer latency to enter the enclosed arm and less spontaneous alterations in Y-maze task consistent with earlier reports.

The biochemical alterations and neuro-behavioral disturbances could be associated with hippocampal neuronal damage. Therefore, we performed HE staining of hippocampal CA 1 regions of normal and cerebral I/R injured rats. The neurons in sham group were healthy, round shaped with clear cytoplasm, spherical nucleus and large nucleolus, while the pyknotic neurons in I/R group were darkly stained, showed marked disorganization and severe cell loss. The possible reason for the formation of darkly stained neurons in the hippocampus region may be due to alterations in the endoplasmic reticulum and mitochondrial dynamics that may divide and accumulate resulting in darkly stained cells (Kirino et al., 1985). Therefore, cerebral I/R

injury with 20min of ischemia followed by 72 hrs of reperfusion was considered to induce cerebral I/R injury and associated cognitive deficits in future studies in this thesis.

6.2   *Model validation of remote ischemic postconditioning (RIPOC)*

Extensive work on RIPOC protection against focal cerebral ischemia was first demonstrated by Ren et al and his colleagues in 2009 (Ren et al., 2009). However, RIPOC algorithms were not clear from the previous studies. Moreover, invasive unilateral (left/right) and bilateral RIPOC was reported with different algorithms in earlier studies indicating that the RIPOC algorithm for neuroprotective potential was inconclusive (table2.2). Data from the earlier studies provided the clue that the overall duration of remote ischemic postconditioning was in the range of 45 min to 90 min. Thus, we designed four algorithms for RIPOC by an elevation of ischemia and reperfusion times with reduction in the number of cycles. We induced RIPOC by four cycles of 5 min ischemia and 5 min reperfusion, three cycles of 10 min ischemia and 10min reperfusion, two cycles of 15 min ischemia and 15 min reperfusion and one cycle of 20 min ischemia followed by reperfusion. In this study, different algorithms of RIPOC were used at early onset of reperfusion to enable maximum potential. Data from our study indicated that RIPOC induced with three cycles of 10 min ischemia and 10 min reperfusion was highly effective against cerebral I/R injury and associated cognitive deficits when compared to other combinations in reducing the oxidative stress, neuroinflammation and hippocampal damage. Later this specific algorithm of RIPOC was standardised and confirmed by various other studies(Gao et al., 2017; Li et al., 2016; Zhang et al., 2017a, 2017b). Further, we further investigated the role of early and delayed RIPOC on cerebral I/R injury and associated cognitive deficits. To achieve this, RIPOC with 3 cycles of 10 min ischemia and 10 min reperfusion was applied at early onset, 10min, 30 min, 1 hr, 3 hrs, 6 hrs of reperfusion to simulate clinical conditions. We found that RIPOC applied at the early onset of reperfusion was found to be protective against cerebral I/R injury that was supported by few recent studies (Ren et al., 2016; Zhang et al., 2017b). Further, early RIPOC reduced the oxidative damage and neuroinflammation in correlation to reduced hippocampal damage which was in harmony with previous studies (Chen et al., 2016; H. Li et al., 2015). Early RIPOC improved the cognitive performance in memory tasks, which was in accordance with earlier studies (Burda et al., 2014; Peng et al., 2012). Moreover, reperfusion in

clinical situations may be performed ideally in hospitals, where RIPOC may be possibly applied at early onset of reperfusion (Non-invasive remote ischemic post conditioning was mentioned as RIPOC rather than NRIPOC in the thesis).

6.3 *Molecular mechanisms of RIPOC*

6.3.1 *Modulation of excitotoxicity pathway by RIPOC*

Cerebral I/R injury induced oxidative stress and energy failure have been found to abnormally concentrate glutamate at synapse causing prolonged stimulation of NMDA, AMPA and metabotropic glutamate receptors (Szydlowska and Tymianski, 2010). NMDA ion channel receptors mediate most of the excitatory impulses of neurotransmitter glutamate in the mammalian brain. NMDA receptors play an important role in synaptic plasticity by long term potentiation and long term depression that is crucial for synaptic modulation for learning and memory (Bear and Malenka, 1994; Malenka and Bear, 2004). Further, the role of NMDA has been implicated in neuronal survival and maturation (Cameron et al., 1995; Platel et al., 2010). Over activation of NMDA receptors during ischemia induced excitotoxicity has been described in previous studies (Besancon et al., 2008; Verkhratsky and Kirchhoff, 2007). However, NMDA antagonists such as gavestinel, selfotel, eliprodil, aptiganel and licostinel failed to show protective effects in clinical trials despite promising preclinical studies. The possible reason for this failure may be attributed to the hindrance of normal physiological actions like neuronal survival and synaptic transmission by NMDA receptors. The second reason may be intolerable side effects induced by NMDA receptor antagonists (Lipton, 2004; Morris et al., 1999; Platel et al., 2010). Further, the NMDA-R subunits often exist in the form of receptor complex with two basic subunits, NR1 has a functional role in regulation of calcium flow role while NR2 has regulatory role. The participation of different NR2 subunits in the NMDA-R complex decides its pharmacological and electrophysiological property (Massey et al., 2004). Therefore, a selective inhibition of NMDA receptor subtype may produce beneficial effects against cerebral I/R injury. Glutamate excitotoxicity via NMDA-R over activation during cerebral I/R injury is well reported by earlier studies (Doyle et al., 2008; Lai et al., 2014; Szydlowska and Tymianski, 2010), however, the specific role of its subunits in ischemic injury remains unclear. Accumulating

evidence on specific role of NMDA-R subunits suggest that activation of NR2A subunits exert pro-survival effects while activation of NR2B subunits resulted in excitotoxic cell death under ischemic conditions (Hardingham et al., 2002; Terasaki et al., 2010). In addition, a recent study published in 2015 demonstrated the pro-survival role of NR2A in ischemic postconditioning against cerebral I/R injury (Zhang et al., 2015). Based on above evidences, it seems that a tailored suppression of NR2B by RIPOC may induce neuroprotection against cerebral I/R injury and cognitive deficits.

To confirm this, we pre-treated cerebral I/R injured rats with NR2B agonist, Quinolinic acid (QA) before RIPOC intervention at early onset of reperfusion. We observed that RIPOC intervention increased oxidative stress, neuroinflammation and neuronal injury in hippocampus in presence of QA, indicating that RIPOC exerts neuroprotection by inhibition of NR2B. These findings were consistent with the earlier report on IPOC where administration of NR2B antagonist prior to IPOC did not show any significant difference when compared to IPOC (Zhang et al., 2015). However, the modulation of cognitive parameters and hippocampal neuronal damage by RIPOC in this context remains unknown. Therefore, it is necessary to determine the downstream modulators of NR2B pathway in RIPOC mediated neuroprotection.

The research during last decade highlighted the importance of posttranslational modifications (PTM) of NMDA-R that are critical regulators of synaptic transmission essential for neuronal survival and synaptic plasticity. These PTM include glycosylation, palmitoylation, phosphorylation ubiquitination and sumoylation. Recent studies reported that phosphorylation (at serine, threonine or tyrosine) is the key regulatory mechanism in controlling surface and synaptic expressions of NMDA-R in a subunit specific manner (Lussier et al., 2015; Qiu et al., 2011). In the hippocampus region, NR2A and NR2B receptors are the most predominant type of NMDA-R subunits. Moreover, it has been suggested that NR2A and NR2B receptors are only 30% identical in sequence, therefore, the nature and subsequent series of events differ in both NR2A and NR2B receptor activation (Kennedy and Manzerra, 2001). The best documented example of NMDA-R (NR2A and NR2B) phosphorylation is through Src family of tyrosine kinases. An earlier study revealed that intracellular application of Src family tyrosine kinases increase activity of NR2A subunits but not of NR2B subunits in HEK 293 cells (Köhr and Seeburg,

1996). This suggests that phosphotyrosine of NR2A play different role than NR2B. Moreover, Src family of proteins are reported to modulate NR2B dependent functions (Salter and Kalia, 2004).

The intracellular signal transduction of NR2B receptors are reported to be mediated through protein-protein interactions that occur between phosphorylated NR2B subunit and various molecules containing SH2 domain to consequently transmit signal to other downstream components. PI3K-Akt is one such protein with SH2 domain and is well reported to bind to phosphotyrosine on cell surface receptors like NMDA to transmit intracellular signals. The crucial role of PI3K-Akt in mediating intracellular signals of NR2B was demonstrated (Habas et al., 2006) in hippocampal neurons during physiological conditions. However, its role in NR2B over activation during conditions of cerebral ischemia remains unclear. It seems that PI3K-Akt may mediate protective effects of NR2B inhibition in cerebral I/R injury and its associated cognitive deficits. In the present study, we observed that the NR2B antagonist, Ifenprodil attenuated cerebral I/R induced oxidative stress, neuroinflammation, memory impairment and hippocampal neuronal damage that are in harmony with earlier reports (Zou et al., 2014). However, the protective effects of NR2B inhibition by Ifenprodil against cerebral I/R injury damage were abolished in presence of PI3K-Akt inhibitor LY294002, suggesting that NR2B exerts its protective effects by activation of PI3K-Akt.

Based on our previous study, we found that RIPOC mediates neuroprotection via NR2B inhibition. Moreover, we observed that protective effects of NR2B inhibition require activation of PI3K-Akt survival pathway. Further, alterations in PI3K-Akt pathway has been implicated in emergency and metabolic disorders like cancer, diabetes, obesity and neurodegenerative disorders (Chen, 2010; Fruman and Rommel, 2014; Heras-Sandoval et al., 2011). In addition, there is an increasing literature showing that ischemia induced oxidative stress and neuroinflammation are closely related to hypo phosphorylation of Akt leading to inactivation of Pi3K-Akt pathway that result in detrimental effects of I/R injury (Lan et al., 2013; Wang et al., 2007b). Moreover, earlier studies suggested the involvement of PI3K-Akt pathway in ischemic tolerance induced by ischemic conditioning(Tu et al., 2015; Yin et al., 2015). Thus, it seems that RIPOC protection might involve modulation of PI3K-Akt pathway to provide ischemic tolerance

against cerebral I/R injury and associated cognitive deficits. Therefore, in this study we evaluated the role of PI3K-Akt in RIPOC protection against cerebral I/R injury and associated cognitive deficits. We observed that RIPOC protection increased oxidative stress and neuroinflammation with significant hippocampal CA1 damage in presence of PI3K-Akt inhibitor. This indicates that RIPOC protects neurons by activating PI3K-Akt pathway which was consistent with recent reports (Li et al., 2016; Qi et al., 2012a; Zhang et al., 2017b). However, the protective role of PI3K-Akt might likely involve activation of certain cell signalling downstream mediators to control PI3K-Akt role ranging from cell survival to cell death.

PI3K-Akt/GSK-3β is an important signaling pathway involved in regulating cell survival, proliferation and growth (Franke et al., 2003). Numerous studies revealed the involvement of PI3K-Akt/GSK-3β pathway in pathological conditions such as cancer, type-II diabetes mellitus, obesity and neurodegeneration(Luo, 2009; Martinez et al., 2002; Zhao et al., 2012). Studies have shown that PI3K-Akt/GSK-3β pathway is involved in oxidative damage, neuroinflammation and apoptosis that is closely related to I/R injury (Reho and Rahmouni, 2017; Zhao et al., 2017). Earlier studies reported that ischemic injury results in inhibition of PI3K-Akt and subsequent activation of GSK-3β (Wu et al., 2012). Further, pharmacological inhibitors of GSK-3β showed improvement in neuronal survival following various neurological disorders (Chen et al., 2013; Collino et al., 2008). Interestingly, an earlier studied revealed the modulation of Akt and GSK-3β by limb remote post conditioning in a focal model of I/R injury (Qi et al., 2012b). The above results raise the possibility that PI3K-Akt/GSK-3β signalling might be involved in RIPOC protection against cerebral I/R injury and associated cognitive deficits. In our study, we observed GSK-3β inhibition by RIPOC, however, this effect was abolished in presence of PI3K-Akt inhibitor LY294002. This indicates that RIPOC results in activation of PI3K-Akt and subsequent inhibition of GSK-3β to mediate neuroprotection against cerebral I/R injury and associated cognitive deficits. Further, earlier studies suggested that modulation of GSK-3β may result in activation or suppression of certain transcription factors to mediates its physiological or pathological actions (Chuang et al., 2011). One of the most important transcription factor regulated by GSK-3β is cyclic-AMP response element-binding protein (CREB). The activation of CREB is implicated in neuronal survival, learning and memory, synaptic plasticity and increased

expression of neurotrophin Brain Derived Neurotrophic factor (BDNF)(Duman et al., 2000; Finkbeiner, 2000). Phosphorylation of CREB at ser-133 is required for recruitment of co-activator, CREB-binding protein (CBP) to exert transcriptional activity and also create a consensus site at serine-129 for phosphorylation by GSK-3β. Thus, GSK-3β inhibition up-regulates transcriptional activity of CREB (Grimes and Jope, 2001; Plátenk et al., 2014). Further, earlier studies reported a decreased CREB phosphorylation and reduced CREB driven transcriptional activity of BDNF in hippocampal neurons following cerebral ischemic injury (Kitagawa, 2007). In our study, we observed a reduction in levels of CREB and BDNF in cerebral I/R injured rats in accordance with earlier studies (Finkbeiner, 2000; Kitagawa, 2007). It is well documented that GSK-3β phosphorylation at ser-9, that leads to its inactivation is regulated either by PI3K-Akt or other pathways. Moreover, GSK-3β negatively regulates CREB activity; therefore, there is a possible signalling from PI3K-Akt/GSK-3β to CREB in cell death/cell survival pathways during ischemic tolerance. In addition, we observed that RIPOC induced PI3K-Akt activation mediated GSK-3β inhibition enhances CREB and BDNF levels to exert neuroprotection against cerebral I/R injury and associated cognitive deficits in harmony with previous reports (Chuang et al., 2011; DaRocha-Souto et al., 2012).

Further, alterations in PI3K-Akt survival signals are known to be involved in pro-inflammatory and multiple oxidative stress and apoptotic insults by modulation of transcription factors such as Nuclear factor-E2-related factor 2 (Nrf2), fork head transcription factors (FOXO), hypoxia-inducible factor-1α (HIF-1α)(Granado-Serrano et al., 2010; Stitt et al., 2004; Zhong et al., 2000). Reports suggest that Nrf2 is a key transcription factor involved in protecting cells against various pathologies ranging from metabolic disorders, cancer and neurodegenerative disorders through neutralization of reactive oxygen species. Moreover, this protective action of Nrf2 is mediated through transcriptional expression of phase-II anti-oxidant enzymes such as heme oxygenase-1 (HO-1), glutathione S transferase, superoxide dismutase (SOD) and NAD(P)H: quinone dehydrogenase 1 (NQO1) (Calkins et al., 2009; Kansanen et al., 2013; Sykiotis et al., 2011). Further, the importance of HO-1 was unveiled by studies on HO-1 knock out animals and HO-1 null mice that displayed increased susceptibility to oxidative stress and neuroinflammation (Poss and Tonegawa, 1997; Ryter and Choi, 2016; Yet et al., 1999).

Moreover, HO-1 knockout was found to worsen outcomes following cerebral ischemic stroke (Sharp et al., 2013). Earlier invitro and in vivo studies revealed that, HO-1 up-regulation during ischemic damage is critical for cytoprotection and blood brain barrier integrity (Y.-H. Li et al., 2015; Wang et al., 2013; Yet et al., 2001). Previous in vitro and in vivo studies suggested that PI3K-Akt activation is essential for Nrf2 transcription and HO-1 induction (Wu et al., 2013; X. H. Xu et al., 2015)[i]. Thus, it seems that HO-1 induction during ischemic tolerance may be modulated by PI3k-Akt pathway. In our study, we found that administration of PI3K-Akt inhibitor, LY294002 prior to RIPOC reduced the levels of HO-1, suggesting that RIPOC mediates neuroprotection by activation of PI3K-Akt and HO-1 induction (PI3K-Akt/HO-1 pathway). This indicates that HO-1 up regulation was mediated by Nrf2 activation that seems to be under the regulatory control of PI3K-Akt in harmony with previous studies (Ryter and Choi, 2016). Based on afore mentioned findings it can be concluded that RIPOC exerts neuroprotective effects against cerebral I/R injury and associated cognitive deficits via modulation ofPI3-Akt/HO-1 and PI3K/Akt/GSK-3β pathways. Therefore, inhibition of GSK-3β and induction of HO-1 may exert beneficial neuroprotective effects against cerebral I/R injury and associated cognitive deficits.

6.3.2   Modulation of apoptotic pathway by RIPOC

Further, earlier studies revealed that GSK-3β predominantly resides in cytoplasm to converge multiple cytoprotective signals under physiological conditions. However, ischemic conditions are reported to induce translocation of GSK-3β from cytosol to mitochondria resulting in mitochondrial dysfunction (Miura and Tanno, 2011). A major mechanism involved in mitochondrial dysfunction is the opening of mitochondrial membrane permeability transition pore (mPTP) that is reported to unfold cristae due to osmotic pressure leading to loss of outer membrane integrity and spillage of cyt c from intermembrane space into cytosol (Capano and Crompton, 2002; Hovius et al., 1993; Kinnally et al., 2011). Thus, mPTP opening is considered as a final common step in oxidant-stress induced cell death. Consistent with this, many investigators have reported the opening of mPTP during ischemia and protective effects of its inhibition in various in vitro and in vivo studies (Li et al., 2000; J. Sun et al., 2014; Zhang et al., 2013). In addition, earlier studies reported that inhibition of mPTP is involved in ischemic tolerance to provide protection against focal model of ischemia (Correia et al., 2010; Kalogeris

et al., 2014; Ye et al., 2012). Considering the detrimental role of mPTP in ischemia induced cell death, we examined the role of RIPOC in modulation of mPTP to exert neuroprotection against cerebral I/R injury and associated cognitive deficits. In our study, we found that the cerebral I/R injury resulted in mPTP opening as evidenced by increased oxidative stress, neuroinflammation along with up-regulation of cytochrome-c levels. In addition, cerebral I/R injury resulted in increased apoptotic neurons as evidenced by TUNEL staining that is consistent with earlier reports(Christophe and Nicolas, 2006; Manzanero et al., 2013). RIPOC intervention at early onset of reperfusion resulted in increased oxidative stress, neuroinflammation, cytochrome-c and apoptotic neuronal count in presence of mPTP opener, atractyloside, indicating that RIPOC exerts neuroprotection against cerebral I/R injury by inhibition of mPTP opening in harmony with earlier study (Sun et al., 2012a).

6.3.3 *Modulation of bioenergetic and epigenetic pathway by RIPOC*

A complex series of events such as oxidative stress, neuroinflammation, excitotoxicity and apoptosis play an important role in the pathological cascade of cerebral I/R injury. During recent years, epigenetic modifications have emerged as an important pathological consequence following cerebral I/R injury. Epigenetic modifications such as histone acetylation are likely to regulate the structure and function of chromatin yielding to different functional consequences (Faraco et al., 2006; Fischle et al., 2003; Holliday, 2006; Lanzillotta et al., 2013; Schweizer et al., 2013). Therefore, histone acetylation is considered to be an important contributor of gene regulation involved in neuronal survival and synaptic plasticity. Aberrant histone acetylation have been linked to various pathological conditions like neurological disorders and neurodegenerative disorders (Kontopoulos et al., 2006; Yildirim et al., 2014). In contrast, the role of histone acetylation in cerebral I/R injury is poorly understood. Further, neuroprotective effects of several HDAC inhibitors against cerebral I/R injury suggest the involvement of histone acetylation during ischemic injury (Kim et al., 2009, 2007). In the present study, we found a decrease in histone H3 acetylation following induction of cerebral I/R injury, which was in harmony with earlier reports (Faraco et al., 2006; Lanzillotta et al., 2013). This suggests that histone acetylation status determines the neuropathological outcomes following cerebral I/R injury. However, histone acetylation is crucially associated with induction of ischemic tolerance

of neurons against prolonged I/R injury is not clear. To our surprise, we observed that RIPOC intervention at the onset of reperfusion increased the histone H3 acetylation levels.

Epigenetic transcriptional regulation by histone acetylation depends upon balance between histone acetyl transferases (HAT) and histone deacetylases (HDAC). A number of recent reports focused the role of Silent Information Regulator (SIRT1), NAD dependent type III HDAC in cell survival under conditions of cellular stress (Giannakou and Partridge, 2004; Peng et al., 2015; Yeung et al., 2004). Further studies revealed the role of SIRT1 in neuronal development, metabolic regulation and learning and memory (Koronowski and Perez-Pinzon, 2015; Michán et al., 2010). Dysregulation of SIRT1 pathway has been linked to cancer, various neurological and neurodegenerative disorders, metabolic complications such as diabetes and obesity (Lin and Fang, 2013; Ng et al., 2015; Shah et al., 2017). The substantial evidence demonstrating the role of SIRT1 in exerting neuroprotective properties in various neurological disorders prompted the investigation of SIRT1 in cerebral ischemia, where its role is not well established. As SIRT1 activity is dependent on NAD, enhancing NAD bioavailability has gained much attention in recent times. NAMPT is the rate limiting enzyme in the NAD biosynthesis and is one of the most important regulators of NAD pools in the cell. Further, due to biosynthetic activity of NAD, NAMPT may influence the activity of NAD dependent enzymes like poly(ADP-ribosyl) polymerases(PARP), sirtuins (SIRT) (Jing et al., 2014; Morris-Blanco et al., 2014; Stein et al., 2014). Earlier invitro and in vivo studies have revealed the importance of NAMPT in various metabolic stress conditions including acute lung injury, atherosclerosis, cancer, diabetes mellitus and rheumatoid arthritis(Houtkooper et al., 2010; S.-N. Wang et al., 2016a; L. Q. Zhang et al., 2011). Rapid accumulating evidences over past decade suggest the role of NAMPT/NAD pathway in vascular repair (P. Wang et al., 2016; Yamamoto et al., 2014), indicating the possible involvement of NAMPT signaling in mediating protection against cerebral ischemic injury. Recent studies have also shown that NAMPT over expression enhanced the functional recovery and survival rate following transient focal ischemia (Jing et al., 2014). Therefore, the activation of NAMPT/NAD pathway may represent an effective way to regulate SIRT1 activity. However, it remains unknown whether NAMPT/NAD/SIRT1 pathway is involved in ischemic tolerance to protect neurons against cerebral I/R injury.

Surprisingly, in this study we observed that RIPOC increased oxidative stress, hippocampal CA1 damage along with memory deficits by RIPOC in presence of NAMPT inhibitor, FK866, indicating that RIPOC mediates neuroprotection against cerebral I/R injury via NAMPT activation. Further, RIPOC improved NAD and SIRT1 levels, while the protective effects were abolished by FK866, suggesting the involvement of NAMPT/NAD/SIRT1 pathway in RIPOC mediated neuroprotection. Moreover, administration of NAMPT activator, provided neuroprotection against cerebral I/R injury and associated cognitive deficits as indicated by reduced oxidative stress and improved memory along with improvement in healthy neurons in hippocampal CA1 region. In addition, P7C3-A20 improved NAD and SIRT1 levels in accordance with recent studies (Balmuth-Loris, 2017; Loris et al., 2017) and (S.-N. Wang et al., 2016b). Further, SIRT1, an NAD dependent deacetylase is reported to regulate the activity of various transcription factors like FOXO, heat shock factor-1 (HSP-1), nuclear factor kappa beta (NF-κB) and transcriptional co-activators such as Peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α)(Lee and Goldberg, 2013; Nemoto et al., 2005; Oeckinghaus and Ghosh, 2009; Westerheide et al., 2009) in response to stress. In a growing list of transcription factors that mediate changes in SIRT1 expression, recent studies highlighted the importance of CREB-regulated transcription co-activator 1 (TORC1) for SIRT1 mediated neuronal plasticity and memory (Cui et al., 2012; Jeong et al., 2012). Under physiological conditions, SIRT1 was reported to deacetylase and activate TORC1 to promote its interaction with CREB and thereby enhance BDNF expression to mediate CREB dependent neuronal survival and synaptic plasticity. The TORC1/CREB/BDNF signaling is recognized to play critical role in neuronal development, neuronal survival and maintenance of energy balance (Sasaki et al., 2011; Q. Xu et al., 2015). These observations raised the possibility that SIRT1 may regulate CREB and BDNF expression to induce neuronal survival and improve memory. In our study, we observed that RIPOC up-regulated the levels of CREB and BDNF that was abolished in presence of NAMPT inhibitor FK866. In contrast, NAMPT activation up-regulated the levels of CREB and BDNF, that clearly established that these effects were mediated by NAMPT mediated SIRT1 up-regulation. Thus, RIPOC may exert neuroprotection by activation of NAMPT/NAD/SIRT1 pathway. Further, up-regulation of SIRT1 levels may provide beneficial effects against cerebral I/R injury and associated cognitive deficits.

6.4 *Combination potential of SIRT1 activator (Resveratrol) and GSK-3 inhibitor (lithium chloride) or HO-1 activator (hemin)*

Based on analysis of our previous studies on neuroprotective role of RIPOC against cerebral I/R injury and associated cognitive deficits, we observed that SIRT1 up-regulation, HO-1 induction and GSK-3β inhibition may result in neuroprotective effect against cerebral I/R injury and associated cognitive deficits. Growing knowledge on naturally occurring compounds indicate that resveratrol protect cerebral ischemic damage mainly due to its anti-oxidant and anti-inflammatory properties(Son et al., 2013; Zhuang et al., 2003). Further, recent studies have shown that beneficial metabolic effects of resveratrol may be mediated through activation of SIRT1 (Lakshminarasimhan et al., 2013; Wan et al., 2016). Despite the acceptance of the fact that resveratrol promotes neuroprotection via SIRT1 activation, little is known about its downstream substrates and signalling cascades. Resveratrol has been reported to improve cell survival possibly by induction of Nrf2 mediated HO-1 induction (HUANG et al., 2005; Son et al., 2013). Moreover, earlier reports also suggest a cross-talk between SIRT1 and GSK-3β modulation in mediating cell survival (Lin et al., 2014; Simão et al., 2012). Moreover, data from our study indicated that RIPOC protection against cerebral I/R injury is mediated through modulation of SIRT1, HO-1 and GSK-3β, suggesting a possible cross-talk between excitotoxicity and epigenetic pathway. Therefore, we have investigated the possibility of modulation of GSK-3β and HO-1 by SIRT1 activation to exert neuroprotective properties. Data from earlier studies suggested that resveratrol is well tolerated, however, resveratrol may lead to mild to moderate gastrointestinal symptoms on higher doses (Brown et al., 2010; Cottart et al., 2010). Previous studies demonstrated toxicity with lithium chloride and hemin high dose treatment (Ahmad et al., 2011; Ghosh et al., 2013; Giles and Bannigan, 2006). We observed that resveratrol (SIRT1 activator), lithium chloride (GSK-3β inhibitor) and hemin (HO-1 activator) did not show any significant effect on GSK-3β and HO-1 expression at sub-effective dose. Thus, we designed a study to investigate the beneficial effects of sub-effective dose combination of resveratrol with lithium chloride or hemin against cerebral I/R injury and associated cognitive deficits. We found that a sub-effective combination was protective in providing neuroprotection against cerebral I/R injury and associated cognitive deficits as evidenced by reduced oxidative stress,

neuroinflammation and reduced hippocampal CA1 damage. In addition, we observed significant inhibition of GSK-3β with sub-effective dose combination of resveratrol and lithium chloride, while significant induction of HO-1 with sub-effective dose combination of resveratrol and hemin. Further, we observed that the above-mentioned combinations improved the levels of CREB and BDNF. Altogether, these represent that neuroprotective effect of resveratrol may be mediates through SIRT1 and HO-1 induction, GSK-3β inhibition and subsequent up-regulation of its downstream modulator CREB followed by BDNF thereby resulting in prevention of neuronal damage following cerebral I/R injury and associated cognitive deficits. Our findings provide evidence that this combination could be putatively used to treat cerebral I/R injury and associated cognitive deficits along with the potential advantage of reduced dose dependent side   effects.

7. *Summary and conclusion*

- Cerebral I/R injury of 20 min ischemia and 72hour reperfusion provided consistent neuronal damage and cognitive deficits as evidenced by increased oxidative stress, neuroinflammation and hippocampal neuronal damage that is well correlated with cognitive deficits

- Likewise, we have standardised RIPOC and found that by 3 cycles of 10 min ischemia and 10 min reperfusion was most effective when compared to other algorithms. Further, RIPOC induced at early onset of reperfusion is most effective than delayed RIPOC

- During cerebral I/R injury, oxidative stress, neuroinflammation and glutamate mediated NR2B activation is the critical step in the pathology of cerebral I/R injury

- To explore the molecular mechanism of RIPOC in cerebral I/R injury induced excitotoxicity, we administered NR2B agonist-Quinolinic acid (QA) prior to RIPOC intervention and found that the protective effects of RIPOC were abolished by QA, indicating that RIPOC mediates protection via NR2B inhibition

- This was confirmed by protective effects of NR2B antagonist, Ifenprodil treatment against cerebral I/R injury and associated cognitive deficits. However, the protective effects of Ifenprodil were abolished by PI3K-Akt inhibitor indicating that NR2B inhibition requires PI3K-Akt activation to mediate its neuroprotection against cerebral I/R injury and associated cognitive deficits

- Strikingly, we found that RIPOC protection is abolished by LY294002, indicating that RIPOC mediates neuroprotection by PI3K-Akt activation. Moreover, we noticed that RIPOC inhibits GSK-3β and up-regulates the levels of HO-1, CREB and BDNF, however, this protective effect was abolished by LY294002, indicating that RIPOC mediates neuroprotection by PI3K/Akt/GSK-3β and PI3K/Akt/HO-1 pathways

- However, multiple complex pathways in pathological cascade of cerebral I/R injury converge at mitochondrial dysfunction. To explore of role of RIPOC in mitochondrial

dysfunction, we pre-treated rats with mPTP opener atractyloside and found that RIPOC protective effects were abolished by atractyloside. This indicates that RIPOC mediates its neuroprotection against cerebral I/R injury by preventing the opening of mPTP

➤ Energy failure/deficit and excitotoxicity results in changes in gene expression leading to epigenetic modifications particularly histone acetylation. In this context, we observed a global reduction of histone H3 acetylation levels following cerebral I/R injury. However, RIPOC intervention at early reperfusion improved the histone H3 acetylation levels

➤ Histone acetylation is dynamic process that may be modulated due to altered levels of HAT and HDAC particularly NAD dependent HDAC III (SIRT1). Further, NAMPT is the rate limiting enzyme in the NAD synthesis, therefore we explored the role of NAMPT/NAD/SIRT1 pathway in cerebral I/R injury and associated cognitive deficits

➤ We noticed that cerebral I/R injury resulted in reduced levels of NAD and SIRT1, while RIPOC early intervention improved the levels of NAD and SIRT1. However, the protective effect of RIPOC was abolished in presence of NAMPT inhibitor FK-866, indicating that RIPOC exerts neuroprotection via NAMPT activation. This was supported by protective effects of NAMPT activator A-20 against cerebral I/R injury and associated cognitive deficits

➤ Based on the above results, it is clear that RIPOC mediates protection by two different pathway i.e modulation of epigenetics (SIRT1 activation), and excitotoxicity (GSK-3β inhibition and HO-1 up-regulation).

➤ Therefore, we designed a study to explore the possible potential of sub-effective dose combination of i) resveratrol (SIRT1 activator) and lithium chloride (GSK-3β inhibitor), ii) resveratrol and hemin (HO-1 activator) and found that the combination exerts strong neuroprotective effect against cerebral I/R injury and associated cognitive deficits.

In a nutshell, cerebral I/R injury induce bioenergetic failure/deficit and subsequent excitotoxicity may act as trigger to mediate disease progression via increased oxidative stress, neuroinflammation and inhibition of survival kinases that ultimately result in mitochondrial dysfunction. All these processes during cerebral I/R injury caused changes in gene expressions

leading to epigenetic modulation. However, RIPOC intervention at the early onset of reperfusion modulated the trigger, mediator and end-effector pathways to induce neuroprotection against cerebral I/R injury and associated cognitive deficits. To conclude, RIPOC may be developed as a therapeutic intervention to treat emergency conditions like cerebral I/R injury. A sub-effective dose combination of resveratrol and lithium chloride or hemin could be used for long term therapy of cerebral I/R injury and associated cognitive deficits. However, future studies are warranted to investigate the molecular mechanism in the neuroprotection of RIPOC and to explore the underlying changes in the disease pathology of cerebral I/R injury.

Salient findings of the study:

- In this study, we found cerebral I/R injury induced consistent neuronal damage and cognitive deficits

- RIPOC intervention at the early onset of reperfusion modulated the trigger, mediator and end-effector pathways in the pathological cascade of cerebral I/R injury to induce neuroprotection against cerebral I/R injury and associated cognitive deficits.

- RIPOC mediated neuroprotection by modulation of trigger and mediator pathway via inhibition of NR2B and subsequent activation of PI3K-Akt via modulation of its downstream regulators GSK-3β and HO-1

- In addition, RIPOC modulated the end-effector pathway by preventing mitochondrial dysfunction via inhibition of mPTP

- Further, RIPOC modulated the epigenetic pathway by improving histone H3 acetylation levels and via activation of NAMPT/NAD/SIRT1 pathway

- Sub-effective dose combination of resveratrol with lithium chloride or hemin exhibits strong neuroprotective action against cerebral I/R injury and associated cognitive deficits compared to either drug alone.

8. Future Scope and limitations of the study

The current research work analyzed the neuroprotective effects of RIPOC against cerebral I/R injury and its associated cognitive deficits. Based on these findings, utilizing the evidence obtained in this work future research can be performed in following areas

➢ Future studies may include various time points of reperfusion following induction of cerebral I/R injury to get a more detailed view of pathology involved in the disease progression

➢ A detailed study on role of I/R injury and RIPOC on other epigenetic modifications such as DNA methylation and other histone modifications are required to understand the epigenetic modifications under normal and pathological conditions

➢ Although NAMPT activators and inhibitor were used in the present to study to investigate NAMPT role in cerebral I/R injury, expression of NAMPT at various time point of reperfusion may improve our understanding on the multifaceted and complex role of NAMPT

➢ Studies to investigate the toxicological and safety profile of tested drugs should be performed to determine its long-term usage. Further, the combination index of the tested drugs should be calculated to determine synergistic or additive effect of combination

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Other publication

- Sharma, S., Taliyan, R. & Ramagiri, S. Histone deacetylase inhibitor, trichostatin A, improves learning and memory in high-fat diet-induced cognitive deficits in mice. J. Mol. Neurosci. 56, 1–11 (2015).

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- Ramagiri, S & Taliyan, R. Beneficial role of HO-1 upregulation against cerebral ischemic stroke. Cerebrovasc. Dis. 1–2 (2016).

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- Sruthi Ramagiri, Rajeev Taliyan: Mitochondrial KATP channel role in neuroprotective effect of RIPOC against cerebral I/R injury (MPDDNP- Chitkara university-Punjab), Mar-27-28, 2015 (**Best Poster-First prize**).

- Sruthi Ramagiri, Rajeev Taliyan: Role of GSK-3β and mPTP in RIPOC against cerebral stroke, (NDCS-BITS Pilani), Oct 16-18, 2015.

- Sruthi Ramagiri, Rajeev Taliyan: Neuroprotective effect of HDAC inhibitor against cerebral I/R injury (MENA stroke conference- Dubai), Oct 22-23, 2015.

- Sruthi Ramagiri, Rajeev Taliyan: Beneficial neuroprotective effect of GSK-3β inhibitors against brain stroke and associated cognitive impairments in diabetic rats (INDO Diabetes EXPO-Bangalore), Nov 23-25, 2015.

- Sruthi Ramagiri, Rajeev Taliyan: Beneficial role of HO-1 upregulation against cerebral ischemic stroke (25th European Stroke Conference – Venice), Apr 13-15, 2016.

- Sruthi Ramagiri, Rajeev Taliyan: Neuroprotective Effect of RIPOC Via Mitochondrial KATP Opening Against Cerebral I/R Injury (WCDDC-IISC Bangalore), Nov 23-25, 2016.

- Sruthi Ramagiri, Rajeev Taliyan: RIPOC attenuates cerebral I/R injury and associated cognitive deficits in rats: Role of GSK-3β and BDNF (Neuroscience School-IBRO-Chandigarh), Dec 14-22, 2016 (**Best poster**).

- Sruthi Ramagiri, Rajeev Taliyan: Protective effect of RIPOC against Cerebral Ischemic Reperfusion injury and associated cognitive deficits (ICCD3-2017- Pilani), Mar 2-4, 2017.

- Sruthi Ramagiri, Rajeev Taliyan: Neuroprotective role of RIPOC against cerebral stroke: Role of PI3K-Akt pathway (NRIPSCON 2017-Ghaziabad), 1-2 Sep, 2017.

Biography of Dr. Rajeev Taliyan

Dr. Rajeev Taliyan is currently working as an Assistant Professor in the Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Pilani-campus, Rajasthan. He has earned his PhD (Pharmacology) under the supervision of Prof. P.L Sharma (Emeritus, Prof. PGIMER) and Late Prof. Manjeet Singh (Ex Head, Dean, Punjabi University) from ISF college of Pharmacy, Punjab Technical University, Punjab. He has been involved in teaching and research for past one decade. He has vast experience in the field of Neuropharmacology, Cardiovascular pharmacology and Drug toxicity. He has been awarded with various research projects from DST, UGC, ICMR and BITS-Pilani. He has also several collaborations and projects with Pharmaceutical Industry such as Etica Clin Pharm Pvt Ltd. He has guided more than 15 students for their post-graduation dissertation. He has been awarded with many prestigious awards at international and national level including, Prof. Manjeet Singh Gold medal award at IPSCON-2015; PP SuryaKumari Gold Medal Award at IPSCON-2014. He has been invited by several Research and Academic institutes for delivering guest lectures including GLA University, Mathura, Rayat Bahra, Ram-Eesh University, and Shoolini University. He has published several papers in peer reviewed international and national journals and in conferences of international and national repute. He is life-time member of Indian Pharmacological Society and British Pharmacological Society, UK.

Biography of R. Sruthi

Mrs. R. Sruthi enrolled as a PhD student in September, 2013 under the supervision of Dr. Rajeev Taliyan in the Department of Pharmacy, Birla Institute of Technology and Science, Pilani-campus, Rajasthan. Her area of interest is to explore the molecular mechanisms involved in pathological cascade of neurological and neurodegenerative disorders such as stroke and Parkinson's. She completed her graduation (B. Pharmacy) from Kakatiya University and was honored with Masters in Pharmacy from NIPER Mohali. She has been awarded with many prestigious awards including, travel grant from IBRO to attend Neuroscience School (2016) and IBRO/APRC Associate School (2017), DST travel grant to attend European Stroke Conference (2016), DBT travel grant to attend MENA stroke conference, Best Poster Award at Chitkara university (2015) and at Neuroscience School at Punjab University (2016). She has published

papers in peer reviewed international journals and delivered presentations in conferences of international and national repute.

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