CHAPTER I

INTRODUCTION

Cerebral stroke is the second leading cause of death worldwide and accounts for about 5.7 million deaths per year (Anthony et al. 2013, Alan et al. 2014). In India, the average annual incidence of stroke is about 84-262 in 100,000 and 334-424 in 100,000, in rural and urban populations respectively (Jeyaraj et al. 2013). Nearly 85% of all strokes are ischemic in origin and the remaining 15% is hemorrhagic in nature (Christine, 2014). Cerebral ischemia is the consequence of transient or permanent occlusion of the middle cerebral artery (MCA) in humans (Jin et al. 2014). Cerebral ischemia is a condition in which there is reduced blood supply to the brain leading to hypoxic state. Decreased blood supply ensues metabolic insufficiency and energy crisis in the brain tissues leading to neuronal death ultimately resulting in enduring physical disabilities or mortality (Vespa et al. 2009).

Cerebral ischemia is caused due to various reasons such as vascular diseases (arteriosclerosis), atherosclerosis, trauma, hypertension, severe hypotension, formation of thrombus or embolus, congenital heart defects, diabetes, hypercholesterolemia, stress, increasing age and sickle cell anaemia. Cerebral ischemia can cause cognitive, sensory and motor function deficits. Clinical signs of cerebral ischemia include apnoea, headache, abdominal pain, sudden loss of memory, difficulty in speech, writing or reading, brief or prolonged loss of consciousness, numbness, paralysis, difficulty in walking or moving body parts, in coordination, visual loss or impairment, vomiting, profuse sweating, unusual anxiety, cold, blue or darkened extremities of the body (Alvarez and Gustavo, 2013).
Cerebral ischemia is of two types (i) focal, where the insult is confined to a specific area (ii) global ischemia, where major part of the brain is affected. Focal cerebral ischemia is caused due to the blockade of one of the major cerebral arteries, hence the insult is confined (Durukan et al. 2007), whereas, global ischemia crops up due to reduced or ceased blood flow to the brain due to complete obstruction of the arteries usually elicited by cardiopulmonary arrest. Restoration of the blood flow within short duration may exhibit brief symptoms and restoration after long duration of ischemia may result in permanent cerebral damage. Focal ischemia is again classified into three categories, thrombotic, embolic and hypoperfusion of which thrombotic and embolic stroke are the most common (Kensaku et al. 2009).

1.1 Pathophysiology of cerebral ischemia

The severity and progression of ischemic injury depends upon various factors such as rate of onset and duration of ischemia, the intensity of collateral circulation, systemic hypotension, hematological factors, hypothermia and glucose metabolism (Chang et al. 2006). Cerebral blood flow (CBF) is approximately 50-60 ml/100g/min and this differs in various parts of the brain (Sang-Pil et al. 2001). The cerebral auto regulatory mechanisms compensate the reduced blood flow by vasodilation, enhanced collateral circulation and increased the utilization of glucose and oxygen from the blood. Yet, the decrease in CBF below 20 ml/100g/min results in synaptic silence in an attempt to conserve the energy stores and when the CBF reaches below 10ml / 100g/ min, irreversible neuronal injury occurs (Kumar et al. 2008). Ischemia triggers the release of proteolytic enzymes from endothelium, leucocytes, and thrombocytes inducing an domino effect i.e., resulting in the formation of thrombi, which in turn results in the mechanical plugging of capillaries (Kristian et al. 2008).

The progression of neuronal damage is crucially influenced by the overactivity of the excitatory neurotransmitters glutamate and aspartate. The phenomenon of increased extracellular accumulation of glutamate is called as excitotoxicity. The released glutamate is normally cleared from the extracellular space by an energy dependent process. Under ischemic condition, the depletion of energy reservoir leads to reduced clearance of glutamate from the extracellular space leading to the accumulation of glutamate and its over activity (Excitotoxicity). Increased extracellular glutamate and aspartate activates N-methyl1-
D-aspartate (NMDA) and Alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate (AMPA) receptors and leads to membrane depolarization which in turn triggers increased influx of calcium, sodium and chloride ions and efflux of potassium ions in the post synaptic neurons (Chrysanthy, 2002).

The reduction in the energy stores alters the sodium potassium gradient and also distorts the cellular calcium homeostasis which in turn causes substantial increase in intracellular calcium. Increased intracellular calcium activates various proteolytic enzymes such as proteases, endonucleases and lipases, and triggers the generation of cytokines and other inflammatory mediators thereby hammering the cellular damage (Diana et al. 2009). Cytokines and other inflammatory mediators further elicits the production of many other inflammatory mediators such as tumour necrosis factor (TNF-α). TNF-α produced activates TNF-R1, that develops a complex I on the plasma membrane. Complex I recruits other proteins such as fas-associated protein with a death domain (FADD and pro-caspase 8) (Complex II), where in pro-caspase 8 is transformed into caspase 8. Activated caspase 8 interacts with Bcl-2-associated X protein (BAX) and forms pores on the mitochondrial outer membrane leading to the liberation of cytochrome c (CYT C) which initiates apoptotic cascades ultimately leading to neuronal death (Heather et al. 2015) (Fig. 1.1).

Infiltration of leukocytes occurring due to increased intracellular accumulation of calcium, activates vasoactive substance such as nitric oxide and arachidonic acid metabolites. These mediators ensues in vasodilatation, vasoconstriction, increased vascular permeability, increased platelet aggregation and adherence of leukocyte to endothelial wall resulting in the swelling of endothelial cells and formation of microvilli. Furthermore this results in the mechanical plugging of RBCs, WBCs and platelets in the capillary beds. In addition injured endothelial cells also activates endothelin peptides, eicosanoids and induces the production of nitric oxide and in turn initiates the inflammatory process (Diana et al. 2009).

Increased intracellular calcium in post synaptic neurons, causes overloading of calcium in the mitochondria, which inturn impairs the process of oxidative
phosphorylation and thereby produces mitochondrial mutilation. Mitochondrial calcium accumulation also activates membrane phospholipases and protein kinases and triggers the production of free fatty acids (FFA’s) and other membrane degraders (Kuresh et al. 2000). Mitochondrial membrane failure further increases the intracellular calcium overload thereby leading to various putative mechanisms such as production of free radicals. In turn the mitochondrial calcium overload leads to decreased ATP generation and impaired oxidative phosphorylation which triggers the formation of free fatty acids (FFA’s) and other long chain fatty acids that are deleterious to various mitochondrial enzyme systems ultimately leading to DNA damage and neuronal death (Massimo and Paolo 2014). The intracellular calcium increase triggers free radical production by inducing the conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase produces superoxide and other free radicals by combining with oxygen during reperfusion (Klas et al. 2006). Reoxygenation during reperfusion also restores ATP thereby increasing the calcium uptake by mitochondria, further leading to mitochondrial calcium uptake and overload (Safar 1986). Mitochondrial calcium overload and free radicals generation contributes to mitochondrial structural changes such as the formation of mitochondrial membrane transition pore and mitochondrial matrix swelling (Sesso et al. 2012).

Generation of reactive oxygen species triggers caspase dependent and caspase independent neuronal death pathways (McManus et al. 2014). In addition to the cellular oxidative damage to biomolecules, ROS (reactive oxygen species) also reacts with β-Amyloid (Aβ ), a kinase activator that protects cells from oxidative stress, (Baruch and Fischer 2009) thereby leading to neuronal death. ROS also triggers the release of Cytochrome c from the mitochondrial membrane indirectly via the translocation of p53 which controls the expression of genes, namely Bax, Bid and p53 upregulated modulator of apoptosis (PUMA) (Endo et al. 2006). They act directly on the mitochondria and instigate neuronal apoptosis.

Oxidative stress also mediates apoptosis and ROS-induced cell death by another caspase independent pathway by decreasing calcium and / or calmodulin-dependent protein kinase, and cAMP responsive element-binding protein (CREB). Another conduit of oxidative damage is prompted by a transcription factor FOXO3, a wide spread neurotrophic
Inhibition of FOXO3 by the insulin like growth factor – I (IGF-I) during ischemia reduces the generation of ROS (Sonntag et al. 2006, and Aleman, 2005). Certainly, IGF-I protects against neuronal insult and is an arbitrator in revival of the ischemic injury (Fernandez et al. 1999). Activation of FOXO3 by a burst of ROS rapidly attenuates IGF-I mediated AKT inhibition of FOXO3 through p38 MAPK. FOXO3 is also activated secondarily by Jun-kinase 2 (JNK2), which is activated by remarkable blockade of IGF-I signaling through p38 MAPK (Davila and Torres 2008). Recent investigation also revealed that alternatively, adiponectin, a polypeptide hormone mediates the activation of pro-apoptotic signaling cascade of p38 mitogen activated protein kinase (p38MAPK) and AMP-activated protein kinase (AMPK) (Thundyil et al. 2010). Reactive oxygen species causes activation of pro matrix metalloproteinases (proMMP) thereby leading to degradation of the neurovascular matrix and ultimately leading to destruction of BBB (Jian & Rosenberg et al. 2005). Also, under the conditions of oxidative stress, a main transcription factor binds to the promoter region of MMP-9 called NF-κB. This results in degradation of collagens and laminins in the basal lamina leading to disruption of vascular wall integrity and BBB permeability.

Within an hour of ischemic episode, an area of infarction (core) and the penumbra is formed. Ischemic penumbra is characterized by compromised energy metabolism with intact cells and cellular functions due to the 25 to 50 % normal CBF in this region. The crucial time point at which the brain tissues at risk can be partly or completely protected from ischemic insult by reperfusion is known as “window of opportunity”. Ischemic penumbra is also characterized by the production of spontaneous waves of depolarization (SWD), which originates from various foci in the ischemic foci or the peri infarct zone (penumbra). This SWD sustains the increase in synaptic glutamate and extracellular potassium. However, rapid depolarizations eventually precedes irreversible neuronal damage (Timothy et al. 2008). Progression to neuronal death involves two processes, necrosis and apoptosis. In necrosis, individual cells die without inducing inflammatory response in other cells. Necrosis occurs due to physical, chemical or osmotic damage of the cell membrane. The neurons swells and then shrinks and nuclear shrinkage (pyknosis) occurs followed by pan-necrosis, where in the myelin sheaths degenerate and the cytoplasm becomes eosinophilic. This type of neuronal death happens by 6-12 hours of ischemic insult (Isin et al. 2004). The other mode of cell death
in ischemia is apoptosis or programmed cell death, wherein the nuclear damage happens first followed by the release of autolytic enzymes due to DNA damage. Finally the neurons undergo death by the activation of apoptotic mediators such as Bax and caspase. Apoptotic cell death is instigated within 1 hour of ischemic insult whereas coagulation necrosis happens after 6 hours of ischemia (Brad et al. 2009).

1.2 Reperfusion Injury

Although treatment of ischemia depends on the root cause, it is normally aimed at restoring blood flow and reducing further tissue injury and death. Though reperfusion of blood is essential to reduce cerebral damage, reperfusion itself can cause injury (Jie et al. 2007, Jaroslaw et al. 1997). If the ischemic episode is brief and the blood flow is restored immediately, the core and penumbral region recovers from the ischemic damage. If reperfusion occurs after moderate ischemia, selective neuronal death occurs (Schaller et al. 2004). The degree and extent of the neuronal damage depends on the duration and severity of ischemic insult.

Reperfusion is putative when it happens late after the ischemic event (Aronowski et al. 1997, Kuroda et al. 1997). Reperfusion injury may result into aggravation of vasogenic edema or hemorrhagic infarction, loss of vasoreactivity, disruption of the blood brain barrier, free radicals generation, release of inflammatory mediators. Leukocytes play a key role in cerebral reperfusion injury. Activated leukocytes roll on the endothelium and firmly adheres to it and extravasate into the interstitial spaces (Schaller et al. 2004, Harlan et al. 2002) blocks the capillaries and disrupts the BBB. Collapse of BBB during reperfusion progresses to the development of edema, hemorrhagic transformation and infarction (Gary et al. 2007).

On the other hand, generation of superoxide free radicals due to reperfusion upregulates endothelial P-selectin and this reacts with leukocytes (Manabu et al. 2002). Leukocytes and endothelial cell reaction, triggers the expression of platelet-endothelial cell adhesion molecule –1 (PECAM-1) (Jacek, 2013). After infiltration into the parenchyma, leukocytes release, neutrophils, reactive oxygen species (ROS), leukotrienes and
prostaglandins resulting in increased vascular permeability of the capillaries, edema, thrombosis and cell death (Carden et al. 2000). Investigations have revealed the crucial role of complement systems in reperfusion injury (Ambrosio et al. 2001). Activation of complement system results in the formation of several inflammatory mediators such as C3a, C5a and C5b-9 (membrane attack complex (MAC) (Collard et al. 1999).

C5a triggers leukocyte infiltration in the penumbra and amplifies the inflammatory response by triggering the release of several proinflammatory cytokines such as Tumour necrosis factor α (TNF-α), IL-1 and IL-6 (Meyer et al. 2001). MAC injures cell membrane and increases cell membrane permeability. Furthermore, MAC attracts and infiltrates leukocytes into the reperfused tissue through IL-8 (Fung et al. 2003, Horstick 2002).
Figure 1.1 Pathophysiology of Cerebral Ischemia

Diminution of blood flow leads to the depletion of oxygen and ATP which leads to the failure of ionic pumps and reduced uptake of extracellular glutamate and increased activation of the receptors, NMDA (A), AMPK (B) and Kainate (C) which in turn leads to the accumulation of extracellular glutamate and intracellular calcium increase. Intracellular calcium increase leads to the following (1) Activation of enzyme phospholipases and proteases (2) Production of free fatty acids and arachidonic acid (3) Release of inflammatory mediators (4) Membrane disintegration. Intracellular calcium increase also leads to the generation of reactive oxygen species in the mitochondria. (5) Oxidative and nitrosative stress due to increased oxidative and nitrosative stress.

1.3 Current treatment strategies in cerebral ischemia

Treatment of cerebral ischemia still remains daunting due to its complex pathophysiology and lack of time dependent therapeutic intervention (Trent et al. 2012). Till date there is no highly effective and safe treatment available for acute ischemic stroke. Though
treatments such as thrombolytic agents (tissue plasminogen activator and pro-urokinase) and other endovascular procedures are currently available, pitfalls like long and poor recanalization rates, vascular rupture and vasospasm restricts their use (Paramdeep et al. 2013; Arnaout et al. 2012; Davalos et al. 2005), and the scope of such treatment therapies is limited especially in developing countries (Jian et al. 2014).

Extensive investigations over the past few decades, reveal membrane damage and generation of overt nitric oxide as the crucial ground in devastating effects of cerebral ischemia and reperfusion. The increase in glutamate level activates phospholipases leading to the hydrolysis of phospholipids and release of arachidonic acid (Ava and Sandra 2002). Subsequent oxidation of arachidonic acid contributes to the generation of reactive oxygen species (Rao et al. 2007). Increased reactive oxygen species leads to mitochondrial mutilation, which in turn results in the imbalance between the pro-oxidant and anti oxidant levels augmenting oxidative stress (Chan, 2001). Massive release of nitric oxide triggered by the calcium dependent activation of neuronal NOS (nNOS) and inducible form of NOS (iNOS) leads to nitrosative stress which in turn leads to neuronal death due to the formation of putative peroxynitrite. In particular, the concentration of NO is augmented about 50% in the ischemic regions within 30 minutes of reperfusion (Iadecola et al. 1995). Hence, protection of neuronal membrane and inhibition of generation of nitric oxide is considered as a potential target in acute ischemic stroke treatment (Nicole et al. 2012). Though the neuroprotection afforded by NOS inhibitors and membrane stabilizers are demonstrated in many studies, these molecules tend to fail in clinical trials due to lack of knowledge on their suitable therapeutic time window.

Since, Citicoline and L-NAME has been revealed as most promising candidates for acute ischemic stroke in preclinical research by various researchers (Nicole et al. 2012, Sahota et al. 2011, Alvarez and Gustavo 2013, Diederich et al. 2012; Xu et al. 2011, Bemeur et al. 2004), current study has been designed to investigate the therapeutic time window of citicoline and L-NAME. Although extensive investigations were carried out to establish the optimum therapeutic time window of citicoline and L-NAME in acute ischemic stroke (Margaill et al. 1997; Bemeur et al. 2004), a closer time point investigation i.e. at pre
(before the ischemia, during (on-going ischemia and post ischemic (after reperfusion) state is yet to be studied. In the present study, citicoline and L-Name were administered at three different time points to determine their therapeutic time window in transient focal cerebral stroke rats. Apart from the evaluation of citicoline and L-NAME for the appropriate time window, combination of citicoline and L-NAME has also been evaluated as a multitarget therapeutic approach, since pathophysiological events occurring in acute ischemic stroke is multicomplex in nature (Strong et al. 2007, Lloyd-Jones et al. 2009).

1.4 N’-Nitro-L-arginine-methyl ester hydrochloride (L-NAME)

L-NAME is a nonselective nitric oxide synthase (NOS) inhibitor. The increase in intracellular calcium triggers the generation of nitric oxide from neuronal NOS (nNOS) and inducible form of NOS (iNOS), which augments the oxidative stress burden further, ultimately leading to neuronal death. Nitric oxide formed by the activation of nitric oxide synthases reacts with the reactive oxygen species and produces peroxynitrite, thereby increasing the nitrosative stress. Peroxynitrite induces apoptotic neuronal death through the activation of caspase 3 (Dohi et al. 2003). Nitric oxide can also inhibit enzymes of mitochondrial respiration, glycolysis and DNA synthesis (Moro et al. 2005). Inhibition of NOS is considered as a potential therapeutic target in acute ischemic stroke (Nicole et al. 2012). L-NAME being a non selective inhibitor of nitric oxide synthase, inhibits the formation of nitric oxide and thereby reduces the neuronal injury ensuing cerebral ischemia (Fig. 1.2). Hence, the non selective NOS inhibitor, L-NAME was evaluated for its appropriate therapeutic time window in focal cerebral ischemia in the present study.
Figure 1.2 Mechanism of Action of L-NAME
L-NAME inhibits Nitric Oxide Synthase (NOS) and thereby prevents the formation of peroxynitrite, massive DNA damage and Cell death

1.5 Citicoline (cytidine-5′-diphosphocholine)

Several investigation revealed that drugs with potency to improve neuronal plasticity and neuronal repair reduces neuronal damage and improves neurological and behavioural functions in animal models of cerebral ischemia even when they are administered at the late phase after ischemia (Sahota et al. 2011, Saver, 2010). Citicoline is an important intermediate in the generation of phosphatidylcholine and in the biosynthesis of membrane phospholipids. Phospholipids are degraded into fatty acids and free radicals during cerebral ischemia (Davalos et al. 2011). Citicoline is shown to restore the mitochondrial ATPase and Na\(^+\)K\(^-\)ATPase by inhibiting the activation of phospholipase A2 and it also reduces cerebral edema in experimental models of cerebral ischemia (Secades, 2011). Citicoline is also reported to be a safe and well tolerated (Gutierrez et al. 2012) (Fig. 1.3). In the present study, the suitable therapeutic time window of citicoline was determined by administering it at three different closer time points, such as pre, during and post.
Figure 1.3 Mechanism of Action of Citicoline
Citicoline inhibits the formation of free fatty acids and preserves phosphatidyl choline and sphingomyelin and thereby restores membrane stability.
1.6 Objectives

- To determine the neuroprotective effects and therapeutic time window of L-NAME, a non-selective NOS inhibitor
- To determine the neuroprotective effects and therapeutic time window of Citicoline, a membrane stabilizer
- To determine the neuroprotective effects and therapeutic time window of combination of L-NAME and Citicoline