CHAPTER II

LITERATURE SURVEY

2.1 Pathophysiology of Cerebral Ischemia

Astrup et al. (1981) defined the region of reduced blood flow (below a critical threshold), demarcated with irreversible neuronal damage as the infarct core and the other region where there is residual blood flow near to normal exists to supply sufficient oxygen for maintaining moderate ATP concentration or the area under risk deprived of neuronal death, as the ischemic penumbra. Penumbra is the area with mild to moderate energy imbalance and collapse of ionic pump that encircles the ischemic core.

Reperfusion occurring within 3 hours of ischemia was identified to be beneficial and less detrimental, whereas reperfusion after 3 or 4.5 hours of ischemia exhibited mild or no benefit or might even worsen the injury. The severity of reperfusion injury also could vary, based on the duration and intensity of reperfusion (Hacke et al. 1995).

James (1996) demonstrated their therapeutic time window as the period of extent to which the ischemic penumbra remains viable and also revealed that the spontaneous reperfusion occurring after the event of cerebral ischemia was not beneficial unless it happened within the therapeutic time window, as it will not result in the survival of penumbral or peri-infarct neurons. It was also stated that the therapeutic time window becomes brief depending on the severity of decreased blood flow. It was also demonstrated that severe neuronal death occurred after 10 minutes of decreased flow and a blood flow of about 15 cc/100g tissue/minute keeps the therapeutic time window essentially longer.

Cerebral ischemia is a debilitating disorder involving multicomplex pathophysiological cascades such as glutamate accumulation, failure of ionic pumps, abnormal calcium influx, free radicals formation, oxidative stress, lipid peroxidation, accumulation of nitric oxide, nitrosative stress, mitochondrial membrane damage, damage of blood brain barrier and necrotic / apoptotic neuronal death. The increase in glutamate level
activates phospholipases leading to oxidative stress and neuronal membrane damage (Servitja et al. 2003).

Helen and Dalton (2004) differentiated the pathophysiology of cerebral ischemia and brain trauma. It is known till date that cerebral ischemia and cerebral trauma exhibited the same mechanisms of action that leads to neuronal injury. Mechanisms such as excitotoxicity, oxidative stress, apoptosis and inflammation leads to cellular death in both the cases. Yet, multiple diverse cellular mechanisms were involved in the cellular death depending on the mode of primary insults such as primary neuronal membrane damage, white matter damage, changes in the vasculature, metabolic distress and ionic imbalance occur in cerebral ischemia ultimately leading to neuronal death. Hence, it was stated in the present research that same type of neuroprotective strategies may be employed for the treatment of both the type of brain injuries.

Jie et al. (2007) expounded that though reperfusion is the ultimate target to overcome ischemic injury in cerebral ischemia, reperfusion itself might cause excitotoxicity and inflammatory responses in the ischemic penumbra called ‘Reperfusion Injury’ and therefore restoration of the blood flow to a crucial threshold to attain better beneficial effect has been considered important.

Lai et al. (2011) revealed that reperfusion injury triggered various physiological and biochemical events such as increased extracellular glutamate, increased activation of glutamate receptors (Glutamate Excitotoxicity), increase in intracellular calcium and generation of free radicals and emergence of oxidative stress. Oxidative stress further led to nitrosative stress and activation of PARP and finally neuronal death. Hence, amelioration of glutamate excitotoxicity was considered to be important and crucial in cerebral ischemia.

Cerebral ischemia / reperfusion triggers inflammatory response. Inflammatory response induced by reperfusion is rapid and more potent than ischemia. Intracellular calcium activates complement system, leucocytes and platelets. Stimulated leucocytes release proinflammatory cytokines and proteases which leads to the formation of thrombus. Cytokines
and other inflammatory mediators leads to the breakdown of Blood Brain Barrier (BBB) and further infiltration of leucocytes which ultimately led to the formation of oedema and vasospasm (Pundik et al. 2012).

Activation of inflammatory system in the brain leads to oxidative stress in stroke. Especially complement system plays key role in the inflammatory pathway in cerebral ischemia. Investigation on the role of complement component in oxidative stress after ischemic stroke was studied in mice using DNA array. It was studied whether the up regulation of complement component 3 is directly or indirectly related to oxidative stress after transient focal cerebral ischemia. Persistent up regulation of complement component 3 was reduced in copper/zinc superoxide dismutase transgenic mice and manganese superoxide dismutase knock-out mice after transient focal cerebral ischemia. Small interfering RNA specific for complement component 3 transfection showed significant increase in brain cells after ischemia. Hence, it was concluded that complement component 3 plays direct role in inflammatory pathway in transient focal cerebral ischemia (Yang et al. 2013).

2.2 Use of animal models in stroke

Stefan and Christoph (2009) reviewed the current animal models used in stroke research and also their advantages and disadvantages. According to them, rodent models were the necessary tools in the investigation of stroke. However, many molecules which showed promising results in the preclinical trails were failed in the clinical trials. Hence, they have identified few points to be considered while planning a preclinical research in stroke experiments such as, selection of species, strain, sex, stroke model, anesthetic agent used and the method of anesthesia, randomization of animals before research, determination of pharmacokinetics and dose response curves, monitoring and controlling of physiological parameters such as brain temperature, multiple outcome measurement, combination therapies and performing the experiment in different labs.

2.3 Treatment strategies in cerebral ischemia

The effect of MK-801 in cerebral ischemia and its dose response relationship was studied in a rat model of permanent middle cerebral artery occlusion. MK-801 was
intravenously administered before occlusion followed by an infusion at 4 h to maintain a steady state plasma concentration throughout the study. MK-801 was administered at doses of 0.04 mg/kg + 0.6 µg/kg infusion (Group I); 0.12 mg/kg + 1.8 µg/kg infusion (Group II); 0.4 mg/kg + 6 µg/kg infusion (Group III) respectively. Plasma concentration of MK-801 and infarction size was determined in all the groups to study the dose response relationship. 10 % reduction in the infarction volume was observed in group I followed by significant reduction of 50 and 60 % group II and group III respectively. The highest plasma concentration of MK-801 (113.2 ng ml⁻¹) resulted in a significant reduction of cerebral infarct volume of about 35%. Hence, MK-801 exerts dose dependent neuroprotective effect in cerebral ischemia (Gill et al. 1991).

Anoxic depolarization and failure of ion homeostasis play a key role in ischemic induced neuronal death. Studies show that blockade of sodium influx improves the neuronal outcome. The effect of altered ion homeostasis on ischemia was studied using tetrodotoxin (10 µm) induced cerebral ischemia in isolated perfused rat brain. Tetrodotoxin inhibited EEG activity with delayed ischemic induced tissue acidication and postponed the occurrence of anoxic depolarization by 65%. The elevation of calcium prior to anoxic depolarization was attenuated from 17.8 to 6 % with increase sodium efflux from 209 to 7.3%. These findings implied that the ischemia-induced early cellular sodium load and the corresponding shrinkage of the extracellular space was counteracted by tetrodotoxin. Hence, it is revealed that Na⁺ influx via voltage-dependent channels preceding complete breakdown of ion homeostasis is one of the major factor in cell depolarization. The massive Na⁺ influx coinciding with anoxic depolarization, however, may be mainly via non-selective cation channels or/and receptor-operated channels. Persistent Na⁺ influx deteriorates neuronal tissue integrity by favouring Ca²⁺ influx and edema formation. Blockade of ischemia-induced excessive Na⁺ influx is, therefore, a promising pharmacological approach for stroke treatment (Xie et al. 1994).

The effects of 1-[7-(4-benzyloxyphenoxy)heptyl]piperidine hydrochloride (SB 206284A) was investigated on invitro calcium and sodium in rat cultured dorsal root ganglion (DRG) neurons and potassium mediated calcium influx in rat synaptosomes. Effect of (SB
206284A) was also investigated in in vivo gerbil common carotid occlusion and rat middle cerebral artery occlusion model. In the DRG cells, it caused almost total blockade of calcium influx suggesting it to be an effective calcium channel blocker. It also reduced locomotor hyperactivity in the gerbil model without damaging hippocampal CA1 region and reduced lesion volume in the posterior fore brain in middle cerebral artery occlusion model of rat. Hence, SB 206284 was considered to be a novel calcium channel blocker exhibiting neuroprotective effect (Wood et al. 1997).

Effect of 3-nitropropionic acid (3-NPA) on neuronal survival in global ischemia model of male Wistar rats was studied. 3-NPA was administered in a single dose intraperitoneally before inducing global ischemia and neuronal survival was studied in both neocortex and hippocampus. Expression of Bcl-2 was determined in all the regions at 3, 12 and 24 hours after the administration of 3-NPA and occlusion of middle cerebral artery. Immunohistochemistry was performed to compare the Bcl-2 immunoreactivity in the hippocampus, dentate gyrus and parietal neocortex. 3-NPA caused significant increase in the expression of Bcl-2 protein immunoreactivity in hippocampal neurons and neocortex in a time dependent manner. Neuronal expression of Bcl-2 remained unchanged in CA2 and dentate gyrus. Bcl-2 immunoreactivity might be increased due to the increased levels of reactive oxygen species (ROS) (Brambrink et al. 2004).

Zhao et al. (2006) studied the effect of preconditioning against stroke protection. Ischemic post conditioning was performed after 10 sec of common carotid artery occlusion and 30 sec of reperfusion. Cerebral infarction was measured after 2 days using TTC staining. Post conditioning after common carotid artery occlusion and reperfusion reduced the infarct size and reduced terminal deoxynucleotidyl transferase mediated uridine 5'-triphosphate-biotin nick end labeling positive cells after 2 days in the penumbra.

Jesus et al. (2014) developed a new theranostics system for treatment in cerebral stroke. Theranostics were not currently used due to lack of ability to cross the blood brain barrier (BBB). A series of studies such as proteomic, blotting and histological investigations were carried out to characterize the molecular bio marker expression in the peri-
infarct tissue. HSP72 was identified as the suitable marker for the peri infarct region assessment for up to 7 days after ischemia. Anti-HSP72 vectorized immunoliposome with imaging probes were developed to trace using fluorescence and MRI scanning was used to encapsulate citicoline for the treatment in cerebral ischemia. In vitro and in vivo methods were performed to test the recognition ability of these encapsulated nano molecules for their diagnostic and therapeutic properties. MRI was used to scan the vectorized liposomes and it was observed that around 80% of the same was located in the penumbra of the infarction and the lesion size was reduced to 30% in citicoline treated group. Hence, it was concluded that nanotechnology could potentially aid in the identification of effective therapeutic tools in the treatment of neurological diseases.

2.4 Role of Nitric oxide in cerebral ischemia

Huang et al. (1994) studied the induction of brain injury by nitric oxide and its metabolites. Since, NO formation in the neurons and glia was blocked by non selective agents, it still controversial whether inhibition of NO formation is beneficial or detrimental. Middle cerebral artery occluded / reperfused n-NOS and e-NOS knockout mice were used in the current study to evaluate the effect of inhibition of n-NOS and e-NOS in cerebral ischemia. The infarct volumes were decreased markedly at 24 and 72 hours with improved neurological functioning after occlusion of middle cerebral artery in n-NOS knockout mice. In the e-NOS knock out mice, neurological functioning was improved. However, the infarction size was increased after the inhibition of e-NOS. Hence, it was concluded that nNOS plays crucial in both infarct size and in neurological functioning.

Hideki et al. (1997) investigated the time and cell type dependent immunohistochemical activity of nitric oxide synthase following cerebral ischemia in the cerebral cortex of rat. Nitric oxide content was measured following ischemia and it was observed that its level was increased after 2 min of ischemia. NOS positive neurons were increased after 5 min of ischemia. NOS positive neurons were first detected in the endothelial cells of the blood vessel and then in the astrocytes after 5 min of ischemia. NOS positive cells were increased progressively through 60 min to 4 days in the astrocytes after ischemia. NOS
immunoreactive cells were identified at the penumbra in astrocytes, vascular endothelium and microglia after 2-4 days of cerebral ischemia.

Karen et al. (1997) proposed the mechanism of synaptic regulation of neuronal NOS (nNOS). Many proteins interact with nNOS and target them to the neurons and skeletal muscles. Intracellular calcium influx play a crucial role in regulation of these interactions. One such protein interaction was that, PSD-95 propels nNOS to NMDA in the central nervous system and sarcolemma in the skeletal muscle.

Kazunori et al. (1998) investigated whether nitric oxide synthesized by nNOS using 7-nitroindazole (7 NI) is neurotoxic in global cerebral ischemia in rats. Four vessel occlusion method was adopted and 20 min of global ischemia was induced in the test rats. 7-NI was administered at a dose of 25 mg/kg, intraperitoneally after 1 hour of inducing global ischemia. Electroencephalogram and temperatures of body and brain was monitored throughout the procedure. Histology of the brain sections were performed seven days after global ischemia to assess the damage in hippocampal CA1 region and neuronal count was performed. Decreased infarction size and increased neuronal count was observed in the group administered with 7-NI. It was concluded that 7-NI was neuroprotective in global ischemia and its neuroprotective effect is due to the blockade of nitric oxide synthesis.

Shigero et al. (1998) studied the effect of inhibition of inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase - 2 (COX-2) in focal cerebral ischemia. Focal cerebral ischemia induces both iNOS and cyclooxygenase-2 and thereby ultimately leads to neuronal death. It was also tested the nitric oxide generated by the induction of iNOS activates COX-2. Middle cerebral artery occlusion / reperfusion method was used to induced focal cerebral ischemia in the experimental mice. Twenty four hours after ischemia, iNOS immunoreactive cells were observed near COX-2 positive cells in the penumbra of the infarction and the olfactory bulb only COX-2 was expressed. Ischemia also induced prostaglandin E-2 (PGE₂) in ischemic infarct and also in the ipsilateral olfactory bulb. The iNOS inhibitor amino guanidine, has reduced the expression of PGE₂. Post ischemic expression of PGE₂ was also reduced in the iNOS knockout mice than the normal mice. Therefore, it was concluded that
nitric oxide produced by iNOS plays crucial role in the activation of COX-2 and further increased expression of PGE₂.

Elizabeth et al. (1999) studied the effect of iNOS after traumatic brain damage in iNOS knock out mice. iNOS plays a critical role in triggering inflammatory process triggered following cerebral ischemia and neuronal damage. Controlled cortical impact (CCI) model was used and rats were treated with 2 iNOS inhibitors (Aminoguanidine and L-N-Iminoethyl-lysine) for 5 days and 1.5 days respectively. Motor activity and cognition was assessed for first 20 days after drug administration and also histopathological evaluation was performed on day 21 after inducing traumatic brain injury (TBI). In both the treatments functional deficit was exacerbated with increased neuronal damage in the hippocampal CA1 and CA2. Severe worsening of the hippocampal neuronal damage was observed in iNOS knock out mice than the control.

Juan and Angeles (1999) suggested generation of nitric oxide as a key factor in pathophysiology of stroke (hypoxic ischemia). It was proposed that though several studies have been performed earlier to demonstrate the influence of nitric oxide and conflicting conclusions were proposed, the biochemical studies and selective inhibition of NOS or the use of transgenic animals might provide better understanding about the specific role of NOS inhibition in cerebral ischemia. Cerebral ischemia induces several pathophysiological events through extracellular glutamate accumulation, intracellular calcium accumulation, thereby leading to the activation of calcium dependent NOS isoforms. It was also reported that selective inhibition of nNOS exhibits neuroprotection, whereas, inhibition of eNOS exerts neurotoxic effects. Activation of calcium independent iNOS occurs mainly in the glial cells after prolonged ischemia or reperfusion. Hence, it was proposed that inhibition of nNOS and iNOS ameliorates cerebral damage and activation of eNOS enhances vasodilation leading to improved blood flow thereby reducing cerebral damage.

Mahony (1999) studied nitric oxide as a potential target in the treatment of cerebral ischemia. It was suggested that the neuroprotection was afforded by selective inhibition of nNOS plays crucial role only during first two hours of ischemia. Cerebral
ischemia triggered the accumulation of glutamate in the extracellular space which activates glutamate receptors such as NMDA/Ca\textsuperscript{2+}. Accumulation of intracellular calcium activates many calcium dependent enzymes and neuronal NOS which ultimately leads to the biosynthesis of nitric oxide. Nitric oxide produced may combined with super oxide radicals and forms peroxynitrite, a highly putative molecule. Neuronal NOS plays major role in cerebral hyperemia in hypoxic ischemia. On the other hand inhibition of neuronal NOS might also produce deleterious effects on synaptic plasticity and signaling in neurons. Inhibition of nNOS also activates Nuclear factor-κ β (NF-κ β ), which induces iNOS and in turn increases the inflammatory mediators and leads to cellular and membrane damage in the neurons.

Mikael et al. (1999) reported nitric oxide as a naturally occurring molecule in the brain and has a physiological role. Cerebral ischemia leads to the over production of nitric oxide which reacts with super oxide radical and forms peroxynitrite. Cerebral ischemia was induced in mice by middle cerebral artery occlusion / reperfusion (MCAO/R) method. Citrulline (marker for NOS activity) and 3-nitrotyrosine (marker for peroxynitrite) was assessed in the MCAO/R mice. Citrulline was expressed more in the peri infarct region of the brain than the ischemic core, which attributed to the activation of NOS by cerebral ischemia, whereas 3-nitrotyrosine was found more in the cerebral infarct core than the penumbra. Hence, it was concluded that nitric oxide itself is not neurotoxic and the formation of peroxynitrite from nitric oxide was reported to be more putative.

Dong et al. (2003) studied the benefit of generation of new neurons in the hippocampus of mammalian adults in cerebral ischemia. Focal cerebral ischemia was induced in Sprague Dawley rats by middle cerebral artery occlusion / reperfusion method with 90 min of ischemia. The animals were observed for 7 days after ischemia and the infarction volume, iNOS gene expression was determined after 7 days of observation. Brain infarction volume was determined by performing triphenyl tetrazolium chloride (TTC) staining of brain sections. Gene expression of iNOS was assessed following Reverse Transcriptase Polymerase Chain Reaction (RT PCR) and iNOS activity was assessed by immunoprecipitation method. No increase in the dentate gyrus neurons was observed in the control animals. Whereas, it was observed that the generation of new neurons was increased to seven folds in the dentate gyrus.
which was associated with the activation of iNOS. Inhibition of iNOS by aminoguanidine principally decreased the generation of new neurons (neurogenesis) and also in iNOS knock out mice, no increase in new neurons were observed. Hence, activation of iNOS may possibly be a therapeutic target in cerebral ischemia.

Yan et al. (2004) investigated the role of Ca2+/Calmodulin-dependent protein kinase II (CaMKII) in inducing the phosphorylation of neuronal nitric oxide synthase (nNOS) and their role in cerebral ischemia. Bilateral common carotid artery occlusion method was followed to induce global ischemia. Immunoblotting and immunoprecipitation was performed to assess interaction of proteins after global cerebral ischemia. Phosphorylation of nNOS was determined in both membrane and cytosolic fraction. Serine phosphorylation of nNOS was increased in the ischemic animals and phosphorylation was decreased in the KN-62 (CaMKII - selective inhibitor). These results show that CaMKII interacts with nNOS and phosphorylates nNOS and induces the production of nitric oxide.

Noboru et al. (2009) reported the beneficial roles of iNOS, eNOS and nNOS in cerebral blood flow and neuroprotection. Cerebral blood flow was increased by nitric oxide synthesized in endothelial cells by eNOS. Activation of eNOS was mediated through cyclic GAMP (cGAMP). Neuronal NOS plays major role in the vasodilatation during cerebral ischemia. Oxygen and carbondioxide modulated cerebral blood flow with the help of nitric oxide formed. Endothelial dysfunction reduces the generation of nitric oxide and thereby reduces the bioavailability of nitric oxide and triggered the production of free radicals leading to oxidative stress. On the other hand overactivation of iNOS and nNOS and the production of nitric oxide along with the production of free radicals augment neurodegeneration. Further understanding is required about physiological and pathophysiological roles of nitric oxide and reactive oxygen species to develop novel preventive and therapeutic approaches for neurodegenerative and stroke disorders.

Ito et al. (2010) investigated the effect of nitric oxide production in the hippocampal CA1 region in global fore brain ischemic / reperfusion knockout mice. Neuronal injury was classified as severely ischemic, moderately ischemic and surviving. The ratio of
survival to degenerated neurons was calculated to determine the rate of survival. Results revealed that increased nitric oxide was due to the activation of both e-NOS and n-NOS and was principally due to n-NOS after reperfusion.

Kanaiyalal et al. (2010) reviewed the role of nitric oxide synthases in cerebral ischemia. Nitric Oxide is a gaseous signaling molecule that plays crucial role in normal physiology and pathophysiology of cerebral ischemia and it is also produced by inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (e-NOS) and neuronal nitric oxide synthase (nNOS). Either of these nitric oxide synthase activated by cerebral ischemia and further produced nitric oxide during different phases of cerebral ischemia. iNOS is expressed in cerebral ischemia in relation various proinflammatory stimuli such as cytokines. Normally the astrocytes and the microglial cells do not express iNOS, where as cerebral ischemia triggers the expression of iNOS in all brain cells. In addition to mRNA and protein expression of iNOS is increased following cerebral ischemia along with increased activity of iNOS. Activation of eNOS requires calcium and the increase in intracellular calcium due to cerebral ischemia triggers the synthesis of nitric oxide by eNOS. Cerebral ischemia also activates nNOS and induces the production of nitric oxide. The beneficial and detrimental role of nitric oxide is related to the type of nitric oxide synthase producing it, amount produced and further oxidation of it. Thus nitric oxide produced exhibits dual role i.e. neuroprotective and neurotoxic in cerebral ischemia depending on various factors explained above.

Intracellular calcium level was sustained under normal physiological condition by Voltage Gated Cation Channel (VGCC). Under ischemic condition, persistent depolarization activates NMDA channel receptors and thereby increase the influx of calcium. Increased intracellular calcium leads to calcium overload in the mitochondrial and triggers the formation of free radicals. Increased generation of free radicals leads to oxidative stress. Intracellular calcium also activates neuronal nitric oxide synthase and triggers the production of nitric oxide, which combines with free radicals and forms peroxynitrite thereby increases nitrosative stress. Intracellular calcium also activates various enzymes such as phospholipases and nucleases which degrades the cell membrane and damages DNA respectively (Crawford et al. 2011).
Marisol et al. (2013) studied the ability of candidates to reduce oxidative stress and inflammation as potential therapeutic targets in the treatment of ischemic stroke. It was suggested that nitric oxide donors (NOD), candidates having potent antioxidant and anti-inflammatory property might exhibit promising therapeutic effect in cerebral ischemia. Besides, it was revealed that NOD is suitable candidate for protection in early phase of cerebral ischemia due to its short therapeutic time window.

2.5 Effect of L-NAME on cerebral ischemia

Kader et al. 1993 investigated the role of nitric oxide in increasing the extracellular glutamate (excitotoxicity) in cerebral ischemia. Cerebral ischemia was induced in rats using bilateral common carotid artery occlusion method. Nitrite content, cyclic Guanosine monophosphate (cGMP) level and Nitric oxide synthase (NOS) was determined in occluded rats. Brain nitrite was significantly increased after 5, 10 and 20 min after cerebral ischemia and returned to baseline after 60 min. Activity of nitric oxide synthase was also increased about 10 fold after the induction of cerebral ischemia and decreased to baseline after 60 min. Cortical and cerebellar levels of cGMP was increased at 10, 20 and 60 min after cerebral ischemia. Rats pretreated with L-NAME did not show increase in the nitrite and cGMP after the induction of cerebral ischemia. It was concluded that increase in NOS activity was observed during the first hour of ischemia that induces the production of nitric oxide that activates cGMP.

Ashwal et al. (1994) investigated the effect of nitric oxide in cerebral ischemia and neuronal injury in permanent focal cerebral ischemia / reperfusion. Nitric oxide was reported to be both neuroprotective and neurotoxic. L-NAME was administered at different low doses, such as 0.1 mg/kg and 0.01 mg/kg. Infarct volume was measured using 2,3,5-triphenyltetrazolium chloride. Cerebral infarct volume was decreased after 180 min of ischemia and 120 min of reperfusion by 33%. Same type of results were obtained when L-NAME was administered 30 to 60 min before restoration of blood flow. Prior administration of L-NAME reduced the infarct volume by 69% in the ipsilateral brain hemisphere. Hence, it was concluded that nitric oxide plays deleterious role during reperfusion in cerebral ischemia by increasing the infarct size.
Sanessario et al. (1994) studied the influence of nitric oxide formation in cerebral ischemia and its effect on hippocampus. In addition to this, the effect of nitric oxide synthase activity on the neurons of sensori motor cortex, hippocampus and the striatum was studied. L-NAME was administered intraperitoneally at doses of 5 and 50 mg/kg twice daily for four days in gerbils. Occlusion of the carotid arteries were performed for 10 min under ether anaesthesia. Body temperature of the gerbils were monitored and controlled during the procedure. After 5 days of post treatment, the gerbils were sacrificed and Nissl-staining of hippocampal sections were performed to determine neuronal damage. Neurons were also stained to determine the activity of reduced NADPH diaphorase. Prolonged treatment of L-NAME had worsened the neuronal loss in hippocampal CA1 and CA4 regions at 50 mg/kg than the other treatment groups. While comparing the acute treatment and chronic treatment with lower dose of 5 mg/kg of L-NAME, Gerbils showed no variation in both the treatments in the neurons of hippocampus, striatum and cortex. Thus, it was concluded that inhibition of e-NOS and n-NOS did not alter the survival of NOS producing neurons during transient cerebral ischemia and also that inhibition of nitric oxide worsens neuronal death in hippocampus post ischemically.

Zhang et al. (1995) studied the effect of L-NAME on nitric oxide synthase inhibition and extracellular glutamate concentration. Cerebral ischemia was induced by systemic hypotension and 15 min occlusion of the two carotid arteries. Reperfusion for 60 min was allowed after ischemia by unclamping the carotid arteries. Extracellular glutamate level was measured using HPLC and a microdialysis probe was introduced in to the cortex and hippocampus to measure the blood flow. L-NAME at a dose of 1, 4 and 20 mg/kg was administered intraperitoneally 30 min before inducing ischemia and changes in vascular flow was assessed using laser-Doppler and microdialysis. During ischemia, vascular flow decreased to 5% and increased after reperfusion to four fold and Glutamate level was transiently increased to ten fold and decreased to the baseline by 30 min of reperfusion. Whereas in the group treated with L-NAME prior to ischemia, glutamate levels increased during ischemia and remained elevated even during reperfusion. However, decrease in the extracellular glutamate was observed in the group administered with combination of L-NAME and L-arginine. Hence, it was concluded that inhibition of nitric oxide prevents extracellular glutamate accumulation and ameliorated excitotoxicity.
Regli et al. (1996) investigated the effects of inhibition of nitric oxide synthase on brain pH, vascular flow, NADH activity using *in vivo* fluorescence imaging and acidosis. Experiment was conducted in rats before and during 3 h of ischemia. L-NAME was administered 20 min prior to ischemia at the dose levels of 0.1, 1 and 10mg/kg individually and one group of animals were administered with 1mg/kg of L-NAME with 5mg/kg arginine in thirty rabbits. It was revealed that brain pH decreased to 6.73 at 30 min and remained acidic throughout the ischemic period in the ischemic group. Whereas, in 1mg/kg, pH decreased to 6.76, 30 min after ischemia and then increased later. In the 10mg/kg and combination with arginine group, pH decreased after 30 min of ischemia and remained only acidic, throughout the study. Cortical blood flow increased in a dose dependent manner in 0.1 and 10 mg/kg group after three hours of ischemia. However, L-NAME did not change the activity of NADH throughout the study in any of the group. Hence, it was concluded that L-NAME prevents acidosis of the brain during cerebral ischemia irrespective to the change in cortical blood flow.

Jeffrey et al. 1997 studied the impact of L-NAME and L–Arginine on neurological functioning after transient global cerebral ischemia in cats. L-NAME was administered intravenously at 5 and 10 mg/kg dose and L-Arginine at 300 mg/kg dose at 30 min and 10 min before inducing ischemia. During reperfusion, cats were administered with additional dose of L-Ariginine (300 mg/kg). The isoelectric electroencephalography was similar in all the groups and reperfusion injury was very severe after 72 hours in all the groups. Neurological deficits was also same in all the treated groups. Hence, it was concluded that nitric oxide does not play important role in global cerebral ischemia.

Esor et al. (1997) reported nitric oxide (NO) as a mediator of glutamate excitotoxicity through the activation of N-Methyl-D-Aspartate (NMDA) receptors. Focal cerebral ischemia was induced by permanent occlusion of right middle cerebral artery occlusion / reperfusion in rats. Nitric oxide was measured in terms of nitrite and cGMP was also determined in the ipsilateral and contralateral cortex and cerebellum after 0, 10 & 60 min of cerebral ischemia. Treatment with L-NAME had significantly reduced the nitric oxide content and cGMP levels in the ipsilateral cortex and cerebellum.

Richard et al. (2001) investigated the effect of long term inhibition of NOS in
middle cerebral artery occluded Sprague Dawley rats. L-NAME was administered for 2 weeks in one group and for 6 weeks in the other group and the reactivity of middle cerebral arteries (MCA) and cortical infarct volume was determined. No significant difference in the reactivity of MCA and cortical infarct volume was observed. Hence, it was concluded that progression of infarction was restricted by the cerebral artery response to vasoconstrictive neurotransmitters associated with guanylate cyclase.

Ding-Zhou et al. (2002) studied the effect of nitric oxide on post ischemic cerebral infarction. The temporal evolution of infarction, nitric oxide generation, neurological functioning and BBB degradation was investigated in the study using mice model. L-NAME was administered after 3 h of ischemia at a dose level of 3 mg/kg. It was observed that 3 mg/kg of L-NAME has reduced the infarction volume by 20% and inhibited the generation of nitric oxide in the brain after 48 h of ischemia, which was determined by evaluating their metabolites. L-NAME also improved the neurological functioning determined using grip strength test in the test animals. BBB breakdown was also significantly reduced by 65% which was evident from the extravasation of evans blue. It was ultimately concluded that nitric oxide plays putative role in cerebral ischemia.

Mohammadi et al. (2011) revealed that cerebral ischemia and hypertension triggers the activity of nitric oxide synthase (NOS). The role of NOS inhibition in the formation of edema and the disruption of Blood Brain Barrier during cerebral ischemia was investigated in hypertensive rats. Middle cerebral artery occlusion / reperfusion (MCAO/R) was performed in hypertensive rats (60 min ischemia and 12 h reperfusion) and the regional blood flow was measured using Laser Doppler. In addition to this, brain infarction, edema and BBB disruption was assessed. L-NAME at a dose of 1 mg/kg was administered intraperitoneally before MCAO/R surgery. About 75 to 85% reduction in the cerebral blood flow occurred during MCAO and it returned to the baseline during reperfusion. L-NAME improved neurological functioning, reduced cerebral edema and also blood brain barrier damage.

Vaibhav et al. (2011) investigated the neuroprotective effect of hesperidin in cerebral ischemic / reperfusion rats. Rats were administered with hesperidin (50, 100 mg/kg,
po) with and without L-arginine (100 mg/kg) or L-NAME (10 mg/kg) for 7 days, and behavioral tests, antioxidant enzyme activity and mitochondrial enzyme complex 1, 2, 3 and 4 dysfunctions were assessed. Both the doses of Hesperidin improved neurological behavior and other antioxidant and mitochondrial enzyme complex. Hesperidin with L-arginine and L-NAME potentiated the protective effect than individual administration.

Saeed et al. (2013) assessed the neuroprotective effect of NOS inhibition using L-NAME in common carotid artery occluded rats. L-NAME was administered intraperitoneally 15 min prior to left common carotid artery occlusion. Malondialdehyde, NO metabolites and antioxidants were assessed. Pretreatment of L-NAME improved neurological deficit and ameliorated oxidative stress in ischemic brain.

Victor et al. (2013) evaluated the neuroprotective effect of zinc (2.5 mg/kg b.wt) and L-NAME (10 mg/kg b.wt) by administering intraperitoneally 1 hour before common carotid artery occlusion in male Wistar rats. Zinc, nitrites and lipid peroxidation was assessed in the cortex and hippocampus of the treated rats. It was observed that combination administration of zinc and L-NAME increased brain injury than individual administration of zinc alone.

Zheng et al. (2014) investigated the role of nitric oxide synthases on blood brain barrier following transient focal cerebral ischemia in a mouse model. Ischemia was induced following middle cerebral artery occlusion/reperfusion method, wherein the artery was ligated at M2 segment. Middle cerebral artery diameter, arterial anastomoses and collateral arteries was imaged and measured using real time. Evans blue and sodium fluorescein staining method was used to assess the blood brain barrier damage after three hours of reperfusion. After 3 hours of reperfusion, intensive vasodilatation and hyperemia was observed with no change in NOS expression. L-NAME inhibited vasodilatation and hyperemia and reduced the damage to blood brain barrier, which was evident from excess extravassation of Evans blue and Sodium fluorescein. L-NIO (eNOS inhibitor) has ameliorated vasodilatation, however no effect on the blood brain barrier damage was observed. Whereas, L-NPA and 7-NI (nNOS inhibitor) had reduced the damage of BBB with no effect on vasodilatation. Combination of L-NAME and papavarine significantly reduced
the BBB damage, improved vasodilatation and reduced hyperemia. It was concluded that nNOS plays critical role in the disruption of BBB in focal cerebral ischemia which is evident from evan blue staining.

2.6 Effect of Citicoline on cerebral ischemia

Adibhatla et al. (2002) revealed that citicoline or CDP-Choline exerts beneficial effects by acting as an intermediate in the biosynthesis of phosphatidylcholine (Ptd-Cho), inhibition of lipid peroxidation, maintaining cardiolipin of mitochondrial membrane, augmenting glutathione synthesis and glutathione reductase, maintaining arachidonic acid and phosphatidylethanolamine, activity, and by reinstating Na+/K+-ATPase activity. Citicoline was also observed to produce enhanced neuroprotective effect in several phase-III clinical trials of stroke.

Richard and Alexander (2004) reported citicoline as a neuroprotectant in clinical trials of the elderly with cognitive and memory impairment. It was also reported that the components of citicoline are easily absorbed in the intestine and it also easily crosses the blood brain barrier. Investigations performed on the use of exogenous citicoline has revealed beneficial results in the animal experimental models. Citicoline also preserved the membrane integrity by conserving the phospholipid content of the cellular membrane and it is also exerts better neuroprotection when administered within 24 hours from the onset of cerebral stroke. It was reported to improve cognition and memory deficits in elderly patients with dementia and Alzheimer’s disease. Moreover, it has been regarded as a safe molecule with less toxicity when therapeutically used. Citicoline was also clinically proven in many conditions and the side effects observed were stomach pain, diarrhea, hypotension, tachycardia and bradycardia.

Jeffrey (2008) revealed that citicoline augments neuro repair and stabilizes cellular membranes in neurological diseases and it confers neuroprotection and neuronal plasticity. It was also observed that citicoline was proven to be a safe and neuroprotective candidate in many clinical trials. It was also stated that the current investigation on neuroprotective effect of citicoline using imaging techniques and clinical trials would offer more confirmative and promising results.
Hyun et al. (2009) evaluated the effect of citicoline on white matter lesions responsible for cognitive impairment in cerebrovascular diseases. Bilateral common carotid artery were occluded in rats. One group of rats received phosphate buffered saline (control) and the other group received citicoline (500 mg/kg) intraperitoneally for 21 days. Cognitive function was assessed using eight arm radial maze test in the experimental rats. Results showed that citicoline prevented white matter damage and improves cognition even at a later stage of cerebral ischemia.

Bi et al. (2010) studied the combined effect of citicoline with rehabilitation training in middle cerebral artery occluded / reperfused male Sprague Dawley rats of three months old. Four groups of rats were used, in which one group was retained as control, second group received citicoline, third group received only rehabilitation training and the last group received both citicoline and rehabilitation. Rats were administered with 500 mg/kg citicoline daily after three days of reperfusion. Rats in the rehabilitation group received only motor training such as balancing, grasping, rotating and walking. Citicoline combined with rehabilitation training was observed to exhibit improved motor functions than the control, citicoline and rehabilitation group.

Irfan et al. (2010) reviewed that citicoline is an endogenous compound acting as an intermediate in the synthesis of phosphatidyl choline exhibiting beneficial functions in neurological disorders. It is also proposed that citicoline plays vital role in protecting the cellular membrane structures and augmenting the synthesis of acetylcholine and betaine. It is also acts as an intermediate in the synthesis of phospholipids via Kennedy cycle. Citicoline also plays crucial role in inhibition of production of free radicals and augments the synthesis of glutathione and glutathione reductase. Citicoline also decreases the duration of coma and ameliorates severe motor function deficits.

Grewal et al. (2012) studied the efficacy and safety of citicoline in cerebral ischemia. The investigation was performed in Medicine Department (Emergency Unit) of Sri Guru Ram Das Institute of Medical Sciences and Research, Amristar for duration of 12 weeks. About 40 patients suffering from stroke were randomized into two groups and one group received standard stroke treatment and the other group received citicoline until discharge from
the hospital. Follow up investigation was carried out during 3, 6 and 12 weeks. No significant
treatment related improvement were observed in both the groups of standard stroke treatment
and citicoline treatment. However, no side effects or other toxic effects were reported in the
citicoline group. Hence, it was concluded that citicoline was safe however it did not exhibit
any better neuroprotection than the standard treatment in the stroke patients.

Krupinski et al. (2012) studied the potency of citicoline to induce angiogenesis
in stroke patients. In vitro angiogenesis assays such as migration, proliferation, differentiation
and spheroid development were performed in human brain microvessel endothelial cells
(hCMEC/D3). Citicoline did not reveal any mitogenic or chemotactic effects in hCMEC/D3
cells, whereas it increased the wound recovery and augmented spheroid development.
Citicoline also augmented the number of new active CD105-postive microvessels in in vivo
middle cerebral artery occlusion studies.

Nipunjot et al. (2012) performed clinical trial in 40 patients to study the role of
citicoline in acute ischemic stroke. One group received standard stroke treatment and the other
group received citicoline with standard treatment. Patients were assessed during admission
and at every 24 h till discharge and follow up investigation was performed at 3, 6 and 12
weeks using National Insitute of Health Stroke Scale (NIHSS), Modified Rankin Scale (MRS)
and Modified Barthel Index (MBI). Patients treated with citicoline along with standard
treatment showed improved neuroprotection.

Pathan et al. (2012) evaluated the neuroprotection of citicoline and piracetam
combination. Citicoline was used in patients with chronic cerebrovascular diseases to
improved cognition and memory, and piracetam was used to increase the blood flow and
oxygen supply in stroke and Alzheimer’s disease. Both citicoline and piracetam when taken
together improves cognition. Whereas citicoline enhanced cognition and then proceeds to
depression, if not taken with piracetam. While, Piracetam induced headache was reduced
when it is combined with citicoline. Hence, citicoline and Piracetam has proven to be
beneficial if taken together.
Martynov et al. (2013), evaluated the neuroprotection of citicoline (Ceraxon) when administered intravenously and orally in 89 patients and the neurological symptoms were determined using Scandinavia Stroke Scale, functional result with Barthel index and modified Rankin scale and compared with 52 patients who received same treatment without citicoline orally and results revealed that citicoline significantly improved the ischemic outcome when administered during first hours of ischemia.

Massimo (2013) considered citicoline to be a potent candidate for neuroprotection in cerebral ischemia and neuro-degenerative conditions. Citicoline was proven to be effective when administered within 24 hours after the onset of cerebral ischemia and it might be considered as a potent candidate for coadjuvant treatment in various neuro-degenerative disorders such as Alzheimer’s disease, Parkinson disease and ischemic stroke.

Karsten et al. (2014) reviewed whether early restoration of blood flow was crucial in cerebral ischemia and stated that approximately 80 to 85% of cerebral ischemia sufferers do not satisfy the conditions for revascularization and hence there is no effective acute treatment. Citicoline, a membrane stabilizer has been studied widely for is neuroprotective effect in clinical trials and has been revealed as a promising candidate in the treatment of acute ischemic stroke treatment.