DISCUSSION

The present investigation was undertaken to investigate the role of NADPH oxidase in cerebral ischemia and reperfusion injury. Cerebral ischemia is clinically known as stroke and is characterized by transient or permanent reduction in cerebral blood flow, which triggers a complex sequence of events that may result in major neurological impairment either acutely or chronically with associated sensorimotor and cognitive deficits, which negatively impact quality of life. The sequel of brain ischemia is characterized by altered voluntary movements (apraxia), aphasia, memory loss, confusion and altered vision.

Several invasive and non-invasive *in vivo* models of stroke have been reported, which simulate the clinical settings of stroke (Buchan et al., 1992; Tamura et al, 1981; Markgraf et al, 1994; Longa et al, 1989). The rat MCAO model has been in use for over last 40 years (Stern et al, 1975) and gained increasing acceptance in recent years owing to its relevance to the human clinical settings of stroke (Garcia et al 1984). In 1989, Longa *et al* modified this novel and relatively noninvasive method of achieving reversible MCA occlusion by use of an intraluminal suture and demonstrated that this technique reliably produced regional infarcts. It offers the advantage of allowing reperfusion as seen in clinical situations and at the same time is non-invasive in contrast to other techniques, such as cauterization (Tamura et al, 1981), mechanical clipping (Buchan et al, 1992) and photo-thrombotic occlusion (Markgraf et al, 1994).

Brain damage produced by MCA occlusion in rodents varies considerably in its size and distribution depending upon the severity of ischemia and duration of reperfusion (Manhas, 2006; Sharma, 2008). Moreover, the vulnerability of brain damage to ischemic insult also differs between brain regions and neuronal population. The striatum in general being most affected and prone brain region to neuronal death following ischemic insult because it receives direct supply from the MCA and lies far away from the collateral arterial connections (Gonzales and Grotta, 2006). Whereas cortical areas do receive some blood from the collateral circulation and moreover, lie farthest from the MCA supply (Gonzales and Grotta., 2006) and reduction in the CBF and ATP is not so severe. Several studies demonstrate that different neuronal populations within striatum also show a differential vulnerability towards an ischemic insult in transient global ischemia in rats

(Meade et al, 2000; Larsson et al, 2001) and in focal cerebral ischemia in rats (Goto et al, 1993; Yamada et al, 1995). A recent study also suggested that striatal interneurons are resistant to focal cerebral ischemia in mice (Katchanov et al, 2003). However, recently it has been, reported that a brief transient cerebral ischemia in gerbils can cause a loss of both parvalbumin (PV)- and neuronal nitric oxide synthase (nNOS)-immunoreactive interneurons in the hippocampal CA1 sector (Himeda et al. 2005). A previous interesting study has also demonstrated that non-pyramidal neurons, like interneurons, in the hippocampal CA1 sector, can survive after transient ischemia, but undergo degenerative changes following complete loss of CA1 pyramidal neurons (Fukuda et al. 1993). These observations suggest that interneurons are not resistant to prolonged periods of cerebral ischemia. Sakuma et al, (2008) showed that transient focal cerebral ischemia in rat causes time dependent damage of interneurons as well as spiny projection neurons in the striatum.

Further, among three types of cholinergic, GABAergic PV-positive and nNOS-positive interneurons, the damage of cholinergic and GABAergic interneurons was more pronounced than that of nNOS-positive interneurons. Furthermore, GABAergic nNOS interneurons in the striatum after focal cerebral ischemia undergo cellular death in a delayed manner. Thus, the regional differences in focal ischemic damage may be described in terms of vascular supply, whereas the differential vulnerability of the neurons is more likely due to intrinsic properties of the neurons themselves. Nearly 85% of strokes are caused by occlusion of a cerebral artery by a thrombus. Thus, effective stroke therapy requires recanalization of occluded cerebral blood vessels. Reperfusion strategies have proven to be the most effective therapies for stroke treatment. However, reperfusion of ischemic brain tissue can have harmful consequences, including the breakdown of the blood-brain barrier, which led to cerebral edema and/or brain hemorrhage, as well as neurovascular injury and neuronal death (Zhu et al, 2006).

Under normal conditions, brain mitochondria constantly produce low levels of $O_2^{\cdot-}$ and hydrogen peroxide, which are effectively scavenged by endogenous SOD, glutathione peroxidase, and catalase. Reflow to a previously ischemic brain region results in a massive increase in oxygen levels that together with a perturbed antioxidant defense

mechanism results in the overproduction of oxygen free radicals. This overproduction significantly limits the benefits of stroke therapies, and these free radicals trigger many cellular and molecular events, including protein oxidation/nitrosylation/nitration, lipid peroxidation, and DNA damage, which can induce cell death following cerebral ischemia and reperfusion (Sugawara et al, 2003; Mehta et al, 2008; Nakka et al, 2008). Although it is clear which oxygen free radicals do play a role in mediating reperfusion injury in the brain but their precise source remains yet to be elucidated. There are number of enzyme systems viz, xanthine oxidase, NO synthase, and NADPH oxidase, that can generate number of different free radicals like, $O_2^{\cdot-}$, $HO^{\cdot}$, and NO. These enzyme systems originate or are present in different cell types like neurons, endothelial cells, macrophages, cardiomyocytes, and nephrons.

Abramov et al (2007) had observed that NADPH oxidase is mainly responsible for ROS generation following reperfusion, whereas mitochondria and xanthine oxidase are involved during ischemic period. NADPH oxidase was first characterized in neutrophils and play role in cellular defense. Recent studies have also suggested that NOX is expressed in the central nervous system and *in vitro* studies have shown NOX expression in neurons, astrocytes, and in microglia (Bedard et al, 2007). *In vivo* studies using immunohistochemistry had showed that NOX subunits are widely distributed in the cortex, the hippocampus, and in the cerebellum (Serrano et al, 2003; Infanger et al, 2006). Genetic ablation of the gp91phox subunit reduced brain infarction by 46% at 22 h of reperfusion after 2 h of focal ischemia (Walder et al, 1997).

Hence, the present study was undertaken to analyze the expression pattern of NADPH oxidase (NOX) subunits, its activity and how it regulates the cell survival/cell death signaling following I/R injury in striatum and cortical brain regions. Our study used 1hr of ischemia with different time points of reperfusion injury. The reason for selecting 1hr of ischemia was that the previous studies from our lab and studies by me suggested that, severity of damage with increasing reperfusion after 2 hr of ischemia was found to damage nearly most of the ipsilateral brain (Manhas, 2006, Mehta, 2008) and this would not enable us to examine downstream cellular and molecular events. Hence, ischemia of 1 hr duration followed by different time points of reperfusion was finally selected to

study the expression pattern and survival/death signaling pathways of NADPH oxidase and their subunits. It is hoped that moderate cerebral damage as a result of such ischemic insult would activate NADPH oxidase mediated machinery responsible for cell damage. Apocynin was used as inhibitor of NADPH Oxidase, whereas previously numbers of studies were undertaken using diphenyliodonium or gp91stat. The main reason for choosing apocynin was as: it doesn't interfere with the activity of endothelial nitric oxide synthase (Abid et al, 2001; Bao et al, 2001; Stolk et al, 1994; Zhou et al, 1999). Apocynin inhibits NADPH oxidase by reacting with thiol groups required for enzyme assembly, but does not scavenge xanthine oxidase-generated superoxide or inhibit eNOS, or prevent NF-κB activation (Stuehr et al, 1991). Moreover on systemic administration, apocynin is capable of crossing the BBB to reach brain tissue (Tang et al, 2008). Therefore, these characteristics of apocynin made this methoxy-substituted catechol a better choice for us to use in the present study.

Apocynin was preferred among various NOX inhibitors as discussed earlier above (page 99). Further gp91da-tat is another most commonly used NOX inhibitor but it was not used because: as it does not crosses blood brain barrier and intra-cerebroventricularly administration which may exacerbate the ischemic damage. Apocynin is an inhibitor of NOX, and has been shown to reduce histological injury following global (Wang et al., 2006) and focal (Tang et al., 2008; Chen et al, 2009) cerebral ischemia. There is quite ambiguity and controversies about the use of dose of apocynin for NOX inhibition. Recent findings had shown that it was protective at a dose of 5mg/kg given intra-peritoneally (i.p.) 30 min prior to ischemia in a rat model of global cerebral ischemia (Wang et al., 2006). In another study, apocynin given at a dose of 50mg/kg i.p. 30 minutes before reperfusion also significantly reduced infarct size in MCAO model in rats (Tang et al., 2007).

Therefore, based on effective dose of apocynin as above, we have titrated the dose response pattern effect of apocynin (1.0, 2.5, 5.0 and 10mg/kg/i.v.) in experimental focal stroke model, given at the time of reperfusion. The results indicate that apocynin at a dose of 2.5 mg/kg i.v. resulted in maximum reduction in infarct volume leading to improved neurological outcome. Interestingly, higher doses of apocynin did not offer

enhanced protection but rather animals showed increased mortality due to brain hemorrhage. Further, our results showed that dose higher than 2.5mg/kg did not show significant effect on infract size and also higher doses tended to cause brain hemorrhage even within non ischemic hemisphere and leading to death of animals. Our observations with low doses of apocynin are in agreement with Tang et al (2008), who also reported that apocynin improves outcome in experimental stroke with narrow dose range in MCAo model in mice. The possible reasons for this narrow dosage range are unclear, but there are certain points that can explain this: Firstly, variations in dose regimens by different routes of administration may have wider bioavailability. Secondly, the higher doses of apocynin cause brain hemorrhage in rats with MCAO model used by us and similar findings were made in MCAO model in mice (Yenari et al., 2006, Tang et al, 2008) and rats (Neumann-Haefelin et al., 2001, Lee et al., 2005). Thirdly, a pilot *in vitro* studies by Tang et al (2008), suggest that at higher concentrations, apocynin have pro-oxidant properties, so it may worsen ischemic damage at higher doses.

5.1. Effect of I/R injury on MABP, HR, CBF and BBB:

Mean arterial blood pressure (MABP) has been found to be elevated already during the first few minutes after the onset of stroke symptoms (Broderick et al, 1993); it seems more likely to be intimately associated with the vascular occlusion. Cerebral blood flow in the human ischemic brain is passively dependent on the mean arterial pressure, because auto-regulation becomes defective due to ischemic insult; hence there is rise in MABP (Eames et al, 2002). Therefore, low MABP or abrupt decreases are hazardous and risks enlargement of the initial infarction. Elevated MABP, on the other hand, affect to maintain cerebral blood perfusion. This leads to two fundamental patho-physiological dogmas in considering therapeutics of acutely elevated BP. First, post stroke hypertension may be deleterious by facilitating edema and hemorrhage formation in the ischemic tissue, as blood-brain barrier is damaged. Secondly, antihypertensive drugs may reduce the pressure-dependent cerebral blood flow in the ischemic penumbra with poor auto regulation. So, the regulation of MABP following I/R injury should be critically regulated.

We have measured different physiological parameters and found that, the mean baseline MABP was about 103 mmHg in both ischemic and apocynin treated rats but during MCAo, MABP increased dramatically in both groups by 18–21 mmHg. Following reperfusion, MABP decreased in both groups and returned near to pre-stroke levels (106 mmHg). So, MABP was elevated by 15–18% during ischemia but following reperfusion it reverted back to basal level. Previous studies by Fagan *et al* (2006) showed that reducing MABP, animals had a robust improvement in neurologic score, and reductions in neuronal and vascular damage. A clear relationship between elevated blood pressure and microvascular damage has also been demonstrated in experimental animals (Bowes et al 1996; Fagan et al 1998; Brott et al 1998; Tejima et al 2001). Both labetalol (Fagan et al 1998) and hydralazine (Tejima et al, 2001) have been shown to reduce the incidence and/or severity of hemorrhagic transformation, when administered prior to the onset of ischemia, preventing the acute hypertension during occlusion. These observations demonstrate that lowering the BP after the onset of ischemia should be attempted judiciously for improved outcome.

It is well established that resting heart rate have close correlation with blood pressure and that it is prospectively related to the development of hypertension. Moreover, there is growing evidence to indicate that an elevated heart rate is associated with increased cardiovascular morbidity and mortality. In this respect, heart rate can be considered both as a marker of risk and may serve as an independent factor in the induction of risk. (Stamler et al, 1975; Palatini et al, 1999). Following I/R injury, the heart rate was increased by 8-10% from baseline but following 1hr of reperfusion it returned to pre ischemic levels. Apocynin did not affect heart rate following I/R injury. The possible reason for increased heart rate and MABP may due to over activity of sympathetic system or decreased vagal tone.

Brain ischemia occurs, whenever cerebral blood flow (CBF) decreases to level that can no longer sustain normal cell homeostasis or maintain normal ionic gradients across the cell membrane. As mentioned earlier, the severity of damage after transient cerebral ischemia depends mainly on the duration of the ischemia and the degree of CBF reduction. The infarct volume will be function of CBF reduction at a given period of

ischemia i.e. infract volume increases with the time period of reduced blood flow. The CBF in the ischemic core is usually strongly affected as indicated by infract but CBF in the periphery of the affected area (penumbra) can potentially indicate the extent of CBF alterations and, therefore, might give an estimation of the severity of ischemia (Memezawa et al, 1992; Soriano et al, 1997). The decrease of CBF in ischemic brain regions may result in an energy failure and leads to an activation of the damaging intracellular pathway (Jorgensen et al, 1982; Dirnagl et al., 1999; Hou et al, 2002). A number of methods have been used for measuring CBF all these have their own strength and weakness (Kramer et al., 1996). Laser Doppler Flow (LDF), compared with other methods provides a noninvasive and continuous measure of local CBF and is sensitive enough to observe instantaneous changes in cerebral microcirculation. The changes of focal CBF after MCAO can be monitored at defined zones of MCA territory by placing the probe on the dura for recording pulsation to provide the blood flow to that region. LDF doesn't give absolute blood flow but gives the value in term of percentage flow to a specific brain region (100% is for standard solution provided by manufactures) and it was very sensitive to an altered position of the LDF probe (Kramer et al, 1996). So, we have measured CBF at different time points, before ischemia, at the onset of ischemia and during reperfusion. Our results clearly demonstrated that, there is 80% reduction in CBF following ischemic injury and it remained approximately at the same level throughout the ischemic period. However, peri-ischemic area i.e. penumbra showed reduction of 65-70% of the basal value (i.e. 100%) in I/R and apocynin treated rat groups. After reperfusion, local CBF of the core returned back to 60-75% and in penumbra the CBF gets back to 80-90% in ischemic and apocynin treated rats. Apocynin, at 2.5mg/kg given at the onset of reperfusion had no effect on cerebral blood flow.

Cerebral I/R injury results in disruption of the blood-brain barrier (BBB) and formation of brain edema. These processes are consequence of increased vascular permeability that results from endothelial cell contraction and disassembly of tight junctions. I/R injury increase the formation of ROS (Liu et al, 2003) and ROS, in turn, are thought to alter BBB integrity (Heo et al, 2005). Incubation of endothelial cells with ROS promotes cellular contraction and increases the permeability of endothelial

monolayers (Fischer et al, 2005; Lagrange et al, 1999). Moreover, *in vivo* treatment with superoxide dismutase or antioxidants such as tempol attenuates vascular leakage after ischemia (Kim et al, 2001; Kato et al, 2003). Our results have shown that, NADPH oxidase is one of the major sources of ROS generation after I/R injury and is responsible for ischemic tissue damage. So, efforts were also made to assess BBB function in response to NADPH inhibition. For this purpose BBB damage was assessed by Evans blue permeability and myleoperoxidase assays. Our results indicate that, inhibition of NADPH oxidase by apocynin following I/R, prevented BBB dysfunction due to reduced infiltration of neutrophils, which is reflected by decreased permeability of evans blue to ischemic area. These findings are in agreement with the observation of Kahles et al, (2007), they also showed that genetic deletion of gp91phox prevents the BBB damage after I/R injury. These observations points that, the ROS generated by NADPH oxidase may be responsible for BBB damage in I/R injured rats and inhibition of NADPH oxidase prevents BBB disruption in experimental stroke.

5.2. Role of NADPH oxidase in neuroprotection:

Our results demonstrated an improvement in neurological deficit and decreased infract area by apocynin following I/R injury. Focal cerebral ischemia induced neurological deficit (ND) score are characterized by sensorimotor dysfunction (Bederson, et al., 1986[a], 1986; Yamamoto et al., 1988; Markgraf et al., 1992, Brightwell et al 2007). The striatum, primary motor cortex, the anterior dorsal cortex are involved in the processing of sensori-motor information (Andersen et al., 1991) and corresponds to the hind limb and the forepaw movements. Thus the damage both in the cortical and sub-cortical regions of the brain is reflected in the form of neurological deficits. However, it is often difficult to distinguish the extent to which neuronal damage in each of these two brain regions contributed to altered sensori-motor performance, because the caudate putamen receives extensive input from the sensori-motor areas of the cortex.

We found that administration of apocynin at the onset of reperfusion offered significant neuroprotection by reducing total infract volume and neurological deficit. Walder et al 1997 found that, in $NOX2^{-/-}$ mice, apocynin had no such effect, raising the possibility that the protection afforded by apocynin pretreatment in this model of stroke

occurred via inhibition of NOX2. Further, Chen et al (2009) found that compared with control mice, smaller mean infarct volume in apocynin-pretreated or NOX 2-deficient mice. These observations further support studies reported in mice lacking $Nox2^{-/-}$ showing smaller infarct volume following MCAO (Kahles et al, 2007; Kunz et al., 2007).

Further, we found that a twofold higher dose of apocynin increased mortality while having no beneficial effect on other measures of post-stroke outcome is consistent with another recent finding by Tang et al., 2008. Interestingly, when apocynin was administered 1h after induction of reperfusion, it failed to improve outcome, with no reduction in infarct or edema volume and no improvement in neurological function (Jackman et al, 2009).This suggests that apocynin has quite a narrow therapeutic window and thus may represent a challenge for therapeutic development. So, our findings with Jackman further suggest that the timing of administration of apocynin is critical for its neuroprotective effect.

The outcome of impaired blood supply to the MCA territory as consequences of focal cerebral ischemia was therefore, evaluated using a simple, rapid quantitative behavioral examination. Examination of ND is important not only in stroke patients but also in animal models of cerebral ischemia as it directly serves as a marker of the cerebral injury severity. Result indicates that, following I/R injury, ND observed after 3h post-reperfusion in rats increases with reperfusion period and peaked at 24hr. This neurological deficit was demarcated by varying degrees of flexion, contralateral circling, hemiparesis and non-spontaneity which increases with reperfusion time. Inhibition of NADPH oxidase prevented development of these neurological deficits.

Oxidative stress is an important underlying factor in neuronal death induced by cerebral I/R injury (Chan et al, 2001; Kirino et al, 2000). The close inter-relationship between cerebral ischemia and oxidative stress has generated considerable interest in using antioxidant agents to combat the deleterious consequences of oxidative insults in ischemia and recirculation injury (Simonyi et al., 2005). Maximal tissue damage is observed during reperfusion, which is primarily attributed to oxidative injury resulting from production of oxygen free radicals. This burst of ROS is incorporated with

increased lipid peroxidation (MDA) and decreased glutathione (GSH) levels. These two are detectable at a very early time points of I/R injury even when even there is no definite evidence of neuronal cell death (Candelario-Jalil et al., 2001; Wang et al., 2005). Thus MDA and GSH could serve as good markers of oxidative stress during I/R injury.

With the progression of reperfusion injury, GSH level decreases and reaches minimum at 24hr indicating that, brain is under excessive oxidative stress. This decrease in GSH levels as a result of post-ischemic reperfusion compared to sham animals are in accordance from our laboratory and by several other investigators (Mishra et al 2010, Nakka et al 2010, Manhas 2006; Sharma 2007; Shivakumar et al, 1995). Inhibition of NADPH oxidase by apocynin prevented this depletion of GSH with reduced the cerebral infract and neurological deficit.

Cellular damage in cerebral ischemia is caused by excessive oxidative stress caused by free radicals and lipid peroxidation. Following I/R injury, lipid peroxidation increases with progression of reperfusion. Our result showed that, there is an early increase in MDA levels within 3h after I/R and this is in agreement with Wang et al., 2005 and also with Candelario-Jalil et al., 2001. Further, it progressively increases with the time of reperfusion and peaked at 24hr of I/R injury indicating that there is maximum tissue damage at this time point. Thereafter it decreases gradually and settles down to basal level at 168hr post reperfusion. This implies that after 24hr of I/R injury, the oxidative stress decreases gradually.

Apocynin appeared to prevent the ischemia/reperfusion induced loss of GSH and increased lipid peroxidation following I/R injury. The protective effect of apocynin in the present study could be due to (i) failure in formation of NADPH oxidase assembly hence decreased ROS production. In conclusion, cerebral I/R injury leads to oxidative stress, which resulted in increased lipid peroxidation and reduced glutathione levels in the affected brain tissue and inhibition of NADPH oxidase results in better neurological and cerebral infract outcomes by dwindling oxidative stress markers.

5.3. Inhibition of NADPH oxidases alleviates ROS levels and cell death following I/R injury:

ROS are well known to play a pivotal role in cerebral ischemia-reperfusion-induced cell injury (White et al, 2000) and increased ROS production worsens ischemic damage. In a rat model of MCAO there was an increased concentration of hydroxyl ions after ischemia/reperfusion. Further, during the 2hr MCAO period, the levels of 2,5 and 2,3-DHBA, indicators of hydroxyl radicals were also increased gradually to 2- to 2.5-fold above basal level and further enhanced to nearly four-fold at 30 minutes post reperfusion after recirculation (Morimoto et al., 1996). Recent studies indicate that NADPH oxidase is a predominant source of superoxide generation in the vasculature (Al-Mehdi et al, 1998, Cai et al, 2003) as well as the central nervous system (Serrano et al, 2003).

In the present study, we have used flow cytometry (FACS) to monitor the production of ROS at different time points of I/R injury. We found that, there is an early increase of superoxide radicals in the striatal and cortical regions which progressively increases and reached at peak at 12hr of reperfusion, thereafter it decreases and again showed a smaller rise at 168hr of reperfusion. Inhibition of NADPH oxidase with apocynin suppresses the ROS generation after I/R injury and prevents ischemic damage. This clearly indicates that, NADPH oxidase derived ROS plays a major role in reperfusion injury and is in agreement with the observation of Abramov et al (2007), who showed that, during reperfusion NADPH oxidase is mainly responsible for ROS burst. Initial increase of ROS following I/R injury can activate expression of certain genes and pathways, which can induce cell death via apoptosis (Griendling et al, 2000), whereas the second burst of ROS may activate cell survival pathways (Stone et al, 2002; Luczak et al, 2004).

More recently, the classical view that reactive oxygen species are merely destructive molecules have been recently challenged and new concept has evolved, that ROS have dual role and plays an important role in cell survival signaling and redox regulation of stress-induced signaling pathways (Mackey et al, 2008, Groeger et al, 2009). There is now substantial evidence suggesting that ROS can cause direct modifications such as disulphide bond formation and glutathionylation of signaling

proteins and these modifications can alter the activity of certain proteins and alter their sensitivity to apoptosis (Hurd et al, 2005, Hurd et al, 2005[a]). ROS have been shown by a number of investigators to enhance cell survival by triggering the activation of certain signaling pathways that protect cell damage. These include activation of the mitogen-activated protein kinase pathway (Wang et al, 1998), ROS-induced activation of phospholipase C-gamma-1 (PLC- γ1) (Wang et al, 2001), epidermal growth factor receptor-dependent activation of Akt (Wang et al, 2000), activation of VEGFR-3 signaling in response to H_2O_2 (Wang et al, 2004), activation of NF-kB (Wang et al, 2001) and H_2O_2 activation of PI3K/Akt (Qin et al, 2003).

It is clear from these observations that, there is burst of ROS following I/R injury. Apocynin inhibits this ROS burst and prevents ischemic damage. Further, concentration of ROS play an important role in regulating cell survival signaling and redox regulation of stress-induced signaling pathways. So, selective inhibition of early-but not late phase generated ROS is neuroprotective in focal cerebral ischemia in rats. These results showed that NADPH oxidase could be a promising drug target against stroke damage in the brain by activating repair mechanisms after injury.

5.4 NADPH oxidase activity & expression following I/R injury:

ROS are well known to play a pivotal role in cerebral ischemia-reperfusion-induced cell injury (White et al, 2000). Superoxide is the first ROS generated among the oxygen free radical chain during the early phase of reperfusion after cerebral ischemia (Fabian et al, 2000). The interaction of superoxide with NO results in the production of peroxynitrite, one of the most harmful ROS species, which causes neuronal tissue damage via lipid peroxidation, protein oxidation, nitration, and DNA breakage (Kidd et al, 2005, Virag et al, 2003).

Recent studies indicate that NADPH oxidase is a predominant source of superoxide generation in the vasculature (Cai et al, 2003; Abramov et al 2007) as well as in the central nervous system (Serrano et al, 2003). However, the temporal profile and regulation of NADPH oxidase activity during cerebral ischemia-reperfusion was not extensively studied so far. This prompted us to analyze the activity of NADPH oxidase following different time points of injury. Result showed that, NADPH oxidase activity

alters in a time-dependent manner with increasing reperfusion period. Further, we found that it was significantly higher in the striatum as compared to the cortex.

During ischemic period, NADPH oxidase activity was slightly reduced as compared to the normal brain tissues. This reduced activity of NADPH oxidase may be attributable to the complete blockade of blood flow to the ischemic core area, where most of the cells die quickly from energy exhaustion. In contrast, the NADPH oxidase activity in the penumbral region remained relatively stable even during the ischemic phase; this may be owing to less reduction in blood supply as compared to core region as blood flow in this area which might have been compensated by supply from collateral blood vessels.

During reperfusion, NADPH oxidase activity progressively increased and peaked at 12hr post reperfusion thereafter it got subsided at 24hr and again a mild increase was noticed at 168hr. The initial peak indicates that, there is excessive oxidative burst due to reperfusion and most of the cells or neurons die due to extreme oxidative stress. Further, NADPH oxidase activity subsides at 24hr because the bulk of cells in core region are almost dead so there is absolutely meager enzyme activity, which was clearly shown by the TTC staining as well. NADPH oxidase activity was again slightly enhanced at 168hr post reperfusion; the possible reason for this may be that damaged cells start regenerating in damaged area by trying to overcome oxidative stress by activating survival machinery. Inhibition of NADPH oxidase with apocynin subdues NADPH oxidase activity following I/R injury. The increased penumbral NADPH oxidase activity may play an important role in neuronal damage and contributes to the enlargement of the infarct size in the early phase of reperfusion but during late phase NOX activation seems to stimulate survival signaling cascade that helps in protection of damaged area. Therefore, prevention of NADPH oxidase activation in the early phase of reperfusion may represent a useful strategy for protection against oxidative stress-induced neuronal injury in ischemic stroke however the late phase inhibition of NADPH oxidase may worsen the ischemic damage.

It has been reported that, expression of $gp91^{phox}$ has been shown to be augmented in ischemic stroke (Hirabayashi et al, 2000) and $gp91^{phox}$ mutant mice exhibit reduced cerebral infarct volume (Walder et al, 1997). But so far no study has analyzed the relative contribution of different sub-units of NOX and their participation in I/R injury. The

present study has demonstrated the protein expression profile of the catalytic subunits gp91, p22 and the cytosolic component p47 and p67 in the striatum and cortical brain regions at different periods of reperfusion. Our results further delineated that expression of gp91, p22, and p47 parallel with augmented NADPH oxide enzymatic activity following I/R injury. The expression of p67 remained constant throughout the reperfusion injury and there is no change in the expression of p40 subunit in striatum and cortex. Thus the expression pattern of gp91, p22 and p47 subunits bears a direct co-relation with the NADPH oxidase enzymatic activity.

Interestingly, inhibition of NADPH oxidase with apocynin impeded the expression of gp91, p22, p47 proteins, which may accounts for the decrease in NADPH oxidase activity. Further, the expression of p67 remained constant. These findings suggest that, p67 provide supporting role and helps in assemblage of NADPH oxidase complex during activation. Hence, gp91, p22 and p47 subunits play a major role in generation of oxidative stress, which leads to cerebral infract and neurological deficit, suggesting that NADPH oxidase plays major role in reperfusion injury and protection against cerebral I/R injury may be mediated partly by inhibition of NADPH oxidase.

5.5. NADPH oxidase and cell survival/death proteins:

Reactive oxygen species (ROS) are associated with multiple cellular functions such as cell proliferation (Buggisch et al, 2007), differentiation (Corzo et al, 2009), and apoptosis (Nakamura et al, 2008). However, the direct role of endogenous ROS production still remains to be elucidated. The vascular endothelial growth factor (VEGF) is a major angiogenesis inducer and is regulated at transcriptional level by hypoxia-inducible factor 1 (HIF-1) in response to hypoxia (Forsythe et al, 1996). In this study, we found that following I/R injury, there is elevated levels of ROS production and the results indicate that it modulates VEGF expression leading to angiogenesis and cell repair.

Hence attempt was made to assess the role of endogenous ROS in regulating angiogenesis and cell repair process following I/R injury in rats as this would provide a new insights in the underlying repair mechanisms. Our results showed that, VEGF is activated just after I/R injury in ischemic rats but it showed an enhanced expression after 72hr and even much higher at 168hr post reperfusion. These findings suggest that, at

early time points of injury i.e. 12hr post reperfusion, cell is under excessive oxidative burst and it can't start repair process but later on, when there is a decrease in oxidative stress then it activates survival pathways and angiogenesis by upregulating VEGF expression. Inhibition of NADPH oxidase prevents ROS burst but the attenuated ROS levels promotes an early repair process following I/R injury. Thus our study provides strong evidence that ROS also play an important role in cell repair.

Endothelial nitric oxide synthase (eNOS), play a pivotal role in the regulation of angiogenesis as well. Endothelial NOS (eNOS) and its final by-product nitric oxide (NO) represent a downstream imperative for the angiogenic response elicited by VEGF (Papapetropoulos et al 1997; Ziche et al 1997; Murohara et al 1998). NO plays a critical role in vascular biology including vascular tone and vascular permeability (Moncada et al 1999). A functional link between VEGF and eNOS has been established *in vitro* by Ziche and co-workers (1997), they first demonstrated that VEGF-induced angiogenesis was selectively blocked by eNOS inhibitors. Murohara and co-workers (1998) demonstrated that *eNos*$^{-/-}$ mice showed a reduced neo-angiogenesis after surgically induced hindlimb ischemia, and this phenotype was not rescued by VEGF treatment, indicating that eNOS is a downstream target of VEGF. More recently, it has been shown that VEGF up-regulates eNOS expression through the binding and activation of Flk-1/KDR receptor (Fukumura et al, 2001; Bussolati et al, 2001) and NO itself modulates VEGF release from endothelial cells (Tsurumi et al, 1997), smooth muscle cells (Namba et al, 2003), and macrophages (Ramanathan et al, 2003) through an autocrine and/or paracrine loop. But, the role of eNOS in response to NOX in angiogenesis has been studied by analysing the expression of eNOS, following I/R injury in ischemic rats. Our results showed that, there is activation of eNOS at late hours of I/R injury and this expression is significantly enhanced at 168hr post I/R injury, indicating that at this time point of injury, there is an active repair process. Inhibition of NADPH oxidase suppresses the production of ROS and activates eNOS expression at early hours.

Reperfusion following ischemia induces high level of free radicals, which leads to increased oxidative stress and culminates in cell death. This ROS burst during reperfusion stimulate mitogen-activated protein kinases viz. stress-activated protein

kinases, including c-jun NH2-terminal kinases (JNK) and Akt and ERK in the mammalian cells and regulates pathophysiology of stroke. Previous studies have shown that, these kinases play role in regulating cell survival (Cowley et al, 1994; Mansour et al, 1994; Leppa et al, 1998) and cell death mechanisms (Liu et al, 1996; Nemoto et al, 1998). However, the majority of studies are focused on particular protein kinase or their knockouts or their substrates illustrating their role in pathophysiological situation. Recent findings affirm that ROS act as a signaling molecule and regulates kinase activity and expression (Fialkow et al, 2007; Powers et al, 2010) which in turn regulates redox regulation of cell. This is of interest to investigate as how NADPH induced ROS regulates these kinases after cerebral I/R injury. For this purpose, we have selected two time points viz: 12hr and 168th hr of post reperfusion time periods. The reason for selecting these time points is that we got maximum NADPH oxidase activity and expression at these two time points. Our results suggest that, rats subjected to cerebral I/R injury resulted in activation of all three mitogen-activated protein kinases viz. ERK, Akt, and JNK at 12hr and 168hr of reperfusion.

The activation of ERK pathway has been shown to be involved in cell survival signaling pathways (Junttila et al 2008) and it gets activated to cope up with oxidative stress generated due to pathological conditions (Gardner et al, 1996; Parrizas et al, 1997). But recent studies have also suggested that the ERK pathway is involved in the regulation of cell death as well (Murray et al, 1998; Sakata et al, 1995). Therefore, we examined the role of NADPH oxidase and ERK in the cerebral I/R injury. Results showed that expression of phosphoERK and ERK2 proteins were decreased at 12hr of reperfusion but rats subjected to NADPH oxidase inhibition with apocynin tends to restore and showed higher expression of phosphoERK and ERK2. Therefore decreased expression of ERK and pERK at 12hr in ischemic rats may be due to increased NADPH oxidase expression and activity which culminates in extreme cellular oxidative stress.

However, no significant change was observed in phosphoERK and ERK2 levels at 168hr post reperfusion in ischemic rats. But treatment with apocynin significantly upregulated phosphoERK and ERK2 levels; this may suggest that it participates in recovery processes by activating cell survival mechanisms. Our findings support the view

of other workers, who have showed that inhibition of ERK, enhances ischemia/reoxygenation induced apoptosis in cultured cardiac myocytes (Arany et al, 2004; Epling-Burnette et al, 2004; Rasola et al, 2010).

After, observing the enhanced expression of pERK and ERK-2, we also studied another cell survival signaling protein Akt. The apoptotic pathway is regulated by a variety of factors and is based on the balance between cell death and survival factors (Goyal et al 2001). Serine/threonine kinases, such as Akt (protein kinase B), are key regulators of neuronal cell death and survival after cerebral ischemia (Noshita et al, 2001). Akt functions as a major downstream target of the phosphatidylinositol 3-kinase (PI3-K) pathway, and after the phosphorylation of Akt, it phosphorylates many substrates on the serine or threonine residue, including glycogen synthase kinase-3, and activate survival mechanisms. So, we have checked the expression of Akt following I/R injury in control ischemic animals and also rats treated with NADPH oxidase inhibitor. Our findings showed that expression of Akt of PI3-K signaling pathway is suppressed at 12hr of reperfusion in control rats but inhibition of NADPH oxidase by apocynin, its expression is upregulated and offered neuroprotection following I/R injury. But at 168hr of reperfusion, expression of Akt in ischemic animals was higher compared to 12hr ischmeic and sham rats, indicating that there may be activation of survival mechanism at late hour of reperfusion and which seems to hasten recovery process. Further, inhibition of NADPH oxidase prevented and activated Akt expression at earlier hour. So, our findings have demonstrated that, inhibition of NADPH oxidase promotes the cell survival pathways by activating PI3-K signaling pathways in focal cerebral ischemia.

Once we have observed the expression of cell survival proteins, we were interested in finding how the stress associated protein kinases respond at these time points of I/R injury. So, we have detected the expression of stress protein i.e. SAPK/JNK at 12hr and 168hr post reperfusion. Interestingly, we found that when the cell is under excessive stress at 12hr and NADPH oxidase expression and enzymatic activity is maximum then expression of these stress associated protein kinase is also high, but on inhibiting NADPH oxidase there is attenuation of oxidative stress and which directly

assuage SAPK/JNK expression. But at late time points of I/R injury, the expression of these proteins was decreased indicating that oxidative stress in the cells has subsided.

In conclusion, these findings suggest that, following I/R injury, the expression of stress associated protein kinases increase but on the other side expression of survival molecules get decreased. These results confer that during early hours of I/R injury, cell is under excessive oxidative stress and cell death pathways are activated. Further, these findings also suggest that, at late time points of I/R injury, the survival pathways get activated and repress the stress kinases. This late phase activation of survival proteins is responsible for repair and regeneration processes.

Further, we have studied the expression of spectrin protein, which is responsible for marinating plasma membrane integrity. It is a cytoskeletal protein that lines the intracellular side of the plasma membrane, forming a scaffolding and cell structure. Cleaved spectrin can cause cytoskeleton destruction and bleb formation in the cell membrane, ultimately causing degradation of cells and leading to cell death. Ischemic insult leads to excess of oxidative burst and lipid peroxidation. This leads to change in stability and structure leading to cell death. The use of spectrin antibody in our experiments recognizes both cleaved spectrin (150 and 120 kDa) products to assess brain injury after I/R. Our results showed that, there is an increased expression of spectrin following I/R injury at 12hr, which is significantly higher in ischemic than apocynin treated rats. These results indicate that cell integrity and structure was damaged due to excessive oxidative stress. Increased oxidative stress following reperfusion caused lipid peroxidation of membrane protein and protein carbonylation which in turn cause cleavage of spectrin, leading to damage of cell membrane integrity causing cell blebbing and death. But the expression of spectrin decreased at late time points i.e. 168hr post reperfusion; this decreased expression of spectrin may be co-related with decreased oxidative stress at 168hr post reperfusion. From above findings it may be concluded that, apocynin significantly reduced the expression of spectrin by reducing oxidative stress and prevented cell damage caused by lipid peroxidation and protein carbonylation. Further, expression of spectrin bears a direct relation with NOX expression and activity.

Lastly, we studied; Bcl-2 which is a proto-oncogene that promotes cell survival in a variety of tissues including the brain (Schratt et al 2004; Guo et al 2010). It has been known for some time that Bcl-2 overexpression can protect cells from apoptosis mediated by ROS (Hockenbery et al, 1993). However, the mechanism by which Bcl-2 prevents ROS-induced apoptosis is not clearly known. Bcl-2 itself does not possess antioxidant activity; rather, it may act indirectly to increase the levels and/or activities of endogenous antioxidants e.g., glutathione or superoxide dismutase within cells (Voehringer et al, 2000; Lee et al, 2001; Ellerby et al, 1996). Thus, over-expression of Bcl-2 may allow cells to cope better with the oxidative stress, possibly by allowing increase in endogenous antioxidant enzymes and activating survival mechanism. So, based on these reports and our findings, we tested whether, inhibition of NADPH oxidase with apocynin may decrease cell death by influencing Bcl-2 expression in ischemic brain injury. Our results showed that, Bcl-2 is significantly down-regulated during early reperfusion and inhibition of NADPH oxidase upregulates the Bcl-2 expression after I/R. The possible explanation for this may be that, inhibition of NADPH oxidase suppresses ROS burst, which may be responsible for major tissue damage by ischemic insult. Due to less oxidative stress, detoxification of ROS is easy and fast and the natural antioxidants present in the body reverse the ROS-induced decline in Bcl-2 and may prevent apoptosis. These findings are in agreement with Pugazhenthi et al, 2003. Further, inhibition of NADPH oxidase activates survival mechanism by activating phosphatidylinositol 3-kinase (PI-3 K) and Akt and prevents cell death. These results also indicate that NADPH oxidase also modulates the Bcl-2 expression, which protects from ischemic injury.