INTRODUCTION

Stroke is the rapidly developing loss of brain functions due to interruption in the vessels supplying blood to the brain. In practice, it is usually referred to a condition caused by the occlusion or hemorrhage of blood vessels supplying the brain (Eng et al., 2003). According to WHO stroke is a 'rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24hr or longer, or leading to death, with no apparent cause other than of vascular origin'. This definition includes the two main pathological subtypes: ischemic and hemorrhagic strokes.

Estimates indicate that stroke is responsible for nearly half of all the patients hospitalized for acute neurological disorders (Dirnagl et al., 2003). It is the number two cause of death and leading cause of disability world over (Feigin, 2005). According to the WHO estimates over 50 million healthy life-years across the globe will be lost by the year 2015 as a result of stroke and 90% of this burden will be borne in low-income and middle-income countries (Strong et al., 2007). Once damage from stroke has plateaued, little can be done to recover premorbid functions. Therefore, as the leading cause of adult disability, stroke poses substantial economic and psychological burden on human population around the world. In contrast to stroke, most of neurodegenerative disorders are insidious with substantial neural injury present well before symptoms are revealed. This is not true in case of stroke, where the onset of injury and symptomatology typically coincides. Moreover, there is a distinct and relatively brief temporal pattern of injury which makes stroke as a devastating and debilitating event – one associated with high mortality and a high emotional cost and financial burden to patients, families and society.

The only FDA approved drug for stroke treatment is recombinant tissue plasminogen activator (r-tPA; alteplase) or prolyse (pro-urokinase) with a narrow therapeutic time window of 3 hours (Brinker et al. 1999a). Recent clinical trials have failed to demonstrate unequivocal benefit using later times in stroke patients (Hacke et al., 1998). Secondly, the diagnosis of stroke is also a challenge and a computed tomography scan needed to confirm that the stroke is ischemic and not haemorrhagic, so care should be taken while giving r-tPA as it exacerbate damage in hemorrhagic condition. Thirdly, r-tPA increases the incidence of cerebral haemorrhage (Wardlaw et al., 1997), by increasing the level of matrix metalloproteinase-9 (MMP-9) in the

endothelium (Lapchak et al., 2000; Pfefferkorn et al, 2003). Therefore, models of ischemia/reperfusion can provide significant insights into early and late cerebral injuries and may help in understanding the molecular mechanisms resulting in cell death during cerebral ischemia and reperfusion (I/R) injury and that may provide a more realistic picture for future stroke treatments.

The brain has poor capacity to store energy; therefore it requires a constant supply of oxygenated blood to maintain its structural and functional integrity. A drop in cerebral blood flow (CBF) after ischemia or hypoxia can cause critical shortage of cellular energy, which starts cell death. Cell death during periods of ischemia and subsequent reperfusion is caused by the loss of the energy supply due to deprivation of oxygen, glucose and cerebral damage is aggravated by the effects of excessive accumulation of neurotransmitters. Oxygen and glucose deprivation results in reduced production and depletion of ATP. A pivotal event is the loss of ATP-dependent Na^+- K^+- ATPase function, resulting in profound membrane depolarization and sodium, calcium dyshomeostasis. It has been proposed that increased intracellular calcium causes increased release of glutamate (Martin et al 1994) and that ATP depletion causes changes in ionic balance that drive ion-dependent glutamate transport in reverse, leading to further extracellular glutamate accumulation (Rossi et al 2007). Glutamate release contributes to a vicious cycle, as the activation of glutamate receptors further increases intracellular calcium concentration, causing calcium overload which can trigger cell death (Rossi et al 2007).

Restoration of blood flow following ischemic stroke can be achieved by means of thrombolysis or mechanical recanalization. However, for some patients, reperfusion may exacerbate the injury initially caused by ischemia, producing the so-called "cerebral reperfusion injury". The exacerbation of ischemic or hypoxic injury by reoxygenation represents a major clinical challenge in treating episodes of cerebral ischemia; as it causes burst of reactive oxygen species (ROS) which overwhelms cellular antioxidant defenses and results in oxidative stress and leading to cellular damage. ROS have been implicated in cerebral ischemic damage since 1970s, yet the full complexity of their generation and their precise role in stroke pathogenesis is still not fully understood. It is

worth noting that one of the first papers to describe a role for oxidative damage in stroke (Flamm et al, 1978) proved that free radicals are major culprit to injury and identified their role through diminution of antioxidant defenses in cat brain following focal ischemia. Major enzymes involved in the clearance of ROS include superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase converts superoxide to hydrogen peroxide, which may then be decomposed by either catalase or glutathione peroxidase. The importance of ROS in instigating neuronal injury has been highlighted more recently through transgenic animal studies. Mice with transgenic absence of both SOD1 and glutathione peroxidase show even larger infarcts, suggesting that glutathione peroxidase is also important in reducing neural injury in ischemia (Crack et al, 2003). On contrary, mice over expressing SOD1 have approximately 35% smaller infarct volume after focal ischemia (Kinouchi et al, 1991). These accumulating evidences suggest that ROS is detrimental to the biological tissues but these finding were challenged after the recent discovery that the ROS can function as signaling molecules to regulate long-term vascular processes, such as cell growth and division, by acting as mediators for the effects of mitogens, such as angiotensin II and platelet-derived growth factor (PDGF), on protein kinase (Meerson et al 1982; Tosaki et al 1993; Ushio-Fukai et al 1998; ; Rosette et al, 1999; Herrlich et al, 2000) and transcription factor (Olivetti et al 1997, Nishio et al 1998) activities and on gene expression (Das et al 1998).

The major sources of ROS in the brain are the mitochondrial respiratory chain, xanthine oxidase (XO) and NADPH oxidase (NOX). Genetic and pharmacological studies have provided information about enzymatic ROS sources, complimenting each other with their specificity and acuteness, respectively. It is now clear that different sources of ROS combine in complex ways to produce the total damage. It is unlikely that inhibition of any one source of ROS would be sufficient to totally protect neurons from death, yet some inhibitors have proved encouraging results in clinical trials and more general antioxidant therapy offers some hope.

Abramov et al (2007) examined the kinetics of ROS production during hypoxia and reoxygenation in primary cultures of neurons from rat hippocampus and cortex. The nature and time course of these changes were somewhat unexpected; three distinct phases

of ROS generation were identified, occurring with a specific temporal relationship to metabolic events taking place within the cells. These findings suggest that, three phases of ROS production in neurons were attributable to three distinguishable ROS sources, viz., mitochondria, xanthine oxidase and NADPH oxidase. Further, they found a role for all these three sources at different time points during ischemia and reperfusion and demonstrated that they all, having been previously implicated independently, have an important and interactive role to play in generating ROS during hypoxia–reoxygenation. Mitochondria respond first to ischemia/reperfusion but are quickly limited by the insufficient oxygen, thereafter; xanthine oxidase becomes an important source of superoxide during ischemic period, whereas during reperfusion NADPH oxidase is a major source of superoxide.

The physiological function of NOX enzyme is to generate reactive oxygen species and play critical role in cellular defense mechanism, which may differentiate this enzyme system from others, whose ROS production abilities are often considered epiphenomenal. NOX is a multi-subunit complex composed of membrane associated subunits of gp91phox, p22phox and cytosolic subunits of p47phox, p67phox and p40phox. On Activation NOX, p47phox phosphorylation first occurs which subsequently causes cytosolic subunits p67phox and p40phox translocate into membranes and fuse with catalytic subunit gp91phox and p22phox (Bedard et al, 2007). The activated enzyme complex transports electrons to oxygen, thus producing superoxide anion, which is a member of oxygen radicals (Bedard et al, 2007). During ischemia, the stimulus activating NADPH oxidase is not clear and could include calcium influx mediated by neurotransmitter receptors or substances released by dying neurons.

NOX has been implicated in ischemia-reperfusion injury. Mice lacking a functional NOX2 and subjected to acute middle cerebral artery occlusion (MCAO) and reperfusion have a significantly reduced infarct size compared to controls (Kahles et al, 2007; Walder et al, 1997) In gerbils, the effects of occlusion of the common carotid arteries were reduced through administration of the NOX inhibitor apocynin (Wang Q, 2006). The role of NOX seems mainly linked to reperfusion; microglial ROS production was reduced during hypoxia and stimulated by reoxygenation (Spranger et al, 1998). This

is consistent with a role for NOX in ROS production during reperfusion, rather than ischemia and highlights the importance of NOX inhibitors during this later stage during which the patient is more likely to be under medical care. However, these studies failed to reveal the regulation, modulation of NADPH oxidase, role of its various sub-units and its contribution to ischemic brain damage and cell survival signaling.

Present study, therefore, is an attempt to revealing the role of NADPH oxidase and ROS derived from NADPH oxidase at varying time points of ischemia/reperfusion injury using rat model of transient focal cerebral ischemia and also to elucidate role of NADPH oxidase in cell death/survival mechanism in early and late phase of cell injury. These findings are expected to help in understanding the basic events involved in NADPH oxidase mediated cell survival/death signaling pathways during I/R injury.