Chapter VI

Neuroprotective effect of Ellagic Acid in experimental stroke
Introduction

Stroke is a leading cause of severe chronic mortality and morbidity and has become a major health concern, not only as it is a threat for life but because of the aftermath linked to it (Tsagalis et al., 2009). Extensive evidence from clinical studies suggests that majority of them are occlusive in nature (Lloyd-Jones et al., 2008). Despite the enormous effort made, only one approved therapy exist today, the effectiveness of which is moderate and can only be used in 10% of the cases because of the risk of intracerebral hemorrhage and narrow time window to use it (Kleinschnitz et al., 2010; Lapchak et al., 2010). To overcome this lack of clinically effective trails, innovative strategies are need to be elucidated, to identify the molecular pathways that can be targeted with innovative therapies.

Ischemic stroke is caused by cessation blood flow to the brain tissue. Disrupted blood flow to the brain in focal cerebral ischemia affects only limited part of the brain (blood clot occluding major artery unilaterally). Transient ischemia has a reperfusion process, which provides oxygen as a substrate for numerous enzyme oxidation reactions that produce free radicals such as superoxide anion (O$_2^-$), perhydroxy radical (HOO$^-$), hydroxyl radical (OH), and free radical nitric oxide (NO$^-$). Thus, free radicals are closely related to this type of injury (Lewen et al., 2000; Cuzzocrea et al., 2001; Kontas et al., 2001). Ischemia/reperfusion activates lipid peroxidation, protein oxidation, and nitric oxide synthase (NOS) (Dringal et al., 1999; Lewen et al., 2000; Cuzzocrea et al., 2001; Kontas et al., 2001). Antioxidant molecules, glutathione, and enzymatic defense system, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-6-phosphate dehydrogenase (G6PDH) are depleted after ischemia/reperfusion (I/R).

It has been a major challenge to develop effective therapeutics for stroke. Many antioxidants are reported to reduce reactive oxygen species (ROS) mediated reactions and rescue neurons from ischemia-reperfusion-induced neural loss in animal models of cerebral ischemia (Ahmad et al., 2006; Al-Majed et al., 2006). Ellagic acid (C$_{44}$H$_{66}$O$_{14}$) (EA) is a polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries. It has anti-carcinogenic, antioxidant and anti-fibrosis properties (Boyuk et al., 2011; Vadhanam et al., 2011). However, the effects of ellagic acid on ischemic stroke have not been studied. Furthermore, the mechanisms
mediating anti-oxidative potential of ellagic acid, in general, remain unknown. Thus, EA with the above properties has stimulated us to study its neuroprotective role in ischemia-reperfusion injury. Accordingly, we designed this study to investigate the effects of EA on the post ischemic deterioration in stroke model of rat.

**Experimental procedures**

**Chemicals and reagents**

As described in material and methods, chapter-II

**Animals and treatment**

As described in material and methods, chapter-II

**Drug administration**

Ellagic Acid (60 mg/kg body weight in saline) was administered orally once daily for 21 days before MCAO (Umesalma et al., 2011).

**Middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia**

As described in material and methods, chapter-II

**Post-operative care**

As described in material and methods, chapter-II

**Experimental design**

To investigate the neuroprotective effects of Ellagic Acid in an experimental model of cerebral ischemia, we used the rat MCAO model (Longa et al., 1989). Animals were divided into four groups each having eight animals. The first group served as sham and saline was given orally, the second was middle cerebral artery occluded (MCAO), i.e., ischemia was induced for 2h followed by reperfusion for 22h, the third was pretreated for 21 days with EA (60 mg/kg, orally) followed by MCAO for 2h and reperfusion for 22h, (i.e., EA-MCAO group) and the fourth was pretreated for 21 days with drug alone, i.e., EA group (60 mg/kg, orally). After the completion of the reperfusion period, the
animals were assessed for neurobehavioral activity and then sacrificed. The brains were taken out to dissect the frontal cortex and striatum for biochemical estimations.

Behavioral studies
The behavioral test in each group was performed before and after occlusion and reperfusion. The experiment was performed between 9:00 A.M. to 4:00 P.M. at standard laboratory conditions. All tests were performed and analyzed by subject blind to the experiment.

Rota rod (muscular coordination)
As described in material and methods, chapter-II.

Grip Strength
As described in material and methods, chapter-II.

Biochemical studies
Tissue preparation for the assays
After behavioral study, the animals were sacrificed and their brains were taken out to dissect frontal cortex and striatum for the biochemical assays (TBARS, GSH, GPx, GR, G6PDH, SOD, catalase) as described in material and methods, chapter-II.

Histopathological examinations
After 24 h of MCAO, the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused as previously described by Nakayama et al. (1998). The brains were removed quickly and kept in the same buffer containing 30% sucrose. The brains were cut into 10-μm-thick coronal sections on crystat (Leica, Germany). Every 10 sections of the cortex region, was mounted on glass slides, and processed for hematoxylin and eosin staining.
Immunohistochemistry for p53, Apaf-1, Caspase-9
As described in material and methods, chapter-II

**Determination of protein**
Protein was determined by the method of Lowry et al. (1951) using BSA as a standard.

**PARP**
As described in material and methods, chapter-II.

**GEL electrophoresis**
As described in material and methods, chapter-II.

**Statistics**
As described in material and methods, chapter-II.

**Results**

**Effect of ellagic acid on behavioral output**

**Rota Rod**
Motor function impairment caused by cerebral ischemia was evident in the vehicle-treated MCAO group as compared to sham group (p < 0.001). Ellagic acid was found to be effective in partial recovery of muscular in-coordination in ellagic acid pretreated MCAO (EA+MCAO) group (p < 0.001) as compared to MCAO group. No significant difference was observed between sham and ellagic acid treated sham group (EA+Sham) (Fig. 1).

![Graph](image)

Fig.1. MCAO leads a significant decrease in motor coordination in MCAO group as compared to Sham group and significantly recovered in EA+MCAO group as compared to MCAO group. Values are expressed as mean±S.E.M of six animals. *p < 0.001, MCAO vs. sham; †p < 0.001, EA+MCAO vs. MCAO.
Grip strength

The mean score was decrease significantly (p < 0.001) in the vehicle treated MCA occluded group as compared to the sham group rats. The ellagic acid pre-treatment has increase the mean score significantly (p < 0.001) in EA+MCAO group as compared to the vehicle treated MCAO group (Fig. 2). However no significant alteration was observed in ellagic acid treated sham (EA+Sham) group as compared to sham group.

Effect of ellagic acid on endogenous antioxidant system

EA pretreatment decreased the TBARS contents in striatum and frontal cortex

The effect of Ellagic acid on TBARS content was measured to demonstrate the oxidative damage in frontal cortex and striatum of MCAO group. A significant increased (p < 0.001) level of TBARS was observed in MCAO group animals as compared to Sham group. Rats of EA+MCAO group exhibited significant attenuation (p < 0.05, p < 0.001) in TBARS content as compared to MCAO group (Fig. 3). Ellagic acid alone pre-treated group showed significant changes in TBARS as compared to sham group.
EA pretreatment restored the GSH level in striatum and frontal cortex

Protective effect of Ellagic acid on GSH level in frontal cortex and striatum was observed. The level of GSH was depleted significantly in frontal cortex ($p < 0.001$) and striatum ($p < 0.001$) in MCAO group as compared to Sham group. Ellagic acid pretreatment increased its level significantly ($p < 0.05$, $p < 0.001$) in EA+MCAO group as compared to MCAO group. Ellagic acid alone pretreated group exhibited no significant changes in GSH level as compared to sham group (Fig. 4).

Ellagic Acid pretreatment attenuated the activities of glutathione dependent antioxidant enzymes in striatum and frontal cortex

The activities of antioxidant enzymes (GPx, GR and G6PDH) were decreased significantly in frontal cortex and striatum of MCAO group animals as compared to sham group animals and their activities were restored significantly in frontal cortex as well as in striatum of ellagic acid treated MCAO group (EA+MCAO) animals as compared to MCAO group animals. No significant change was observed in ellagic acid pretreated sham group (EA+Sham) animals as compared to sham group animals (Tables I and II).
Table I. Effect of cerebral ischemia on the activity of various enzymes in Frontal cortex of different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx (nmol NADPH oxidized/min/mg protein)</th>
<th>GR (nmol NADPH oxidized/min/mg protein)</th>
<th>G6PD (nmol NADP reduced/min/mg protein)</th>
<th>SOD (nmol epinephrine protected from oxidation/min/mg protein)</th>
<th>CAT (nmol H₂O₂ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>275.72 ± 18.75</td>
<td>320.57 ± 32.39</td>
<td>81.13 ± 4.60</td>
<td>339.89 ± 25.85</td>
<td>7.21 ± 0.14</td>
</tr>
<tr>
<td>MCAO</td>
<td>194.95 ± 7.95*</td>
<td>206.62 ± 24.63*</td>
<td>40.30 ± 2.29**</td>
<td>187.28 ± 8.43***</td>
<td>5.29 ± 0.15***</td>
</tr>
<tr>
<td></td>
<td>(- 29.29%)</td>
<td>(- 35.54%)</td>
<td>(- 50.43%)</td>
<td>(- 44.89%)</td>
<td>(- 54.36%)</td>
</tr>
<tr>
<td>EA+MCAO</td>
<td>250.30 ± 19.44*</td>
<td>270.18 ± 7.75*</td>
<td>72.52 ± 3.29**</td>
<td>274.75 ± 7.97**</td>
<td>5.41 ± 0.15***</td>
</tr>
<tr>
<td></td>
<td>(28.39%)</td>
<td>(15.71%)</td>
<td>(79.95%)</td>
<td>(46.70%)</td>
<td>(64.43%)</td>
</tr>
<tr>
<td>EA+Sham</td>
<td>270.16 ± 12.45</td>
<td>323.07 ± 11.70</td>
<td>80.22 ± 5.57</td>
<td>340.06 ± 13.09</td>
<td>7.27 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>(-2.0%)</td>
<td>(0.77%)</td>
<td>(-1.12%)</td>
<td>(0.05%)</td>
<td>(0.83%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E in nmol/min/mg protein. Significance was determined as *p<0.05, **p<0.01, ***p<0.001 when compared with sham group; *p<0.05, **p<0.01, ***p<0.001 when compared with MCAO group. Values in parentheses show the percentage increase or decrease with respect to their control.

Table II. Effect of cerebral ischemia on the activity of various enzymes in striatum of different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx (nmol NADPH oxidized/min/mg protein)</th>
<th>GR (nmol NADPH oxidized/min/mg protein)</th>
<th>G6PD (nmol NADP reduced/min/mg protein)</th>
<th>SOD (nmol epinephrine protected from oxidation/min/mg protein)</th>
<th>CAT (nmol H₂O₂ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>283.04 ± 14.42</td>
<td>300.83 ± 9.60</td>
<td>81.70 ± 6.99</td>
<td>344.92 ± 17.10</td>
<td>7.19 ± 0.15</td>
</tr>
<tr>
<td>MCAO</td>
<td>174.12 ± 12.90*</td>
<td>202.87 ± 9.52*</td>
<td>43.91 ± 2.75**</td>
<td>220.16 ± 26.74***</td>
<td>3.37 ± 0.13***</td>
</tr>
<tr>
<td></td>
<td>(-38.48%)</td>
<td>(-32.56%)</td>
<td>(-64.25%)</td>
<td>(-36.17%)</td>
<td>(-53.12%)</td>
</tr>
<tr>
<td>EA+MCAO</td>
<td>229.57 ± 6.17*</td>
<td>258.45 ± 15.99*</td>
<td>68.03 ± 4.43**</td>
<td>287.30 ± 15.38**</td>
<td>6.52 ± 0.37***</td>
</tr>
<tr>
<td></td>
<td>(49.07%)</td>
<td>(27.39%)</td>
<td>(54.49%)</td>
<td>(30.49%)</td>
<td>(93.47%)</td>
</tr>
<tr>
<td>EA+Sham</td>
<td>285.40 ± 6.47</td>
<td>298.56 ± 20.83</td>
<td>80.71 ± 3.90</td>
<td>338.04 ± 5.41</td>
<td>7.20 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>(0.83%)</td>
<td>(-0.75%)</td>
<td>(-1.21%)</td>
<td>(-1.99%)</td>
<td>(0.13%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E in nmol/min/mg protein. Significance was determined as *p<0.01, ***p<0.001 when compared with sham group; *p<0.05, **p<0.01, ***p<0.001 when compared with MCAO group. Values in parentheses show the percentage increase or decrease with respect to their control.
Ellagic Acid pretreatment attenuated the activities of SOD and catalase level in striatum and frontal cortex

The activity of SOD and catalase in frontal cortex and striatum was decreased significantly in MCAO group as compared to the sham group. The ellagic acid pretreatment has restored there activity significantly in frontal cortex and striatum in ellagic acid treated MCAO (EA+MCAO) group as compared to the MCAO group. No significant change was observed in the ellagic acid pretreated sham (EA+Sham) group as compared to the sham group (Tables I and II).

Effect of ellagic acid pretreatment on histopathological changes in MCAO rats

Figure. 5 shows the histopathological changes after 2 h of occlusion and 22 h of reperfusion. Sections of the brain passing from frontal cortex of MCAO, EA+MCAO and sham groups were examined. Sections of MCAO group showed neuronal loss and presence of numerous vacuolated spaces. Intact neurons were absent in that area. The corresponding area in the sections of EA+MCAO group showed partial neuronal loss and the presence of intact neurons in between the vacuolated spaces. The section of the sham group showed normal neurons with no pathological change.

Fig. 5. Cortical area of sham group animal showing uniform distribution of neuron (A). Normal neurons with the characteristic conical outlines with no abnormal features are seen. Tissues around infarcted area in MCAO group showing a focal area of vacuolation and neuronal loss (B). Ellagic Acid-pretreated group EA+MCAO shows partial neuronal loss (C). Magnification at 20X.
Effect of ellagic acid pretreatment on p53, Apaf-1 and caspases-9 expression in MCAO rats

The activation of p53 protein is associated with neuronal cell death in cerebral ischemia. p53 expression was found to be remarkably high in ischemic hemisphere of the vehicle treated MCAO group rats (Fig. 6B). A noticeable reduction in p53 expression was observed in ellagic acid pretreated group as compared to MCAO group (Fig. 6C). Expression of p53 was seen to be very scarce in sham group (Fig. 6A). Ellagic acid pretreatment did not show any remarkable effects in the EA+Sham group compared with the sham group (data not shown).

Fig.6. Effect of ellagic acid pretreatment on p53, Apaf-1 & Caspase-9 expression respectively. The profound expression of SOD, Hsp70 & NF-kB was observed in MCAO group (B) compared to sham group (A), while the MCAO group pretreated with EA (C) has shown a moderate staining of p53, Apaf-1 & Caspase-9. However, the sham group has shown almost negligible staining. Magnification at 20X.
The expression of Apaf-1 was increased strongly in the ischemic hemisphere of the vehicle control rats. In EA+MCAO group Apaf-1 was decreased as compared to MCAO group (Fig. 6C). Ellagic acid pretreatment did not show any remarkable effects on EA+Sham group as compared with sham group (data not shown).

Caspase-9 expression was significantly increased in MCAO group as compared to sham group. Caspase-9 activity was significantly attenuated by the administration of silymarin (Fig. 6C). Ellagic acid pretreatment did not show any remarkable effects in the EA+Sham group compared with the sham group (data not shown).

Effect of ellagic acid on poly (ADP-ribose) polymerase activity

The activity of PARP was assessed by radioactive assay whether this key regulator of apoptosis was involved in ischemia reperfusion induce stress. The activity of PARP was increased significantly (p < 0.01) in MCAO group as compared to sham group. EA supplementation significantly (p < 0.05) recovered its activity in EA+MCAO group as compared to MCAO group (Fig. 7).

Effect of ellagic acid on DNA fragmentation

Figure 8 shows the pattern of DNA degradation in cortex following ischemia reperfusion. In sham group no DNA fragmentation was detected in cortex, whereas MCAO group rats resulted in a shearing pattern of DNA fragmentation (lane 3).
However, pre-treatment of animals with EA before MCAO resulted in no apparent DNA shearing (lane 2).

Fig.8. Representative agarose gel electrophoresis exhibits DNA fragmentation in cortex following MCAO. Lane 1, DNA isolated from cortex after sham operation. Lane 2, pre-treatment of animals with EA before MCAO resulted in no apparent DNA shearing DNA obtained from cortex. Lane 3, DNA isolated from cortex after MCAO.

Discussion

The present study demonstrates the beneficial effects of EA in MCAO rats. In this context, we have evaluated middle cerebral artery occlusion (MCAO) rats model for the behavioral and biochemical tests which is widely used to study neuroprotective effect of drugs because it recapitulates the biochemical and pathological features of stroke in humans (Khan et al., 2009; Longa et al., 1989) such as oxidative stress, mitochondrial dysfunction and apoptosis.

The behavioral impact is closely linked to the degree of neuronal dysfunction (Schwarting et al., 1991). Functional deficits are common neurological sequel in patients with brain injuries and animal models of cerebral ischemia (Yousuf et al., 2009). In the present study, it is suggested that EA which is a potent antioxidant, has reduced neurobehavioral deficits significantly in EA treated animals by scavenging free radicals, which are thought to cause behavioral deficits in experimental animals (Beckman et al., 1990). Earlier studies have shown an improvement in various behavioral outputs like motor coordination skill as a result of antioxidant treatment (Zafar et al., 2003; Kovalenko et al., 2006). In this study, the motor function was found to be disturbed in rota rod and grip strength task. We proved that the motor functions were impaired after ischemic insults in rat and were significantly ameliorated by supplementation with EA.

The biochemical mechanism immersed in the thriving of ischemia-induced neuronal injury is well studied (Kirino, 1982 and Evans, 1993). ROS are critical determinants in
brain injury (Chan et al., 2001). We found that cerebral ischemia caused a significant increase in lipid peroxides and protein carbonyl accompanied by significant depletion in brain GSH. These results are in agreement with other studies (Candelario et al., 2001; Al-Omar et al., 2006; Saleem et al., 2006). The overproduction of ROS can be detoxified by endogenous antioxidants, causing their cellular stores to be depleted (Candelario et al., 2001). GSH, which is considered the most prevalent and important intracellular non-protein thiol, has a crucial role as a ROS scavenger.

The loss of GSH and formation of PrSSG in the brain results the various membrane dysfunction, such as inhibition on Na⁺ K⁺ ATPase activity. The enzyme is relevant for cellular excitability and is very susceptible to free radical reaction and lipid peroxidation because it is wedged in cell membrane and requires phospholipids for the maintenance of the activity (Furui et al., 1990; Ildan & Polat, 1996). There are several reports about the modulatory effect of EA on lipid peroxidation, glutathione and antioxidant enzymes following brain injury (Yang et al., 2008). In agreement with this finding, we also observed that EA significantly reduced the TBARS level along with increase in level of glutathione and antioxidant enzymes. SOD scavenges superoxide radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen (Freeman and Crapo, 1982). The hydrogen peroxide formed by SOD and by other processes is scavenged by GPx and CAT, a ubiquitous protein that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen. GPx uses hydrogen peroxide to oxidize GSH (Beckman et al., 1990). Thus, GPx is low molecular weight antioxidant and play a key role in detoxifying hydrogen peroxide (Imam and Ali, 2000).

Glucose-6-phosphate dehydrogenase enzyme (G6PDH) is known to produce NADPH which acts as the reducing potential for the output of reduced glutathione (GSH), as well as the controller of the activity of plasma membrane (PM). NADPH oxidase under stress, which results in H₂O₂ accumulation. H₂O₂ acts as a signal in regulating G6PDH activity and expression, and the activities of the enzymes in the glutathione cycle as well, through which the ability of GSH regeneration is known to increase under stress. Thus, G6PDH plays a critical role in maintaining cellular GSH levels under long-term stress. In
the current work, GSH content was significantly reduced due to ischemic insult and also significant decline in activity of the endogenous antioxidant enzymes were observed. However with the supplementation of EA all these alterations were significantly attenuated.

Besides defending against oxidant stress, another existing and encouraging finding was that EA significantly attenuated histological changes i.e. it caused minimal glial cell infiltration, less neural damage with small vacuolated space along with presence of intact neuron in the neuronal tissue as compared to ischemic cortical neuronal loss. The section of sham group exhibited normal neuronal staining and did not show any significant histological changes. Our findings are consistent with other studies where antioxidants have been used to improve the morphological changes after stroke (Yousuf et al, 2009 and Khan et al, 2009).

Apoptotic and anti-apoptotic signaling pathways are activated after cerebral ischemia, and it is generally accepted that a shift in the balance between pro- and anti-apoptotic protein factors toward the expression of proteins that promote cell death may be one mechanism underlying apoptotic cell death (Saito et al, 2004). It has been known that apoptosis is one of the major neuronal cell death mechanisms in the experimental model of cerebral ischemia (Shin et al., 2006; Wang et al., 2008; Broughton et al., 2009). Various signalling pathways are involved in apoptotic cell death. Induction of p53, Apaf-1 and caspase-3 activation is associated with stroke-induced apoptotic neuronal cell death (Ji et al., 2007; Dohare et al., 2008; Brahma et al., 2009) and inhibition of p53, Apaf-1, expression and caspase-inhibition reduced ischemic injury (Xu et al., 2003; Chen et al., 2009; Luo et al., 2009). In the present study, we showed that elevations of caspase-3 activity and p53, Apaf-1, caspase-9 expressions occurred after ischemic injury, and these expressions could be significantly suppressed by EA pre-treatment.

PARP, activated by cleaved DNA strands, utilizes nicotinamide adenine dinucleotide (NAD⁺) as its substrate and synthesizes long branched and negatively charged polymers of ADP-ribose which are covalently bound to chromatin associated proteins and to PARP itself. Resynthesis of NAD⁺ leads to ATP depletion and necrotic cell death. There are also proposition that PARP induced NAD⁺ utilization may first release apoptosis inducing factor (AIF) from mitochondria and activate caspases independent mechanisms.
of apoptotic cell death (Yu et al., 2002). PARP and caspase-3 activation, in the ischemic stroke induces apoptotic processes in the cortical region via a mitochondria-mediated pathway. PARP is also known for its interaction with several transcription factors engrossed in the regulation of apoptosis, one among them is p53 (Hassa & Hottiger, 1999; Oliver et al., 1999; Zhu et al., 2009). Both factors play a critical role in transcription of many proteins, regulation of DNA repair and in immune or inflammatory responses. PARP inhibited by EA protected cells against death provoked by brain ischemic/reperfusion injury. So, EA act as PARP inhibitor, besides their specific inhibitory action on the enzyme, may possess some anti-oxidative properties, affecting their pharmacological specificity. P53 might primarily serve a protective role by activating DNA repair mechanisms after mild to moderate insults but could become detrimental in response to severe ischemic insults (Tomasevic et al., 1999). A fraction of induced p53 translocates to the mitochondria at the onset of p53-dependent apoptosis in cerebral ischemia (Khan et al., 2009). Bypassing the nucleus by targeting p53 to mitochondria is yet profuse to launch apoptosis by direct signaling at the mitochondria. They propounded that mitochondrial translocation of p53 triggers a rapid pro-apoptotic response that jump starts and amplifies the slower transcription-dependent response. EA plays an innovative role for treating neurologic ailments that involve caspase-mediated cell dysfunction and cell death. It has been reported that blockade of p53 activation and PARP inhibition by pharmacological agents are associated with inhibition of apoptosis (Culmsee & Mattson, 2005; Graziani & Szabo, 2005). Together our data and similar evidence in MCAO model of cerebral ischemia further support the neuroprotective potential of ellagic acid.
Stroke is one of the leading causes of morbidity and mortality. It is the acute severe manifestation of cerebro-vascular disease. WHO defined stroke as ‘rapidly developed clinical signs of focal disturbance of various cerebral functions, lasting more than 24h or leading to death, with no apparent cause other than vascular origin. Ischemic stroke is the most frequent form of stroke encountered in clinical studies. It is caused by a reduction or complete blockade of blood flow, resulting in the deficiency of glucose and oxygen supply in the territory of the affected region.

Increased levels of reactive oxygen species (ROS) are the major cause of tissue injury after cerebral ischemia, in which inactivation of antioxidant enzymes and consumption of antioxidants such that endogenous antioxidant defense mechanisms fail to protect neurons from oxidative damage. Oxidative stress is the state of imbalance between the level of antioxidant defense mechanism and production of the free radicals that favor the latter leading to potential damage. Brain tissue is particularly susceptible to oxidative damage. Therefore, it is believed that pharmacological modification of oxidative damage is one of the most promising avenues for stroke therapy.

The use of appropriate animal models is essential to predict the value and effect of therapeutic approaches in human subjects. There is a great need to understand the mechanisms of ischemic injury and neuroprotection, if we are ever to learn new target sites to treat ischemia. There are many animal models available to investigate injury mechanisms and neuroprotective strategies; however the success lies only when we can reproduce the result. This has prompted us to critically review the current animal models and discuss how these models may yield fresh insights into the pathogenesis associated to ischemia reperfusion, as well as new preventive/therapeutic opportunities.

Based on current knowledge regarding the ethology, pathogenesis and mechanism of cell death in cerebral ischemia, numerous neuroprotective strategies might be devised. Neuroprotection might be provided by agents that interfere with factors involved in pathogenesis. To date, most basic and clinical trials have focused on antioxidants and anti-inflammatory agents.

Chapter-III

The present study was undertaken to investigate the neuroprotective and anti-inflammatory properties of hesperidin against ischemia reperfusion induced brain insult in Middle cerebral artery occlusion (MCAO) model of cerebral stroke. Rats were divided into three sets of experiment each having four groups. In the first set of experiment 8 animals per group were used while in the rest experiments each group consisted of 6 animals. Hesperidin (50 mg/kg body weight in saline) was administered orally (p.o.) once daily for 15 days before MCAO. On day 16, MCAO was performed.
for 2h and reperfusion for 22h. After 22h of ischemia the animals were assessed for neurobehavioral activity and then sacrificed for the estimation of superoxide dismutase, catalase, glutathione content, lipid peroxidation and to assess the anti-inflammatory potential of hesperidin etc. This finding indicated that MCAO cause behavioral deficits and oxidative stress due to abrupt generation of free radical. However hesperidin treatment offered significant protection against transient focal cerebral ischemic induced characteristic behavioral and biochemical alteration, which is further corroborated by reduced brain edema and increased endogenous antioxidant defence system, which may be attributed to its anti-oxidative and anti-inflammatory property.

Thus findings of this study demonstrated that hesperidin protected neurons against ischemia reperfusion induce brain injury.

Chapter-IV
The aim of this study was to identify the effect of Silymarin on ischemia reperfusion induced oxidative and apoptotic neuronal damage. Animals were divided into four groups each having eight animals. The first group served as sham (S) and received vehicle (0.3% sodium carboxymethyl cellulose) orally, second was middle cerebral artery occluded (MCAO) group and received vehicle only, third was MCAO group pretreated with silymarin (200 mg/kg in 0.3% sodium carboxymethyl cellulose, orally) (Sil + MCAO), once daily for 15 days and fourth was pretreated with drug alone (Sil + S) i.e., silymarin group. After the completion of the reperfusion period, the animals were assessed for neurobehavioral activity and then sacrificed. The brains were taken out to dissect frontal cortex and striatum for biochemical estimations. Here we provide evidence that MCAO induces oxidative stress and provokes apoptosis by a common oxidative mechanism involving free radicals and production of by product, which in turn triggers a specific cell signalisation through the activation of p53, apaf-1 and caspase-3 activation pathway. The present study demonstrated that silymarin supplementation potentially reverses ischemia-reperfusion induced alterations in behavioral, biochemical and immunohistopathological parameters in rats. These beneficial effects of silymarin are attributed to its anti-oxidantive properties.

Chapter-V
The most important findings of this study suggest that the neuroprotective effect of naringenin on cerebral ischemic damage in MCAO-reperfusion rats is probably mediated by the inhibitionof
Summary of Conclusion

neurological deficits and oxidative damage followed by the inhibition of inflammatory responses. To investigate the neuroprotective effects of naringenin in experimental model of cerebral ischemia, we used the rat MCAO model (Longa et al., 1989). Animals were divided into four groups each having eight animals. The first group served as sham, second was middle cerebral artery occluded (MCAO), i.e., ischemia was induced for 2 h followed by reperfusion for 22 h, third was treated with naringenin (50 mg/kg in saline, orally) for 21 days before the onset of ischemia and fourth was treated with drug alone, i.e., naringenin group (50 mg/kg, orally). After the completion of the reperfusion period, the animals were assessed for neurobehavioral activity and then sacrificed. The brains were taken out to dissect frontal cortex and striatum for biochemical estimations and immunological assays. It has been well known that ischemia-reperfusion induce brain injury in rodents induces neuronal loss which was well ameliorated by the supplementation of naringenin. These observations suggest that naringenin may be a clinically viable protective agent against a variety of conditions where cellular damage is a consequence of oxidative stress. Further understanding the mechanism underlying the neuroprotection of naringenin will provide an avenue to disclose both the pathogenesis and therapeutic mechanisms underlying ischemia-reperfusion injury. We believe that our results will contribute to the clinical applications in the treatment of stroke.

Chapter VI

This chapter provides evidence that prophylactic treatments with ellagic acid has prophylactic potential in that it protects against ischemia reperfusion induced oxidative stress in MCAO model of stroke. Animals were divided into four groups each having eight animals. The first group served as sham, second was middle cerebral artery occluded (MCAO), i.e., ischemia was induced for 2 h followed by reperfusion for 22 h, third was treated with ellagic acid (60 mg/kg, orally) for 21 days before the onset of ischemia and fourth was treated with drug alone, i.e., ellagic acid group (60 mg/kg, orally). The behaviour tests were monitored after 22 h of ischemia and then animals were sacrificed for evaluation of biochemical parameters. The present study provided evidence that the pre-treatment with ellagic acid resulted in better performances in behavioral tests. Furthermore, pre-treating rats with ellagic acid before ischemia lead to a restoration of compromised behavioural activity and cellular integrity by reversing the effect and restoring near normal levels of the various enzymatic and non-enzymatic markers of lipid peroxidation and GSH. These findings are further strengthened by the normalization of PARP receptors and by immunological evidences. Thus
findings of this study demonstrated that ellagic acid protected cortical and striatal neurons against ischemia reperfusion induce brain insult.

So these selected neuroprotective agents: hesperidin, silymarin, naringenin and ellagic acid can be used as favoured remedies in ischemia reperfusion brain injury pending elucidation of proper molecular mechanisms and deciphering appropriate genetic pathways.