Chapter III

Hesperidin improves functional and histological deficits and reduces neuroinflammation in ischemic brain injury in rats
Introduction

Cerebral stroke sets off a series of pathophysiological events that are linked to primary morbid consequence - death of neurons and elicit inflammatory responses which are believed to participate in secondary injuries. It is one of the leading causes of mortality and morbidity, with astronomical financial repercussions on health systems worldwide (Allen and Bayraktutan, 2009). Presently it has turned into a major public health hazard in developing countries of Asian subcontinent.

Oxidative stress has always been implicated in the machinery of ischemia-reperfusion injury after cerebral ischemia in a number of studies (Chan et al., 1994; Kinouchi et al., 1991) consequently producing surplus amounts of biphasic players, reactive oxygen species (ROS). ROS plays a role in normal physiology and is also caught up in a number of disease processes in pathological condition. A substantial body of evidence has been produced that links the production of ROS and subsequent oxidative damage to the pathogenesis of ischemia-reperfusion (Sun et al., 2009; Saleem et al., 2006).

Cerebral ischemic insult usually causes irreversible deterioration of central nervous system (CNS) behaviors (Kim et al., 2007). After the onset of cerebral ischemia, inflammatory process triggers the acceleration of the early onset and functions as a determinant factor in severity of cerebral damage, morbidity and mortality (Berner et al., 2005). Neuroinflammatory mediators have always been pivotal in the pathophysiology of the brain ischemia, exerting either beneficial role during recovery and repair or deleterious effects on the progression of tissue damage. Both, local and systemic pro-inflammatory and anti-inflammatory signals are known to be activated in the event of stroke (Amantea et al., 2009; Planas et al., 2007). Cytokines are thought to be the major mediators behind these complex inflammatory responses. Furthermore iNOS is known to express in ischemia-reperfusion induce inflammatory cells. They are known to participate in ischemia-reperfusion neuronal damage (Iadecola et al., 1995). Moreover, involvement of astrocytes have always been linked with many pathologies of the CNS including cerebral ischemia (Pekny and Nilsson, 2005) which markedly upregulate expression of glial fibrillary acidic protein (GFAP).

Hesperidin (3',5,7'-trihydroxy-4'-methoxy-flavanone-7- rhamnoglucoside), a member of the flavanone group of flavonoids, mainly isolated from citrus fruits is known to exhibit antioxidative, anti-inflammatory activities (Galati et al., 1994), anti-hypercholesterolemic
activity (Bok et al., 1999), anti-hypertensive and diuretic effects (Galati et al., 1996). The neuroprotective efficacy of hesperidin is attributed to its ability of inhibiting Fe$^{2+}$-induced linoleate peroxidation and auto-oxidation of cerebral membranes (Saija et al., 1995), scavenging peroxynitrite radicals (Kim et al., 2004) and inhibition of ROS generation, including hydroxyl radical (Jung et al., 2003). It protects the neurons against various types of insults associated with many neurodegenerative diseases (El-Sayed et al., 2008; Kumar and Kumar, 2010). However, very little is known about its effect on behavioral deficits, biochemical parameters and inflammatory changes associated with ischemia-reperfusion. Keeping in view the above mentioned facts and to understand the complex mechanism behind oxidative stress and neuroinflammation and to develop sensible therapeutic strategies, we sought to investigate the antioxidative and anti-inflammatory potential of hesperidin in focal ischemic models of rats.

**Material and methods**

**Chemicals and reagents**

As described in material and methods, chapter-II.

**Animals and treatments**

As described in material and methods, chapter-II.

**Drug administration**

Hesperidin (50 mg/kg body weight in saline) was administered orally (p.o.) once daily for 15 days before MCAO.

**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

Experiment # 1
This experiment was carried out to evaluate the pre-treatment effect of hesperidin on neurobehavioral activity, content of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and enzymatic assays. The rats were divided into 4 groups, each having 8 animals. Group 1 was vehicle treated sham rats (S), Group 2 was vehicle treated MCAO rats (MCAO), Group 3 was hesperidin pretreated MCAO rats (H+MCAO) and Group 4 was hesperidin pretreated sham rats (H+S).

Experiment # 2
This experiment was carried out to evaluate the pre-treatment effect of hesperidin on ischemic damage through infarct volume assessment. The rats were divided into 4 groups as in experiment # 1 but each having 6 animals.

Experiment # 3
This experiment was carried out to evaluate the pre-treatment effect of hesperidin on the modulation of proinflammatory cytokines (IL-1β, TNF-α) and GFAP expression. The rats were divided into 4 groups as in experiment # 2, each having 6 animals.

Behavioral studies

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

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

Adhesive-removal test
As described in material and methods, chapter-II.
Biochemical studies

Tissue preparation for the assays of TBARS, GSH and antioxidant enzymes

In experiment # 1, 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, SOD and catalase) as described in material and methods, chapter-II.

Experiment # 2

Evaluation of ischemic damage

The pre-treatment effect of hesperidin on ischemic damage was evaluated through infarct volume assessment in the animals of experiment # 2.

Extent of ischemic damage was assessed using TTC and cresyl violet staining

As described in material and methods, chapter-II.

Infarct volume analysis

As described in material and methods, chapter-II.

Assessment of neuronal damage

After 24 h of MCAO, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, kept in the same fixative for overnight at 4 °C and immersed in 0.1 M PBS containing 20%, 30% sucrose overnight at 4 °C, respectively. Sections were incubated in 0.5% cresyl violet solution for 3–5 min. The sections were rinsed in distilled water and differentiated in 95% alcohol, followed by dehydration in 100% alcohol. The sections were cleared in xylene and mounted in DPX mounting medium. Images were acquired using the light microscopy (BX51, Olympus, Tokyo, Japan).
Measurement of cytokines

Brains from MCAO, hesperidin treated MCAO and sham-control animals were removed without fixation after cervical dislocation 24 h following surgery and processed as describe in material and methods, chapter-II.

Immunofluorescence for GFAP

After 22 h of reperfusion the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, and processed as describe in material and methods, chapter-II.

Immunohistochemistry for iNOS

As described in material and methods, chapter-II.

Determination of protein

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Statistics

As described in material and methods, chapter-II.

Results

Experiment # 1

Effect of hesperidin on behavioral output

Adhesive Removal Test

To test whether the reduction in infarct volume actually improves functional outcome, we performed the tape removal test, a technique that assesses sensory and motor impairments in forepaw function. Twenty-four hours after MCAO, an increase in the time needed to remove adhesive tape from forepaws was observed in MCAO group as
compared with sham group rats (Fig. 1), which was in consistent with the previous reports (Bouet et al., 2007).

Interestingly, hesperidin treated MCAO group (H+MCAO) significantly shortened the time to remove the adhesive tapes from the forepaws compared with MCAO group (p<0.001) indicating a profound improvement in sensorimotor performance of the rats pretreated with hesperidin. Hesperidin alone pre-treated group (H+S) showed no significant changes as compared to sham group. Taken together, these results show a protective effect of hesperidin when given before cerebral ischemia.

Rota Rod
Motor function impairment caused by cerebral ischemia was evident in the vehicle-treated MCAO group as compared to sham group. Hesperidin was found to be effective in partial recovery of muscular incoordination in hesperidin pretreated MCAO (H+MCAO) group (p<0.01) as compared to MCAO group. No significant difference was observed between sham and hesperidin treated sham group (H+S) (Fig. 2).
Grip strength
The mean score was decrease significantly (p<0.01) in the vehicle treated MCA occluded group as compared to the sham group rats. The hesperidin pre-treatment has increase the mean score significantly (p<0.05) in H+ MCAO group as compared to the vehicle treated MCAO group (Fig. 3). However no significant alteration was observed in hesperidin treated sham (H+S) group as compared to sham group.

Fig 3. The grip strength was decreased significantly in the MCAO group animals as compared to sham group animals. Pretreating the animals with hesperidin followed by MCAO has protected motor deficit as compared with MCAO group. Values are expressed as mean ± S.E.M of 6 animals. *p <0.01, MCAO vs. sham; #p <0.05, H + MCAO vs. MCAO.

Effect of hesperidin on endogenous antioxidant system
Hesperidin pretreatment decreased the TBARS contents in striatum and frontal cortex
The effect of hesperidin on TBARS content was measured to demonstrate the oxidative damage by LPO in frontal cortex and striatum of MCAO rats. We noticed a significant increase (p<0.001) in TBARS level in MCAO group animals as compared to sham group. Rats of hesperidin pretreated MCAO group exhibited significant attenuation (p<0.05 & p <0.01) in TBARS in MCAO rats (Fig.4). Hesperidin alone pre-treated sham (H+S) group showed no significant changes in TBARS as compared to sham group.

Fig 4. TBARS content was significantly increased in MCAO group as compared to sham group (p<0.001 MCAO vs. sham group). Hesperidin pretreatment significantly decreased TBARS content in the H + MCAO group as compared to MCAO group (t p<0.05 &## p<0.01 MCAO vs. H + MCAO group). Values are expressed as mean ± SEM.
Hesperidin pretreatment restored the GSH level in striatum and frontal cortex

Protective effect of hesperidin on GSH level in frontal cortex and striatum was observed. The level of GSH was depleted significantly (p<0.01 & p<0.001) in MCAO group as compared to sham group. Hesperidin pretreatment increased its level significantly (p<0.05) in hesperidin pretreated MCAO (H+MCAO) group as compared to MCAO group. Hesperidin alone pretreated (H+S) group exhibited no significant changes in GSH level as compared to sham group (Fig. 5).

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

The activities of antioxidant enzymes (GPx and GR) 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 hesperidin treated MCAO group (H+ MCAO) animals as compared to MCAO group animals. No significant change was observed in hesperidin pretreated sham group (H+S) animals as compared to sham group animals (Tables I and II).

Hesperidin 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 hesperidin pretreatment has restored there activity significantly in frontal cortex and striatum in hesperidin treated MCAO (H+ MCAO) group as compared to the MCAO group. No

53
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-I oxidized/min/mg protein)</th>
<th>SOD (nmol epinephrine protected from oxidation/min/mg protein)</th>
<th>CAT (nmol H$_2$O$_2$ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>318.5±10.36</td>
<td>392.29±15.25</td>
<td>268.5±14.33</td>
<td>7.33±0.77</td>
</tr>
<tr>
<td>MCAO</td>
<td>195.2±18.36*</td>
<td>187.46±13.52*</td>
<td>165.74±13.60*</td>
<td>3.96±0.18**</td>
</tr>
<tr>
<td>H+MCAO</td>
<td>264.16±8.3*</td>
<td>287.94±20.76*</td>
<td>269.61±26.34**</td>
<td>6.03±0.08*</td>
</tr>
<tr>
<td>H+sham</td>
<td>319.5±17.43</td>
<td>393.7±7.03*</td>
<td>269.58±19.84*</td>
<td>7.34±0.54*</td>
</tr>
</tbody>
</table>

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

significant change was observed in the hesperidin pretreated sham (H+S) group as compared to the sham group (Tables I and II).

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-I oxidized/min/mg protein)</th>
<th>SOD (nmol epinephrine protected from oxidation/min/mg protein)</th>
<th>CAT (nmol H$_2$O$_2$ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>261±27.52</td>
<td>320.81±10.79</td>
<td>307.98±20.09</td>
<td>6.46±0.36</td>
</tr>
<tr>
<td>MCAO</td>
<td>130±4.07**</td>
<td>155.74±12.34**</td>
<td>160.35±17.70**</td>
<td>3.37±0.13**</td>
</tr>
<tr>
<td>H+MCAO</td>
<td>217.66±8.14*</td>
<td>214.28±10.95**</td>
<td>257.51±14.89**</td>
<td>4.62±0.09*</td>
</tr>
<tr>
<td>H+sham</td>
<td>262.66±33.43</td>
<td>319.52±10.89</td>
<td>308.83±15.81</td>
<td>6.5±0.43</td>
</tr>
</tbody>
</table>

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

Experiment # 2

Effect of hesperidin on TTC stain and infarct volume

We evaluated the role of hesperidin in ischemic stroke hypothesizing that it has a protective role. Indeed, MCAO group rats have shown a significantly increased infarct volume as compared with sham. TTC staining of MCAO brain sections showed reproducible and readily detectable lesions in the areas that are supplied by the MCA at 24 h after thereperfusion (Fig. 6A). The lesions were present in striatum and the overlying cortex. Hesperidin reduced the infarct volume significantly (p<0.001) as compared to the MCAO group rats (Fig. 6B).

Effect of hesperidin on cresyl violet staining

To access the neuronal damage induced by ischemia-reperfusion we evaluated the MCAO group against the hesperidin pretreated MCAO animals. Sham group animal

Fig6. Representative photographs of brain sections stained with 0.1% TTC. (A) measurement of infarct volumes and (B) MCAO and hesperidin pre-treated MCAO group are presented. MCAO group produced a significant lesion over sham group (figure not shown). However hesperidin treated MCAO group showed a significant (p<0.001) reduction in tissue damage as compared to MCAO group.

Fig7. Hesperidin reduces the neuronal injury after MCAO. Cresyl violet staining shows the neuronal alterations in the ipsilateral brain after 24 h of MCAO. (A) Normal morphologic features of neurons are present in sham (arrow showing the normal neuron). (B) Characteristic features of hyperchromia, shrinkage of nucleus and cytoplasm in neurons present in MCAO groups (arrow showing the damaged cell). (C) Administration of hesperidin clearly ameliorates ischemia-induced neuronal damaged in H+MCAO. Magnification is 40X.
Chapter III

exhibited no sign of neuronal injury (Fig. 7A). After MCAO, animals showed significant neuronal loss, neuronal shrinkage and marked vascular changes throughout the cortex and striatum. The impairment was reduced with the pretreatment of hesperidin (Fig. 7C). No remarkable effect was observed in hesperidin pre-treatment sham group (H+S) compared with the sham group (data not shown).

Experiment #3

Effect of hesperidin on IL-1β and TNF-α

Proinflammatory cytokines IL-1β and TNF-α are reported to be overexpressed under ischemic conditions (Bergur et al., 2002, Rezaie et al., 2002). Their overexpression in the brain is considered as a detrimental response during cerebral ischemia. We hypothesized that the hesperidin pretreatment would decrease the expression of these proteins as a protective measure. Quantification of IL-1β and TNF-α in frontal cortex and striatum by ELISA showed a significant increase in the level of IL-1β (p < 0.001) and TNF-α.

![Graphs showing IL-1β and TNF-α levels](image)

Fig 8. Values are mean ± S.E.M. Interleukin (IL)-1β and tumour necrosis factor (TNF)-α protein levels were increased significantly in MCAO rats after the stroke. Bar graphs of IL-1β (A) and TNF-α (B) protein in sham, MCAO and hesperidin pretreated MCAO groups are shown. Concentration of IL-1β (p < 0.001 MCAO vs. sham) & TNF-α (p < 0.001 MCAO vs. sham) was significantly increased in MCAO group as compared to sham group. Hesperidin pretreatment significantly reduced IL-1β (p < 0.001 MCAO vs. H + MCAO group) & TNF-α (p < 0.001 MCAO vs. H + MCAO group) protein concentrations in H+MCAO as compared to MCAO group.

(p < 0.001) in MCAO group as compared to sham group. Interestingly, hesperidin supplementation has significantly decreased the levels of IL-1β (p < 0.001) and TNF-α (p < 0.05 & p < 0.001) in hesperidin pretreated MCAO group (H+ MCAO) when compared with MCAO group (Fig. 8A & 8B). However no significant alteration was
observed in hesperidin treated sham (H+S) group as compared to sham group (data not shown).

**Effect of hesperidin pretreatment on GFAP and iNOS expression in MCAO rats**
The activation of astrocyte upregulation is associated with neuronal cell death in cerebral ischemia. GFAP expression was found to be remarkably high ($p<0.001$) in ischemic hemisphere of MCAO group (Fig. 9 B). A noticeable reduction ($p<0.01$) in GFAP expression was observed in hesperidin pretreated group as compared to MCAO group. Expression of GFAP was seen to be very scarce in sham group animals (Fig. 9 A). Hesperidin pre-treatment did not show any remarkable effects in the H+S group as compared to sham group (data not shown).

![Immunofluorescence staining](image1)

Fig9. Immunofluorescence staining from cortical region of brain from sham, MCAO and hesperidin pretreated MCAO (H + MCAO) group showing neurons with GFAP expression. The profound expression of GFAP was observed in MCAO group (B) compared to sham group (A), while hesperidin pretreated MCAO group (C) has shown a moderate expression of GFAP level. However, the sham group has shown almost negligible GFAP positive cells. Magnification is 20X.

![Immunohistochemistry staining](image2)

Fig10. Immunohistochemistry staining from cortical region of brain from sham, MCAO and hesperidin pretreated MCAO (H + MCAO) group showing neurons with iNOS expression. The profound expression of iNOS was observed in MCAO group (B) compared to sham group (A), while hesperidin pretreated MCAO group (C) has shown a moderate expression of iNOS level. However, the sham group has shown almost negligible iNOS positive cells. Magnification is 20X.
Numbers of iNOS positive cells are remarkably high (p < 0.001) in ischemic hemisphere of MCAO group, which was significantly (p < 0.01) attenuated with the pretreatment of hesperidin. However iNOS expression was found to be almost negligible in sham group (Fig. 10 B). Hesperidin pre-treatment did not show any remarkable effects in the H+S group as compared to sham group (data not shown).

Discussion

We have demonstrated the ability of hesperidin to partially protect the dying neurons following focal cerebral ischemia. The MCAO model with reperfusion that mimics many features of the stroke in humans was used since the MCA, which is the specific occlusion site in this model, is the most commonly affected vessel in both embolic and thrombotic strokes in humans (Saleem et al., 2006; Longa et al., 1989). It is well documented that MCAO results in behavioral, neurochemical and histological abnormality in rat brain and generation of free radicals has been implicated to be one of the main contributing factors (Ahmad et al., 2005). Our results clearly demonstrate the scavenging effect of free radicals by hesperidin as evidenced by different neurochemical and neurobehavioral parameters which was well correlated with reduced expression of proinflammatory cytokines.

Functional deficits are common neurological sequelae in animal models of cerebral ischemia (Chen et al., 2008). Behavioral parameters are useful measures of functional and sensorimotor deficits following experimental focal cerebral ischemia. In the present study, the batteries of neurobehavioral tests were assessed to characterize the functional and sensorimotor performance in cerebral ischemic model. The alteration observed in motor coordination and adhesive removal test in MCAO treated group might be due to the necrosis induce in sensorimotor cortices and caudate-putamen especially the putamen as it is evident in cerebral ischemia (Hunter et al., 1998) and has a command on motor and sensorimotor activities. Furthermore, the free radicals are known to play key role in neurobehavioral deficit in experimental models through oxidative stress (Fukui et al., 2002; Fukui et al., 2001), so the poor neurobehavioral outcome in MCAO group rats might be attributed to oxidative stress induced free radicals. Earlier studies have shown an improvement in various behavioral outputs as a result of antioxidant treatment (Yousuf et al., 2005). In line with above studies, we observed that hesperidin treated
MCAO group has shown significant improvement in behavioral and functional outcome reflecting the antioxidative potential of hesperidin. The reduction in functional deficits seen in the hesperidin pretreated rats correlate well with the biochemical findings.

One of the universally accepted etiologies of stroke is the imbalance between free radical formation and the maintenance of the neuronal integrity through the endogenous antioxidant defense system resulting in oxidative stress. Production of free radicals in the ischemic brain depends on the intensity, stage and site of ischemia and occurrence of reperfusion. Surplus amount of free radical generation is thought to be the key module of neuronal damage in the brain. ROS threaten neuronal survival by their ability to propagate the initial attack on lipid rich membranes of the brain to cause LPO. However, cell damage can be prevented by detoxification of free radicals, which eventually prevent the progress of LPO. We have observed an elevated level of TBARS accompanied by depleted GSH level in MCAO rat brain, which was in agreement with the previous observations (Al-Omar et al., 2006; Chan et al., 2001; Saleem et al., 2006). The results indicate that peroxidative stress takes its toll on the GSH content. However our experimental finding reveals that pre-treatment with hesperidin partially attenuates the elevated level of TBARS and depleted level of GSH which is in concomitant with the previous observations where antioxidants were used as a remedy in experimental stroke models (Ansari et al., 2004; Roghani and Behzadi, 2001). GPx plays a predominant role in removing excess free radicals and hydroperoxides and is a major defense system against oxidative stress in the brain (Imam and Ali 2000). SOD converts superoxide into H$_2$O$_2$ (Freeman and Crapo 1982) and catalase, which is found at a very low activity in the brain, detoxifies H$_2$O$_2$ into H$_2$O. Evidence has been presented that the neuronal defense against H$_2$O$_2$, which is the most toxic molecule to the brain, is mediated primarily by the glutathione system. The increase in the content of GSH and decrease in the extent of lipid peroxidation with the treatment of hesperidin, in our study, is in concordance with earlier reports, where flavonoids had been used for the treatment of different type of brain diseases (Jungsook, 2006; El-Sayed et al., 2008; Khan et al., 2009).

Undoubtedly histology can correlate the development of ischemic-reperfusion injury and the extent of neuroprotection by pharmacological intervention. Infarction volume in the brain is an important determinant in assessing the consequences of stroke (Yousuf et al.,
2009). To identify infarcted areas we employed TTC and Nissl staining. In the present study we observed that MCAO group has a prominent infarct size which was ameliorated significantly with hesperidin pre-treatment. These observations were further boosted with histopathological changes seen in cresyl violet staining. The cortex of MCAO group rats has shown clear demarcated infarcted areas with regular architecture destruction by the necrosis of the cells, which in turn was directly related to neurological outcome. Studies have revealed that flavonoids administered following ischemia are effective in reducing infarct volume and lead to improvements in neurological outcome (Shah et al., 2010; Yamazaki et al., 2010; Khan et al., 2009).

Focal brain ischemia caused a significant induction of cytokines and peaks within hours of reperfusion. Here we sought to observe the changes in the levels of IL-1β and TNF-α with reference to cerebral ischemia. TNF-α is involved in systemic inflammation and induce apoptotic cell death. IL-1β is an important mediator of the inflammatory response and apoptosis. Together both represents crucial mediator of neurodegeneration induced by ischemic brain injury (Shohami et al., 1996; Touzani et al., 1999). The increased content of IL-1β and TNF-α in MCAO group was significantly protected in H+MCAO group rats suggesting the neuroprotective effect of hesperidin following cerebral ischemia may be in part, mediated through modulation of the injury caused by proinflammatory cascades. Pretreatment of hesperidin significantly counteracted the upregulation of proinflammatory cytokines; a piece of evidence attributed to its anti-inflammatory property of hesperidin which was consistent with the previous finding (Choi et al., 2007; Rizza et al., 2011).

It is well established that astrogliosis and failure of astrocytic glia function appears to play an important role in the pathogenesis of stroke (Basu et al., 2002). GFAP expression is known to be high in inflammation (Bradley et al., 1997) and can be induced by cytokines (Anderova et al., 2001). Increasing evidence points to a correlation between cerebral ischemia and GFAP over activation (Karin et al., 2007). Flavonoids have been reported to act on glial cells (Vafeiadou et al., 2009). A specific receptor for flavonoids has not been identified yet. However, putative flavonoid binding sites have been described, such as adenosine, glutamate and GABA receptors present in postmitotic neurons. Astrocytes are believed to be responsible for most glutamate uptake in synaptic areas and consequently are the major regulators of glutamate homeostasis (Matute et al.,
2006). An over expression of GFAP in MCAO rats was observed as compared to hesperidin pre-treated rats where GFAP expression significantly reduced which again indicates that hesperidin might interfere with the cytokines upregulation. The above protection offered by hesperidin may be attributed to its regulatory mechanism of glutamate upregulation and thus reducing glial mediated ischemic injury.

The inducible form of NOS (iNOS) has been implicated as an important mediator of inflammatory responses during ischemia and reperfusion (Samdani et al., 1997). Astrocytes elaborate iNOS in response to a series of proinflammatory mediators, including cytokines such as IL-1β and TNF-α (Hu et al., 1995). Nitric oxide (NO) derived from iNOS in astrocytes and its oxidative by-product peroxynitrite are thought to contribute to neuronal death due to oxidation of structural neuronal proteins during ischemia (Vaughan and Delanty, 1999). It has been demonstrated that flavonoids inhibit the expression of isoforms of inducible nitric oxide synthase which are responsible for the production of nitric oxide, as well as inflammatory mediators such as cytokines, chemokines or adhesion molecules (Tunon et al., 2009). Further, hesperidin has also been reported to cause inhibition of iNOS by L-arginine-NO (L-NAME) signaling pathway (Gaur and Kumar, 2010). Our finding shows that iNOS overexpression in MCAO rats were significantly attenuated with the supplementation of hesperidin. These findings are in harmony with the earlier studies carried out by others (Vaughan and Delanty, 1999; Zhang et al., 2005).