Neuroprotective effects of naringenin in experimental stroke are mediated through suppression of NF-κB proinflammatory signaling pathway
Introduction

Cerebral Ischemia, a major subtype of stroke has turned up into a global public hazard as it has become the leading cause of disability (Rossi et al., 2007). It is a disorder of arterial function characterized by insufficient oxygen supply to the brain to meet its demand. The stroke cases up to 87% are ischemic in nature, with the most common type being thrombosis (Lloyd-Jones et al., 2009). Ischemic stroke is characterized by the sudden loss of blood circulation to an area of the brain, resulting in a corresponding loss of neurologic function. The complex pathobiological mechanisms of this medical problem include excitotoxicity, inflammation, apoptosis, oxidative damage and ionic imbalances (Kumar and Dogra, 2008; Ozbal et al., 2008; Yousuf et al., 2009).

Reperfusion after ischemia, although essential for cell survival may result in numerous negative consequences like microvascular damage, cell dysfunction and death etc. It can bring leukocytes and several pro-inflammatory mediators in addition to induction of microglial and macrophages. During reperfusion the generation of reactive oxygen species (ROS) is greatly elevated which could overcome the antioxidant capacity of the ischemic brain, and thus results in oxidative stress. Thus disturbances in the redox equilibrium of tissues can lead to a proinflammatory state, a condition classically evident in ischemia reperfusion injury. However the mechanism of redox stress induces inflammation is not fully elucidated; there are numerous evidences that advocate a possible role of nuclear factor-κB (NF-κB). The reduction/oxidation (redox)-sensitive transcription factors NF-κB is present in the cytoplasm in its resting stage. Once activated, subunits of NF-κB translocate to the nucleus and mediate transcription of various inflammatory gene or their products such as inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Largo., 2003; Ding et al., 1998). NF-κB activation has been shown to regulate the expression of more than 500 different gene products linked with inflammation, tumor cell transformation, survival, proliferation, invasion, angiogenesis, metastasis, and chemoresistance(Sung et al., 2008). Thus, inhibitors of NF-κB activation may have therapeutic potential and are actively being researched.

Due to complex nature of stroke pathophysiology only few compounds have shown potential in animal trials and subsequent efficacy in human trials in combating stroke. Even though a treatment for ischemic stroke has been developed, the risk of intracerebral hemorrhage makes this treatment inappropriate (Lapchak, 2010). As a result, there is a great need for an alternative approaches to manage the high risk patients. One such approach involves understanding the role of pathways leading to inflammation such as nuclear factor-κB (NF-κB) activation in controlling the brain’s response to ischemia and their potential for pharmacological modulation.
Interestingly, growing attention is being paid to traditional medicines, as they have been proved therapeutically/prophylactically fruitful against manifold diseases, which can be evaluated by the dependence of 3.2 billion people (64%) of the whole world population on traditional medicines (Yousuf et al., 2010). Since inflammatory damage is implicated in the etiology of ischemia reperfusion induce complications, flooding the system with anti-inflammatory herbs may be a sensible step. Naringenin [5, 7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one], a flavonoid from grapefruit is now being actively investigated as a possible candidate to protect the inflammatory injuries. To account for the diverse effects of Naringenin, it has been proposed that its biological activities involve downregulation of the expression of proinflammatory markers, including inducible nitric oxide synthase and COX-2, by reducing the activities of NF-kB (Hamalainen et al., 2007). Neuroprotection offered by Naringenin is attributed to its free radical scavenging, anti-oxidant and anti-inflammatory properties and its ability to move across blood brain barrier (BBB) (Cavia-Saiz et al., 2010; Kanaze et al., 2007; Youdime et al., 2004). Vafeiadou et al (2009) have reported that the flavanone naringenin effectively reduces LPS/IFN-c-induced glial cell activation and resulting neuronal injury. Similarly, Chao et al (2010) have shown the inhibitory effects of naringenin on lipopolysaccharide (LPS)-induced inflammation in macrophages and microglia, indicating a possible beneficial effect of Naringenin in cardiovascular and cerebrovascular studies. Thus Naringenin with its above properties has prompted us to test the hypothesis that it offers a significant neuroprotection by suppressing the oxidative stress responsive transcription factor, NF-kb induced inflammation.

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
Naringenin (50 mg/kg body weight in saline) was administered orally (p.o.) once daily for 21 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 naringenin on neurobehavioral activity and biochemical assays. The rats were divided into 4 groups, each having 8 animals. Group 1 was vehicle treated sham rats (sham), Group 2 was vehicle treated MCAO rats (MCAO), Group 3 was naringenin pretreated MCAO rats (Nar+MCAO) and Group 4 was naringenin pretreated sham rats (Nar+Sham).

Experiment # 2
This experiment was carried out to evaluate the pre-treatment effect of naringenin on the modulation of proinflammatory cytokines (TNF-α, IL-1β & IL-6) and protein expression. The rats were divided into 4 groups as in experiment # 2, each having 8 animals.

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

Evaluation of ischemic damage
As described in material and methods, chapter-II.

Biochemical studies
Tissue preparation for the enzymatic assay SOD
After behavioral study, the animals were sacrificed and their brains were taken out to dissect frontal cortex and striatum for the biochemical assay for SOD, as described in material and methods, chapter-II.
Measurement of cytokines
As described in material and methods, chapter-II.

Immunohistochemistry for SOD, Hsp70, NF-kB, iNOS, COX-2
As described in material and methods, chapter-II.

Immunofluorescence for GFAP
As described in material and methods, chapter-II.

Expression of iNOS and COX-2 by Western Blot
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.

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

Results
Behavioral outputs
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). Naringenin was found to be effective in partial recovery of muscular in-coordination in naringenin pretreated MCAO (Nar+MCAO) group (p<0.01) as compared to MCAO group. No significant difference was observed between sham and naringenin treated sham group (Nar + Sham) (Fig. 1).

![Fig. 1. MCAO leads a significant decrease in motor coordination in MCAO group as compared to Sham group and significantly recovered in Nar + 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 < 0.01, Nar + MCAO vs. MCAO.](image-url)
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 naringenin pre-treatment has increase the mean score significantly ($p<0.05$) in Nar + MCAO group as compared to the vehicle treated MCAO group (Fig. 2). However no significant alteration was observed in naringenin treated sham (Nar + Sham) group as compared to sham group.

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. 3), which was in consistent with the previous reports (Bouet et al., 2007). Interestingly, naringenin treated MCAO group (Nar + MCAO) significantly shortened the time to remove the adhesive tapes from the forepaws compared with MCAO group-up ($p<0.001$) indicating a profound improvement in sensorimotor performance of the rats pretreated with naringenin. Naringenin alone pre-treated group (Nar + Sham) showed no significant changes as compared to sham group. Taken together, these results show a protective effect of naringenin when given before cerebral ischemia.
Effect of naringenin on endogenous antioxidant system

Naringenin pretreatment attenuated the activities of SOD level in striatum and frontal cortex. The activity of SOD in frontal cortex and striatum was decreased significantly in MCAO group as compared to the sham group (p<0.001). The naringenin pretreatment has restored there activity significantly in frontal cortex and striatum in naringenin treated MCAO (Nar+MCAO) group as compared to the MCAO group (p<0.01). No significant change was observed in the naringenin pretreated sham (Nar+Sham) group as compared to the sham group (Fig.4).

Effect of naringenin on TNF-α, IL-1β and IL-6

Proinflammatory cytokines TNF-α, IL-1β and IL-6 are reported be over expressed under ischemic conditions (Bergur et al., 2002, Rezaie et al., 2002). Their over expression in the brain is considered as a detrimental response during cerebral ischemia. We hypothesized that the naringenin pretreatment would decrease the expression of these proteins as a protective measure. Quantification of TNF-α, IL-1β and IL-6 in frontal cortex and striatum by ELISA showed a significant increase in the level of TNF-α (p<0.001), IL-1β (p<0.01) and IL-6 (p<0.001) in MCAO
Fig. 5. Quantification of interleukin-1 (TNF-α), (IL-6) and (IL-1β) by ELISA in the frontal cortex and striatum of sham, MCAO and Nar+MCAO groups are shown. Concentration of IL-1β (⁎p<0.01 MCAO vs. sham), IL-6 (⁎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. Naringenin pre-treatment significantly reduced IL-1β (##p<0.05 Nar+ MCAO vs MCAO), IL-6 (⁎p<0.01 Nar+ MCAO vs MCAO) & TNF-α (⁎p<0.05 &##p<0.001 Nar+ MCAO vs MCAO) protein concentrations in Nar+MCAO as compared to MCAO group. Values are mean ± S.E.M.

Interestingly, naringenin supplementation has significantly decreased the levels of TNF-α (p<0.05), IL-1β (p<0.05) and IL-6 (p<0.01) in naringenin pretreated MCAO group (Nar+ MCAO) when compared with MCAO group (Fig. 5). However, no significant alteration was observed in naringenin treated sham (Nar+Sham) group as compared to sham group (data not shown).

Fig. 6. Effect of naringenin pretreatment on SOD, Hsp70 & NF-kB 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 naringenin (C) has shown a moderate staining of SOD, Hsp70 & NF-kB. However, the sham group has shown almost negligible staining. Magnification at 20X.
Effect of naringenin pretreatment on SOD, Hsp70, NF-kB expression

To examine a functional relationship between elevation of the SOD, Hsp70 and suppression of NF-kB activation in response to naringenin we did immunohistochemistry. Moderate staining of SOD, Hsp70 and negligible positive NF-kB neurons were evident in sham group (Fig. 6A). The expression of SOD was seen to be decreased however Hsp70 and NF-kB were found to be elevated strongly in the ischemic hemisphere of the MCAO group rats. This suppression of SOD and the elevation in Hsp70 along with the NF-kB expression is associated with ROS generation in cerebral ischemia. In Nar+MCAO group, SOD expression was observed to be increased while Hsp70 and NF-kB expression was decreased as compared to MCAO group (Fig. 6C). Naringenin pretreatment did not show any remarkable effects on Nar+Sham group as compared with sham group (data not shown).

Effect of naringenin pretreatment on iNOS, COX-2 expression

It is known that NF-kB regulates the expression of iNOS and COX-2. The expression of both NF-

![iNOS](image1)

![COX-2](image2)

Fig. 7. Effect of naringenin pretreatment on iNOS & COX-2 expression correspondingly. The profound expression of iNOS & COX-2 was observed in MCAO group (B) compared to sham group (A), while the MCAO group pretreated with naringenin (C) has shown a moderate staining of iNOS & COX-2. However, the sham group has shown almost negligible staining. Magnification at 20X.
kB dependent genes were increased strongly in the ischemic hemisphere of the MCAO group rats. However, the expressions for both were significantly attenuated by the administration of naringenin (Fig. 7C). Naringenin pretreatment did not show any remarkable effects in the Nar+Sham group compared with the sham group (data not shown).

**Effect of naringenin pretreatment on GFAP expression**

The activation of astrocyte upregulation is associated with inflammation mediated neuronal cell death in cerebral ischemia. GFAP expression was found to be remarkably high in ischemic hemisphere of the vehicle treated MCAO group (Fig. 8B). The GFAP expression was most pronou-

![Fig.8](image)

Fig.8. Immunofluorescence staining from cortical region of brain from sham, MCAO and naringenin pretreated MCAO (Nar + MCAO) group showing neurons with GFAP expression. The profound expression of GFAP was observed in MCAO group (B) compared to sham group (A), while naringenin 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.
-nced in areas of robust tissue inflammation and microglial activation, as also evident from iNOS expression. A noticeable reduction in GFAP expression was observed in naringenin pretreated group as compared to MCAO group (Fig. 8C). Expression of GFAP was seen to be very scarce in sham group animals (Fig. 8A). Naringenin pre-treatment did not show any remarkable effects in the Nar+Sham group as compared to sham group (data not shown).

Effect of naringenin pretreatment on western blot expression of iNOS and COX-2
Significant increase in the expression of iNOS was observed in MCAO group after 22 h. Expression was found to be reduced in the naringenin pretreated group as compared to MCAO group (Fig. 10a). Significant increase in the expression of COX-2 was observed in MCAO group after 24 hrs. Expression was found to be decreased in the naringenin pretreated treatment group and was almost equal to sham-operated group (Fig. 10b).

![Western Blot Images](image)

Fig.9. iNOS & COX-2 expression by immunoblotting. Bar chart shows the quantification of bands. Upper Panel showing the representative bands for iNOS, COX-2 and β-Actin as lane 1: sham-operated, lane 2: ischemic group, and lane 3: Nar pre-treated ischemic. Data are expressed as mean ± S.E.M of five animals per group.
Effect of naringenin pretreatment on histopathological changes in MCAO rats

Figure 10 shows the histopathological changes after 2 h of occlusion and 22 h of reperfusion. Sections of the brain passing from frontal cortex of MCAO, Nar+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 Nar+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.

Discussion

Co-morbidities of stroke involve inflammation, which can directly contribute to ischemic damage and has adverse impact on recovery (Emsley and Hopkins, 2008; Denes et al., 2009; McColl et al., 2009). The neuroprotection observed in MCAO induce ischemic rat raises a question that which molecular pathway(s) may be involved and what intervention could be done. We have critically evaluated MCAO rats model for the behavioral, biochemical and immunological tests which is widely used to study neuroprotective effect of drugs because its recapitulation of the biochemical and pathological features of human stroke (Longa et al., 1989; Yousuf et al., 2009; Khan et al., 2009). We demonstrated that prophylactic treatment of naringenin partially attenuates the inflammatory responses by suppressing ROS induced NF-κB mediated downstream signaling.

Nuclear factor-κB (NF-κB) is an oxidative stress responsive transcription factor (Piette et al., 1997) and their involvement in ischemia reperfusion injury has been known for several years (Botchkina et al., 1999; Blondeau et al., 2001; Jeong et al., 2010). According to the present findings, naringenin has
inhibitory activity on NF-κB activation and downstream proinflammatory signaling pathway (Hämäläinen et al., 2007). Our results demonstrate that NF-κB transcription factor is activated after transient MCAO (Botchkina et al., 1999; Clemens et al., 1998; Clemens et al., 1997). Moreover, on the basis of morphological experiments, we assume that this activation occurred at penumbra region, suggesting an inflammatory process (Rami et al., 2008). This fits well with our results since NF-κB activation was previously suggested to induce inflammation (Jeong et al., 2010) by inducing transcription of pro-inflammatory genes (Olivier and James, 2010). However, naringenin supplementation significantly counteracted all the alteration observed in the expression of NF-κB in the striatum as well as cerebral cortex of I/R rats. This occurs often due to Naringenin's anti-inflammatory and neuroprotective properties (Zbarsky et al., 2005; Chao et al., 2010; Yang et al., 2011) by its potential to avert ROS generation in neuron (Moncada et al., 2006).

Earlier reports indicated the effect of naringenin administration on neurobehavioral scoring (Heo et al., 2004; Baluchnejadmojarad et al., 2006). To characterize the behavioral and functional performance in cerebral I/R after naringenin supplementation we have used the rota rod and grip strength test. The alteration observed in motor coordination, grip strength and tape adhesive test in MCAO treated group might be due to the ROS induced NF-κB mediated inflammation 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 activities. In this study, we proved that the motor function was impaired after ischemic insults in rat and could be significantly ameliorated by supplementation with naringenin through inhibiting ROS generation and thus suppressing NF-κB downstream signaling.

Regulation of NF-κB is known to be redox-sensitive which depends on availability of ROS. The ROS has been implicated as one of the factor involved in deleterious effects of reperfusion via effects on cerebral vasculature on nerve cells, and reperfusion. It appears to promote neutrophil recruitment into affected brain tissue (McNeil et al., 1992; Clark et al., 1994). It is well appreciated that under ischemia, ROS burden is premilary contained by SOD. To determine the effect of naringenin supplement to the induction of antioxidant pathways, which detoxify ROS, we measured the activities of antioxidant enzyme SOD. The activity of SOD was found to be significantly increased in naringenin pretreated MCAO rats as compared to MCAO group alone. Herein, we concluded that elevated level of SOD through naringenin pretreatment attenuated the oxidative stress. This finding was further corroborated by the immunological staining of SOD observed in our study, which are in agreement with the previous observations (Zafar et al., 2003; Ahmad et al., 2005; Bora et al., 2011).
A considerable notion is that redox-sensitive transcription factor NF-κB inhibition is closely associated to the activation of 70-Kd injury-associated chaperon, heat shock protein (Hsp70) in cerebral I/R (Rossi et al., 1998). To check out the causal relationship between NF-κB suppression and Hsp70 activation, we did the expression studies of Hsp70 and NF-κB. We observed an elevation in Hsp70 and suppression in NF-κB expression with the supplementation of naringenin, which is known to be able to cross the in vitro and in situ (rat) BBB (Youdim et al., 2004). This finding suggests that pretreatment of naringenin upregulates Hsp70 expression which exerts its protective effect by inhibiting NF-κB-mediated inflammatory reaction (Dokladny et al., 2010) and so, overexpression of Hsp70 inhibits the translocation of NF-κB, attenuating the release of inflammatory factors. Herein, we conclude that increased expression observed in Hsp70 through naringenin pretreatment led to the downregulation of NF-κB after cerebral I/R injury, which is in concordance with earlier reports (Seegers et al., 2010).

Naringenin is known to inhibit the activation of LPS induce NF-κB by more than 80% (Hamalainen et al., 2007) thus inhibiting the expression of iNOS and COX-2, the downstream target genes of NF-κB. These downstream target genes of NF-κB catalyze oxidative stress inducible production of NO and prostaglandins respectively, which plays important role in the pathogenesis of ischemia (Perez-Polo et al., 2011). In our model, the observed activation of NF-κB in rat brain was accompanied by elevated protein expression of iNOS and COX-2 (Yang et al., 2005; Tu et al., 2009). Our results confirm earlier observations on the inhibitory effects of naringenin on iNOS, COX-2 expression (Chao et al., 2010), and provide a mechanism for the effect through suppression of NF-κB activations. These observations were further confirmed with the help of western blot analysis. Naringenin pretreatment significantly decreased the ischemia induced expression of iNOS and COX-2 when compared with MCAO group after 22 h.

Moreover, the pro-inflammatory transcription factor, NF-κB is known to promote GFAP expression in injured human astrocytes (Bae et al. 2006), and increases astrocytic swelling (Sinké et al. 2008). Injured astrocytes can release proinflammatory factors, reactive oxygen species and reactive nitrogen species, leading to abnormal breakdown of molecules. Though, in the line of previous findings we observed that naringenin down-regulate GFAP expression in the cortex (Vafeiadou et al., 2009). We conclude here that naringenin by virtue of its anti-inflammatory properties, decreases the level of astroglial activation.

Above findings were further boosted by the result obtain from downstream proinflammatory mediators of NF-κB. It is widely accepted that NF-κB transcriptional activation regulates inflammation by exerting its effect on TNFa, IL-1β, and IL-6 (Olivier and James, 2010). These
cytokines possess kB-binding motifs in their promoter regions, and so their transcriptions are thought to be under the control of NF-kB. Thereby, modulation of NF-kB activity might provide a means of reducing inflammatory factors too. Our data suggest that 21 days pretreatment of naringenin, which is found in high concentrations in citrus fruits, efficiently attenuates the level of these proinflammatory cytokines, data were consistent with the literatures where inflammatory processes have been attenuated by anti-inflammatory supplementations (Khan et al., 2009; Tu et al., 2010).