

# Partial role of multiple pathways in infarct size limiting effect of quercetin and rutin against cerebral ischemia-reperfusion injury in rats

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**Abstract. – BACKGROUND:** Reperfusion therapy used in the treatment of cerebral ischemia often causes reperfusion neurological injury. Multiple pathological processes are involved in this injury including oxidative stress and components of the inflammatory response appear to play key roles in these deleterious effects. Thus new therapeutic strategies aimed at neutralization of OS-induced neurotoxicity support the application of natural antioxidant bioflavonoids. Both experimental and epidemiological evidence demonstrate that bioflavonoid such as quercetin and rutin are neuroprotective in models of cerebral ischemia reperfusion injury. However, recent studies indicate that the radical scavenger property of quercetin and rutin is unlikely to be the only reason for their cerebroprotective actions and in fact, a wide spectrum of cellular signaling events may well account for their biological actions.

**AIM:** In this study we attempted to establish the various mechanisms involved in the cerebroprotective activity of quercetin and rutin.

**METHODS:** Adult Sprague Dawley rats were anesthetized with thiopentone and subjected to global cerebral ischemia by occlusion of bicommon carotid arteries. Infarct size (TTC staining), SOD, MDA, CAT and MPO levels was assessed 4 h after the onset of ischemia.

**RESULTS:** Quercetin (50 mg/kg) and rutin (10 mg/kg) administered 10 min before reperfusion resulted in significant reduction of infarct size, MDA, and MPO levels and significant increase in SOD and CAT levels. Administration of L-NAME prior to administration of quercetin and rutin, significantly reduced the cerebroprotection offered by quercetin and rutin.

**CONCLUSIONS:** Possible partial role of antioxidant, anti-inflammatory and involvement of NO in the beneficial effects of bioflavonoids quercetin and rutin against cerebral ischemia reperfusion injury was observed.

*Key Words:*

Reperfusion injury, Cerebral ischemia, Oxidative stress, Bioflavonoids.

## Introduction

Oxygen-derived free radicals produced on reperfusion of ischemic brain could constitute the main cause of reperfusion injury<sup>1</sup>. Brain is the most susceptible organ to the damage due to oxidative stress because neurons are rich in polyunsaturated fatty acids (PUFA), and levels of endogenous antioxidant enzymes [superoxide dismutase (SOD), catalase, glutathione peroxidases] and non enzymes (vitamins C and E) in neuronal tissue are low<sup>2</sup>. Mitochondria are considered to be the main source of reactive oxygen species (ROS) production<sup>3</sup>, but another ROS source are activated inflammatory cells, such as neutrophils<sup>4</sup> and microglial cells<sup>5</sup>. The superoxide anion radicals ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) are the main ROS. These constantly produced ROS are scavenged by superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. Other small molecular antioxidants, including reduced glutathione (GSH), ascorbic acid, and  $\alpha$ -tocopherol, are also involved in the detoxification of free radicals<sup>6</sup>. However, during reperfusion these endogenous antioxidative defences are likely to be disturbed with a resulted overproduction of free radicals causing oxidative damage to biomolecules like lipids, proteins and DNA<sup>7</sup>.

Hydroxyl radical ( $OH^{\cdot}$ ) formed from  $H_2O_2$  (in itself not highly reactive) in the presence of divalent metal ions, especially  $Fe^{2+}$  and  $Cu^{2+}$ , via the Fenton reaction and peroxynitrite ( $ONOO^-$ ) formed by interaction of super oxide ( $O_2^{\cdot-}$ ) with nitric oxide ( $NO^{\cdot}$ ), a strong oxidant, initiates lipid peroxidation (LP)<sup>8</sup>. LP causes damage to mitochondria further and generated ROS. In addition, lipid peroxides degrade to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein<sup>8</sup>. This cascade of reactions induced by

ischemia followed by recirculation causes membrane disintegration and irreversible energy failure, leading to the aggravation of brain edema and loss of neuronal functions<sup>9</sup>.

Since reperfusion injury is associated with an imbalance of oxidative stress and antioxidant defense system, theoretically it would be possible to limit oxidative damage and ameliorate disease progression by supplementing antioxidants. Indeed, numerous natural antioxidants have shown cerebroprotective effect in ischemia-reperfusion induced cerebral injury<sup>10</sup>.

Both experimental and epidemiological evidences demonstrate that bioflavonoids such as quercetin and rutin are neuroprotective in models of cerebral ischemia reperfusion injury<sup>11, 12</sup>. However, recent studies indicate that the radical scavenger property of flavonoids is unlikely to be the only reason for their cerebroprotective actions and in fact, a wide spectrum of cellular signalling events may well account for their biological actions<sup>13</sup>. In this study we attempted to establish the various mechanisms involved in the beneficial health action and cerebroprotective activity of quercetin and rutin.

## Materials and Methods

All experimental protocols were approved by the Institutional Animal Ethics Committee of Acharya & B.M. Reddy College of Pharmacy, Bangalore under the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi.

Sprague-Dawley rats weighing 250-300 g of either sex were used which were obtained from National Institute of Nutrition, Hyderabad. Animals were housed in groups of 6-7 in colony cages at an ambient temperature of 25±2°C and 45-55% relative humidity with 12 h light/dark cycle. They had free access to pellet chow (Pranav Agro Limited) and water *ad libitum*.

Quercetin, rutin, L-N-nitroarginine methyl ester (L-NAME) were procured from Sigma Aldrich (India), and all other chemicals used for the experiments were of analytical grade and procured from regular suppliers.

### Induction of Cerebral Ischemia

Overnight fasted rats were anaesthetized with thiopental sodium (30 mg/kg). A midline ventral incision was made in the throat. Right and left common carotid arteries were located and freed from surrounding tissue and vagus nerve. A cot-

ton thread was passed below each of the carotid artery. Global cerebral ischemia was induced by occluding the bicommon carotid arteries by a knot<sup>14</sup>. After 30 min of global cerebral ischemia, the cotton threads were removed with the help of two knot releasers to allow the reperfusion of blood through carotid arteries for 4 h. Body temperature of rats was maintained at 37°C by heated surgical platform. All surgical procedures were carried out under sterile conditions.

### Determination of Infarct Size

The infarct size was determined in rats as described in previous studies<sup>15</sup>. In brief, animals were killed at the end 4 h reperfusion and brains were removed rapidly by cervical dislocation and frozen at -4°C for 5 min. Coronal slices were made at 1-2 mm and sections were immersed in 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min. TTC is converted to red formazone pigment by NAD and dehydrogenase present in living cells. Hence, viable cells were stained deep red. The infarcted cells have lost the enzymes and thus remained unstained. Whole brain slices were weighed. Infarcted unstained part was dissected out weighted and expressed as % of total weight of brain.

### Preparation of Brain Tissue for Estimation of Biochemical Parameters

Stained tissue were not suitable for estimating the oxidative stress markers, hence a separate group of animals were used for estimating these enzymes.

The brain of each animals was removed after completion of 4 h reperfusion following decapitation and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4) using a Remi homogenizer (Remi Instruments Division, Vasai - 401 208, India). Homogenisation procedure was performed as quickly as possible under completely standardized conditions. The homogenate was centrifuged at 1000 rpm 4°C for 3 min and the supernatant divided into two portions, one of which was used for measurement of malondialdehyde (MDA). The remaining supernatant was again centrifuged at 12,000 rpm at 4°C for 15 min and used for the measurement of superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO). Protein was measured by the method of Lowry et al<sup>16</sup>.

### Estimation of MDA Level

MDA level was measured as previously described by Ohkawa et al.<sup>17</sup>

### **Superoxide Dismutase (SOD)**

The SOD level was estimated by the method described by Kakkar et al.<sup>18</sup>

### **Catalase (CAT)**

The CAT level was measured by the method of Aebi<sup>19</sup>.

### **Estimation of MPO level**

MPO level was estimated by the method described by Mullane et al.<sup>20</sup>

### **Statistical Analysis**

The results were expressed as (Mean± SEM). Differences in infarct size, MDA, SOD, catalase and myeloperoxidase were determined by factorial One-way ANOVA. Individual groups were compared using Tukey's test. Differences with  $p < 0.05$  were considered statistically significant. Statistical analysis was performed using Prism software (Version 5.0).

## **Results**

### **Effect of Quercetin and Rutin on Infarct Size After Ischemia-Reperfusion**

In order to examine the cerebroprotective effect of quercetin and rutin against an ischemia-reperfusion insult, we measured the infarct size with or without administration of quercetin and rutin. As shown in Figure 1, percentage of infarct size was  $65.38 \pm 2.52\%$  in vehicle-treated animals, it was significantly reduced by 83.95% to  $10.49 \pm 1.77\%$  ( $p < 0.05$ ,  $n = 5$ ) and 80.20% to  $12.94 \pm 0.80\%$  ( $p < 0.05$ ,  $n = 5$ ) respectively, in animals given with 50 and 10 mg/kg dose of quercetin and rutin respectively. These observations indicate that quercetin and rutin can reduce ischemia-reperfusion-induced brain injury.

**Table I.** Experimental design for determination of biochemical parameters.

Group	Treatment
Group 1	Served as sham control (without I/R)
Group 2	Rats received 0.2 ml of 50% dimethyl sulfoxide (DMSO) 10 min before reperfusion and served as vehicle control
Group 3	Rats received quercetin 50 mg/kg 10 min before reperfusion
Group 4	Rats received rutin 10 mg/kg 10 min before reperfusion

**Table II.** Experimental design for determination of infarct size.

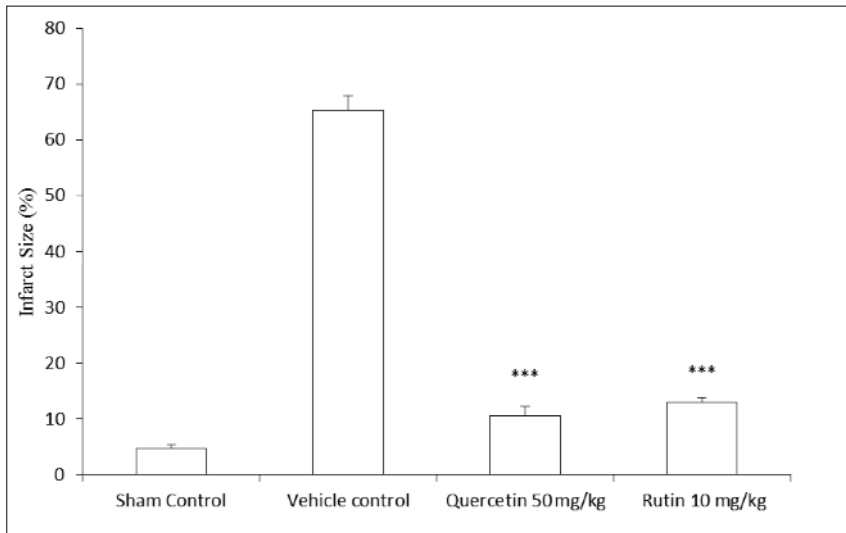
Group	Treatment
Group 1	Served as sham control (without I/R)
Group 2	Rats received 0.2 ml of 50% DMSO 10 min before reperfusion and served as vehicle control
Group 3	Rats received Quercetin 50 mg/kg before reperfusion
Group 4	Rats received Rutin 10 mg/kg before reperfusion
Group 5	Rats received L-NAME (10 mg/kg) before occlusion and received 0.2 ml of 50% DMSO 10 min before reperfusion
Group 6	Rats received quercetin 30 mg/kg 10 min before reperfusion
Group 7	Rats received L-NAME (10 mg/kg) before occlusion and received quercetin 30 mg/kg, 10 min before reperfusion
Group 8	Rats received rutin 5 mg/kg 10 min before reperfusion
Group 9	Rats received L-NAME (10 mg/kg) before occlusion and received rutin 5 mg/kg, 10 min before reperfusion

### **Effect of Quercetin and Rutin on The Content of Malondialdehyde After Ischemia-Reperfusion**

4h after 30 min of ischemia, malondialdehyde content, an index of lipid peroxidation, was significantly elevated in ischemia-subjected rats compared to that in sham-operated group from  $6.39 \pm 0.72$  to  $28.75 \pm 2.22$  nmol/g ( $p < 0.05$ ,  $n = 6$ ). Ischemia-mediated lipid peroxidation was significantly decreased to  $13.66 \pm 0.63$  nmol/g ( $p < 0.05$ ,  $n = 6$ ),  $8.38 \pm 0.74$  nmol/g ( $p < 0.05$ ,  $n = 6$ ), respectively in quercetin (50 mg/kg) and rutin (10 mg/kg) administered rats compared to the vehicle treated ischemic rats, as shown in Figure 2.

### **Effect of Quercetin and Rutin on Activities of Superoxide Dismutase After Ischemia-Reperfusion**

The results of superoxide dismutase activity are summarized in Figure 3. The activity of superoxide dismutase in the brain was decreased by 45.24% from  $25.15 \pm 0.94$  to  $13.77 \pm 0.82$  U/mg protein ( $p < 0.05$ ,  $n = 6$ ) in ischemic rats compared to sham-operated rats. However, when treated quercetin (50 mg/kg) and rutin (10 mg/kg), superoxide activities was significantly increased by 49.45%, 73.49% to  $20.58 \pm 0.39$  and  $23.89 \pm 0.56$  U/mg protein ( $p < 0.05$ ,  $n = 6$ ) compared to the vehicle-treated ischemic rats, respectively.



**Figure 1.** Effect of quercetin and rutin on infarct size in the brain in rat. Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. Values are mean  $\pm$  SEM. Each group consists of 5 rats. \*\*\* $p < 0.05$  vs. vehicle-treated group

**Effect of quercetin and Rutin on Activity of Catalase After Ischemia-Reperfusion**

4h after 30 min of ischemia, catalase levels decreased by 65.70% from  $18.02 \pm 0.56$  to  $6.18 \pm 0.22$   $\mu$ moles/min per mg protein ( $p < 0.05$ ,  $n = 6$ ) compared to those seen in the sham-operated groups. However, when treated quercetin (50 mg/kg) and rutin (10 mg/kg), significantly enhanced catalase levels about  $17.00 \pm 0.47$  and  $17.78 \pm 0.53$   $\mu$ moles/min per mg protein ( $p < 0.05$ ,  $n = 6$ ) compared to the vehicle-treated ischemic rats (see Figure 4).

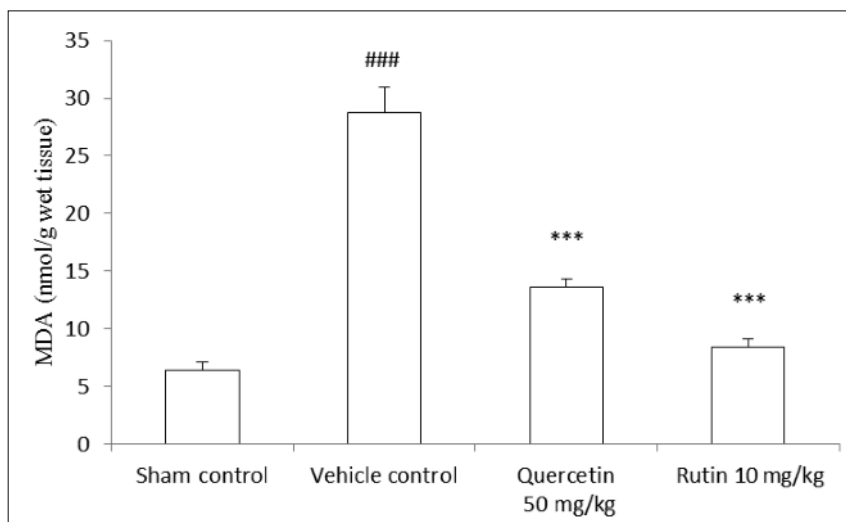
**Effect of Quercetin and Rutin on Myeloperoxidase After Ischemia-Reperfusion**

As shown in Figure 5, 4 h after ischemia-reperfusion, myeloperoxidase levels increased by

59.02% from  $0.118 \pm 0.003$  to  $0.288 \pm 0.007$  U/g ( $p < 0.05$ ,  $n = 6$ ) compared to those seen in the sham-operated groups. However, when treated quercetin (50 mg/kg) and rutin (10 mg/kg), myeloperoxidase activity was significantly decreased to 52.43%, 55.20 % to  $0.137 \pm 0.009$  U/g ( $p < 0.05$ ,  $n = 6$ ),  $0.129 \pm 0.010$  U/g ( $p < 0.05$ ,  $n = 6$ ), respectively compared to the vehicle treated ischemic rats.

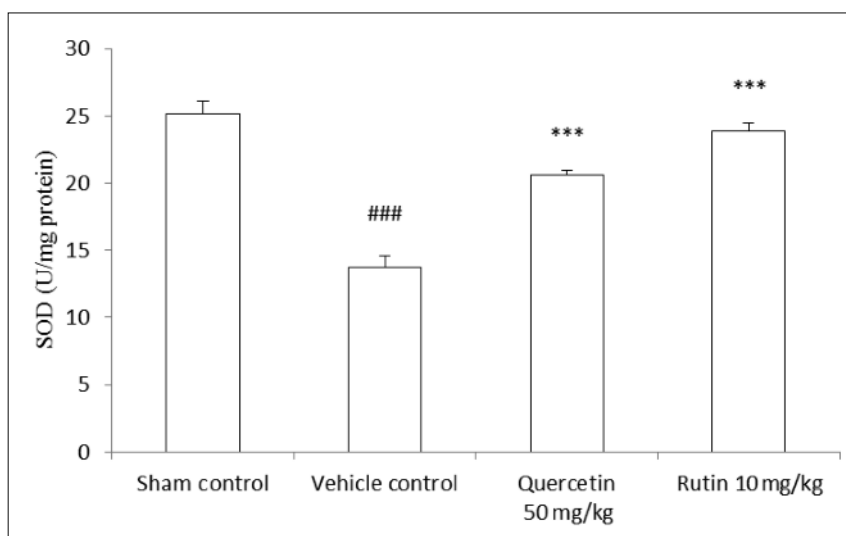
**Effect of Quercetin and Rutin on Percentage of Infarct Size With and Without NO synthase inhibitor (L-NAME) after ischemia-reperfusion**

As shown in Figure 6, infarct size obtained with the administration of L-NAME (NO synthase inhibitor) was almost similar to that of infarct size observed in vehicle control group



**Figure 2.** Effect of quercetin and rutin on malondialdehyde in rat brain. Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Results are expressed as mean  $\pm$  SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. ### $p < 0.05$  vs. sham-operated rat; \*\*\* $p < 0.05$  vs. vehicle-treated ischemic rat.

**Figure 3.** Effect of quercetin and rutin on superoxide dismutase content in rat brain. Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Results are expressed as mean  $\pm$  SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. ### $p$  < 0.05 vs. sham-operated rat; \*\*\* $p$  < 0.05 vs. vehicle-treated ischemic rat.



animals. There was no significant difference between infarct sizes of L-NAME treated group and vehicle control. The cerebroprotection offered by quercetin and rutin (30 mg/kg and 5 mg/kg) was significantly blocked by L-NAME. L-NAME antagonized the cerebroprotective actions of rutin more effectively than quercetin.

## Discussion

### ***Effect of Quercetin and Rutin on Infarct Size, Lipid Peroxidation and Antioxidant Enzymes After Ischemia-Reperfusion***

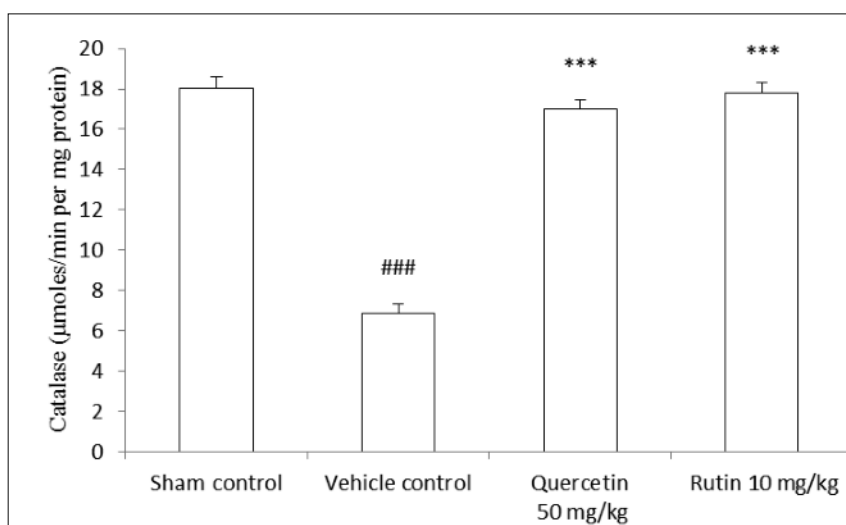
Ischemia and reperfusion cause brain injury via multiple pathways. Previous studies demonstrate that ROS are elevated during cerebral is-

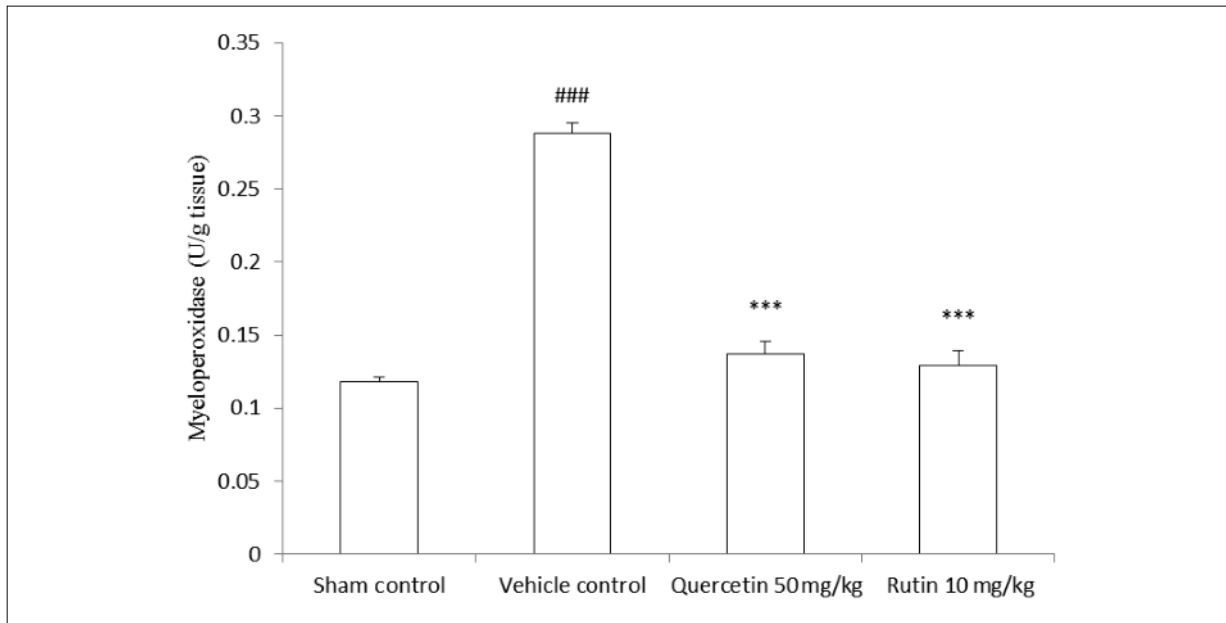
chemia and reperfusion, which plays a major role in the pathophysiology of ischemic stroke or cerebral ischemia-reperfusion related injury<sup>21</sup>. In order to investigate the mechanism of protection induced by quercetin and rutin against the ischemic cerebral injury, in the first part of the experiment infarct size, LP and antioxidant defenses including SOD and CAT in the injured brain tissue of rats were measured.

The results demonstrate an increase in tissue MDA levels in parallel to significant increase in infarct size in the vehicle control group when compared to sham control group.

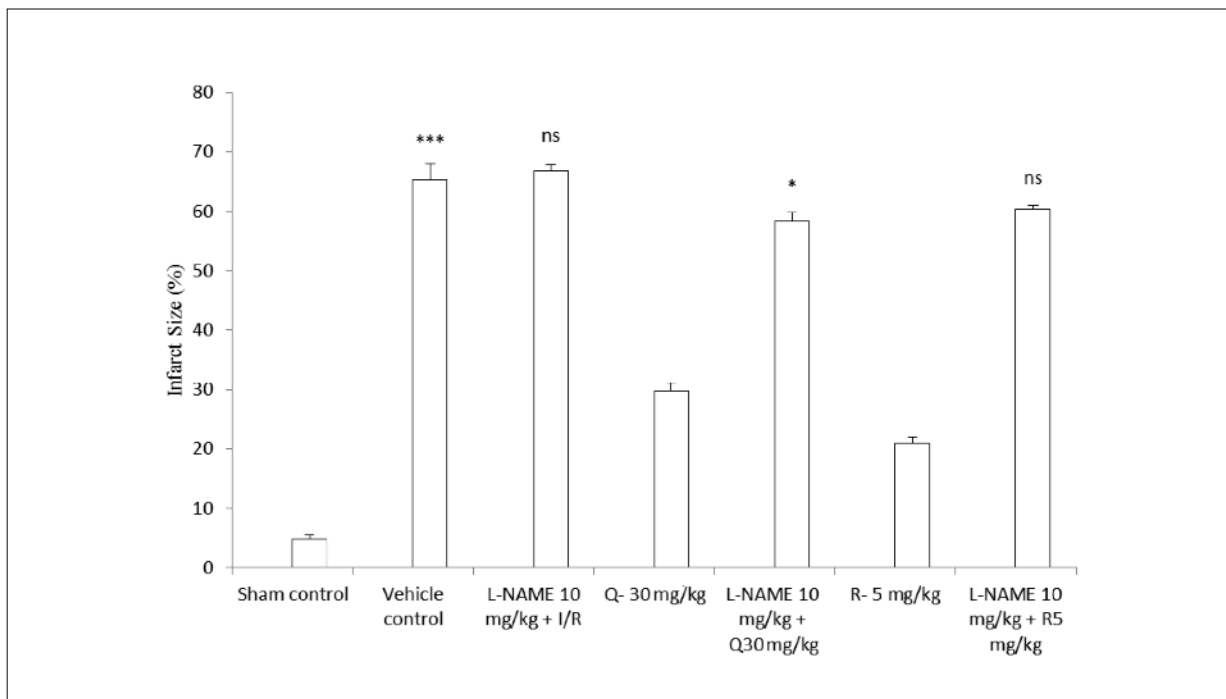
Since MDA is endproduct of LP, the results clearly indicate the cytotoxic effect of free radical by peroxidation on brain tissue, because it contains large amount of phospholipids that are rich in PUFA leading to neuronal death<sup>22</sup>.

**Figure 4.** Effect of quercetin and rutin on catalase levels in rat brain. Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Results are expressed as mean  $\pm$  SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. ### $p$  < 0.05 vs. sham-operated rat; \*\*\* $p$  < 0.05 vs. vehicle-treated ischemic rat.





**Figure 5.** Effect of quercetin and rutin on myeloperoxidase levels in rat brain. Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Results are expressed as mean  $\pm$  SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. ### $p$  < 0.05 vs. sham-operated rat; \*\*\* $p$  < 0.05 vs. vehicle-treated ischemic rat.



**Figure 6.** Effect of quercetin and rutin on percentage of infarct size with and without NO synthase inhibitor (L-NAME). Cerebral ischemia was induced by bicommon carotid artery occlusion for 30 min followed by 4h of reperfusion. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. Values are mean  $\pm$  SEM. Each group consists of 5 rats. \*\*\* $p$  < 0.05 vs. sham control, \* $p$  < 0.05 vs. vehicle control, ns  $p$  < 0.05 vs. vehicle control. R = Rutin; Q = Quercetin.

Quercetin and rutin treatment significantly reduced the elevated tissue MDA levels. Despite complete inhibition of MDA, protection to neuronal damage was partial. This may be because of involvement of multiple pathways in global cerebral ischemia-reperfusion injury.

The beneficial effects of these flavonoids are attributed to their antioxidant and anti-inflammatory properties. The evidence from our study clearly indicates that besides the peripheral organs, these bioflavonoids may also help to prevent tissue damage from oxidative stress in the brain.

Present study demonstrated that SOD and CAT levels were significantly reduced in vehicle control group when compared to sham control.

To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system that include enzymatic activities (SOD, CAT, GPx) and free radical scavengers such as reduced glutathione (GSH), vitamin C and E<sup>23</sup>. SOD catalyzes the dismutation of superoxide radicals forming hydrogen peroxide. GPx and CAT are the unique enzymes scavenging hydroperoxides and, therefore, act in concert with SOD. Decrease in the antioxidant enzyme activities during ischemia and reperfusion is due to the attack of sulphhydryl (-SH) groups of enzymes by oxygen free radicals and interaction of enzymes with peroxidation products, which can affect the active site of the enzyme<sup>24</sup>. Another reason for reduction of enzyme activities can be attributed to the reduction in pH, i.e. acidosis. Ischemia renders the cells to undergo anaerobic metabolism, thereby, producing lactic acid and acidosis. Enzymes that are pH-sensitive will, therefore, be easily affected. Thus, significant alteration in the antioxidant enzyme activities during cerebral ischemia and reperfusion may be responsible for more neurodegeneration than ischemia.

Quercetin and rutin treatment in our present investigation increased the endogenous antioxidant enzymes SOD and CAT indicating enhanced biochemical defenses to scavenge the overproduced free radicals.

Our results on SOD and CAT correlate with earlier reports<sup>25,26</sup>.

However, quercetin and rutin could not completely antagonize the lipid peroxidation observed in vehicle control animals and at the same time, drugs could not completely restore the antioxidant reserves depleted in vehicle control animals. Therefore, results from first part of the experiment indicate that antioxidant effects of these

drugs are partially responsible for cerebroprotective activity. Considering the potential neuroinflammatory modulating nature of bioflavonoids, experimental studies were continued further to evaluate the anti-inflammatory role of quercetin and rutin in their cerebroprotective actions.

### ***Effect of Quercetin and Rutin on Inflammatory Response***

Transient ischemia of the cerebral vasculature followed by reperfusion leads to a secondary cascade of pathophysiologic events, characterized by a complex inflammatory response<sup>27</sup>. Inflammation in stroke has been traditionally identified on histopathology as neutrophil infiltration, which correlates positively with ischemic damage. MPO is the most abundant component in azurophilic granules in neutrophils and has often been used as a histopathological marker for neutrophils<sup>28</sup>. It is also expressed in the myeloid line, especially in monocytes and macrophages/microglia. MPO interacts with hydrogen peroxide to generate highly reactive species including hypochlorite ( $\text{OCl}^-$ ) and radicalized oxygen species ( $\text{O}_2^{\cdot-}$ ,  $\text{ONOO}^{\cdot-}$ ). MPO-mediated radicalization of molecules induces apoptosis<sup>29</sup> and nitro-tyrosination of proteins<sup>30</sup>. Thus, MPO is a key component of inflammation and has been shown to play a major role in animal models of stroke in the posthypoxic inflammatory response<sup>31,32</sup>. MPO genotypes are associated with increased brain infarct size and poorer functional outcome<sup>33</sup>. Therefore, inflammation is a complex cascade of events involving different types of cells and molecules. MPO could be used as an excellent biomarker for inflammation to access the extent of neutrophil infiltration during cerebral ischemia reperfusion injury.

In our present study we demonstrated that MPO activity was increased significantly in vehicle control group when compared to sham control and was correlated positively with infarct size.

The results, clearly indicating an infiltration of polymorphonuclear leukocytes (neutrophils) into the ischemic region of the brain, agree with previous studies<sup>34,35</sup>.

Quercetin and rutin treatment significantly reduced the inflammation characterized by decrease in myeloperoxidase activity in animal subjected to cerebral I/R injury. Flavonoids have also been demonstrated to have inhibitor effect on MPO *in vivo*<sup>36</sup>.

Quercetin and rutin have been shown to have inhibitory effect on myeloperoxidase (MPO) ac-

tivity *in vitro*<sup>37</sup>. Our results are in agreement with the recent report on baicalin<sup>38</sup>. Quercetin directly scavenges hypochlorous acid (HOCl), a chlorinated species generated by the MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup> system<sup>37</sup>. The MPO/nitrite-mediated lipid peroxidation of LDL was effectively blocked by the quercetin, rutin<sup>39</sup>. This may be due to structure-activity relationships of flavonoids as the anti-inflammatory constituents targeting the MPO-derived oxidative reactions *in vivo*<sup>36</sup>. However anti-inflammatory effects of quercetin and rutin are partially attributed to their cerebroprotective against ischemia reperfusion injury in rats.

Overall, these findings clearly indicated that antioxidant, anti-inflammatory effects of quercetin and rutin are not fully enough to explain the extent of cerebroprotection offered by these flavonoids. Therefore, it was thought worthwhile to explore other possible mechanisms involved in the cerebroprotection action of quercetin and rutin in cerebral ischemia reperfusion injury.

#### ***Effect of Quercetin and Rutin with and Without L-NAME on Infarct Size***

Our investigation has revealed a significant increase in infarct size in vehicle control group subjected to 30 min cerebral ischemia followed by 4 h reperfusion when compared to sham control group. Cerebral damage observed in the vehicle control group probably involves multiple mechanisms like free radicals<sup>40</sup>, inflammation<sup>41</sup> and nitric oxide deficiency<sup>42</sup>. Endothelial cell dysfunction occurs during cerebral ischemia reperfusion injury<sup>43</sup>. One of the most sensitive indicators of endothelial cell dysfunction is impaired endothelium-dependent vasodilatation that is mediated by NO<sup>44</sup>. This may be attributed to decrease in nitric oxide synthesis by eNOS or iNOS because of decrease in availability of the precursor L-arginine or by depletion of the cofactor tetrahydrobiopterin (BH<sub>4</sub>)<sup>44</sup>. This is followed by release of potent vasoconstrictor endothelin<sup>43</sup> along with the release of inflammatory mediators and increased expression of adhesion molecules by initiating inflammatory and coagulation cascades that culminate in the occlusion of capillaries, known as the “no-reflow” phenomenon that may maximizes infarct size<sup>45</sup>.

No significant difference in infarct size was observed between L-NAME treated group and vehicle group. In the present investigation L-NAME by itself did not increase infarct size. This may be because of the fact that NOS inhibitor blocks NO synthesis. It also blocks the

free radical formation. It could be that normally there is a balance between the protective effect of NO and the harmful effects of the free radical.

In the present study, quercetin and rutin offered significant cerebroprotection in terms of limiting infarct size. Administration of L-NAME prior to administration of quercetin and rutin, significantly reduced the cerebroprotection offered by quercetin and rutin. This implies the partial role of nitric oxide in the cerebroprotective mechanism of quercetin and rutin. These flavonoids probably act by increasing activity of nitric oxide synthase resulting in enhanced NO synthesis. Huk et al<sup>46</sup> demonstrated that quercetin treatment reduced ischemia-reperfusion injury to skeletal muscle by scavenging destructive superoxide and enhancing the cytoprotective nitric oxide concentration. Since constitutive nitric oxide synthase (cNOS), the enzyme through which endothelial nitric oxide production is regulated, is usually turned off by phosphorylation of one of its serine residues shortly after activation<sup>47</sup>, it is suggested that quercetin and rutin may prolongs cNOS activity and, thereby, nitric oxide production. Nitric oxide is a potent vasodilator<sup>48</sup>, while superoxide is a potent vasoconstrictor, the latter being effected by the superoxides ability to scavenge nitric oxide<sup>49</sup>. By tipping the balance between vasoconstrictor and vasodilator action in the latter direction, the net effect would be attenuation of cerebral ischemia reperfusion injury. Villar et al<sup>50</sup> demonstrated that flavonoids stimulated NO release from endothelial cells leading to vascular cGMP accumulation. Further flavonoids have been demonstrated to improve endothelial function by increasing the bioavailability<sup>51</sup> of NO. Willmot et al<sup>52</sup> in his review suggested that administration of NO in experimental stroke is associated with a reduction in infarct size.

Thus, therapeutic strategies with multiple actions such as attenuation of oxidative stress, inflammation and improvement of endothelial function with a high NO supply can be effective and promising drugs to guard against cerebral ischemia-reperfusion injury.

## **Conclusions**

Possible partial role of antioxidant, anti-inflammatory and involvement of NO in the beneficial effects of bioflavonoids quercetin and rutin against cerebral ischemia reperfusion injury was observed.



### Limitations

Since it was a mechanistic study with NO synthase inhibitor, this mechanism need to be confirmed with further studies estimating NO levels and eNOS expression in the brain.

### Acknowledgements

The Authors are thankful to Mr. B. Premnath Reddy, Chairman Acharya Institutes, Bangalore for providing necessary facilities and Dr. Divakar Goli, Principal, Acharya & B.M. Reddy College of Pharmacy, Bangalore for his support to carry out this Research Project.

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