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PROTECTIVE EFFECT OF NARINGIN AGAINST CEREBRAL INFARCTION INDUCED BY ISCHEMIC-REPERFUSION IN RATS

Orsu Prabhakar *, Koyyada Arun and Yadam Naveen

GITAM Institute of Pharmacy, GITAM Deemed to be University, Visakhapatnam - 530045, Andhra Pradesh, India.

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Correspondence to Author:
Dr. Orsu Prabhakar

Assistant Professor,
GITAM Institute of Pharmacy,
GITAM Deemed to be University,
Visakhapatnam - 530045, Andhra Pradesh, India.

E-mail: orsuprabhakar@gmail.com

ABSTRACT: Background: Leukocytes play an important role in tissue apoptosis as scavengers and vital components of inflammation, which has been generated during ischemia-reperfusion injury. The present study aimed to investigate the cerebroprotective potential of naringin through anti-inflammatory mechanism. **Methods:** Cerebral infarction was induced through bi-lateral common carotid artery occlusion for 30 min and 4 h reperfusion in rats. Cerebral infarction, number of leukocytes/cu.mm, and inflammatory markers (TNF α, IL-6 and IL-10) were measured in ischemic rat brain. **Results:** Percentage of infarction, the number of leukocytes/cu.mm, inflammatory markers (TNF-α, IL-6) and myeloperoxidase were significantly increased, and IL-10 levels were significantly decreased in ischemia-reperfusion injury rats relative to sham control rats. Percentage of infarction, the number of leukocytes/cu.mm, inflammatory markers TNF-α, IL-6, and myeloperoxidase were significantly decreased and IL-10 levels were significantly increased in naringin treated rats relative to ischemia-reperfusion injured rats. **Conclusion:** This study indicates that infiltrating leukocytes and inflammation plays a key role in cerebral ischemia- reperfusion injury and naringin alleviate the inflammation and infiltrating leukocytes.

INTRODUCTION: Stroke is a third leading death causing disease worldwide. Cerebral ischemia-reperfusion injury is a result of stroke, cerebral clot and trauma. Microvascular impairment associated with the ischemia-reperfusion injury, which contributes to increasing of infiltrating leukocytes and pro-inflammation [1]. Inflammation is one of the major pathological events in cerebral ischemic-reperfusion injury, which increases the number of leucocytes in blood circulation; these leukocytes entrapped by vascular endothelial cells can cause the further release of infiltrating leukocytes [2].

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In stroke patients and stroke animal models, post-ischemic brain inflammation is characterized by rapid activation of resident cells followed by circulation of the infiltrating inflammatory cells such as monocyte/macrophages, neutrophils, T cells [3, 4, 5].

In acute ischemic stroke, pro-inflammatory mediators such as cytokines, chemokines, and reactive oxygen species (ROS) are released rapidly from injured tissue [4]. Further, these mediators cause the overexpression of the adhesion molecules on cerebral endothelial cells and on the leukocytes. Thus it promotes the transendothelial migration and adhesion of circulating leukocytes leading to the release of cytokines and chemokines into the extracellular matrix, especially excessive induction/ activation of matrix metalloproteinases (MMPs), mainly MMP-9, and production of ROS.

Further, causing extensive stimulation of resident cells and infiltration of leukocytes by amplifying the brain-inflammatory responses and eventually leading to disruption of the blood-brain barrier, hemorrhagic transformation, brain edema, and neuronal death [4]. Therefore, a strong antioxidant and anti-inflammatory intercession may beneficial in the treatment of cerebral ischemia and reperfusion injury. Naringin is a flavanone glycoside having anti-inflammatory, anticancer, and antioxidant activities [6, 7].

Earlier studies suggested that anti-inflammatory agents have a potential role in treating ischemia and reperfusion injury [8]. Hence, the study was involved in investigating the effects of naringin on cerebral infarction caused by inflammation and infiltrating leukocytes in the animal model of bi-lateral carotid artery occlusion and reperfusion.

MATERIALS AND METHODS:

Chemicals: Rat ELISA MPO (Assaypro, USA), Rat ELISA TNF-α (Assaypro, USA),) Rat ELISA IL-6 (Eiaab,USA) , Rat ELISA IL-10 (Eiaab, USA), 2,3,5-triphenyl tetrazolium chloride (TTC) (Himedia chemicals, India), naringin (Sigma Aldrich, India), were used and other chemicals used were of analytical grade.

Animals: Adult Wistar rats (210-310 g) were obtained from the NIN, Hyderabad, Telangana, India. Animals were maintained under a 12/12-h light/dark cycle in an ambient temperature (24±1 °C) colony room. Animals were provided with an adequate supply of food and water. Animals were taken care in compliance with the CPCSEA, New Delhi and experimental protocols were conducted with the approval of the Institutional Animal Ethics Committee of GITAM Institute of Pharmacy (1287/PO/2009), GITAM deemed to be University.

Experimental Procedure:

Experimental Induction of Cerebral Infarction: Cerebral infarction was induced by bilateral common carotid artery occlusion method described by Iwasaki *et al*. [9] Briefly, rats were anesthetized with thiopental sodium dose 30 mg/kg during a surgical operation. Cervical vertebrae and the common carotid arteries were then exposed carefully are separated from the vagus nerve. These arteries were occluded for 30 min followed by reperfusion for 4 h. The rectal temperature was maintained at 37±0.5 °C with a feedback-controlled heating-pad. Animals that did not lose the righting reflex or convulsed during the ischemic episode were excluded.

Experimental Protocols:

Measurement of Percentage Cerebral Infarct Volume: Naringin was dissolved in 5% DMSO. It was administered at doses of 50,100,150 and 200 mg/kg, intraperitoneally 5 min before reperfusion. Rats were randomly divided into groups such as normal, sham, ischemia-reperfusion rats (I/R), I/R+ vehicle (DMSO), and naringin treated. Animals were subjected to estimation of blood cells (leukocytes), further, the animals were sacrificed and used for biochemical estimation. Brain tissues were isolated for estimation of percent infarct volume. Each group contains 6 animals. After a predetermined time point of ischemia-reperfusion, the brains were quickly removed and sliced into coronal sections of 2 mm thickness. Each slice was immersed in a 1.0% solution of TTC for 30 min. The TTC is converted to red formazone pigment by nicotinamide adenine dinucleotide (NAD) and dehydrogenase present in the living cells. Thus, the viable cells stain deep red, and dead cells (infarcted) remain unstained [10]. Pale necrotic infracted tissue was separated and weighed. Percent of cerebral infarction was calculated.

Measurement of Inflammatory Markers: The selected group of animals was treated with naringin (200 mg/kg dose), brain tissues were isolated and used for the estimation of inflammatory markers such as myloperoxidase [11], TNF-α [12], IL-6 [13], and IL-10 [12,] respectively. MPO, TNF-α, IL-6, and IL-10 were measured by using Rat ELISA kits. These experiments were conducted according to the manufacturer's instructions.

Statistical Analysis: Data has been represented as Mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. All analyses were performed using the prism software. Values of $P<0.05$ were considered to be significant.

RESULTS:

Effect of Naringin on Cerebral Infarction: There was a significant increase in percentage infarction

among I/R group when compared to sham control. Further, treatment with naringin has produced a dose-dependent reduction in cerebral infarction relative to I/R rats, as shown in **Table 1**.

TABLE 1: EFFECT OF NARINGIN ON PERCENTAGE CEREBRAL INFARCTION IN RATS

Groups (n=6)	Percentage cerebral Infarction
Normal	0
Sham control	1.0237 ± 0.316
I/R	66.08 ± 0.9771*
Vehicle treated	66.32 ± 0.8067
Naringin (50 mg/kg, i.p.)	32.49 ± 1.986*
Naringin (100 mg/kg, i.p.)	28.72 ± 1.326*
Naringin (150 mg/kg, i.p.)	20.84 ± 0.8122*
Naringin (200 mg/kg, i.p.)	08.87 ± 1.0807*

Data represent the mean ± SEM; the asterisk indicates P≤0.05, statistically significant difference from control group, I/R indicates ischemia and reperfusion. No of animals used in each group = 6

Effect of Naringin on Leukocytes in Ischemia-Reperfusion Injured Rats: In I/R rats, the number of leukocytes/cu.mm were significantly increased (15090 ± 164.3/cu.mm) when compared to sham control rats (7721 ± 521.4) and significantly decreased (10283 ± 186.71, 9280 ± 342.71, 8518 ± 162.71 and 7575 ± 65.65/cu.mm) in naringin treated (50,100,150 and 200 mg/kg) rats relative to I/R rats. These results were shown in **Table 2**.

TABLE 2: EFFECT OF NARINGIN ON LEUKOCYTES IN ISCHEMIA-REPERFUSION INJURED RATS

Groups (n=6)	No leukocytes/Cu.mm
Normal	7238 ± 137.5
Sham control	7721 ± 521.4
I/R	15090 ± 164.3
Vehicle treated	15180 ± 865.71
Naringin (50 mg/kg, i.p.)	10283 ± 186.71*
Naringin (100 mg/kg, i.p.)	9280 ± 342.71*
Naringin (150 mg/kg, i.p.)	8518 ± 162.71*
Naringin (200 mg/kg, i.p.)	7575 ± 65.65*

Data represent the mean ± SEM, the asterisk indicates P≤0.05, statistically significant difference from control group, I/R indicates ischemia and reperfusion. No of animals used in each group =6

Effect of Naringin on Inflammatory Markers against Cerebral Ischemia-Reperfusion Injury: Pro-inflammatory markers (TNF-α, IL-6) and MPO were significantly increased and anti-inflammatory marker (IL-10) was significantly decreased in I/R rats relative to sham control rats. In Naringin treated rats, pro-inflammatory markers (TNF-α, IL-6) and MPO were significantly reduced, and anti-inflammatory marker (IL-10) was significantly increased compared to I/R rats. The results were shown in **Table 3**.

TABLE 3: EFFECT OF NARINGIN ON CEREBRAL INFLAMMATORY MARKERS IN RATS

ASSESMENTS	Normal	Sham Control	I/R Control	Vehicle Control	Naringin treated (200 mg/kg i.p)
MPO (ng/mg of tissue)	0.4503 ± 0.205	0.8812 ± 0.524	8.484 ± 0.678*	8.342 ± 1.210	0.456 ± 1.210*
TNF-α (ng/mg of tissue)	0.105 ± 0.0053	0.2013 ± 0.008	0.5217 ± 0.015*	0.5183 ± 0.031	0.09335 ± 0.0094*
IL-6 (ng/mg of tissue)	0.1973 ± 0.030	0.395 ± 0.031	0.8167 ± 0.015*	0.8517 ± 0.026	0.1867 ± 0.028*
IL-10 (ng/mg of tissue)	1.986 ± 0.106	1.847 ± 0.102	0.545 ± 0.076*	0.612 ± 0.051	1.892 ± 0.143*

Data represent the mean ± SEM, the asterisk indicates P≤0.05, statistically significant difference from control group, I/R indicates ischemia and reperfusion, MPO indicates Myeloperoxidase, TNF-α indicates Tumor necrosis factor-alpha, IL-6 indicates Interleukin-6, IL-10 indicates Interleukin-10. No of animals used in each group =6

DISCUSSION: In this study, we investigated the cerebroprotective potential of naringin through anti-inflammatory effect against ischemic brain insult. Ischemic stroke involving reperfusion injury does not have standard approved therapy. The ischemia-reperfusion injury occurs in various clinical conditions relevant to adults, including circulating shock, myocardial infarction, and stroke, as well as surgical interventions like organ transplantation and cardiopulmonary bypass. In ischemia-reperfusion, the mechanisms underlying neuronal injury are not well understood yet. However, a recent study found that in ischemic stroke, white blood cell flexibility was increased in the aggressive inflammation condition, which results in the development of pro-inflammatory responses. Furthermore, ischemic insults were increased due to enhanced platelet aggression and fibrinogen formation [14]. Therefore, overwhelming inflammation was generated due to increased peripheral leukocytes in cerebral ischemic-reperfusion injury [15]. In this study, we observed that leukocytes count was significantly increased in I/R treated rats compared to sham control rats. The increased levels of leukocytes/cu.mm in the brain circulation can cause further worsening of the ischemic insult, which leads to increased brain infarction volume.

Earlier studies also suggested the key role of white blood cells in cerebral infarction, using the model of global forebrain ischemia-reperfusion injury [16]. Koren-Morag *et al*., also demonstrated that the higher levels of leukocytes in stroke patients cause a higher mortality rate [17]. In naringin treated group, the levels of leukocytes/cc.mm were decreased relative to I/R treated rats.

In the cerebral ischemic stroke, determination of infarction size is important to assess the different consequences. The infarction volume was quantified by staining slices of the brain with TTC. In this study, a significantly increased cerebral infarction was noticed in I/R treated group rats when compared to sham control, and a significantly decreased cerebral infarction was noticed in the naringin treated rats in comparison with I/R treated. Therefore, these results were in accordance with earlier reports [10, 18]. A significant dose-dependent reduction in the percentage of cerebral infarction was noticed with naringin treatment, indicating the dose-dependent cerebroprotection activity of naringin against ischemia-reperfusion injured rats. Suggesting this, our results demonstrated that the cerebral infarction was increased by the higher circulatory leukocytes in ischemia-reperfusion injury and naringin inverts circulatory leukocytes in ischemic brain tissue.

Naringin exerts multiple biological effects such as antioxidant, anti-inflammatory, anti-proliferative effects [19]. Recent studies also suggested that the naringin had neuroprotection against mitochondrial dysfunction which is caused by 3-nitropropionic acid through the Nrf2 signaling pathway in PC12 cells [20]. According to the supporting evidence, chronic naringin treatment alleviates the oxidative stress and inflammation levels, which were benefited in the recovery of traumatic brain injury in rats [21]. Naringin can prevent a wide variety of circumstances such as cancer, cardiovascular, diabetes, and neurological disorder [19, 22, 23].

After the cerebral ischemic-reperfusion injury, free radicals and inflammatory mediators (TNF-alpha, IL-6, and MPO) are released rapidly from injured tissue. These mediators act on leukocytes, and they promote the transendothelial migration of circulating leukocytes [4]. Leukocyte infiltration is characterized by leukocyte-endothelial interaction, leukocyte activation resulting in an accumulation in the vascular bed, followed by leukocyte extravasation into the interstitial space[24]. Reactive oxygen species are the major components to cause tissue injury in ischemia-reperfusion injury, further reactive oxygen species activate the leukocytes leading to inflammation. TNF-α and IL-6 were the pro-inflammatory markers that can be activated by reactive oxygen species. In naringin-treated rats, TNF-α, IL-6, and MPO levels were significantly reduced as compared to I/R rats. Leukocytes participate in the area of the newly returning blood; further they release a host of inflammatory markers such as TNF-α, interleukins, and free radicals leading to secondary tissue damage. Reperfusion introduces oxygen within tissues that damage cellular DNA, plasma membrane, and proteins. The damaged cell membrane may release a number of free radicals [25]. Pro inflammation can be enhanced by leukocyte activation. Therefore, the results indicate that naringin may be having cerebroprotection by reducing leukocyte activation and inflammation.

CONCLUSION: The present study demonstrated the dose-dependent potential cerebroprotective activity of naringin against cerebral ischemia and reperfusion injury. The significant reduction of leukocytes, inflammatory (MPO, TNF-α, and IL-6) parameters, and increased anti-inflammatory (IL-10) levels shows the anti inflammatory potential of the naringin treatment.

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CONFLICTS OF INTEREST: Not Applicable

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