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Neuroprotective Effect of *Flavonoid,* Hesperetin on Lipid Peroxides and Antioxidants against Cerebral Ischemia-Reperfusion Induced Cerebral Infarction in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Ischemic stroke is one of the important complications of diabetes. Diabetes exacerbates cerebral injury after ischemia and reperfusion. This study was designed to investigate whether the hesperetin has a cerebroprotective action against the ischemic-reperfusion injury via infarct size and anti-oxidant mechanisms in diabetic rats. Diabetes was induced by Streptozocine (45mg/kg., i.p.) intraperitoneal injection at once. Medial carotid artery occlusion (30 min) and reperfusion (5 hr) were employed to induce cerebral infarction in diabetic rats. The animals were divided into groups as normal, sham, ischemia-reperfusion, and hesperetin treated (30, 60, and 90mg/kg., i.p.). These were used for the evaluation of the percentage of cerebral infarction and oxidative stress biomarkers such as malondialdehyde, superoxide dismutase, and myeloperoxidase were studied. Dose-dependent reduction in the percentage of cerebral infarction was observed in hesperetin-treated groups. With hesperetin 90mg/kg dose, percentage infarct size and oxidative stress markers like myeloperoxidase and malondialdehyde levels were distinctively reduced and there was a remarkably increased level of anti-oxidant markers like superoxide dismutase. Conclusion: Collectively, these findings demonstrate that the mechanism (s) responsible for a cerebroprotective effect of hesperetin against the ischemic reperfusion injury in diabetic rats involves anti-oxidant and anti-inflammatory actions.

1. INTRODUCTION:

Stroke is the second leading cause of death and long-term disability in the world [1]. Diabetes is the major risk factor for ischemic stroke. Diabetes in the ischemic-reperfusion state can increase the inflammation and oxidative stress induced by reperfusion [2]. There is an increased mortality rate in patients with diabetes-associated cerebrovascular accidents (ischemic stroke and intracerebral hemorrhage) and are at more risk of suffering from organ damage and ischemic events [3]. In acute stroke, thrombolysis plays a life-saving therapy and helps in reperfusion. Although reperfusion is needed in ischemic stroke, it can exaggerate the condition causing further damage through inflammation and reactive oxygen species released during reperfusion. This can be further augmented by diabetes making the condition worse. These implications associated with reperfusion injury have made the active attention to it. The pathological aspects of reperfusion injury are related to oxidative stress, leukocyte infiltration, and damage to the blood - brain barrier, inflammation, nitric oxide release, platelet activation, and apoptosis [4]. Worsening clinical and laboratory outcomes are seen in ischemic-reperfusion injury patients with diabetes [5]. The intervention with anti-inflammatory and anti-oxidant agents was thought to be beneficial in treating the cerebral ischemia-reperfusion injury.

Epidemiologic studies suggest an inverse association of tea consumption with cardiovascular disease. The antioxidant effects of flavonoids in tea are the potential mechanisms that could underlie the protective effects. Other possible mechanisms include attenuating the inflammatory process in atherosclerosis, reducing thrombosis, promoting normal endothelial function, and blocking the expression of cellular adhesion molecules [6]. Flavonoids, plant-derived antioxidants, are defined as non-nutritive dietary components that are abundant in foods [7]. Consumption of flavonoids containing food and beverages has been proposed as a useful practice to limit oxidative damage in the body [8]. The protective role of flavonoids involves several mechanisms of action: a direct antioxidant effect, inhibition of enzymes of the oxygen-reduction pathway, and sequestration of transient metal cations [9,10,11]. Bioflavonoids comprise a diverse class of polyphenolic compounds with antioxidant activity. Therapeutic effects of bioflavonoids on human health are reported abundantly in scientific literature and include anti-bacterial, anti-viral, anti-inflammatory, anti-allergic, and vasodilator activities [12,13,14]. Work has been done to establish a negative relationship between bioflavonoid intake and heart disease [15,16,17]. It is believed that many of the

therapeutic effects of bioflavonoids result from their potent antioxidant and free radical scavenging properties [18].

Hesperetin is known to act as an antioxidant and scavenger of peroxynitrite at low concentrations [19]. Hesperetin was found to have neuroprotective, anti-inflammatory, and anti-proliferative effects [the 20s]. Hesperetin protects against peroxynitrite-mediated cytotoxicity. This protection was partially mediated by the intracellular scavenging of peroxynitrite by hesperetin. Hesperetin exerts an inhibitory effect on the HMG-CoA reductase [21]. In the present study, we made an attempt to investigate the protective role of hesperetin in cerebral ischemia-reperfusion injury in Wistar diabetic rats by employing medial carotid artery occlusion for 30 min and reperfusion for 5 hours.

2. MATERIALS AND METHODS:

2.1. Drugs and chemicals:

Hesperetin and Streptozotocin were procured from the Sigma- Aldrich chemicals Ltd, St. Louis, USA. 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), and reduced glutathione were obtained from Himedia Laboratories, Mumbai. All the other chemicals procured from Merck laboratories, Nice chemicals, Loba Chemie, and Sd. fine chemicals were of analytical grade.

2.2. Animals:

All the experiments were carried out with adult albino Wistar rats, 150-200g approved CPCSEA vendor. Rats were housed in polyacrylic cages (38X23X10 cm) with not more than four animals per cage. They were housed in an air-conditioned room and were kept in standard laboratory conditions under a natural light-dark cycle (approximately 14 h light/ 10 h dark) maintaining the humidity of 60±5% and ambient temperature of 25±2˚C. All animals were free to access a standard diet (Amrut rat feed) and tap water *ad libitum.* Allowed to acclimatize for one week before the experiments. The commercial pellet diet contained 22 % Protein, 4% Fat, 4% Fiber, 36% Carbohydrates, and 10% Ash w/w. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

2.3. Experimental procedure

2.3.1 Induction of diabetes:

Diabetes was induced in the rats by a single dosage of Streptozotocin (STZ) (45mg/kg, i.p.) and they were also administered with 50% w/v sucrose solution. After 6 days of STZ injection, the animals were subjected to cerebral ischemia-reperfusion injury, followed by a collection of blood samples from the tail vein of rats for the estimation of glucose levels. The blood glucose levels of > 250mg/dl in rats were considered to be diabetic and such rats are included in the study.

2.3.2. Experimental Induction of Focal Cerebral Ischemia

Overnight fasted rats were anesthetized with thiopental sodium (45 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 cotton thread was passed below each carotid artery. Global cerebral ischemia was induced by occluding the common carotid arteries by a knot [22]. 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 5 h. The body temperature of rats was maintained at 37°C by a heated surgical platform. All surgical procedures were carried out under sterile conditions.

2.3.3. Determination of Infarct Size

The infarct size was determined in rats as described in previous studies [23]. In brief, animals were killed at the end of 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-triphenyl tetrazolium 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 the total weight of the brain.

2.3.4. Preparation of Brain Tissue for Estimation of Biochemical Parameters

The brain of each animal was removed after completion of 5 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. The homogenization 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 was 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), and myeloperoxidase (MPO). Protein was measured by the method of Lowry et al [24]. Estimation of MDA Level MDA level was measured as previously described by Ohkawa et al. [25]. Superoxide Dismutase (SOD) Table II. Experimental design for determination of infarct size. The SOD level was estimated by the method described by Kakkar et al. [26]. The estimation of the MPO level was estimated by the method described by Mullane et al. [27].

3. STATISTICAL ANALYSIS

The results were expressed as (Mean± SEM). Differences in infarct size, MDA, SOD, 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).

4. RESULTS

Effect of hesperetin on Infarct Size after Ischemia-Reperfusion in diabetic rats

In order to examine the cerebroprotective effect of hesperetin against an ischemia-reperfusion insult, we measured the infarct size with or without administration of hesperetin. As shown in **table 1**, the percentage of infarct size was 89.75±3.1% in vehicle-treated animals, it was significantly reduced to 65.23±1.3, 51.2±1.23, and 49.34±2.33 in a dose-dependent manner respectively, in animals given with 30, 60 and 90 mg/kg dose of hesperetin respectively. These observations indicate that hesperetin can reduce ischemia-reperfusion-induced brain injury.

Table 1: Effect of hesperetin on % of Brain Infarct in diabetic rats

Groups	% infarct (Focal)
Diabetic Sham-operated control without I/R.	22.12±2.14***
Diabetic vehicle control- Rats received 0.2 ml of 10% DMSO and served as a control subjected to ischemia-reperfusion (with I/R)	89.75±3.1
Diabetic rats received Hesperetin 30 mg/kg before reperfusion	65.23±1.3**
Diabetic rats received Hesperetin 60 mg/kg before reperfusion	51.2±1.23***
Diabetic rats received Hesperetin 90 mg/kg before reperfusion	49.34±2.33***

Each value is expressed as mean ± SD, n=6, and statistical analysis was performed by using one-way ANOVA followed by Dunnett's post hoc test where *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$ compared to saline group.

Effects of hesperetin on biochemical parameters in diabetic rats

After 30 min of global cerebral ischemia and 5 h of reperfusion, a significant reduction in SOD level in the brain was observed in the I/R control i.e. Saline group as compared to the sham group. This reduction was reversed by hesperetin 30, 60, and 90mg/kg (dose-dependent) and showed an increased significant ($p<0.01$) level when compared to the I/R control group of diabetic rats (vehicle).

Treatments with hesperetin at 60 and 90 mg/kg reduced the MPO level compared to I/R treated group i.e. vehicle control group significantly (P<0.01) and moderately significant (P<0.05) with hesperetin at 30 mg/kg dose.

The amount of MDA formed in the vehicle control group was significantly high as compared to the sham group. Treatments with hesperetin 30, 60, and 90 mg/kg reduced the MDA level in a dose-dependent fashion as compared to I/R treated (P<0.05; p<0.01 and p<0.001).

Table 2: Effects of HMC on biochemical parameters in Global model

Groups	**SOD (units/mg)**	**MPO (pg/ml)**	**MDA (pg/ml)**
Diabetic Sham-operated control without I/R.	16.26±1.96***	4.94±1.95***	5.60±1.7***
Diabetic vehicle control- Rats received 0.2 ml of 10% DMSO and served as a control subjected to ischemia-reperfusion (with I/R)	1.623±0.2473	26.7±1.4	51.04±1.8
Diabetic rats received Hesperetin 30 mg/kg before reperfusion	6.23±0.9***	12.56±0.04**	12.32±2.1***
Diabetic rats received Hesperetin 60 mg/kg before reperfusion	10.11±1.3***	10.3±1.1***	10.56±2.3***
Diabetic rats received Hesperetin 90 mg/kg before reperfusion	13.97±1.1***	6.4±1.0***	6.79±2.3***

Each value is expressed as mean ± SD, n=6, and statistical analysis was performed by using one-way ANOVA followed by DUNNET'S post hoc test where *=p<0.05, **=p<0.01, and ***=p<0.001 compared to saline group.

DISCUSSION:

The present study was involved to investigate the possible cerebroprotective mechanisms of hesperetin against ischemia-reperfusion injury in diabetic rats. The clinical outcomes are worsened in diabetic-associated ischemic stroke. Both diabetes and ischemia-reperfusion are involved in the release of ROS and inflammation. This can lead to further serious pathological events in the ischemic tissue injury. Few studies have demonstrated that anti-inflammatory and antioxidant agents may be useful in limiting the reperfusion injury [28,29].

Recent researchers also suggested that medial carotid artery occlusion (MCA) can induce brain ischemia in rats [30]. MCA occlusion and reperfusion are followed by pathological events such as inflammation and free radicals generation to cause tissue apoptosis in diabetic rats. Evaluating the area of infarction in the brain gives the estimate of cerebral damage and thus the consequences of cerebral ischemia which leads to neurological impairment can be determined. The size of the infarct was determined by staining of coronal sections with TTC. TTC helps in distinguishing the viable cells as deep red color and infarct tissue as unstained cells (pale whitish tissue). In the present study, we noticed the percentage of infarction in I/R diabetic rats was significantly increased, whereas a significantly decreased percentage is seen in hesperetin-treated diabetic rats. A dose-dependent reduction in percent infarction was observed in hesperetin (30, 60, 90mg/kg) treated rats. These results were in accordance with the earlier reports. Diabetes in ischemia-reperfusion injury can worsen the exposure of brain cells to free radicals by oxidative metabolism and inflammation. Lipid peroxidation is generated by free radicals and initiates the release of the end product, malondialdehyde (MDA) which determines oxidative stress. SOD is the most important endogenous anti-oxidative enzyme which plays a key role in scavenging the free radicals. However, excess free radicals in the ischemic condition limit the levels of SOD altering the anti-oxidative defensive mechanism. This is supported by the well-known fact that the involvement of increased levels of free radicals in diabetes is associated with ischemia. When compared to normal rats the oxidative stress is greater in ischemic rats associated with diabetes. In the present study, we identified a remarkable reduction of SOD and a significant increase in levels of MDA and MPO in I/R diabetic rats. In contrast, SOD levels were increased and MDA and MPO levels were decreased significantly in hesperetin-treated diabetic rats which demonstrates the strengthened oxidative defense mechanisms and reduced lipid peroxidation by hesperetin. In supporting this, several studies have reported the modulatory effect of hesperetin on lipid peroxidation and antioxidant enzymes following CNS injuries such as ischemia/hypoxia [31, 32]. Therefore, we suggest that hesperetin has cerebroprotective action against cerebral ischemia and reperfusion injury through the anti-oxidative mechanism.

5. CONCLUSION:

The present study reveals the cerebroprotective activity of hesperetin by declining cerebral infarct percentage. Hesperetin has also shown suppressing effects against oxidative stress and inflammation markers which were elevated by cerebral ischemia-reperfusion injury. These findings suggest that hesperetin has a protective potential effect against cerebral ischemic

stroke via antioxidant and anti-inflammatory mechanism(s)and further supports the possible use of hesperetin as a therapeutic agent to ameliorate cerebral infarction.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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