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Possible nitric oxide mechanism in the protective effect of hesperidin against ischemic reperfusion cerebral injury in rats

Vaibhav Gaur, Aditi Aggarwal & Anil Kumar*

Pharmacology Division, University Institute of Pharmaceutical Sciences,
Panjab University, Chandigarh 160014, India

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Stroke is the third leading cause of death and disability around the globe. The aim of the present investigation was to evaluate the protective effect of hesperidin and its nitric oxide mechanism against cerebral ischemia reperfusion injury. Bilateral common carotid artery occlusion for 30 min followed by 24 h reperfusion was given to induce ischemia in rats. Animals were pretreated with hesperidin (50 and 100 mg/kg, po) for 7 days. Various behavioural tests, oxidative stress parameters, endogenous antioxidant system, antioxidant enzyme activity and mitochondrial enzyme complex (I, II, III and IV) dysfunctions in cortex and striatum were assessed subsequently. Hesperidin (50 and 100 mg/kg) significantly improved neurobehavioral alterations (neurological score, locomotor activity, resistance to lateral push and hanging wire latency), attenuated oxidative damage, restored antioxidant and mitochondrial complex enzyme activities in cortex and in striatum regions of the brain as compared to their respective controls. L-arginine (100 mg/kg) or L-NAME (10 mg/kg) pretreatment with lower dose of hesperidin (50 mg/kg) significantly reversed or potentiated its protective effect, respectively which was significant as compared to hesperidin (50 mg/kg). The results highlight the involvement of nitric oxide mechanism in the protective effect of hesperidin against ischemia reperfusion injury induced alterations.

Keywords: Bilateral common carotid artery occlusion, Hesperidin, Ischemia, Oxidative stress, Respiratory enzyme activity, Stroke

Acute ischemic stroke is a very common cause of death and disability in modern society[1]. Stroke occurs dueto obstruction of blood flow in cerebral vessels. Cerebral ischemia followed by reperfusion alters functional properties of brain, induces neuroinflammation and excessive generation of reactive oxygen species (ROS)[2]. However, the entire mechanisms of these several cellular cascades and their interaction are still far from our understanding. A pivotal role of oxidative stress and mitochondrial enzyme complex dysfunction in acute ischemic stroke has been reported[3]. Reports suggest that cerebral hypoperfusion causes oxidative damage in diverse areas of the brain[4,5]. Ischemia reperfusion (I/R) injury causes excessive ROS production which sets off several intracellular cascades causing pathological alterations in diverse regions of the brain[6]. A homeostatic balance exists between formation of oxygen free radicals and their removal by endogenous scavengers in normal physiological conditions[6,7]. During ischemia, free radicals generate causing damage to lipids, DNA, proteins and produce neuronal cell death. They also contribute to the breakdown of the blood-brain barrier and are responsible for brain edema.

Neurones produce nitric oxide (NO) mostly by activation of neuronal nitric oxide synthase (NOS), which is constitutively expressed, in neuronal cells[8]. The three isoforms of NOS have been well characterized in brain cells. Most of these brain cells express NOS isoforms. For examples, neurons and endothelial cells express inducible nitric oxide synthase (iNOS)[2,9] whereas astrocytes express neuronal nitric oxide synthase (nNOS)[10] Glial cells (astrocytes, microglial cells and macrophages) cause iNOS expression[11]. The interaction of cellular cascades and nitric oxide has been suggested during degenerative condition and related pathologies including ischemic damage[12,13].

Reactive oxygen species (ROS) namely superoxide and hydroxyl free radicals, together with H_2O_2 cause neuronal toxicity and initiate a free-radical-mediated chain reaction damaging diverse areas of the brain[5]. Therefore, oxidative reperfusion injury could be one

*Correspondent author
Telephone: +91-172-2534106
Telefax: +91-172-2541142
E-mail: kumaruips@yahoo.com

of the possible cellular cascade affecting all organs and tissues during ischemia. However, the entire mechanisms which trigger and modulate these cellular cascades are so far partially understood[14]. Since reperfusion injury is associated with an imbalance of oxidants and antioxidant defense system, therefore, prevention of cerebral ischemia induced oxidative damage could be as one of the possible therapeutic strategy to manage the stroke[15]. Indeed, many antioxidants have been reported to be neuroprotective in experimental models of cerebral ischemia[16-18].

Hesperidin is a flavanone glycoside abundantly found in sweet orange, lemon and by-product of citrus fruits[19]. Hesperidin is effectively used as a dietary supplement of several degenerative processes in the treatment. Its deficiency has been linked to abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps[20]. Hesperidin was found to be essential in maintaining the permeability and integrity of the micro vascular endothelium[19]. Supplemental hesperidin helps in reducing edema or excess swelling in the legs due to fluid accumulation[20]. Flavonoids are polyphenolic compounds and constitute an important group of antioxidants, which can directly quench free radicals and inhibit enzymes of oxygen reduction pathways[21]. The activity of hesperidin against ischemia reperfusion induced memory dysfunction has been recently reported[18].

Therefore, present study has been designed to reveal possible nitric oxide mechanism in the protective effect of hesperidin against ischemic reperfusion cerebral injury induced neurobehavioral, biochemical and cellular alterations in rat brain.

Materials and Methods

Animals—Male Wistar rats (200-220 g) bred in Central Animal House of Panjab University, Chandigarh were used. The animals were housed under standard laboratory conditions, maintained on 12 h light/dark cycle, had free access to food and water. Animals were acclimatized to laboratory conditions before the test. All the experiments were performed between 0900 and 1700 h. Research protocol was approved by Institutional Animal Ethics Committee and experiments were conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

Induction of cerebral ischemia/reperfusion (I/R) injury in rats—Induction of cerebral ischemia/ reperfusion was carried as per modified method of Jingtao *et al*[22]. The animals were anaesthetized with chloral hydrate (350 mg/kg, ip). Both common carotid arteries were exposed over a midline incision. Dissection was made between sternocleidomastoid and sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. The induction of ischemia was performed by occluding bilateral common carotid arteries (BCCAO) with micro-aneurism clamps for 30 min followed by 24 h reperfusion and the skin was stitched using waxed silk suture.

During BCCAO, animals were observed for following criteria: maintenance of dilated pupils, absence of a cornea reflex when exposed to strong light stimulation, and maintenance of rectal temperature at (37° ± 0.5ºC). Animals did not match these criteria or showed seizures were excluded from study. Sham treated animals underwent surgery, without BCCAO. After the completion of reperfusion period, animals were assessed for neurological outcome after 24 h and then sacrificed for biochemical and cellular assessment.

Drug treatment—All the drugs (purchased from Sigma Chemicals, St. Louis, MO, USA) were suspended in normal 0.25 % of carboxymethylcellulose (CMC) suspension in sterile water for injection. Drugs were prepared freshly just before the administration daily. Animals were divided into different groups of 12 rats each. First group as animals of sham groups were anaesthetized and bilateral common carotid artery was isolated but not occluded. A second group of rats served as controls, received 0.25% (w/v) suspension of sodium carboxy methyl cellulose for 7 days and subjected to I/R on the last day. Hesperidin [HES] (50 and 100 mg/kg, po) pretreatment was given for 7 days before I/R in group 3 and group 4. Hesperidin was suspended in 0.25% solution of sodium carboxy-methyl-cellulose. In Group 5 and 6, L-arginine (100 mg/kg, ip) and L-NAME (10 mg/kg, ip) were administered respectively. L-arginine or L-NAME was pretreated with lower dose of hesperidin (50 mg/kg, po) with in a groups 7 and 8 respectively. L-NAME and L-arginine were administered one hour before hesperidin treatment. All the doses have been selected on the basis of previous studies and pilot studies[18,23-25].

Behavioural parameters

Inclined beam-walking test—Inclined beam walking test was employed to evaluate fore and hind limb motor coordination[26]. Each animal was

individually placed on a wooden bar, inclined at an angle of 60° from the platform. The motor performance of rats was scored on a scale ranging from 0-4. A score of 0 was assigned to animal that could readily traverse the beam. Score 1, 2 and 3 were given to animals demonstrating mild, moderate and severe impairment, respectively. Score 4 was assigned to the animals completely unable to walk on the beam.

Measurement of locomotor activity (ambulation) by actophotometer—The locomotor activity (ambulatory activity) was recorded by using actophotometer (IMCORP, India). Before locomotor task, animal was placed individually in the activity meter for 3 min for habituation. Thereafter, locomotor activity was recorded using actophotometer for 5 min. Ambulatory activity was recorded and expressed in terms of total photo beam counts/5 min[27].

Lateral push test—Animal was placed on a rough surface for firm grip and was evaluated for the resistance to lateral push from either side of shoulder. Animal with increased or decreased resistance to lateral push after ischemia was assigned + or score respectively[28].

Hanging wire test—This task was used to measure griping and forelimb strength of the rats after I/R induced brain injury as per Hunter[29]. In this test, ischemic animals (control and drug treated) were suspended by the forelimbs on a wire stretched between 2 posts 60 cm above a foam pillow. The time (sec) until the animal fell was recorded. The cut off time was taken as 90 sec.

Biochemical tests

Dissection and homogenization—Animals were scarified by decapitation, immediately after behavioral assessment for the biochemical analysis. The brains areas were dissected out and cerebellum discarded. Brain areas (cortex and striatum) were separated out and weighed. A 10% (w/v) tissue homogenates were prepared in 0.1 *M* phosphate buffer (*p*H 7.4). The homogenates were centrifuged at 10,000 *g* for 15 min and aliquots of supernatants were separated and used for biochemical estimation of lipid peroxidation (LPO)[30], nitrite[31], catalase[32], superoxide dismutase activity (SOD)[33], reduced glutathione[34], total glutathione[35], glutathione–S–transferase (GST)[36] and Protein[37]. Rat brain mitochondria were isolated[38] and Complex-I (NADH dehydrogenase activity)[39], Complex-II (succinate dehydrogenase activity)[40], Complex IV (Cytochrome oxidase assay)[41] and MTT assay[42] were performed.

Statistical analysis—All the values are expressed as mean ± SE. Wilcoxon rank sum test was used for the neurological scores and Chi-square test was applied for the lateral push experiment. The data of all the other experiments were analyzed using analysis of variance (ANOVA) followed by Tukey test. In all the tests, criterion for statistical significance was $P < 0.05$.

Results

Effects of hesperidin on neurological score, locomotor activity, hanging latency time and its modification with nitric oxide modulators against I/R injury in rats—BCCAO challenge for 30 min followed by 24 h reperfusion significantly increased neurological score, reduced locomotor activity and hanging latency time as compared to sham operated group. Seven days hesperidin (100 mg/kg) pretreatment significantly reduced neurological score, improved locomotor activity, and delayed hanging latency time as compared to control (BCCAO) group (Table 1). However, 50 mg/kg hesperidin pretreatment did not influence significantly neurological score, locomotor activity and hanging latency time as compared to control. Further, L-NAME (10 mg/kg) pretreatment with hesperidin (50 mg/kg) caused significant potentiation in the protective effect of hesperidin (reduction in neurological score, improvement in locomotor activity and delayed hanging latency time) which was significant as compared to their effect per se. However, L-arginine (100 mg/kg) pretreatment with hesperidin (50 mg/kg) significantly reversed the

Table 1—Effects of hesperidin on neurological score, locomotor activity and hanging time against ischemia reperfusion injury in rats.

[Values are expressed as mean ± SE from 12 animals per group]

Treatment	Neurological score	Locomotor activity (counts /5 min)	Hanging latency time (sec)
Sham	0.00 ± 0.0	249.20 ± 19.7	80.2 ± 3.53
Control	3.17 ± 0.34 [a]	139.37 ± 14.7[a]	19.93 ± 2.98 [a]
HES (50)	2.4 ± 0.2 [a]	166.80 ± 6.07[a]	41.00 ± 2.35 [a]
HES (100)	1.6 ± 0.24 [a,b]	220± 17.88 [b]	67.4 ± 3.01 [b,c]
L-arg (100)	3.2 ±0.2	136 ± 9.13	19.00 ± 3.08
L-NAME (10)	2.6 ± 0.13	142.2 ± 10.31	23.8 ± 1.77
HES (50)+ L-arg(100)	3.0± 0.17 [c]	130.8 ± 3.61[c]	24.00 ± 3.9 [c]
HES (50)+ L-NAME (10)	2.4 ± 0.16[NS]	204.2 ± 16.65 [c,d]	57.2 ± 3.35 [c,d]

$P<0.05$ as compared to [a]sham, [b]control (ischemia reperfusion), [c]HES (50 mg/kg), [d]L-NAME (10)

protective effect of hesperidin. Further, L-NAME (10 mg/kg) and L-arginine (100 mg/kg) per se treatment did not influence significantly neurological score, locomotor activity and hanging latency time as compared to sham animals (Table 1).

Effects of hesperidin on lateral push and its modification with nitric oxide modulators against I/R injured rats—BCCAO challenge for 30 min followed by 24 h reperfusion significantly impaired percentage resistance to lateral push on rough surface as compared to sham operated animals. Seven days hesperidin (100 mg/kg) pretreatment significantly increased percentage resistance to lateral push as compared to control group (Fig. 1). However, hesperidin (50 mg/kg) did not show any significant effect on percentage lateral push resistance on rough surface as compared to control. Further, L-NAME (10 mg/kg) pretreatment potentiated the protective effect of hesperidin (increased percentage resistance to lateral push) which was significant as compared to the effect *per se.* However, L-arginine (100 mg/kg) treatment with hesperidin (50 mg/kg) did not significantly influence percentage resistance to lateral push as compared to hesperidin. L-arginine (100 mg/kg) and L-NAME (10 mg/kg) *per se* treatment did not show any significant effect as compared to sham group (Fig. 1).

Effects of hesperidin on oxidative stress and its modification with nitric oxide modulators against I/R injured rats—BCCAO for 30 min followed by 24 h reperfusion caused significant (P<0.05) oxidative damage as indicated by increase in lipid peroxidation, nitrite concentration, attenuated reduced glutathione, redox ratio as well as depletion of catalase, SOD, and GST activity in the cortex and striatum regions of the brain as compared to their respective sham treated rats. Seven days pretreatment with hesperidin (50 and 100 mg/kg) significantly attenuated these parameters (rise in lipid peroxidation, nitrite concentration, enhanced reduced glutathione, redox ratio and restored depleted catalase, SOD and GST activity) in cortex and striatum areas of the brain as compared to control (Table 2). L-arginine pretreatment (100 mg/kg) with lower dose of hesperidin (50 mg/kg) significantly reversed the protective effect of hesperidin. Further, L-NAME (10 mg/kg) pretreatment with hesperidin (50 mg/kg) significantly potentiated the protective effect of hesperidin which was significant as compared to their own effect alone (Tables 2 and 3). L-arginine (100 mg/kg) and L-NAME (10 mg/kg) *per se* treatment did not show any significant effect as compared to control.

Effects of hesperidin on respiratory enzyme activity and its modification with nitric oxide modulators against I/R injured rats—BCCAO (for 30 min) followed by 24 h reperfusion significantly impaired mitochondrial enzyme complex-I, II and III activity in striatum and cortex as compared to sham group. Seven days hesperidin (50 and 100 mg/kg) treatment significantly restored mitochondrial enzyme complex

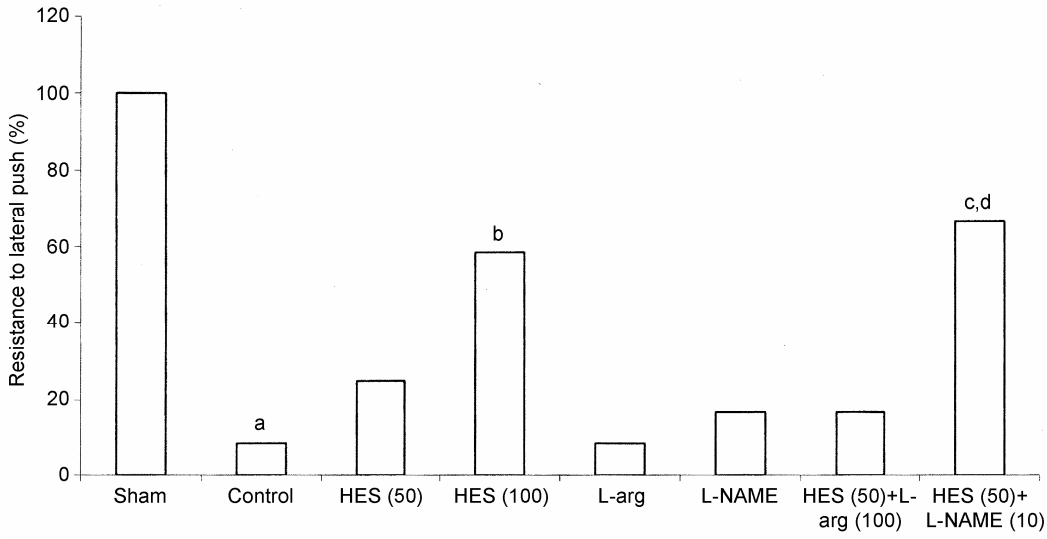

Fig. 1—Effects of hesperidin on percentage of rats showing resistance to the lateral push. Values are mean ± SE from 12 animals per group. Data was analysed by Chi square ($\chi 2$) test. P<0.05; as compared to [a]sham, [b]control (ischemia reperfusion injured), [c]HES(50 mg/kg), [d]L-NAME (10).

Table 2—Effect of hesperidin on oxidative stress and its modification with nitric oxide modulators against I/R injury in rats.
[Values are expressed as mean ± SE from 6 animals per group]

Treatment (mg/kg)	Brain region	LPO (moles of MDA/mg protein)	Catalase (μ Mole of H_2O_2/min/ mg protein)	SOD (μ Moles/min/mg protein	Nitrite (μg/ml)
Sham	Cortex	1.95±0.14	25.82±0.43	30.18±1.36	523.33±16.78
	Striatum	1.96±0.13	26.89±1.74	30.34±1.29	421.67±16.48
Control	Cortex	5.25±0.30[a]	10.79±1.18[a]	15.84±0.78[a]	688.33±9.31[a]
	Striatum	3.90±0.34[a]	11.82±1.35[a]	13.82±0.72[a]	723.33±18.21[a]
HES (50)	Cortex	3.99 ±0.16[a]	14.82±0.44[a,b]	18.70±1.03[a]	630.0±14.14[a]
	Striatum	3.37±0.22[a]	19.05±0.34[a,b]	18.81±0.20[a,b]	620.0±14.14[a]
HES (100)	Cortex	2.97±0.24[a,b]	21.64±0.86[b]	26.45±2.10[a,b]	590.0±21.21[a]
	Striatum	2.22±0.12[a,b]	24.24±2.39[b]	25.60±1.28[a,b]	527.5±12.37 [a,b]
L-arg (100)	Cortex	6.09±0.21	9.70±0.19	14.90±1.14	703.33±22.51
	Striatum	4.20±0.13	9.75±0.65	13.26±0.65	691.67±35.94
L-NAME (10)	Cortex	5.58±0.17	11.62±1.36	16.83±1.13	683.33±17.08
	Striatum	3.93±0.17	12.19±0.47	15.69±0.84	681.67±29.18
HES (50)	Cortex	5.84±0.06 [b,c]	10.4±0.30 [b,c]	15.22±0.74 [b,c]	683.33±17.08[b,c]
+ L-arg (100)	Striatum	4.09±0.12 [b,c]	10.43±0.71 [b,c]	13.96±0.76 [b,c]	663.33±27.96 [b,c]
HES (50)+	Cortex	3.03±0.07 [c,d]	20.3±0.13 [c,d]	23.68±0.67 [c,d]	601.67±20.29 [c,d]
L-NAME (10)	Striatum	2.10±0.27 [c,d]	22.95±0.88 [c,d]	24.94±0.65 [c,d]	576.67±28.75 [c,d]

P<0.05; as compared to [a] sham, [b] control (ischemia reperfusion), [c] hesperidin (50mg/kg), [d]L-NAME (10)

Table 3— Effect of hesperidin on glutathione system and its modification with nitric oxide modulators against I/R injury in rats

[Values are expressed as mean ± SE from 6 animals per group]

Treatment (mg/kg)	Brain region	Total glutathione (nM of GSH/mg protein)	Reduced glutathione (nM of GSH/mg protein)	Oxidized glutathione (nM of GSH/mg protein)	Redox ratio	GST (μ mole of CDNB formed/min/mg protein)
Sham	Cortex	124.77±2.13	87.50±2.16	37.27±4.30	2.35±.30	45.29±4.15
	Striatum	126.44±4.25	87.87±2.73	38.57±5.02	2.41±.32	55.20±1.16
Control	Cortex	120.01±0.71	49.48±2.40[a]	70.53±2.35[a]	0.70±0.06[a]	20.83±2.40[a]
	Striatum	119.62±4.99	42.13±4.28[a]	77.49±9.19[a]	0.59±0.12[a]	21.44±1.44[a]
HES (50)	Cortex	118.0±3.24	58.53±1.47[a,b]	59.46±1.77[a,b]	0.98±0.004 [a,b]	29.35±0.74[a,b]
	Striatum	123.18±3.72	62.13±1.89[a,b]	61.05±1.83[a,b]	1.02±0.001[a,b]	30.06±2.18[a,b]
HES (100)	Cortex	128.87±4.93	79.26±4.13[b]	49.61±9.06[a,b]	1.59±0.40[a,b]	40.95±4.61[a,b]
	Striatum	126.61±2.24	77.10±3.08[b]	49.51±5.32[a,b]	1.56±0.24 [a,b]	44.24±3.66 [a,b]
L-arg (100)	Cortex	123.68±7.03	46.95±1.07	76.73±6.73	0.61±0.05	19.85±2.22
	Striatum	121.48±7.17	41.96±0.42	79.52±7.58	0.53±0.05	19.84±1.74
L-NAME (10)	Cortex	122.10±4.04	51.44±4.39	70.66±0.53	0.73±0.07	23.05±0.99
	Striatum	123.52±2.44	46.16±2.20	77.36±4.15	0.59±0.06	22.59±1.55
HES (50) + L-arg (100	Cortex	122.03±5.18	47.94±2.09 [b,c]	74.08±3.09 [b,c]	0.65±0.0 [b,c]	20.29±2.13 [b,c]
	Striatum	122.24±3.33	44.65±1.8 [b,c]	77.59±2.08 [b,c]	0.57±0.02 [b,c]	20.04±0.27 [b,c]
HES (50) + L-NAME (10)	Cortex	122.15±4.18	74.84±4.90 [c,d]	47.31±3.89 [c,d]	1.58±0.2 [c,d]	38.61±3.21 [c,d]
	Striatum	121.78±1.12	74.49±2.23[c,d]	47.29±2.34 [c,d]	1.57±0.12 [c,d]	40.49±1.06 [c,d]

P<0.05 as compared to [a] sham, [b] control (ischemia reperfusion), [c] HES (50mg/kg), [d]L-NAME (10)

I, II and III activities as compared to control group (Figs 2 and 3). However, mitochondrial enzyme complex IV activity was not affected by ischemia reperfusion injury. L-NAME (10 mg/kg) pretreatment with hesperidin (50 mg/kg) significantly potentiated the protective effect of hesperidin (restored mitochondrial enzyme complex I, II and III) activity which were significant as compared to their effect *per se* (Figs 2 and 3). Further, L-arginine (100 mg/kg) pretreatment with lower dose of hesperidin (50 mg/kg) significantly reversed the protective effect of hesperidin and altered mitochondrial enzyme complex I, II and III activity (Figs 2 and 3). L-arginine or L-NAME *per se* did not produce any significant effect on mitochondrial enzyme complex activity as compared to sham group. However, there was no

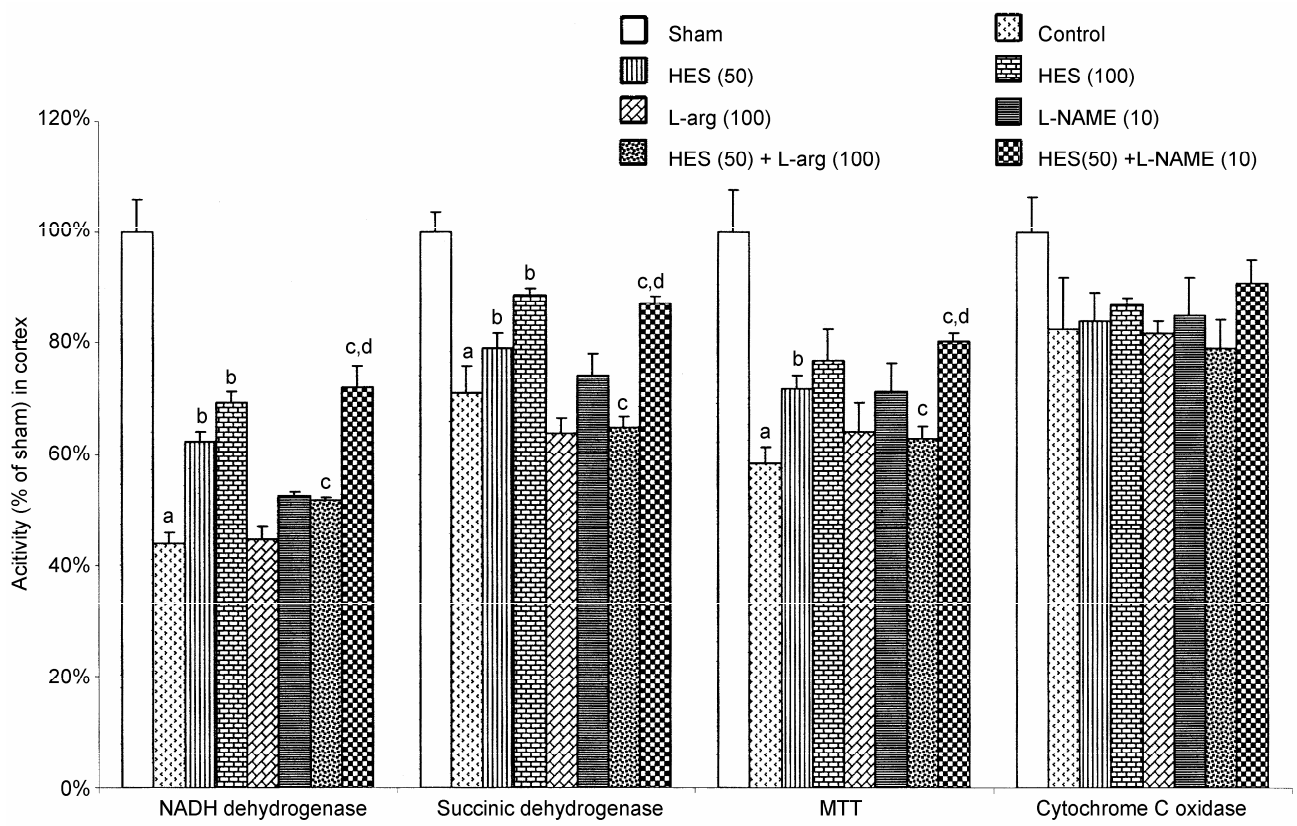

Fig. 2—Effects of hesperidin on respiratory enzyme activity in cortex and its modification with nitric oxide modulators against I/R injured rats. Values are mean ± SE from 6 animals per group. Data was analysed by One-Way Analysis of Variance (ANOVA) followed by *post hoc* Tukey test. $P<0.05$; as compared to [a]sham, [b]control (ischemia reperfusion injured), [c]HES(50 mg/kg), [d]L-NAME (10).

significant alteration is seen in mitochondrial complex-IV in ischemia reperfusion group as compared to sham treated group.

Discussion

Cerebral ischemia is one of the major leading causes of morbidity and mortality worldwide. Ischemic stroke causes death of brain tissue resulting from transient or permanent reduction in intra-cranial blood perfusion. According to the World Health Organization, world's 15 million population suffers from stroke each year leading to significant mortality. One-third does not seem to survive and another one-third remains permanently disabled. Few of them show favorable recovery and a satisfactory lifestyle, if treatment is given on time[43]. The measurement of physical impairment involves an evaluation of motor and sensory functions and the performance of daily routine activities.

Hesperidin is a naturally occurring flavonoid abundantly found in fruits, vegetables, nuts, seeds, and beverages such as tea and wine. Flavonoids act as powerful antioxidants, having a remarkable neuroprotective activity against oxidative and free radical damage. Studies suggested that the antioxidant activities of flavonoids are due to their hydrogen-donating also and free-radical scavenging properties[21]. Flavonoids can inhibit free radical formation and free-radical mediated reactions by chelating the metal-ions[44]. The effects of hesperidin in prevention and treatment of neurological disease have received considerable attention[45]. Hesperidin has been reported to have antioxidant, anti-inflammatory and anticancer properties[46]. Hesperidin has been reported to offer neuro-protection by terminating lipid peroxidation side chain rather than scavenging extra-cellular non-lipid radicals that initiate lipid peroxidation[47].

In the present study, bilateral common carotid artery occlusion (BCCAO) for 30 min followed by 24 h reperfusion showed significant impairment of sensorimotor activities (reduced locomotor activity, inclined beam walking test and increased resistance to lateral push) suggesting deficit in sensorimotor and neurological functions. Global cerebral ischemia and reperfusion induced behavioural alterations are very much similar to clinical symptoms of stroke in patients[48]. In the present study, a marked decrease in locomotor activity and inclined beam walking test has been observed after I/R, which was reversed by hesperidin pretreatment suggesting its therapeutic

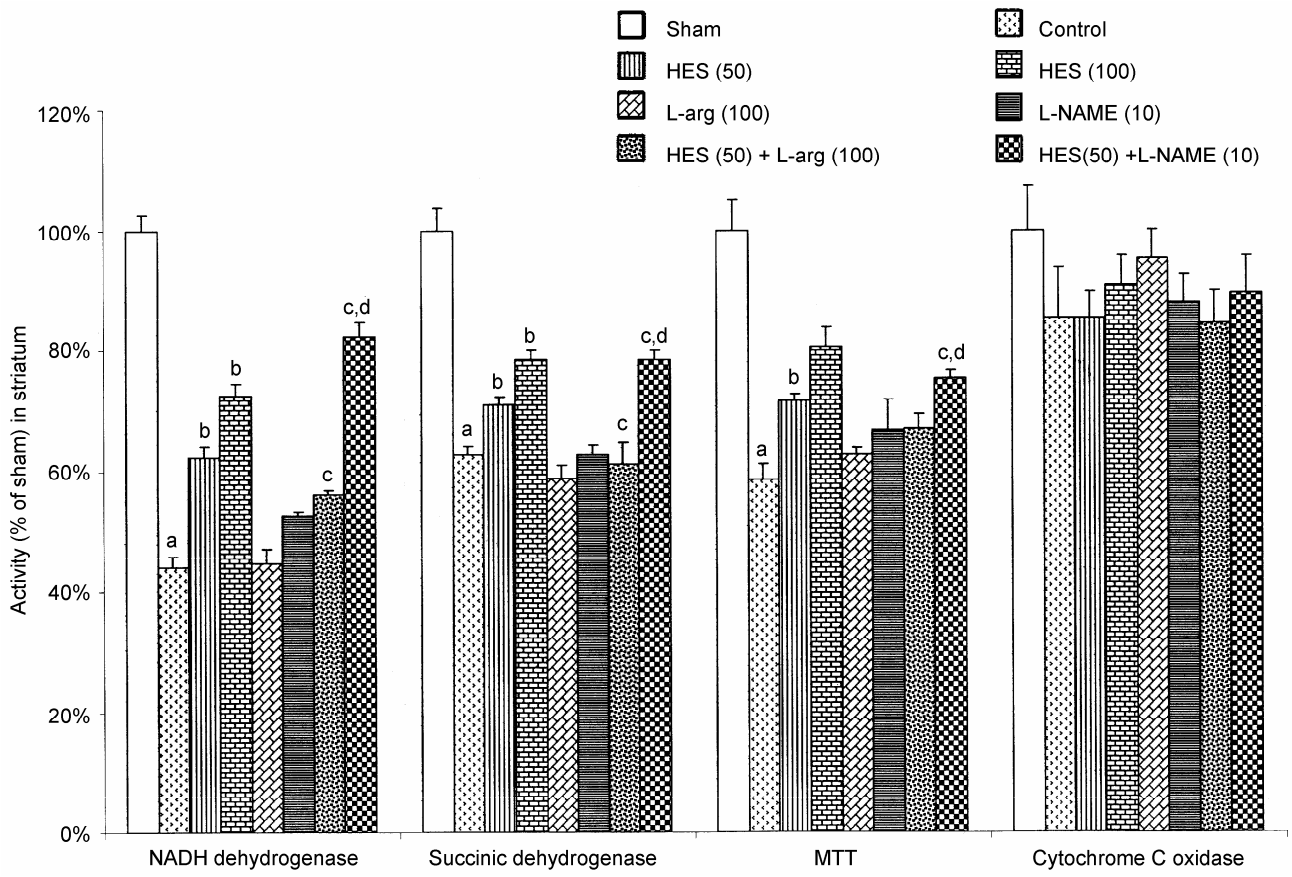

Fig. 3—Effects of hesperidin on respiratory enzyme activity in striatum and its modification with nitric oxide modulators against I/R injured rats. Values are mean ± SE from 6 animals per group. Data was analysed by One-Way Analysis of Variance (ANOVA) followed by *post hoc* Tukey test. $P<0.05$; as compared to [a]sham, [b]control (ischemia reperfusion injured), [c]HES(50 mg/kg), [d]L-NAME (10).

potential. Similarly, global cerebral ischemia has also been reported to cause marked decrease in grip and muscle strength of the limbs as demonstrated by hanging wire test and rota-rod performance[49,50]. Consistent to the above studies, a marked decrease in grip muscle strength performance has been observed in the present study, which was significantly reversed by hesperidin pretreatment. In support, several studies have reported the protective effect of antioxidants against ischemic reperfusion injury[51]. It seems that antioxidants might have some role in ischemia/reperfusion induced neurobehavioral alteration. However, mechanism of their protective effects have not been clearly understood so far against ischemic and reperfusion injury. In the present study, L-arginine and L-NAME *per se* treatment did not produce any significant effect on these neurobehavioural alterations against ischemic reperfusion injury. However, L-arginine pretreatment significantly reversed the protective effect of hesperidin on neurobehavioural alterations. However, L-NAME pretreatment with hesperidin significantly potentiated the protective effect of hesperidin (neurobehavioural changes) on behavioural alterations, suggesting the involvement of nitric modulation in the protective effect of hesperidin.

Ischemia reperfusion injury leads to the formation of several cellular toxic mediators, which contribute to oxidative damage. It is now well documented that oxygen free radical is involved in the pathogenesis of ischemic reperfusion injury[6,13,14,52]. Knowing that lipids are the most susceptible macromolecules to oxidative stress, the present results demonstrated levels of MDA in ischemia reperfusion. This finding reconfirms the observations of Bromont *et al*[53]. In the present study, hesperidin pretreatment significantly improved MDA and nitrite concentration in striatum and cortex regions of the brain suggesting its antioxidant like effect.

Superoxide dismutase, catalase and glutathione peroxidase act together against reactive oxygen species and free radical damage. Superoxide

dismutase (SOD) is a ubiquitous enzyme with an essential function of protection of aerobic cells against oxidative stress. It is primarily a mitochondrial enzyme usually found in the plasma membrane[54]. Superoxide dismutase and catalase are mutually supportive members of defense against reactive oxygen species which have been found to be decreased during ischemia reperfusion injury[49]. In the present study, hesperidin pretreatment significantly restored antioxidant enzyme (SOD and catalase) levels in striatum and cortex regions of the brain, suggesting its potential effect in restoring oxidative defense. However, the exact mechanism of hesperidin's effect in restoration of oxidative defense is still not clear.

Glutathione is a powerful endogenous tripeptide antioxidant that inhibits formation of free radicals and thought to be the most important cellular antioxidant which protects cell from singlet oxygen, hydroxyl radical and superoxide radical damage[55]. Decreased levels of glutathione are associated with cell damage. *N*-acetyl cysteine (NAC), a precursor of glutathione and a potent antioxidant, have been reported to attenuate ischemia/reperfusion injury to brain tissue caused by a focal cerebral ischemia model in rats[56]. This further indicates that antioxidants may be involved in restoring oxidative defense.

In the present study, ischemia reperfusion caused significant attenuation of reduced glutathione levels as well as redox ratio in cortex and striatum regions of the brain. An imbalance between reactive oxygen species and glutathione defense system results in marked oxidative damage. BCCAO followed by 24 h reperfusion significantly decreased level of the GST in cortex and striatum areas of the brain. In the present study, redox ratio and GST levels were significantly restored by hesperidin pretreatment suggesting its effecting restoring oxidative defense. Further, L-arginine pretreatment with lower dose of hesperidin caused reversal of hesperidin's protective effect. However, L-NAME pretreatment with lower dose of hesperidin significantly potentiated its protective effect suggesting that nitric oxide modulation is involved in the protective effect of hesperidin. However, exact influence of nitric oxide system on protective effect of hesperidin is far from elucidation. Kohno *et al.*[12] have also suggested that intracerebraoventricular (icv) administration of NOS inhibitor produces neuroprotective effect, and causes 90% neuron survival *in vivo* as well as *in vitro* following transient forebrain ischemia. Several studies have demonstrated that inhibition of NO synthesis reduces NMDA-receptor activation mediated neuro injury and exhibits neuroprotection in various models of stroke[57]. These investigations are further supported by the evidences demonstrating enhanced formation of NO in various models of stroke[58]. However, the mechanism of cellular injury is still far from elucidation.

The precise mechanisms responsible for mitochondrial dysfunction due to hypoxic-ischemia in the brain are not fully understood. However, mitochondrial respiration and the mitochondrial respiratory chain seem to be especially susceptible to hypoxia-ischemia and reperfusion. Thus, during brain ischemia, complexes I, II, and III of the mitochondrial respiratory chain get damaged[59]. In the present study, ischemia reperfusion significantly impaired mitochondrial enzyme complex I, II and III activity in the ischemic brain. Cytochrome-*c* oxidase enzyme activity is usually not altered with ischemia reperfusion injury for the short period of time however long durations of ischemia can lead to a severe destruction in its activity[13,60]. Hesperidin pretreatment significantly restored mitochondrial enzyme complex activity (I, II and III) suggesting its role in mitochondrial enzyme complex activity. As a toxin, NO• is a well-established inhibitor of the mitochondrial electron transport system (ETS)[61,62], causing mitochondrial adenosine triphosphate (ATP) depletion and loss of mitochondrial respiration. The latter has been directly linked to apoptotic cell death[63]. NO• can also form a variety of toxic reactants, including peroxynitrite anion, $ONOO^-$, a product of NO• and superoxide anion radical, $O_2•^-$. Superoxide anion radical is a normal byproduct of the mitochondrial ETS and is normally harmlessly detoxified by manganese superoxide dismutase (MnSOD) enzyme system. In the present study, L-NAME pretreatment with hesperidin significantly potentiated protective effect of hesperidin in restoring attention in mitochondrial enzyme complex activity. However, L-arginine pretreatment with hesperidin reversed the protective effect of hesperidin in mitochondrial enzyme complex activity, suggesting the involvement of nitric oxide mechanism. Further, involvement of other mechanism could also be possible in the protective effect of hesperidin. It seems that nitric oxide modulation may also be involved in the protective effect hesperidin in reparatory mitochondrial enzyme complex activity.

In conclusion, the present study suggests that (i) BCCAO induced cerebral ischemia and reperfusion injury caused locomotor abnormalities in rats; (ii) hesperidin protects against ischemia reperfusion induced behavioural alterations, biochemical alterations and mitochondrial dysfunctions in the brain; and (iii) the protective effects of hesperidin may be having involvement of nitric oxide pathway.

Conflict of interest

The Authors have no competing interests.

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