

Cerebroprotective effects of extract of *Beta vulgaris* (C.) in middle cerebral artery occlusion (MCAO)-induced cerebral ischemia

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Abstract

Lack of efficient and widely applicable pharmacological treatments for ischemic stroke has necessitated attention towards novel traditional medicines. The effect of *Beta vulgaris* (*B. vulgaris*) extract on neurobehavior, grip strength, serum lactate dehydrogenase and brain SOD activity were studied at the dose of 250 mg/kg body weight in right middle cerebral artery occlusion model. *B. vulgaris* extract significantly improved the neurobehavioral function in rats after cerebral ischemia and reperfusion, increased grip strength as compared to the MCAO group. Moreover, treatment significantly boosted the defence mechanism against cerebral ischemia by increasing SOD activity and decrease in serum LDH. These experimental results suggest that extract of *B. vulgaris* may exert protective effect after cerebral ischemia. Administration of extract significantly reduced focal cerebral ischemic/reperfusion injury by augmenting antioxidants. Thus therapeutic strategies against oxidative stress could serve effective in ischemic diseases.

Key words: Cerebral ischemia; Cerebroprotective; *Beta vulgaris*; MCAO; oxidative stress

Introduction

Ischemic hypoxic brain injury causes irreversible brain damage by activating the cascade of events such as release of cytokines and free radicals, and induction of inflammation, apoptosis, and excitotoxicity which ultimately lead to neuronal injury and death (Kuroda et al., 1997). Reperfusion of ischemic areas could aggravate ischemic brain injury through the generation of reactive oxygen species. Moreover, a growing concern in traditional medicines has raised due to lack of effective and widely applicable pharmacological treatments for ischemic stroke.

Fruits and vegetables which are rich in pigments found as complex mixtures such as anthocyanins, betalains, or carotenoids are potent sources of antioxidants (Fernandez-Lopez et al., 2001). Table beet (*Beta Vulgaris Var conditiva*) has sparked much interest because of availability which contains a mixture of red betacyanin (BC) and yellow betaxanthin (BX) pigments (Gasztanyi et al., 2001) that belong to a group of compounds collectively known as betalain which have shown to be very good sources of dietary folate (Nutrient Data Laboratory 2003). Compelling evidences show that folate may prevent neural tube defects (Czeizel et al., 1992), exhibit antioxidant activity (Josh et al., 2001) and play an important role in prevention of cardiovascular diseases and cancer (Verhoef et al., 1996; Glynn et al., 1994; Gregory et al., 2002).

Beet root juice is considered powerful to prevent infectious and malignant disease and indeed scientific literature exists which supports these considerations. An *in vitro* study using Raji cells has demonstrated inhibitory effect of *Beta vulgaris* root extract on Epstein-Barr virus early antigen induction (Kapadia et al., 1996) and *in vivo* extracts of *Beta vulgaris* var. rubra has also revealed significant tumor inhibitory effects in murine skin and lung cancer (Rauha et al., 2000). These findings suggest that beetroot can be a useful to prevent development and progression of cancer. Moreover, extracts of beetroot has also showed some antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* and also antiviral effect (Prahoveanu et al., 1986; Gutteridge et al., 2000).

The present study investigated effects of *Beta vulgaris* on mortality rate, neurobehavior, grip strength, lactate dehydrogenase and SOD activity in a rat model. These data may therefore help in the development of effective and widely relevant pharmacological treatments for ischemic stroke patients with traditional medicines.

Experimental Methods

Animals

Wistar-kyoto male rats of 8 weeks age with body weight range from 200-250 gms were procured from Central Animal Facility, Nootan pharmacy college, Visnagar, India. They were maintained in essential condition of controlled temperature (<30° C) and humidity (< 70%) with 12 hour day and night cycle according to the norms of CPCSEA. Each rat was housed in plastic box cage individually and had free access to autoclaved, untreated tap water and standard rat chow (Pranav Agro Ltd, Ahmedabad). After surgery; rats were inspected daily for level of activity and healing of surgical wounds. Care was taken to minimize discomfort, distress and pain to the animals.

Extract preparation

50 g of beets was measured and homogenized for 5 min with 80% acetone (1:2 w/v). After centrifugation, 80% acetone supernatant was separated from the residue and evaporated under vacuum at 45 °C until approximately 90% of the filtrate had evaporated. These residues were used for the ethyl acetate extraction. Bound ethyl acetate-soluble phytochemicals of canned beets were extracted by method previously reported (Dewanto et al., 2002b; Chu et al., 2002). Briefly, 2-g equivalents of beets were weighed from the acetone

extraction residues and hydrolyzed directly with 4 N sodium hydroxide at room temperature for 1h under shaking conditions. The bound phytochemicals were extracted with ethyl acetate and this ethyl acetate fraction was evaporated under vacuum at 45 °C to dryness.

Experimental design

Right middle cerebral artery occlusion (MCAO) was performed using an intraluminal filament model and method described by Longa et al. (Longa et al., 1989). In brief rats were anesthetized with chloral hydrate (400 mg/ kg, i.p.), a 4-0 nylon monofilament with a blunt end was introduced into the external carotid artery (ECA) and advanced into the middle cerebral artery via the internal carotid artery (ICA) (17-20 mm), until a slight resistance was felt. Two hours after induction of ischemia, the filament was slowly withdrawn and animals were returned to their cages for a period of 22 hours of reperfusion. Throughout the procedure, the body temperature was maintained at 37° C, with a thermostatically controlled infrared lamp.

Sham Surgery

In sham rats, the ICA was surgically prepared for insertion of the filament, but the filament was not inserted. Similar post-operative care was followed. After surgery rats were placed individually in cages with free access to food and water.

Grouping of animals

Animals were separated into three groups of six rats each. Group I served as sham surgery group (SHAM). Group II was the ischemic group (MCAO treated orally by distilled water for 30 days respectively). Group III were treated orally by *B. vulgaris* extract i.e. BVE (250 mg/kg/day respectively) for 30 days followed by MCAO induced cerebral ischemia.

Neurobehavioral test

The sensorimotor integrity was conducted to assess neurobehavior at 24 h after MCAO in rats (Lee et al., 2002). Five categories of motor neurological findings were scored: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling; 4, forelimb flexion, unable or difficult to ambulate. Animals that showed features of higher scores also showed all the features of lower grades.

Grip strength study

Grip strength in all the animals was measured for evaluation of neuromuscular strength, as described by Ali et al. (Ali et al., 2004) and tests were carried out between 10:00 a.m. to 5:00 p.m. under standard laboratory conditions.

Tissue preparation

After grip strength measurement, blood samples were drawn from the tail vein from all the groups and serum was separated for biochemical estimations. Thereafter, the animals

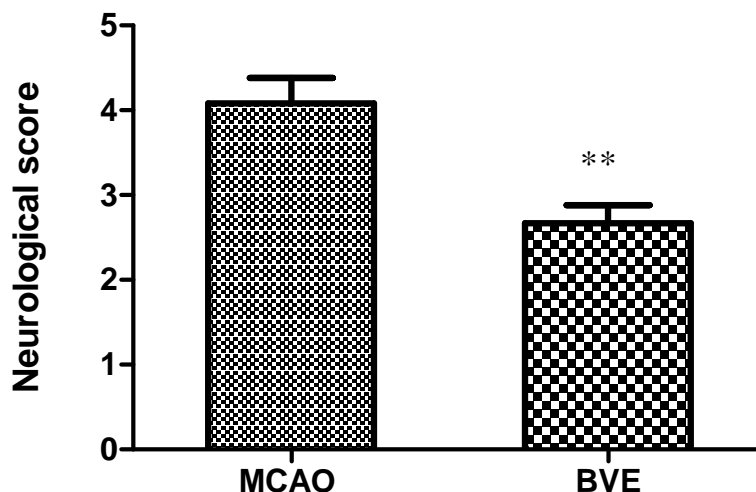


Figure 1. Effect of BVE treatment on the Development of Behavioral abnormalities after middle cerebral artery occlusion. Values are shown as means \pm SEM. $**p < 0.01$ vs. MCAO. (BVE = *B. vulgaris* extract (250 mg/kg/day) treated group, MCAO = MCAO group).

were sacrificed immediately and their brains were taken out to dissect the hippocampus (HIP). Post-mitochondrial supernatant (PMS) obtained from 10% homogenate of tissue was used for the estimation of SOD activity for oxidative stress.

Estimation of serum LDH and brain SOD activity

In serum, lactate dehydrogenase (LDH) was estimated using a method described by Lum et al (Lum et al., 1974). Brain SOD ($U\ mg^{-1}$ protein) activity was measured in MCAO group, SHAM group as well as BVE (250 mg/kg/day respectively) treated group using the

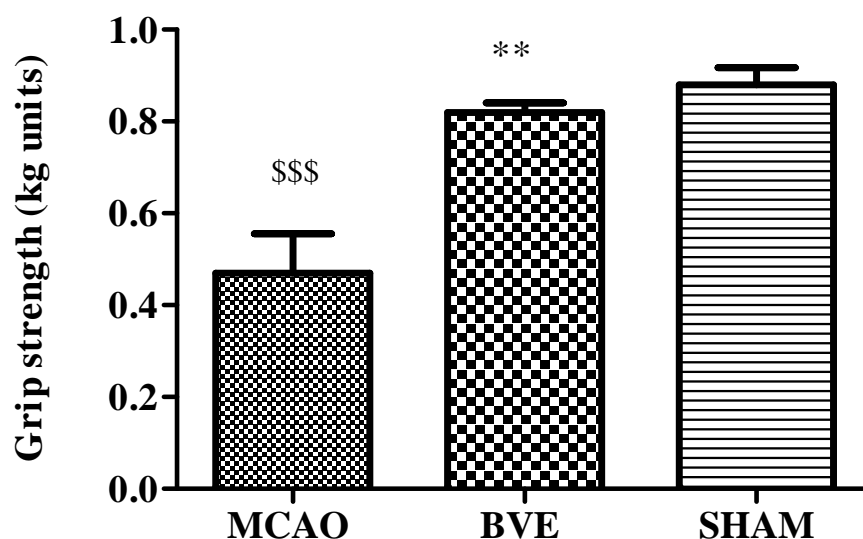


Figure 2. Effect of BVE treatment on the Grip strength (Kg Units) after middle cerebral artery occlusion. Values are shown as means \pm SEM. $***p < 0.001$ vs. SHAM, $**p < 0.01$ vs. MCAO. (BVE = *B. vulgaris* extract (250 mg/kg/day) treated group, MCAO = MCAO group, SHAM = SHAM group).

Superoxide Dismutase Kit (R&D Systems, Inc.) by using brain tissue homogenate and measuring absorbance at 550 nm.

Treatment with extract of *B. Vulgaris* (C.)

Initial testing were carried out with different oral doses (50–250 mg/kg bw) of *B. vulgaris* extract in focal cerebral ischemia induced rats. Four groups of six rats each were used in the experiment in which Group-1 comprised of untreated control, Group-2 at the dose of 50 mg/kg bw, Group-3 at the dose of 100 mg/kg bw and Group-4 at the dose of 250 mg/kg bw. Similarly, out of the three doses tested in rats, 250 mg/kg bw was found to be effective dose. Thus Group 4 was considered for study and animals in BVE group were treated with a dose 250 mg/kg bw o.d. for 30 days.

Statistical analysis

Results were expressed as mean \pm SEM. The value of statistical differences of $p < 0.05$ was considered as statistically significant. All statistical calculations were performed with Prism software package (GraphPad Prism, version 5) using unpaired or paired t-test depending on type of competition.

Result

In this study, the cerebroprotective effect of extract of *Beta vulgaris* (C.) on ischemic neuronal damage was clearly demonstrated using focal ischemia model. The behavioural tasks adopted in this study were designed to assess impairments consistent with the known functional architecture of the rat brain. Twenty-four hours after MCAO in rats, neurological deficit scores were significantly reduced in BVE (250 mg/kg/day) treated Group III. The neurobehavior for MCAO group was 4.08 ± 0.12 and BVE group was 2.67 ± 0.09 . BVE gro-

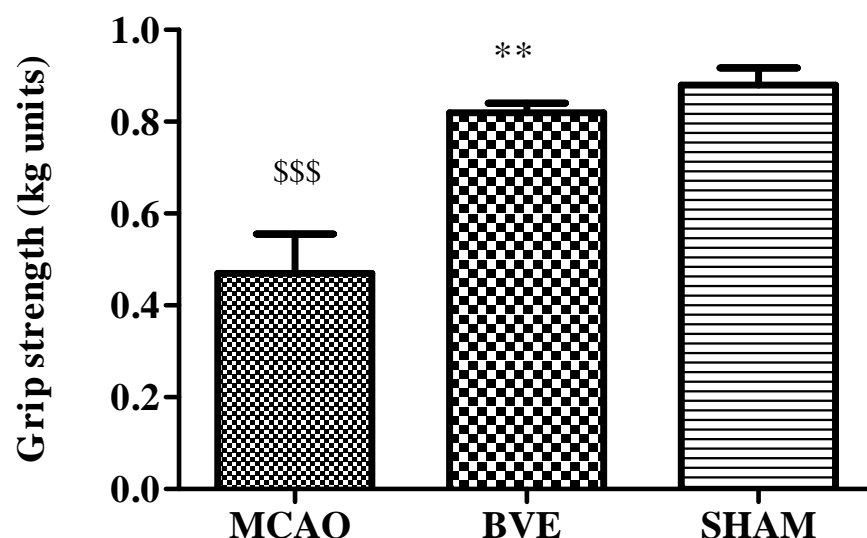


Figure 3. Effect of BVE treatment on SOD (U) levels after middle cerebral artery occlusion. Values are shown as means \pm SEM. \$\$\$ $p < 0.001$ vs. SHAM, *** $p < 0.001$ vs. MCAO. (BVE = *B. vulgaris* extract (250 mg/kg/day) treated group, MCAO = MCAO group, SHAM = SHAM group).

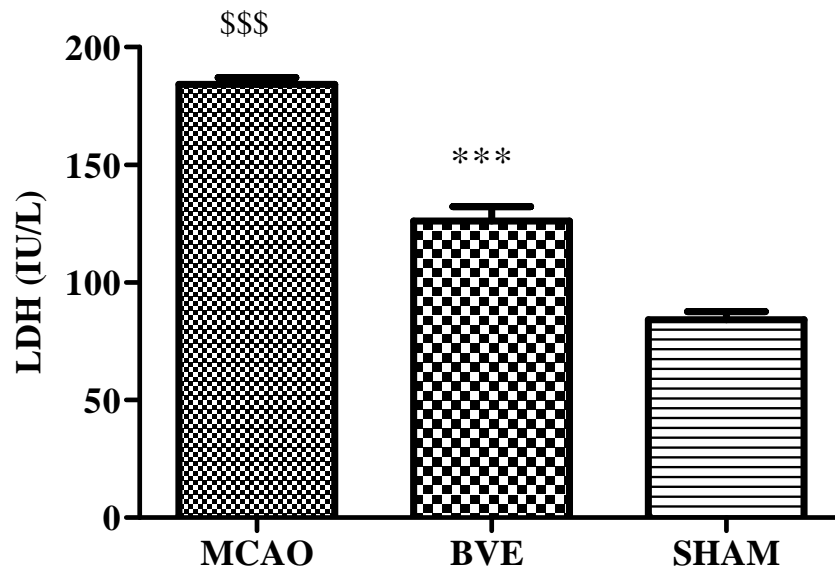


Figure 4. Effect of BVE treatment on the serum LDH (IU/L) after middle cerebral artery occlusion. Values are shown as means \pm SEM. \$\$\$ p < 0.001 vs. SHAM, *** p < 0.001 vs. MCAO. (BVE = *B. vulgaris* extract (250 mg/kg/day) treated group, MCAO = MCAO group, SHAM = SHAM group).

up significantly suppressed the development of behavioural abnormality as compared with the MCAO group (Figure 1). Grip strength in the SHAM group was found to be 0.88 ± 0.01 kg units. A significant decrease in the grip strength was observed in MCAO group, as compared to SHAM rats ($P < 0.01$) which was observed to be 0.47 ± 0.04 . BVE treated rats showed a significant increase in grip strength, as compared to the MCAO group ($P < 0.01$) which was 0.82 ± 0.01 (Figure 2).

Serum LDH levels in SHAM group were found to be 84.17 ± 1.44 IU/L. A significant increase in the activity of LDH in serum was observed in MCAO group which was found to be 184.17 ± 1.19 , as compared to SHAM group; whereas, BVE treatment significantly resulted in decreased serum LDH levels when compared with MCAO group rats, which was found to be 126.0 ± 2.53 (Figure 4). SOD activity in brain homogenate showed a significant decline in MCAO group as compared to SHAM group which was observed to be 6.0 ± 0.33 U. Moreover treatment with BVE extract increased the levels of SOD activity to 9.67 ± 0.10 U which was partially normalized to the levels of SHAM group which was found to be 11.17 ± 0.35 U (Figure 3).

Discussion:

Stroke is the second most common cause of death worldwide and 1/6 of all human beings suffering at least one stroke in their lives thus being the leading cause of adult disability with approximately one third of patients surviving 6 months are dependent on others. Stroke has huge socioeconomic burden absorbing 6% of all health care budgets and with the fact that life expectancy increases globally it is already, and will continue to be, the most challenging disease. Ischemic stroke results from a thrombotic or embolic occlusion of a major cerebral artery (most often middle cerebral artery, MCA) or its branches and accounts for 80% of all strokes. Clinical variability of stroke such as its cause, duration, localization, severity of ischemia and coexisting systemic diseases makes it essential for very

large patient group sizes in clinical research to avoid confounding effects of the diversity. Many experimental focal cerebral ischemic models have been developed to mimic human stroke as well as variables may be taken under strict control and researchers may address specific questions about either pathologic events occurring after ischemic stroke and how to develop novel stroke therapies which have shown to serve as an indispensable tool in the stroke research field. There has been tremendous increase in the number and diversity of experimental focal ischemia models over recent decades and these animal studies have provided most of the knowledge on pathophysiological mechanisms involved in focal cerebral ischemia (NINDS, 1995).

Among experimental ischemic stroke models, intraluminal suture MCAO in rats is the most frequently used model being less invasive and easy to perform both permanent and transient ischemia in a controlled manner. It involves inserting a monofilament into the internal carotid artery and advancing until it blocks blood flow to MCA. This model provides reproducible MCA territory infarctions involving both frontoparietal cortex and lateral caudoputamen and retraction of the suture allows reperfusion as well. MCAO model also provides higher ischemic lesion growth size within 24 h thus may be beneficial for studies of neuroprotective strategies. Moreover, depending on the shape, size, and insertion length of the thread, the MCA can be either occluded selectively or in conjunction with the posterior communicating artery, branches of internal carotid artery and common carotid artery (Woitzik et al., 2006). Taken into consideration various aspects of the study, we employed MCAO injury model for screening the cerebroprotective effect of *B. vulgaris* extract.

Although it is desirable to prevent strokes in the first place though unattainable, it might be possible to develop neuroprotectant drugs minimizing the residual damage and hence the disability burden after ischemic strokes. In fact there is evidence that neuroprotection could be valuable which has been demonstrated in a study where accelerating reperfusion with thrombolytics given within 3 hours of the onset of symptoms and longer lead to a clinically relevant improvement in outcome which suggested that there may be viable neurons in the hypoperfused penumbra for at least 6 hours after occlusion. Thus a drug that protects neurons from toxic effects of ischemia may therefore increase the number of neurons that could recover their normal function when blood flow returns (Marshall et al., 1999).

Furthermore, it has been well documented that abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades, leading to spread of neuronal death via numerous pathways which includes excessive activation of glutamate receptors, accumulation of intracellular calcium cations, abnormal recruitment of inflammatory cells, excessive production of free radicals and initiation of pathological apoptosis. Thus, interruption in the propagation of these cascades can protect at least part of the brain tissue. However, neuroprotective agents designed to block these cascades have been investigated in animal models of cerebral ischemia for last two decades. Numerous agents have also been found to reduce infarct size in rodent, rabbit and primate stroke models (Marshall et al., 2001) but translation of neuroprotective benefits from the laboratory bench to the emergency room has not been successful. According to a recent review, of 178 controlled clinical trials that were published in the stroke-related literature, more than 100 were related to neuroprotection thus reasons for failures has led to intense discussion for last several years (Kidwell et

al., 2001; Gladstone et al., 2002). Though, one important success rt-PA is approved for acute stroke management in many countries.

Twenty-four hours after MCAO in rats, neurological deficit scores were significantly reduced in *B. Vulgaris* treated rats as compared to vehicle treated group. Behavioral abnormality that was developed in the MCAO group was significantly different from SHAM group. But treatment with extract suppressed the development of behavioral abnormality which was observed when compared to vehicle treated MCAO group (Figure 1). Moreover, a significant increase in grip strength in BVE extract treated group as compared to sham and MCAO group suggests a beneficial effect on neuromuscular strength (Figure 2). Compelling evidence has shown involvement of oxidative stress in ischemia/reperfusion that may potentiate ischemic injury. The activities of SODs are depicted in Figure 3. Brain SOD activity was measured in extract treated group and were compared with MCAO group as well as SHAM group to which a significant rise in value was observed in the extract treated group as compared to vehicle treated group (Figure 3). Increasing evidence has indicated that ischemia/reperfusion occurs due to oxidative stress that may potentiate ischemic injury (Traystman et al., 1991). Lactate dehydrogenase was measured to evaluate the role of antioxidative stress in the protection of BVE to which we obtained significant difference between MCAO group and SHAM group. Treatment with BVE extract significantly resulted in decreased serum LDH levels when compared with MCAO group rats.

A great deal of exertion has been directed toward searching for a new drug that can be used for protection of cerebral ischemia-reperfusion injury. Although *Beta vulgaris* has great therapeutic value with many natural benefits and curative properties, it lacks scientific grounds for its efficacy and to the best of our knowledge, this is the first study to report its possible protective mechanisms against cerebral ischemic damage. Table beet (*Beta vulgaris* C.) contains important bioactive agents (betaine and polyphenols), which have a wide range of physiologic effects. Because nutritive antioxidants may reduce occurrence of complications and postoperative mortality, dietary intake of polyphenols and vitamins before surgery may greatly contribute to the survival of patients (Vali et al., 2007).

Here we showed that *Beta vulgaris* extract significantly improved outcome in rats after cerebral ischemia and reperfusion in terms of neurobehavioral function. At the same time, supplementation of *Beta vulgaris* extract significantly boosted the defense mechanism against cerebral ischemia by increasing antioxidants activity related to lesion pathogenesis. Restoration of the antioxidant homeostasis in brain after reperfusion may have helped the brain recover from ischemic injury.

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