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Research Article

FLAVONOID RICH FRACTION OF WILLOW LEAVED SEA BUCKTHORN BERRIES ATTENUATED ISCHEMIA REPERFUSION INDUCED NEUROBEHAVIORAL DEFICITS, EXCITOTOXICITY AND ASSOCIATED NEURONAL DAMAGE

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ABSTRACT
The present study aimed to evaluate the protective effect of flavonoid rich fraction of willow leaved Sea buckthorn (SBT) berries against cerebral ischemia reperfusion induced neurobehavioral deficits and excitotoxicity. Briefly, the rats were randomly divided into sham, ischemia- reperfusion (I/R) control, SBT (250 and 500 mg/kg) treated groups. The rats were received their respective treatment orally by gavage once daily for 7 days prior to middle cerebral artery occlusion (MCAO) for 2 h and reperfusion for 22 h. To evaluate the severity of neurobehavioral deficits, a battery of tests were employed such as neurological deficit score, grip strength score and adhesive tape removal test. The excitotoxicity mediators like glutamate and calcium were measured. The neuronal damage was quantified in terms of infarcted area, which was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. SBT treated rats showed substantial improvement in neurobehavioral deficit, significantly inhibited the increased levels in glutamate and calcium, significantly prevented the associated neuronal damage. From our results, it can be concluded that supplementation of SBT significantly mitigated the ischemia- reperfusion induced neuronal damage by inhibiting excitotoxicity.
Keywords: Ischemia/reperfusion, Neurobehavioral deficit, Willow leaved Sea buckthorn, Neuroprotection

INTRODUCTION

Cerebral ischemia- reperfusion (I/R) injury is the leading cause of death worldwide[1]. The current therapeutic drug of choice is tissue plasminogen activator (tPA). But, its therapeutic approach is limited due to time constraint. Intensive research has been diverted on the development of traditional herbal medicines for effective therapeutic approach. The severity of I/R injury is examined symptomatically by assessing neurobehavioral severity. The severity is measured using a battery of tests like neurological deficit score, grip strength score and adhesive tape removal test. The neuronal damage is quantified by assessing the area of infarction. The more the infarcted area, the more the damage and vice versa. Multiple consequences have been implicated in I/R damage. The primary consequence is excitotoxicity[2,3]. Targeting and preventing the excitotoxicity will provide basis for the neuroprotective effect of a drug. Willow leaved Sea buckthorn *(Hippophae salicifolia)*, a traditional herbal drug found in Himalayan region. Its berries are rich in vitamin-c, vitamin-k, omega-3 fatty acids, omega-6 fatty acids, carotenoids[4]. Previous reports demonstrated that antioxidants played a protective role against I/R damage[5-10]. SBT is recognized as rich source for antioxidants and berries are used as food by local people in Himalayan region. The present study evaluated the protective effect of flavonoid fraction of SBT berries against cerebral-ischemia reperfusion injury in rat model of middle cerebral artery occlusion.

MATERIAL AND METHODS

Plant Material and extraction

Willow leaved Sea buckthorn berries powder was obtained from Organic Changsha Herb Inc. China. The berries were subjected to extraction of flavonoid fraction. The percentage yield was found to be 4.57 % w/w.

Animals

Male rats of Wister strain weighing 280-300 g were used for the study. The rats were maintained under conditions of 12 h light/dark cycle and had free access to food and water. All experimental procedures were carried out after obtaining approval from the Institutional Animal Ethical Committee (No. 1677/PO/a/12/CPCSEA/02).

Experimental design

After acclimatization, the rats were randomly separated into four groups; each group consisted of 9 rats, treated with drug or vehicle orally by gavage once daily for 7 days prior to MCAO. The first group attended to as sham control and had 2 % tween 80 orally. The second group attended to as a MCAO model group and had 2 % tween 80 orally. The third and fourth groups were treated with SBT 250 and 500 mg/kg/p.o respectively for 7 days. After 7 days of pretreatment, rats were subjected to 2 h right MCAO via the intraluminal filament technique and 22 h reperfusion. Briefly, the right common carotid artery was exposed at the level of the external and internal carotid artery bifurcation. A 4-0 nylon suture with a blunted tip was inserted into the internal carotid artery and advanced into the anterior cerebral artery to block the middle cerebral artery (MCA). After closing up the MCA for 2 h, nylon suture was removed carefully to restore blood flow and then the skin was sutured. The animals were then returned to their cages and closely monitored for 22 h. The body temperature was maintained at 37^0C with a thermostatically controlled infrared lamp. In sham-operated group, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted. After 22 h of reperfusion, a battery of tests was employed to measure the neurobehavioral severity. Then the animals were sacrificed by cervical decapitation to obtain brain samples for further studies.

Neurobehavioral studies: Neurological score

A neurological examination is blindly performed at 22 h after reperfusion (before killing) according to the method described by Longa *et al*[11]. The neurological findings were scored using a 5-point scale. No neurologic deficit= score 0, failure to extend forepaw fully= score 1, circling to the left = score 2, falling to the left or no spontaneous motor activity= score 3, and do not walk spontaneously and experience a lowered stage of consciousness= score 4.

Grip strength

Grip strength was done according to the method of Moran *et al*[12]. The apparatus with a string of 50 cm length, pulled out between two vertical supports at height 1 m and evaluated according to the following scale: 0 – fall off; 1 – hangs onto the string by two forepaws; 2 – as for 1 but attempts to climb on the string; 3 – hangs onto the string by two forepaws plus one or both hind limbs; 4 – hangs onto the string by forepaws plus tail wrapped around string and 5 – escape. The highest reading of three successive trials was recorded for each animal.

Adhesive-removal test

The adhesive removal test was performed by the method of Schallert *et al*[13]. For the adhesive-removal somatosensory test, animals were tested 22 h after MCAO. Two small adhesive-back paper dots each 8-mm diameter were placed on each forepaws to cover the hairless part of the forepaws. The animal was placed in a box (40 cm × 30 cm) and the time required by the animal to remove the pieces of tape from the paw was recorded. The animals were given a maximum of 180 seconds to sense the tapes and remove them and were scored as 180 seconds if they did not succeed. The highest reading of three successive trials was recorded for each animal.

Biochemical analysis: Brain tissue preparation

The animals in all groups (six from each) were sacrificed by cervical decapitation under anesthesia and brains were quickly dissected out, homogenized in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to yield 5 % (w/v) homogenate. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 0^0C in cold centrifuge; the resulting supernatant was used for further studies.

Determination of protein content

Protein was determined according to the method of Lowry *et al*.,[14] using bovine serum albumin (BSA) as standard protein.

Measurement of glutamate levels

Glutamate levels were measured according to the method described by Bernt and Bergmeyer[15] with minor modifications. To 1 ml of supernatant, 2 ml of Perchloric acid was added and the pH was adjusted to 9.0 with phosphate buffer. The resulting mixture was subjected to centrifugation at 1500 × g for 15 minutes and was allowed to stand for 10 minutes in an ice bath and then filtered through fluted filter paper. Absorbance was measured at 340 nm. The glutamate levels are expressed as µmol/g tissue.

Measurement of total calcium levels

The total calcium levels were measured according to the manufacturer's instructions using commercially available kits (Span Diagnostics Ltd., India).

Cerebral infarct area by 2, 3, 5-triphenyltetrazolium chloride staining

After reperfusion for 22 h, rats were decapitated under anesthesia with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and the brains were kept at -20^0C for 40 minutes. Frozen brain was sliced into uniform coronal sections of about 2 mm thickness each. Brain slices were incubated with 2 % 2, 3, 5-triphenyltetrazolium chloride (TTC) in 0.2 mol/L phosphate buffer (pH 7.4) at 37^0C for 30 minutes and fixed in 10 % neutral buffered formalin for overnight. The unstained areas of the fixed brain sections were defined as infarcted. After 24 h of fixation, the slices are photographed. The infarct area was calculated by measuring the unstained and stained areas in each slice, multiplying this by the slice thickness (2 mm).

RESULTS

Effect of SBT on neurobehavioral changes

The animals were scored for neurologic deficit after 22 h of reperfusion using a five point scale. The MCAO model group showed considerably increased ($P < 0.001$) neurologic scores as compared to the sham group. SBT pretreated animals showed substantial improvement in neurologic deficit score ($P < 0.001$) in a dose dependent manner as compared with model group (Table 1). The tape removal test is a technique that assesses sensory and motor impairments in forepaw function. After 2 h MCAO/22 h reperfusion, an increase in the time needed to remove adhesive tape from the contra lateral forepaw was observed in the MCAO group as compared with sham group rats (Table 1). Interestingly, dose dependently, SBT pretreated animals showed significant shortened time to remove the adhesive tapes from the forepaws compared with a MCAO group ($P < 0.001$). The grip strength was found to be significantly decreased ($P < 0.001$) in the MCAO group as compared to the sham group. Whereas significantly ($P < 0.001$) improved grip strength was observed in SBT pretreated groups dose dependently as compared to MCAO group (Table 1).

Effect of SBT on glutamate and calcium levels

A significant increase ($P < 0.001$) in glutamate and calcium levels was observed in MCAO groups as compared to the sham group (Table 2). The SBT pretreated group offered significant restoration of glutamate and calcium levels in comparison to MCAO group ($P < 0.001$)

Effect of SBT on Infarct area

Figure 1 showed typical pictographs of TTC stained coronal sections of sham, MCAO and SBT pretreated rats. MCAO group showed significant increase in infarct area as compared with Sham group. SBT pretreatment showed a significant reduction in the infarct area as compared with MCAO group. The percentage of infarction is calculated and given in Table 3.

Table 1: Effect of SBT on neurobehavioral changes after I/R injury

Groups	Neurological deficit score	Grip strength score	Adhesive tape removal time (sec)
Sham	0.0 ± 0.00+++	3.47 ± 0.03+++	19.12 ± 6.28+++
MCAO	3.03 ± 0.04***	1.42 ± 0.10***	126.11 ± 20.33***
SBT 250 mg/kg	1.81 ± 0.06**+	1.83 ± 0.06**++	60.27 ± 7.45*++
SBT 500 mg/kg	1.42 ± 0.09*+++	2.99 ± 0.04+++	41.39 ± 5.52+++

Values are expressed as mean ± SEM (n = 6); *(P < 0.05), **(P < 0.01), ***(P < 0.001) vs sham group; +(P < 0.05), ++(P < 0.01), +++(P < 0.001) vs MCAO group

Table 2: Effect of SBT on glutamate and calcium levels

Groups	Glutamate levels (µg/mg protein)	Calcium levels (µg/mg protein)
Sham	18.26 ± 0.03+++	0.818 ± 0.02+++
MCAO	38.99 ± 0.08***	2.147 ± 0.10***
SBT 250 mg/kg	30.97 ± 0.09**+	1.363 ± 0.06**,++
SBT 500 mg/kg	21.47 ± 0.06+++	1.098 ± 0.04+++

Values are expressed as Mean ± SEM [n = 6]; **(P < 0.01), ***(P < 0.001) Vs Sham group; ++(P < 0.01), +++(P < 0.001) vs MCAO group

Table 3: Effect of SBT on percentage of infarcted area

Groups	Percentage of infarct volume
Sham	0.36 ± 0.02
MCAO	43.62 ± 0.07***
SBT 250 mg/kg	24.82 ± 0.04*++
SBT 500 mg/kg	2.24 ± 0.01+++

Values are expressed as Mean ± SEM [n = 3]; *P < 0.05, ***P < 0.001Vs Sham group; ++P < 0.01, +++P < 0.001 vs MCAO group

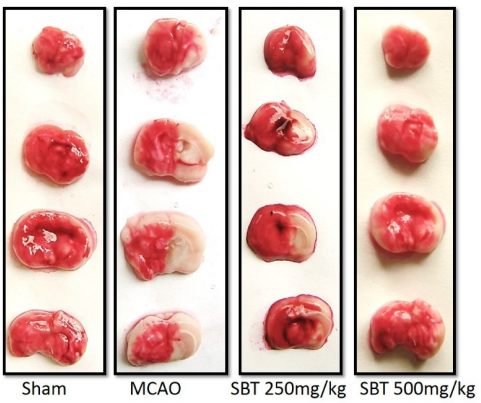

Figure 1: Effect of SBT on infarct area using 2, 3, 5-triphenyl tetrazolium chloride

DISCUSSION

The present study employed preliminary screening of neuroprotective effect of flavonoid fraction of willow leaved sea buckthorn in transient focal cerebral ischemia-reperfusion induced by employing MCAO model. Since the focal cerebral ischemia model with transient MCAO followed by reperfusion in experimental animals is generally accepted as the most appropriate model for human stroke[16], we used the MCAO reperfusion model to induce ischemic injury. SBT exhibited neuroprotection, as evident from improvement in the neurobehavioral deficit, reduction in infarct volume, calcium and glutamate levels. Cerebral ischemia reperfusion injury produced significant impairments in the neurological function and coordination, impairing the sensory-motor system. Motor sensory deficits are evaluated based on a neurological scale. It is observed that flavonoid fraction of SBT showed significant improvement in the neurological scoring compared to the ischemic control group suggesting the efficacy of the SBT flavonoid fraction in reversing the MCAO induced cerebral ischemia reperfusion injury. Ischemia leads to the release of cellular toxic mediators and increases the permeability of the blood brain barrier. Hyper permeability of the BBB leads to brain cellular swelling and causes brain infarction and edema[17]. Triphenyltetrazolium chloride (TTC) staining has been employed in the present study to determine the area of infarction in brain tissue. TTC is a water soluble dye that is reduced to formazone by the enzyme succinate dehydrogenase and cofactor NAD, present in mitochondria and stain viable tissue deep red in color. Ischemic tissue with damaged mitochondria remains unstained. Present investigations revealed that pre-treatment with SBT flavonoid fraction offered effective protection dose dependently compared to neuronal damage induced by MCAO, which could be the one of the protective mechanism of SBT against I/R injury. Excitotoxicity through over activation of NMDA receptors by the extensive release of glutamate is well established as an important trigger to the tissue damage in focal cerebral ischemia[18,19]. Activation of NMDA receptors elevates the influx of Ca^{2+} and that of non-NMDA glutamate receptors promotes the influx of Na^{+}, both of which can lead to membrane depolarisation. In turn, depolarisation can activate plasma membrane voltage-dependent Ca^{2+} channels, leading to additional Ca^{2+} influx[20]. Furthermore increased calcium activates enzymes, such as xanthine oxidase and NOS that are involved in the ROS generation leading to lipid peroxidation and neuronal damage[21,22]. In the present study, the glutamate levels were estimated, pre-treatment with flavonoid fraction of SBT effectively reduced the excitotoxicity by decreasing the glutamate levels thereby reducing the tissue damage. This ability of flavonoid fraction of SBT to reduce the glutamate induced tissue damage has contributed to the neuroprotection from the cerebral ischemia reperfusion injury. Calcium released due to excitotoxicity and depolarized neurons and glia due to energy depletion, enters into damaged neurons through voltage-gated calcium channels, via NMDA and AMPA receptor operated channels, and also from intracellular stores. Cytoplasmic calcium activates enzymes and second messenger cascades that contribute to cell death. Activated proteolytic enzymes break down elements of the

cytoskeleton, leading to protein aggregation. Calcium-mediated lipolysis damages membranes and along with nitric oxide synthase activation provides nitric oxide and fatty acid substrates for free radical production. Apoptotic cascades are stimulated by the rise in calcium through mitochondrial permeability. Glutamate release is stimulated by calcium-dependent excitotoxicity and the released glutamate in turn causes Ca^{2+} channels to open, leading to further Ca^{2+} overload forming a vicious cycle. Thus calcium plays a unique role in the ischemic pathophysiology. In the present study results demonstrated that calcium levels were elevated in Ischemic control group compared to sham group rats which is in consonance with the earlier reports[23]. A prominent reversal of MCAO induced elevation in calcium levels by SBT flavonoid fraction may also be attributed to its protective effects. It can be concluded that flavonoid rich fraction of SBT significantly ameliorated the ischemia-reperfusion induced neurobehavioral deficit, excitotoxicity. This preliminary report provides the scientific basis for the in-depth evaluation of neuroprotective mechanism of willow leaved sea buckthorn berries.

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