Original Article

Protective Effect of Some Medicinal Plant Phytoconstituents against Transient Middle Cerebral Artery Occlusion Induced Focal Cerebral Ischaemia and Consequent Cerebral Infarction in Mice

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ABSTRACT

The present study was designed to evaluate the effect of major phytoconstituents of three important and wildly growing medicinal plants of forests of Punjab region in India for their possible protective effect against focal cerebral ischaemia and associated cerebral infarction. Ajugarin I I, Punarnavine and Marmesinin obtained from *Ajuga bracteosa*, *Boerhaavia diffusa* and *Aegle marmelos* respectively were used in the present protocol. Cerebral ischaemia was induced in mice using transient middle cerebral artery occlusion method. Evaluation of infarct volume and histomorphology was performed. Authenticity of stroke was evaluated by measuring neurological deficit score. A standard neuroprotective agent edavarone was used as control. Cerebral ischaemia resulted in infarction as evidenced by histopathology of the cerebral cortex sections of the animals and a subsequent increase in the neurological deficit score. Administration of the medicinal substances resulted in a significant reduction in the infarct volume and neurological deficit score. The results indicate the possible protective potential of selected phytoconstituents in cerebral ischaemia induced stroke.

Keywords: Cerebral ischaemia, Stroke, Infarction, Phytoconstituents, Anti-oxidants.

INTRODUCTION

Stroke has increasingly become a widespread medical problem accounting for 9% of all deaths worldwide[1]. It is also becoming one of the primary reasons of adult long-term disability [2]. In the current medical practice, tissue-type plasminogen

activator (tPA) is the most widely used and the only FDA-approved therapy for ischaemic stroke[3]. Unfortunately, tPA therapy finds its usefulness in only a limited number of patients. The primary reason for this handicap is that the treatment needs to be initiated within 3–5 h post-stroke[4]. Moreover; some potential hazards are associated with the use (especially delayed use) of tPA. It is now widely known that tPA therapy (especially in the later stages of therapy) may lead to haemorrhage and oedema[5], thus resulting in an increase in mortality. Due to this narrow therapeutic window, delivery of a safe and effective therapy with tPA have achieved only limited success even in the animal models[6, 7]. This underscores the need for new therapeutic approaches to the treatment of stroke.

Ischemic stroke is characterized by an interruption or marked reduction in the cerebral blood supply. This results in initiation of a complex cascade of pathological mechanisms involving loss of ionic homeostasis, cellular energy failure, unregulated release of excitatory amino acids, severe increase in oxidative stress and associated free radicals and apoptosis which finally culminates in irreversible tissue damage[8-10]. Experimental evidence shows that reperfusion alone does not prevent brain injury[11]. As a result, different steps of the ischemic cascade have been targeted for therapeutic interventions[12]. Unfortunately, no neuroprotective agents have found to be clinically effective for ischemic brain injury[13]. This lack of effective and safe drug for treatment of ischaemic stroke prompted us to examine the role of natural anti-oxidants in preventing the stroke associated cerebral damage. The three phytoconstituents namely Ajugarin I I, Punarnavine and Marmesinin chosen for the current study have been a subject of high anti-oxidative potential. Preliminary studies were performed to evaluate the most potent anti-oxidant fraction of the different plant extracts of *Ajuga bracteosa*, *Boerhaavia diffusa* and *Aegle marmelos* in-vitro. The results showed that methanolic extract of *A. bracteosa* and *B. diffusa* and aqueous extract of *A. marmelos* had most potent anti-oxidant action in-vitro. Therefore major anti-oxidant phytoconstituents found in the selected extracts were purchased and used for the present study.

MATERIALS AND METHODS

Swiss albino mice (Punjab Agriculture University, Ludhiana, India) of either sex weighing 25±5g and adult Sprague-Dawley Rats (Punjab Agriculture University, Ludhiana, India) of either sex weighing 250±20g were employed in the present study. Animals were fed on standard laboratory feed (Kisan Feeds Ltd., Chandigarh, India) and water *ad libitum*. They were housed in the departmental animal house and were exposed to natural cycles of light and dark. The experimental protocol was approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No.:–911/ac/05/CPCSEA).

The standard phytoconstituents (95%) pure Punarnavine and Ajugarin I both were obtained as gift samples from Biogen Extracts Pvt. Ltd., Bangalore, India. Marmesinin and Edavarone (95% Pure) were obtained as gift samples from Dabur India Ltd. Baddi, India. An 7-0 Ethilon nylon filament was purchased from Ethicon, Inc., Somerville, NJ, USA, having 5 mm of the tip coated with silicone (Cutter Sil Light and Universal Hardener, Heraeus Kulzer, GmbH, Hanau, Germany), 2,3,5-

triphenyltetrazolium chloride (TTC) was purchased from Sigma-Aldrich, USA. All other chemicals and regents were purchased from Loba Chem., Mumbai, India and SD Fine Chem., Mumbai. All the reagents and chemicals used in the experimental protocol were of analytical grade.

Experimental Procedure

Induction of Focal Cerebral Ischaemia by transient Middle Cerebral Artery Occlusion

The middle cerebral artery occlusion procedure was carried out using an established method (Shah *et al*., 2006). Mice were anesthetized with halothane (Nicholas Piramal, India; 3% initial, 1 to 1.5% maintenance) in O_2 and air (80%:20%). Mice were turned to the supine position, and a midline incision was made in the neck to clear and expose the right common carotid artery (CCA), external carotid artery, and internal carotid artery. Care was taken not to disturb the vagus nerve and the adjoining tissue. A 7-0 Ethilon nylon filament which had 5 mm of the tip coated with silicone was slipped into the internal carotid artery through the external carotid artery stump to block blood circulation to the middle cerebral artery (MCA) territory. The filament was carefully advanced up to 11 mm from the carotid artery bifurcation or until resistance was felt. Mice that did not attain at least an 80% decrease in cerebral blood flow were terminated from the further study. Animals were kept in a humidity/ temperature-controlled chamber at 32 °C to maintain their body temperature at 37 °C during the 90 min of MCA occlusion. For reperfusion, mice were briefly anesthetized under halothane anesthesia, and the filament was withdrawn carefully without rupturing the arteries; open ends of arteries were immediately cauterized to prevent bleeding. After the neck incision was sutured, mice were again placed in a humidity/temperature-controlled chamber for 2 h for recovery and then returned to their respective home cages.

Evaluation of Neurologic Deficit Score (NDS)

Forty-eight hours after *t*MCAO, neurologic deficits were evaluated by an investigator blinded to treatment group using a previously modified 28-point scoring system (Saleem et al., 2009, Zeynalov et al., 2009). Motor deficits were evaluated by tests for body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling; sensory deficits were evaluated by a whisker response test. Each test was graded from 0 (no deficit) to 4 (greatest deficit), establishing a maximum NDS of 28. After assessing weight loss, mice were sacrificed for measurement of infarct volume.

Infarct Size and Infarct Volume Analysis

After 48 h of reperfusion, mice were anesthetized, and their brains dissected out. Coronal brain slices (2 mm) were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) and fixed in 10% buffered normal saline for 24 h. The slices were scanned individually by a video image analyzing system, and the infarct lesions were measured and analyzed by image analysis software SigmaScan pro 4 and 5 (Systat Inc., San Jose, CA, USA).

Experimental Protocol

Toxicological Study and Selection of Dose

In the primary toxicological studies, when Ajugarin I I, Punarnavine and Marmesinin were given as intraperitoneal injection to mice, the maximal dosage (5000 mg/kg) showed no toxic effects, the median lethal dose (LD50) could not be determined, and the maximum tolerable dose (MTD) mice was 5 g/kg in all three cases. Therefore, we selected the dose of 110

mg/kg *i.p* for all three phytoconstituents under study, which was 1/45 of the MTD in mice

Swiss albino mice of either sex were randomly divided into five groups each consisting of ten (10) animals.

Group I (Control)

Animals in this group were given focal cerebral ischaemia by occlusion of transient middle cerebral artery for a period of 90 minutes followed by reperfusion. Forty-eight hours after *t*MCAO neurological deficits were evaluated. Ninety minutes later, mice were sacrificed, and their brains were dissected out for valuation of infarct size and infarct volume.

Group II (Ajugarin I treated)

Animals in this group were pretreated with Ajugarin I (110 mg/Kg *i.p*) for a period of seven days. On 8th day animals were subjected to focal cerebral ischaemia by occlusion of transient middle cerebral artery for a period of 90 minutes followed by reperfusion. Rest of the protocol remained same as that of group I.

Group III (Punarnavine treated)

Animals in this group were pretreated with Punarnavine (110 mg/Kg *i.p*) for a period of seven days. On 8th day animals were subjected to focal cerebral ischaemia by occlusion of transient middle cerebral artery for a period of 90 minutes followed by reperfusion. Rest of the protocol remained same as that of group I.

Group IV (Marmesinin treated)

Animals in this group were pretreated with Marmesinin (110 mg/Kg *i.p*) for a period of seven days. On 8th day animals were subjected to focal cerebral ischaemia by occlusion of transient middle cerebral artery for a period of 90 minutes followed by reperfusion. Rest of the protocol remained same as that of group I.

Group V (Edavarone treated)

Animals in this group were pretreated with a standard neuroprotective agent Edavarone (8 mg/Kg *i.p*) for a period of seven days. On 8th day animals were subjected to focal cerebral ischaemia by occlusion of transient middle cerebral artery for a period of 90 minutes followed by reperfusion. Rest of the protocol remained same as that of group I.

RESULT AND DISCUSSION

Mice randomized into different treatment groups were pretreated for 7 days with test drugs (Ajugarin I, Punarnavine, Marmesinin) and subjected to *t*MCAO. After 48 h of reperfusion, infarct volumes as analyzed by TTC staining were significantly lower in groups treated with Ajugarin I (40.6 ± 2.5%; $p = 0.021$), Punarnavine (37.5 ± 2.4%; $p < 0.005$), Marmesinin (39.4 ± 2.0%; $p = 0.011$), and Edavarone (41.2 ± 2.1%; $p = 0.007$) than in the control group (51.9 ± 2.9%) (Fig. 1A& 1B)

The NDS was also significantly reduced in the groups treated with Ajugarin I (9.3 ± 0.9; $p < 0.026$), Punarnavine (6.8 ± 1.1; $p = 0.010$), or Marmesinin (7.3 ± 1.7; $p = 0.030$) compared to the control group (13.5 ± 1.1). However, Edavarone treated groups did not show a statistically significant difference (Table 1).

Focal cerebral ischaemia was produced in the experimental animals by transient middle cerebral artery occlusion. Focal cerebral ischaemia resulted in occurrence of ischaemic cerebral infarcts in the mice brain with severe neurological deficit. Treatment with Ajugarin I (110 mg/kg *i.p*), Punarnavine (110 mg/kg *i.p*) and Marmesinin (110 mg/kg *i.p*) resulted in significantly lower neurological deficit score and significantly smaller infarct volume in focal cerebral ischaemia model

Reactive oxygen and nitrogen (such as peroxynitrite) species are hypothesized to be important in brain injury after cerebral ischemia. Multiple studies show that there is release of reactive oxygen species (ROS) after

experimental and human cerebral ischemia[14-19]. Neuronal biochemical composition is mainly susceptible to ROS since it involves pool of unsaturated lipids those are labile to peroxidation and oxidative modification. Double bonds of unsaturated fatty acids are hot spots for attack by free radicals those initiate cascade or chain reaction to damage neighboring unsaturated fatty acids[20]. Brain contains high level of fatty acids which are more susceptible to peroxidation that consumes an inordinate fraction (20%) of total oxygen consumption for its relatively small weight (2%). In addition, it is not particularly enriched in antioxidant defenses. Brain is lower in antioxidant activity in comparison with other tissues, for example, about 10% of liver. Several researchers considered brain to be abnormally sensitive to oxidative damage and many studies demonstrative of the ease of peroxidation of brain membranes supported this notion[21-23]. Moreover, human brain has higher level of iron in certain regions and in general has high levels of ascorbate. As evident from above data, neural cells are considered to be more susceptible to oxidative damage as compared to other body tissues [23].

Reactive oxygen species can exacerbate inflammation and generalized oxidative stress after cerebral ischemia by increasing lipid peroxidation, causing direct DNA damage and protein oxidation. These processes in turn activate apoptotic signals and inflammatory cascades that further damage the brain[18]. A major source of ROS after cerebral ischemia is thought to be oxidation reactions catalyzed by the heme groups of hemoglobin that are obviously abundant in the subarachnoid space after cerebral ischemia[15].

Free radical scavenging activity of the phytoconstituents used in the present study can be directly implicated for their evident neuroprotective potential. There are clinical evidences that neurodegenerations can be ameliorated upon dietary intake or supplementary intake of natural antioxidants. A variety of antioxidants including vitamin supplements play a vital protective role in neuroprotection in variety of neurological disorders[17].

CONCLUSION

It can be concluded from the present study that the phytoconstituents selected showed potent protective effect against ischaemic focal cerebral injury. Although the antioxidant and free radical scavenging property of the drugs under consideration appears to be the most promising mechanism for their observed effect, yet further studies are warranted to delineate the exact mechanism of action of the phytoconstituents. Moreover, this lead also inspires us to investigate the effect of these selected plant extracts in other models of global cerebral ischaemia and associated neurodegeneration.

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Table 1. Changes in Focal cerebral ischaemia induced Neurological Deficit Score (NDS)

Sr. No.	Group	Neurological Deficit Score (NDS)
1	Control	13.5 ± 1.1
2	Ajugarin I Treated	9.3 ± 0.9[a]; $p < 0.026$
3	Punarnavine Treated	6.8 ± 1.1[a]; $p = 0.010$
4	Marmesinin Treated	7.3 ± 1.7[a]; $p = 0.030$
5	Edavarone Treated	11.8 ± 0.7

Changes in Focal cerebral ischaemia induced Neurological Deficit Score (NDS) of Control, Ajugarin I treated, Punarnavine treated, Marmesinin treated and Edavarone treated groups. Results are here represented as the mean ± S.E.M. with n = 10 in each group. [a] $p<0.05$, as compared to the control group.

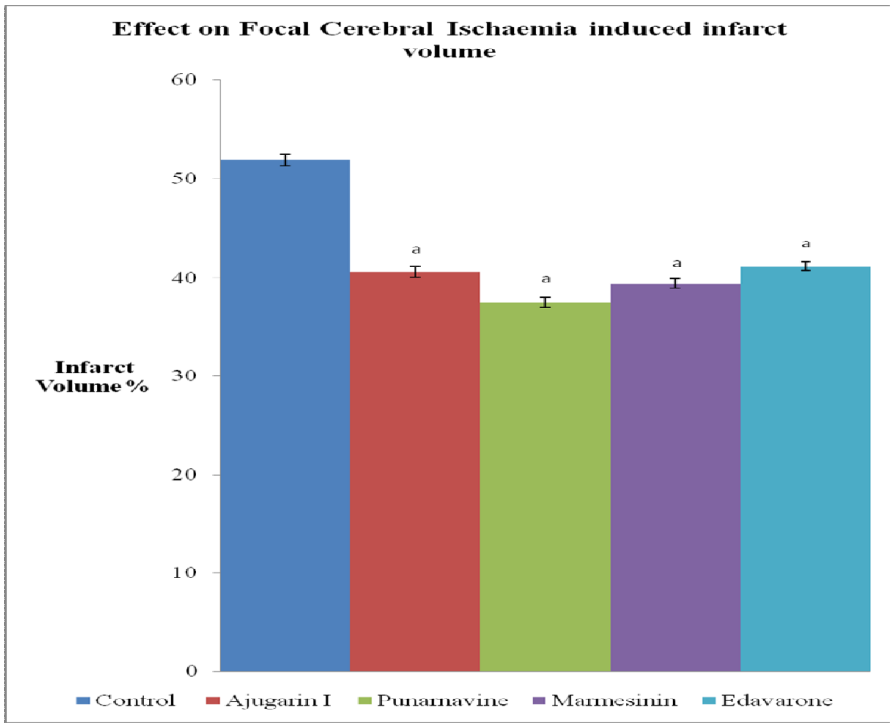

Figure 1A. Changes in Focal cerebral ischaemia induced infarct volume of control, Ajugarin I treated, Punarnavine treated, Marmesinin treated and Edavarone treated groups. Results are represented as the mean ± S.E.M. with n = 10 in each group. [a] $p<0.05$, as compared to the control group.

Control **Ajugarin I treated** **Punarnavine treated** **Marmesinin treated** **Edavarone treated**

Figure 1B. Sections of cerebral cortex of rat brain showing infarct lesions by tMCAO induced focal cerebral ischaemia and the effect of administration of various phytoconstituents on the infarct volume.