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ORIGINAL ARTICLE

Effect of embelin in middle cerebral artery occlusion-induced focal cerebral ischemia in rats

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Antioxidant; Cerebral ischemia; Embelin; MCAO

Abstract
Objective: Cerebral ischemia induced by stroke is one of the major causes of death and disability in the world and reactive oxygen species play key role in pathogenesis of cerebral ischemia. Chemical structure of embelin is similar to antioxidant çoenzyme Q_{10} and embelin is reported to have potent antioxidant activity. Therefore, the present investigation is carried out to study the effect of embelin using the middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia model.
Methods: Male Wistar rats were treated with embelin (50, 75 and 100 mg/kg, *p.o.*) for 20 days, followed by MCAO-induced focal cerebral ischemia. Measurement of neurological score, infarct size and malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) in brain homogenates were carried out at the end of study.
Results: Embelin decreases MDA level whereas increases SOD and CAT level as compared to ischemic control group. Moreover, embelin decreases infarct size, but there was no improvement in neurological score.
Conclusion: Pretreatment of embelin enhances the antioxidant defense and thereby ameliorates cerebral ischemia/reperfusion induced injury in the rat MCAO model.

INTRODUCTION

Cerebral ischemia induced by stroke is recognized as a worldwide problem as it is one of the major causes of death and disability. It has great effect on public health which account for largest number of hospitalization [1]. Ischemic brain damage involves complex cascade of events like neuron depolarization, glutamate receptors activation and glutamate hyperactivity, increases intracellular calcium concentration, up-regulation of enzymes such as lipases, proteases, endonucleases and over-production of reactive oxygen species (ROS) [2]. Several components of ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH^-$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) that are generated due to ischemia-reperfusion injury play key role in neuronal loss after cerebral ischemia. These ROS are constantly scavenged by endogenous antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). When endogenous anti-oxidant mechanisms are suppressed or production of free radical overweighs these mechanisms, a chain of reactions cause severe oxidative damage to lipids, proteins and nucleic acids [3, 4]. Increasing evidence has indicated that ischemia/reperfusion, which occurs either due to blockage of the middle cerebral artery (MCA) or due to recirculation of blood flow, causes oxidative stress that may potentiate ischemic injury [5]. Therefore, inhibition of production, enhanced degradation of ROS or strengthening the antioxidant system have been found to limit the extent of brain damage after stroke like events [6].

Some herbal medicines or their products having anti-oxidant activity has been suggested to protect against ischemic reperfusion injury, and thus justifying their use in ischemic stroke patients [7, 8]. Recently, antioxidant and neuroprotective activity of *Embelia ribes* Burm fruits in MCA occlusion (MCAO)-induced focal cerebral ischemia has been reported in rats [9, 10]. Embelin is found to be an active constituent of fruit of *Embelia ribes* Burm [11] and it shows potent antioxidant activity [12]. Moreover, it also possesses central analgesic, antifertility, antipyretic, anti-inflammatory [13, 14], antimicrobial [15], insecticidal [16], antitumor [17], anti-diabetic and anti-dyslipidemic activity [12, 18, 19]. Chemical structure of embelin is having quite resemblance with the structure of natural coenzyme Q_{10} (ubiquinones), a well known antioxidant agent and reported to have neuroprotective activity (Fig.1) [20, 21].

In light of above facts, the current investigation is carried out to study the effect of embelin in MCAO-induced focal cerebral ischemia in rats.

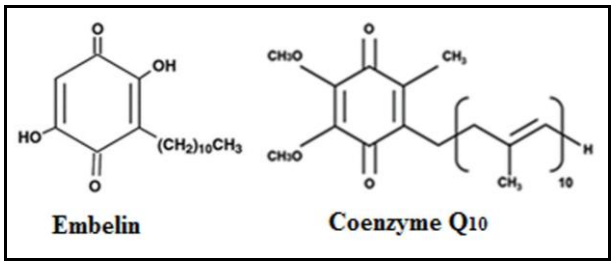

Figure 1. Chemical structure of embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) and coenzyme Q_{10}

MATERIAL AND METHODS

Ethical approval

The study protocol (SKPCPER-IAEC-197) was approved by Institutional (Shri S. K. Patel College of Pharmaceutical Education & Research, India) Animal Ethics Committee (IAEC) under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experiments were conducted in accordance with the international standards on animal welfare.

Animals

Male Wistar rats weighing 200-250 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12/12 h light/dark cycle. They were maintained under standard environmental conditions and were fed a standard rat chow diet with water given *ad libitum* throughout the study.

Chemicals

2,3,5-Triphenyl tetrazolium chloride (TTC) dye and poly-L-lysine used in study were obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Isolation of embelin

Isolation of the Embelin was carried out according to Indian herbal pharmacopoeia. The *Embelia ribes* fruits were collected from Gir Forest, Gujarat, India, and authenticated by Department of Bioscience, Ganpat University, India. A voucher specimen (Voucher No C-0256) was deposited in Department of Pharmacognosy, Shree S. K. Patel College of Pharmaceutical Education and Research, India. Dried fruits of *Embelia ribes* Burm were powdered (100 g) and extracted with n-hexane in soxhlet apparatus for half an hour to remove fatty materials. The marc after drying was again extracted in the soxhlet apparatus with diethyl ether until the extract was no longer in color and did not show pink color with ammonia. The diethyl ether was concentrated at room temp to yield (3.5% w/w) crude embelin. Crude embelin was washed with cold pet-ether to remove remaining fatty materials and isolated embelin was purified by recrystallisation several times to yield shiny orange yellow crystalline embelin. Thin layer chromatography (TLC) using mobile phase ethylacetate:formic acid:acetic acid:water (94:1:1:2) was performed to confirm embelin (R_f value: 0.65) [11].

Induction of cerebral ischemia-reperfusion

The 3-0 ethilon surgical sutures (Ethicon; Johnson and Johnson Ltd., Mumbai, India) were used to prepare filaments. Surgical suture were cut in length of 30 mm by keeping a precaution that edge of suture remain uniform. The 30 mm sutures were deep in poly-L-lysine solution for 10 min. and allowed to dry in an oven at 60°C for 45 min [22].

MCAO was induced in rats as described by Longa *et al* [23]. In brief, the rats were anesthetized with chloral hydrate (350 mg/kg, *i.p.*). The left common carotid artery (CCA) was exposed through a midline incision. The external carotid artery (ECA) was then isolated and dissected further distally and coagulated along with the terminal lingual and maxillary artery branches. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve. Filament was tied loosely around the mobilized ECA stump, and a curved microvascular clip was placed across both the CCA and the ICA adjacent to the ECA origin. A 30 mm length of poly-L-lysine coated 3-0 ethilon monofilament nylon suture was introduced into the ECA lumen through a puncture and advanced from the ECA to the ICA lumen. After a variable length of 20-22 mm nylon suture had been inserted into the ECA stump, resistance was felt indicating that the tip of the suture had passed the MCA origin and effectively occluding the MCA and inducing a ischemia. After 2 h of MCAO, the filament was slowly withdrawn and the animals were returned to their cages for a period of 22 h of reperfusion. The body temperature was maintained at 37°C with thermostatically controlled infrared lamp throughout the procedure.

Experimental design

The rats were divided into five experimental group (n = 6 each): group I was served as control and given orally 2% Tween 80 for 20 days; group II was the ischemic control group in which ischemia was induced for 2 h, followed by reperfusion for 22 h; group III, IV and V were treated by embelin 50, 75 100 mg/kg, respectively for 20 days, followed by MCAO procedure. Immediately after the neurological assessment, animals were sacrificed by cervical dislocation and their brains were taken out for infarct size measurement and malondialdehyde (MDA), SOD and CAT estimation.

Neurological score assessment

Neurological score of each rat was evaluated carefully after ischemia, after reperfusion and before sacrifice. A grading scale of 0-4 was used to assess the effects of occlusion [24] (Table 1).

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Quantification of infarct volume

Isolated brains were placed in a freezer at -20°C for up to 20 min to facilitate sectioning. The sections (thickness: 2 mm) were put in a glass Petri dish containing 2% TTC (2 g of TTC powder in 100 ml of phosphate buffer prepared by mixing 50 ml 0.2 M KH_2PO_4 and 39.1 ml 0.2 M NaOH and diluting up to 1000 ml with distilled water, pH 7.4) solution and were allowed to stain for 30 min at 37°C in water bath. After staining, sections were removed from TTC solution and placed in 4% formalin solution for fixation. The stained sections were scanned for infarct measurement using computer software Scion Image [25].

MDA, SOD and CAT estimation

Isolated brain were homogenized in phosphate buffer (prepared by mixing 50 ml 0.2 M KH_2PO_4 and 39.1 ml 0.2 M NaOH and diluting up to 1000 ml with distilled water, pH 7.8) and centrifuged at 2-8°C at 15,000 rpm for 10 minutes. The supernatant was used for assay of MDA [26], SOD [27] and CAT [28].

Statistical analysis

The data were expressed as mean ± SEM. Statistical differences were determined by analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of embelin on neurological score

No significant difference has been found in the neurological score amongst all groups (Table 2).

Effect of embelin on infarction and edema

The 50 and 75 mg/kg doses of embelin did not show any significant difference in decreasing the infarction and edema as compared to ischemic control group. Embelin administered at the dose of 100 mg/kg significantly ($P < 0.05$) decreased the infarction and edema (Figs.2&3).

Effect of embelin on MDA, SOD and CAT

MDA level was found to be significantly ($P < 0.01$) higher in ischemic control group compared to normal control group. Administration of 75 and 100 mg/kg embelin significantly ($P < 0.01$) decreased MDA level compared to ischemic control group. However, no significant change was observed in rats treated with 50 mg/kg embelin.

SOD and CAT activities were significantly ($P < 0.01$) lower in ischemic control group compared to normal control group. 100 mg/kg embelin administration significantly ($P < 0.01$) increased SOD and CAT activities compared to ischemic control group. However, no significant change was observed in rats treated with 50 and 75 mg/kg embelin.

Table 1. Neurological score

Score	Finding/Observation
0	Animal remain normal, no abnormality
1	Forelimb flexion and thorax twisting
2	Circling towards contra-lateral side by pulling the tail and keep the forelimb on ground
3	Spontaneous circling movement toward contra-lateral side
4	Docile

Table 2. Effects of embelin on neurological score

Groups	After ischemia	After Reperfusion	Before Sacrifice
Group II	2.5 ± 0.4	2 ± 0.4	2 ± 0.4
Group III	2.5 ± 0.4	2.25 ± 0.25	2 ± 0.0
Group IV	2.5 ± 0.4	2.25 ± 0.4	2 ± 0.4
Group V	2.5 ± 0.4	2.25 ± 0.25	2 ± 0.4

Each value represents the mean ± SEM (n = 6). Group II, MCAO; group III, embelin (50 mg/kg) + MCAO; group IV, embelin (75 mg/kg) + MCAO; group V, embelin (100 mg/kg) + MCAO. Neurological score of group I was 0.

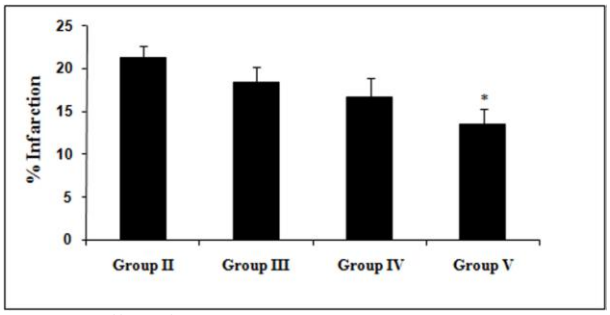

Figure 2. Effect of embelin on edema. Group II, MCAO; group III, embelin (50 mg/kg) + MCAO; group IV, embelin (75 mg/kg) + MCAO; group V, embelin (100 mg/kg) + MCAO. Group I is not shown due to no edema. Each bar represents the mean ± SEM (n = 6 each). *****P < 0.05 compared to group II.

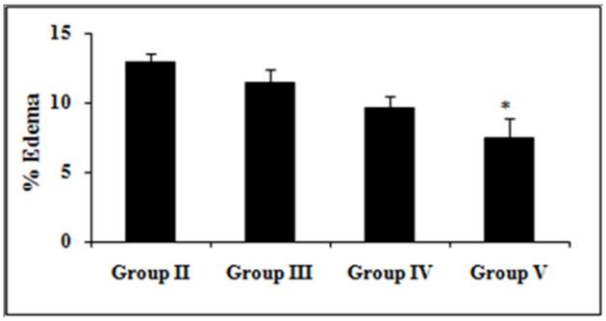

Figure 3. Effect of embelin on % infarction. Group II, MCAO; group III, embelin (50 mg/kg) + MCAO; group IV, embelin (75 mg/kg) + MCAO; group V, embelin (100 mg/kg) + MCAO. Group I is not shown due to no infarction. Each bar represents the mean ± SEM (n = 6 each). *****P < 0.05 compared to group II.

Table 3. Effects of embelin on malondialdehyde, superoxide dismutase and catalase levels

Groups	SOD (U/mg protein)	CAT (μmol/mg protein)	MDA (nmol/mg protein)
Group I	12.18 ± 1.34	1.50 ± 0.06	2.83 ± 0.11
Group II	7.39 ± 0.68**	0.85 ± 0.04**	4.35 ± 0.06**
Group III	7.92 ± 0.51	0.94 ± 0.1	3.95 ± 0.13
Group IV	9.48 ± 0.74#	1.03 ± 0.16	3.8 ± 0.1#
Group V	11.36 ± 0.48##	1.46 ± 0.08##	2.99 ± 0.13##

Each value represents the mean ± SEM (n = 6 each). Group 1, normal control; Group II, MCAO; group III, embelin (50 mg/kg) + MCAO; group IV, embelin (75 mg/kg) + MCAO; group V, embelin (100 mg/kg) + MCAO. #P < 0.05 and ##P < 0.01 *vs* group II; **P < 0.01 *vs* group I.

DISCUSSION

Middle cerebral artery occlusion is most commonly used model to induce experimental focal cerebral ischemia because of its reproducibility, production of significant ischemic penumbra and similarities with human stroke [29]. Therefore, MCAO model is preferred in present investigation to find out the effect of embelin.

Brain is highly susceptible to oxidative stress due to its enrichment with non-heme iron that is catalytically involved in the production of free radicals [30]. Free radical generation is the central event for the cellular injury in cerebral ischemia. Increasing evidence has indicated that ischemia/reperfusion occurs due to oxidative stress that may potentiate ischemic injury [5].

Decreased activity of SOD and CAT in ischemic rat brain leads to excess availability of $O_2\bullet^-$ and H_2O_2, which in turns generate $\bullet OH^-$ resulting in the propagation of lipid peroxidation, which increases the MDA content. In present study, ischemic control rats showed significant increase in MDA level as compare to normal control rats. Administration of embelin at 100 and 75 mg/kg doses showed significant decrease in MDA level whereas 50 mg/kg embelin did not show change as compared to ischemic control rats. Contrary to MDA level, ischemic control rats showed significant decrease in SOD and CAT activity levels as compared to normal control rats. Administration of embelin at 100 mg/kg caused significant increase in SOD and CAT level whereas 75 and 50 mg/kg embelin did not change those enzymes levels as compared to ischemic control rats.

The neurological score indicates the extent of cerebral damage induced by MCAO. Neurological score was determined after ischemia, after reperfusion and before sacrifice but there was no significant difference found between ischemic control rats and embelin treated rats. Furthermore, cerebral damage induced by MCAO caused local infarction and edema which is commonly detected by TTC stain [31, 32]. In the present study, administration of embelin at 100 mg/kg showed significant difference in infarction and edema as compare to ischemic control rats. No significant difference in infarction and edema was observed in rats treated with 50 or 75 mg/kg embelin.

In conclusion, pretreatment with embelin enhances the antioxidant defense and thereby ameliorates cerebral ischemia/reperfusion injury in the used MCAO rat model. One of the possible mechanisms for this effect of embelin seem to be the restoration of altered antioxidant enzymes activity as well as decreasing the production of lipid peroxides. However, there was no improvement in neurological score. Therefore, more extensive work using higher dose of embelin as well as other models of cerebral ischemia are needed to explore the use of embelin in the treatment of stroke.

CONFLICT OF INTEREST

The author does not have any conflict of interests related to this paper.

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