Nepeta Dschuparensis Bornm Extract Moderates COX-2 and IL-1β Proteins in a Rat Model of Cerebral Ischemia

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Abstract

Background: *Nepeta dschuparensis Bornm (NP)* is used as a medicinal herb in Iran. In traditional medicine, this herb is extensively employed for curing ailments such as cardiovascular diseases. *NP* has antioxidant and anti-inflammatory properties. This project examined the effects of the *NP* extract on cyclooxygenase-2 (COX-2) and interleukin-1β (IL-1β) protein levels and its efficacy in neuroprotection in a cerebral ischemia-reperfusion model.

Methods: Twenty-six male rats were randomly divided into 3 groups: 1) sham (n=6): no middle cerebral artery occlusion (MCAO) procedure, 2) control (n=10): MCAO procedure and treatment with normal saline, and 3) *NP* extract (n=10): MCAO procedure and treatment with the *NP* extract (20 mg/kg, i.p.) at the beginning of reperfusion. To examine the injury caused by cerebral ischemia, we measured motor coordination and the infarct area using the rotarod test and triphenyl tetrazolium chloride staining, respectively. IL-1β and COX-2 protein levels, as inflammatory markers, were measured by immunoblotting assay. The statistical analyses were performed using SPSS, version 16, and the data are expressed as means±SEMs. Statistical difference was evaluated using the one-way ANOVA, followed by the post hoc LSD test (P<0.01).

Results: Treatment with the *NP* extract significantly diminished the infarct volume and alleviated the motor coordination disorder induced by cerebral ischemia. The *NP* extract administration significantly attenuated the increase in IL-1β and COX-2 protein levels too (P<0.01).

Conclusion: The beneficial effects of the *NP* extract are related to its ability to decrease the levels of IL-1β and COX-2.

What's Known

• *Nepeta dschuparensis* has been used as a traditional medicine for the treatment of gastrointestinal, respiratory, and cardiovascular diseases. Research has also shown the antioxidant and anti-inflammatory effects of this herbal medicine.

What's New

• This is the first study to provide evidence of the efficacy of NPE in an experimental model of cerebral ischemia by reducing the protein levels of both IL-1β and COX-2.

Introduction

Stroke is mainly caused by the sudden occlusion of a blood vessel, followed by the inauguration of a number of biochemical events and ultimately neuronal death.[1] Although reperfusion in ischemic brain tissue is vital for maintaining normal function, it may cause a secondary injury, in which oxidative stress mediators have a significant part.[2] Furthermore, cerebral

damage is an effective stimulant of inflammatory cytokines and protease secretion by leukocytes, microgliae, and neurovascular unit resident cells. The breaking of the neurovascular unit activates multiple neuroinflammatory cascades, which might lead to additional secondary brain injury.[3] Cyclooxygenase (COX) is a main enzyme in this incident.[4] It catalyzes the first committed step of arachidonic acid conversion into the unstable intermediate prostaglandin G2, which is transformed quickly into prostaglandin H2 sequentially by COX, and lastly, into a series of biologically active prostaglandins and thromboxane A2.[5] There are 2 known isoforms of COX: COX-1 and COX-2. Unlike COX-1, COX-2 expression is significantly induced by ischemia, which has been proposed to intensify brain damage.[6] In addition, interleukin-1 (IL-1), as a proinflammatory cytokine, has been recognized as an important neurodegeneration mediator caused by cerebral ischemia.[7] IL-1α and IL-1β ligands are produced quickly in the rodent brain following cerebral ischemia,[8] and treatment by recombinant IL-1β enhances ischemic and other damage.[9] In contrast, blocking IL-1 actions, by the administration of the IL-1 receptor antagonist, obviously, attenuates inflammation and neuronal loss caused by a number of brain insults.

The genus *Nepeta* (catmint) is comprised of about 300 species, occurring in Asia and Europe. The greatest variety of species is found in 2 areas: South-western Asia, particularly Iran, and the Western Himalayas. Some species, particularly *Nepeta dschuparensis Bornm* (*NP*), are used as medicinal herbs in Iran. In traditional medicine, *NP* is extensively employed for curing different ailments of gastrointestinal, respiratory, and cardiovascular systems.[10] The medicinal properties of the *Nepeta* species are usually attributed to their flavonoids. In the current study, we evaluated the effects of the *NP* extract on COX-2 and IL-1β protein levels and its efficacy in neuroprotection in a cerebral ischemia-reperfusion model.

Materials and Methods

Animals

The current study was approved by the ethics committee for the animal experimental protocols of Kerman University of Medical Sciences (EC/KNRC/91-28). Adult (3 mon) male healthy Sprague–Dawley rats (supplied by the Neuroscience Research Animal Center, Kerman, Iran), weighing 220–260 g, were kept in a controlled environment at a room temperature of 24±1.0 °C and automatic day–night schedule (12-h cycle). The animals were selected based on simple sampling method and divided into 3 groups: 1) sham (n=6): animals which underwent the same surgical procedures as the rats subjected to middle cerebral artery occlusion (MCAO), but with the exception of MCAO, 2) control (n=10): animals subjected to 15 minutes of ischemia, 24 hours of reperfusion, and treatment with saline solution, and 3) *NP* extract (n=10): animals subjected to 15 minutes of ischemia, 24 hours of reperfusion, and treatment with the *NP* extract (20 mg/kg, i.p.) at reperfusion commencement. The mortality rate of the animals was 40%, 40%, and 0% in the *NP* extract, control, and sham groups, respectively. The *NP* dose was selected based on a pilot study. Three different doses (i.e. 0.2, 2, and 20 mg/kg) were injected to the animals, and the best effective dose was selected for the main study.

Establishment of Cerebral Ischemia/Reperfusion

Left middle cerebral artery occlusion was induced using the intraluminal filament model and the method described by Longa et al.[11] In brief, the animals were anesthetized with chloral hydrate (360 mg/kg, i.p.). With the rats in the supine position, a midline ventral incision was made to expose the left common carotid artery, which was carefully separated from the vagus nerve. All the branches of the external carotid artery and the extracranial internal carotid artery were blocked. Then, 3-0 nylon suture was introduced into the internal carotid artery and advanced intracranially to block the blood flow into the middle cerebral artery. After 15 minutes of ischemia, the suture was withdrawn to restore blood flow for 24 hours (reperfusion).[12] Rectal temperature was maintained at 37±0.5 °C using a thermistor coupled to a heating blanket during surgery. The animals were returned to their cages after recovery.

Preparation of the Ethanolic Extract of NP

Dried leaves of *NP* were collected from the Rabor area, Kerman Province, Iran, in May, 2011. A voucher specimen was stored at the herbarium of the Pharmacy Faculty of Kerman Medical University (#KF1384). The dried leaves (100 g) were packed in a Soxhlet apparatus and were extracted with ethanol over 48 hours. A vacuum drier was used to evaporate the filtrate, and the obtained remnant brown mass was stored at 4 °C for further use. An average yield of roughly 10.1% was obtained for the ethanolic *NP* extract. The ethanolic *NP* extract (20 mg/kg) was dissolved in 1% of normal saline and given to the animals.

Rotarod Activity

With the rotarod test, the animals were evaluated for balance and grip strength. Each animal was given a training session prior to the beginning of the therapy in order for them to adapt to a rotarod apparatus (Technical and Scientific Equipment, GmbH, Germany). The rats were put on the rotating rod with a 7-cm diameter (speed=20 rpm). Three trials were conducted for each animal at 10-minute intervals, and the cut off time (300 s) was followed throughout the trial. The average of falling time was recorded.[13]

Cerebral Infarct Volume Evaluation by Triphenyl Tetrazolium Chloride Staining

Twenty-four hours after reperfusion, 2 animals from each group were anesthetized with sodium chloral hydrate (400 mg/kg weight, i.p.) and decapitated. Whole brain tissue was carefully removed, submerged in cold saline for 10 minutes, and sliced into 2.00-mm thick segments using a brain matrix apparatus. The segments were incubated in 2% (w/v) triphenyl tetrazolium chloride (Sigma), and immersed in distilled water for 15 minutes at room temperature. The infarct area on each brain segments was measured (blinded) using a digital scanner and an image tool program (UTHSCSA Image Tool for Windows, version 3.00, Department of Dental Diagnostic Science at the University of Texas Health Science Center, Texas, USA). In order to reduce any artifacts caused by post-ischemic swelling in the infarct tissue, we directly measured the infarct area in the left cortex by subtracting the non-infarct area in the left cortex from the total cortical area of the right hemisphere. The total infarct volume was calculated from the result of the average segment thickness and the sum of the infarct area in all brain segments. The infarct volume was determined according to the following formula:

right hemisphere volume–(left hemisphere volume–infarct volume)=corrected infarct volume

Immunoblotting Analysis

The dissected brains (n=4) were retrieved 24 hours after reperfusion to measure COX-2 and IL-1β protein levels, respectively. The dissected brain tissues were homogenized with an ice-cold buffer containing 0.1% SDS, 0.1% Na deoxycholate, 10 mM of Tris–HCl (pH=7.4), 1 mM of EDTA, 1% NP-40 with protease inhibitors (2.5 μg/mL of leupeptin, 1 mM of phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin), and 1 mM of sodium orthovanadate.

The homogenate was centrifuged at 14,000 rpm at 4ºC for 15 minutes. The subsequent supernatant, as the whole cell fraction, was retained. The Bradford method was used for the measurement of protein concentrations (Bio–Rad Laboratories, Munich, Germany). The same quantities of protein were resolved electrophoretically on 9% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Having been blocked (overnight at 4 °C) with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM of NaCl, 20 mM of Tris–HCl, pH=7.5, and 0.1% Tween 20), the membranes were explored with COX-2 and IL-1β rabbit polyclonal antibody COX-2 (H-3): sc-376861 and IL-1β (H-153): sc-7884 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000, for 1 hour at room temperature. Having been washed in TBS-T (3 times, 5 min), the blots were incubated at room temperature for 60 minutes with a horseradish peroxidase-conjugated secondary antibody (1:15000, GE Healthcare Bio-Sciences Corp.). All the antibodies were diluted in blocking buffer. The antibody–antigen complexes were discovered by the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roche Applied Science, Mannheim, Germany). To explore the intensity of the expression, we utilized the Lab Work analyzing software (UVP, Cambridge, UK). β-Actin immunoblotting (antibody from Cell Signaling Technology, Inc.; 1:1000) was used as a loading control.

Statistical Analysis

The statistical analyses were done using SPSS, version 16. All the values are expressed as means±SEMs. The data from the rotarod test and protein levels were calculated using the one-way ANOVA, followed by the post hoc LSD test. The infarct volume data were analyzed using a nonparametric test (Kruskal–Wallis), followed by the Bonferroni test. The statistical difference was determined by a value of $P<0.05$.

Results

NP Extract Treatment Reduced Falloff Time in the Rotarod after MCAO in the Rats

The falloff time is measured for rotarod evaluation to measure motor incoordination. A significant decrease was observed in the falloff time in the control group as compared to the sham group, showing motor incoordination and muscle weakness. The *NP* extract

significantly (P<0.01) improved the fall off latency time as compared to that of the control group (figure 1).

NP Extract Treatment Reduced the Infarct Volume after MCAO in the Rats

The injury produced by 15 minutes of ischemia plus 24 hours of reperfusion showed an infarct area of 144.76±17.36 mm^3. The administration of the NP extract at the beginning of reperfusion significantly decreased the infarct area to 39.58.1±9.24 mm^3 (P<0.01) (table 1 and figure 2).

NP Extract Treatment Moderated the Increased Level of COX-2 after MCAO in the Rats

The animals subjected to 15 minutes of ischemia and 24 hours of reperfusion (the control group) showed an increase in COX-2 protein levels when compared with the sham animals (P<0.01). The NP extract treatment significantly diminished the increase in COX-2 protein levels induced by cerebral ischemia (P<0.01) (table 1).

NP Extract Treatment Moderated the Increased Level of IL-1β after MCAO in the Rats

The animals subjected to 15 minutes of ischemia and 24 hours of reperfusion (the control group) showed an increase in IL-1β protein levels when compared with the sham animals (P<0.01). The NP extract treatment significantly diminished the increase in IL-1β

Figure 1: NP extract treatment group showed a significant increase in the falloff time compared to the control group (P<0.000). The data were analyzed with the one-way ANOVA, followed by the post-hoc LSD test. NP: Nepeta dschuparensis Bornm.

protein levels induced by cerebral ischemia (P<0.01) (table 1).

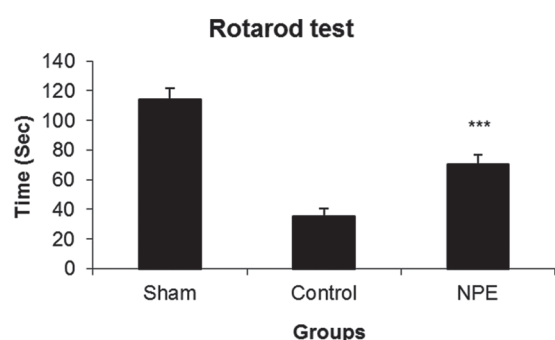## Discussion

The results of the present study are indicative of the neuroprotective effect of the NP extract on ischemia insults induced by cerebral ischemia in male rats.

Our results showed that 15 minutes of MCAO, followed by 24 hours of reperfusion, resulted in severe injury to the cerebral cortex in the control group. This finding is in agreement with that in other studies.[14-16] Since most of the blood supply of the pyramidal tract and motor cortex is provided by the middle cerebral artery, the occlusion of this vessel begets different motor disorders.[17] Motor dysfunction can result from a failure of cortical excitability and/or the inhibition of electrical impulses in the subcortical area. Axonal conduction, however, readily recovers after ischemia and reperfusion. In addition, a steady malfunction in cortical synapses leads to motor dysfunction.[18]

Rotarod is a regularly used procedure for testing coordination and balance constituents of general motor function. It is a responsive index for the evaluation of motor impairment induced by focal cerebral ischemia.[19] Our results showed that the falloff time from rotarod was significantly decreased in the control group compared to the sham group, proving deficits in muscle coordination too. A decrease in muscle coordination has also been shown by different studies on the MCAO model of cerebral ischemia.[13,20]

It has been reported that inflammation induces inflammatory mediators in the brain through glial cell activation and intracellular signaling pathways stimulation, inducing some proinflammatory cytokines such as COX-2.[21,22] Also, the upregulation of inflammatory agents has an important role in neuron loss in some neurodegenerative diseases.[23] In addition, elevated levels of COX-2 are observed within ischemia and neural injury.[24] These alterations cause neurobehavioral and cognitive deficits.[25] Treatment with the NP extract in the current study was found to alleviate muscle weakness,

Table 1: Infarct volume, cyclooxygenase-2 (COX-2), and interleukin-1β (IL-1β) protein levels in the different groups

Groups	Infarct volume		Cox-2		Il-1β	
Sham	0	-	0.62±0.00	-	0.90±0.02	-
Control	144.76±17.36	*(P<0.01)	0.98±0.04	*(P<0.01)	1.21±0.08	*(P<0.01)
NP extract	39.58±9.24	*(P<0.01)	0.64±0.01	*(P<0.01)	0.93±0.01	*(P<0.01)

NP extract group exhibited a significant decrease in the infarct volume (P<0.05) and in the levels of COX-2 (P<0.001) and IL-1β (P<0.001), respectively, in the cerebral cortex. Values are expressed as means±SEMs. The data were analyzed with the one-way ANOVA, followed by the post-hoc LSD test. *P<0.01 as compared to the control group. NP: Nepeta dschuparensis Bornm
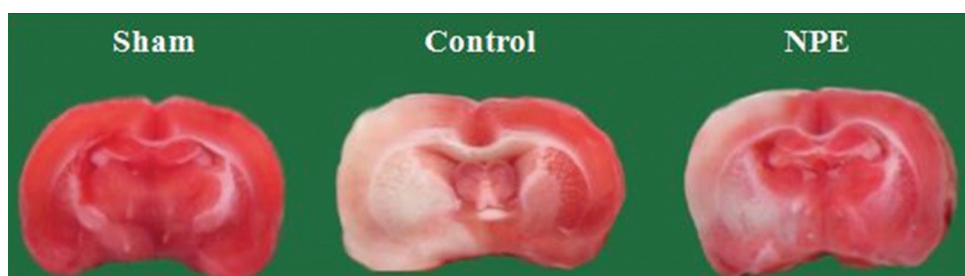
Figure 2: Triphenyl tetrazolium chloride staining of the cerebral cortices in the different groups. Treatment with the *NP* extract markedly decreased the cortical infarction in the rats with cerebral ischemia injury. Marked infarction (white areas) was observed in the rats' left cortex in the control group. *NP*: *Nepeta dschuparensis Bornm*

supporting its positive effect against ischemia-reperfusion damage owing to its ability decrease COX-2 levels.

The pathological mechanisms underlying the neuronal damage in the cerebral ischemia are multifactorial and complex.[26] When there is a loss of blood flow in an area of the brain, the ischemic cascade is quickly initiated, triggering 2 important pathophysiological mechanisms: oxidative stress and inflammation. Crucial hypoperfusion occurs as a result of ischemic stroke; it usually initiates oxidative damage and excitotoxicity. In addition, after ischemic inflammation, microvascular damage also creates a disruption in the blood brain barrier.[1] The induced inflammation can intensify ischemic injury through different mechanisms. In rodent models of cerebral ischemia, enhanced levels of inducible nitric oxide synthase are generated by infiltrating neutrophils, responsible for producing toxic amounts of nitric oxide. Additionally, ischemic neurons express COX-2, an enzyme which mediates ischemic injury by creating superoxide anion and toxic prostanoids.[27] In the current study, the *NP* extract treatment significantly attenuated the increase in COX-2 protein levels caused by cerebral ischemia, indicative of the anti-inflammatory properties of the extract.

It has been shown that IL-1β is the initial form of IL-1 involved in ischemic brain injury.[28] Our results also showed that treatment with the *NP* extract significantly decreased the increase caused by cerebral ischemia in IL-1β protein levels. These effects may be due to the anti-inflammatory effects of the extract. In line with the above findings, the *NP* extract can reduce cerebral infarct volume in rats subjected to MCAO.

The generation of excessive inflammatory cytokines and reactive oxygen species throughout reperfusion has a main role in brain injury associated with stroke. Since the brain has low activities of antioxidant enzymes, it is very susceptible to reactive oxygen species induced by reperfusion injury, which causes oxidative injury to brain structures such as proteins, lipids, and DNA, resulting in neuronal death.[29]

The *NP* extract has some antioxidant and anti-inflammatory agents such as β-caryophyllene, 1.8 cineole, β-eudesmol, thujone, and α- and β-pinene.

β-Caryophyllene is an anti-inflammatory agent that is nonsteroidal in nature. Besides anti-inflammatory properties, it has analgesic, antipyretic, and platelet-inhibitory functions. It acts by blocking the synthesis of prostaglandins through inhibiting COX, converting arachidonic acid into cyclic endoperoxides, precursors of prostaglandins. The inhibition of the synthesis of prostaglandins is responsible for their analgesic, antipyretic, and platelet-inhibitory functions; the anti-inflammatory effects may be intensified by other mechanisms.[30] 1.8 cineole is another component of the *NP* extract, with anti-inflammatory properties that may enhance its protective effects.[31] It has been shown that β-eudesmol, as one of the *NP* extract components, has a potential effect on neuronal functions, including neurite outgrowth, in rat pheochromocytoma cells accompanied by an activation of the mitogen-activated protein kinase pathway.[32] In addition, this component has anticonvulsive properties, as shown by Quintans Júnior et al.[33] Another component of the used extract is thujone, with well-known neuroprotective effects.[34] In addition, α- and β-pinene trepenoid molecules have been identified as therapeutic targets in neurodegenerative disorders: for instance, Alzheimer's disease, cerebrovascular impairment, seizure disorders, head injury, and Parkinsonism. Altogether, these compounds can account, at least in part, for the protective effects of the *NP* extract.

The major limitation of the current work is the insufficiency of relevant data in the existing literature expanding on the effectiveness of the

NP extract. Another drawback of note is that inadequate financial support for this project limited us to concentrating on only 2 molecules (COX-2 and IL-1β). We did not assess glial cell activation, especially astrocyte and microglial activity, in the current work and it could be a target for future research.

Conclusion

To the best of our knowledge, this is the first study to provide evidence of the efficacy of the *NP* extract in an experimental model of cerebral ischemia in reducing the levels of both IL-1β and COX-2. Further studies are needed to be able to propose the potential therapeutic use of the *NP* extract in protecting the brain against ischemia-induced insults.

Acknowledgment

Conflict of Interest: None declared.

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