

The Neuroprotective Effect of *Nepeta menthoides* on Axotomized Dorsal Root Ganglion Sensory Neurons in Neonate Rats

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Background and Objective: Sensory neurons have critical role in improvement of functional outcome of any neuroprotective strategy. The herbal medicine *Nepeta menthoides* has been reported to have anti-apoptotic effect on axotomized spinal motoneurons. In the present study, the putative neuroprotective effect of *Nepeta menthoides* on the axotomized dorsal root ganglion sensory neurons in neonate rats was investigated.

Materials and Methods: In fifteen two-day-old rat neonates, the right sciatic nerve was transected. The animals were subdivided into two experimental groups receiving 250 and 500 mg/kg of *Nepeta menthoides* and a control group treated with the normal saline as the vehicle for three days following the axotomy. At the fourth day the neonates were sacrificed and the L5 dorsal root ganglions of both sides were dissected and prepared for morphometrical cell count and TUNEL assay.

Results: In the control group, four days following axotomy, 38.51% of dorsal root ganglion sensory neurons were lost. Administration of 250 and 500 mg/kg of *Nepeta menthoides* for three days significantly reduced the cell loss to 24.64% and 21.69%, respectively. The findings of TUNEL assay in control group indicated that axotomy significantly increased the apoptotic index from 3.93% to 10.8%, but in both experimental groups the difference of the reduced percentage of apoptotic cells (the apoptotic index) between intact and axotomized sides was insignificant.

Conclusion: *Nepeta menthoides* through attenuating the apoptotic cell death can induce neuroprotective effect on axotomized dorsal root ganglion sensory neurons.

1. Introduction

The term neuroprotection can be applied to any treatment strategy preventing loss of neurons, which is likely to be generic for all harmful factors and neurodegenerative disorders (1), and must be induced before initiation of neuronal loss and appearance of clinical signs (2). Recent studies express the role

of apoptosis versus necrosis in neuronal cell death. These two types of cell death, which share common signal transduction pathways, may be induced by the same stimulators (3). Since apoptosis is a more delayed event, its prevention can be a putative neuroprotection strategy.

Apoptosis also called programmed cell death,

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roots in a Greek word meaning "falling of the leaves" (4). During embryonic development, apoptosis plays an important role in shaping and improving different organs of the body, and after birth the balance between the processes of mitosis and apoptosis maintains the stability of cell turnover. Early apoptosis happens in neuronal pathologies including trauma, ischemic and neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington Diseases (5-7).

Apoptosis morphologically starts in the nucleus as chromatin condensation. It changes to sharp and prominent features attached to the nuclear membrane as dense crescent-like masses. This process will eventually lead to a reduction in nuclear volume. Parallel to the changes in the cell nucleus, also the cytoplasm becomes dense. Cell size diminishes and cell membrane becomes convoluted by protuberances giving the cell a "star-like" appearance. Apoptotic cells finally disintegrate into apoptotic bodies composed of chromatin and membrane organelles, which will be ingested by macrophages and neighboring cells and removed from the tissue (4, 8).

There are many known natural and chemical compounds, such as hormones and pharmacological drugs, with claimed neuroprotective effects. However, because of considerations such as unwanted side effects, availability and effectiveness, there has been considerable interest in investigating the neuroprotective capacity of new agents such as herbal medicines.

The genus *Nepeta* from the family of Lamiaceae comprises about 400 species. Most of them grow wild in Central and Southern Europe, North Africa and Central and Southern Asia, and due to their antispasmodic, diuretic, antiseptic, antitussive, antiasthmatic and febrifuge activities, are widely used in traditional medicine (9). The therapeutic effect of *Nepeta* species are usually attributed to their essential oils and flavonoids. The majority of *Nepeta* species contain lipophilic flavonoids of the flavone group on their leaves (10), which due to their antioxidant and free radical scavenging capacity (11) can be the therapeutic components of these herbs. About half of the existing species of *Nepeta* has been recorded in Iran (12). One of them, the *Nepeta menthoides* Boiss and Bushe commonly known

as *Ostokhodus-e Khorasani* (13) or briefly *Ostokhodus*, has been used as an herbal medicine to treat neural disorders such as epilepsy and melancholia in Iranian Traditional Medicine (14), and it has been reported to have neuroprotective effects on axotomized spinal motoneurons (15,16). Because of the claimed therapeutic capacities of *Nepeta menthoides*, in the present study we investigated its putative neuroprotective and anti-apoptotic effects on axotomized sensory neurons in dorsal root ganglion (DRG) of neonate rats.

2. Materials and Methods

2.1. Preparation of *Nepeta menthoides*

Dried aerial parts of *Nepeta menthoides* were obtained from a local herbal medicine grocery in Mashhad/Iran and was confirmed by the Tehran Medical University Herbarium, where a voucher specimen was deposited under the reference number PMP-302. Then, 100 g of the powdered plant was macerated in 1000 ml of 80% ethanol for 48 hours and filtered by filter papers. After evaporating the solvent by a 50 °C hot tissue bath, a waxy extract with a yield of 16% (w/w) was obtained. The extract was diluted in normal saline, centrifuged for 15 min at 2000 rpm and filtered twice through sterile 0.2 µm filter papers (Whatman-Uk) to obtain a sterile stock which can be used to prepare the desired doses of 250 and 500 mg/kg.

2.2. Animal groups and surgery

The animal care and experimental procedures were accomplished according to ethical guidelines of Ministry of Health and Medical Education of Iran. Fifteen two-day-old Sprague-Dawley rat neonates (Razi Institute, Karaj, Iran) were housed under standard conditions accompanied by their mothers. The neonates were subdivided into two experimental and one control groups, each consisting of 5 animals. In all groups, the animals were anesthetized by hypothermia and under sterile conditions the right sciatic nerve was transected at the mid-thigh and approximately 2 mm of the distal stump was removed. Following axotomy, the neonates were returned to their mothers. The experimental groups (E1 and E2) received 250 and 500 mg/kg of *Nepeta menthoides*, respectively, for three successive days starting at the day of axotomy,

and the control group received equal volume of saline as the dilution vehicle. The *Nepeta menthoides* was administered intraperitoneally and the first injection was performed quickly after recovery from the surgery and repeated at the same hour on the following days. On the fourth day, the animals were deeply anesthetized and transcardially perfused with cold heparin-containing normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the L5-DRG of both sides were dissected through laminectomy and transferred to the same fixative for 24 h. In all samples the contralateral intact DRG was considered as an internal control.

2.3. Cell count and apoptosis assessment

The samples were processed and 8 μm transverse serial sections were obtained. Every tenth section was stained with Cresyl violet and used for morphometry and counting of sensory neurons in DRG of both sides of the spinal cord, where cells with a distinct nucleolus were counted at 400x magnification (Figure 1). In each group, the mean of sensory neurons in both DRGs and the mean percentage of sensory neurons reduction of axotomized side compared to the intact side were calculated.

In all groups three sections, each next to one of the cell-counted sections, were selected for TUNEL assay study.

The selected tissue sections were dewaxed, rehydrated and permeabilized in freshly prepared 0.1% Triton X-100, 0.1% sodium citrate (Sigma, Germany) for 8 min, washed in PBS and the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed as described in the In Situ Cell Death Detection Kit, POD instruction manual (Roche-Germany). Briefly, samples were incubated in 50 μl of TUNEL reaction mixture (5 μl enzyme solution containing TdT from calf thymus in storage buffer, and 45 μl of label solution containing FITC-labeled dUTP nucleotides in reaction buffer) for 60 min at 37 °C in a humidified chamber and in the dark, covered with parafilm. Omission of TdT provided the negative control for the assay, and preincubation of cells with 10 $\mu\text{g/ml}$ of DNase I in 50 mM Tris-HCl, pH 7.4, 1 mM of MgCl₂ and 1 mg/ml of BSA for 10 min at room temperature, served as positive control. Sections were washed with PBS and incubated for 30 min in a humidified chamber, at 37 °C with 50 μl of converter-POD (Anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase). After rinsing in PBS, the samples were incubated for 10 min with 100 μl of diaminobenzidine substrate (Sigma, Germany) at 20-25 °C in the dark. Following washing again with PBS, the samples were mounted and analyzed under light microscope, where the apoptotic cells could be seen as highly condensed shrunk dark brown representations (Figure 2).

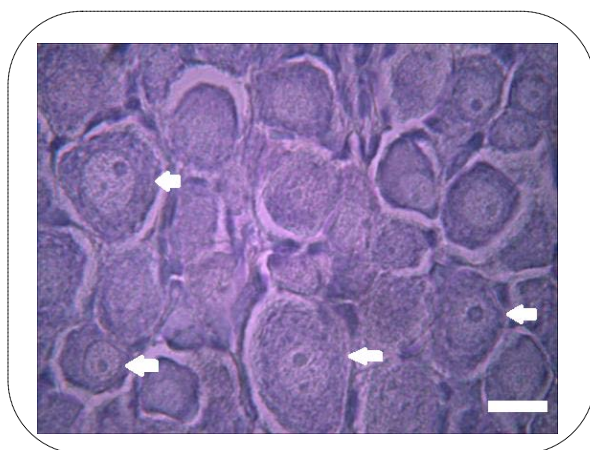


Figure 1.A. Section of L5 dorsal root ganglion in the axotomized side of control group stained with cresyl fast violet. The arrows demonstrate large sensory neurons with a distinct pale nucleus and prominent nucleolus. Scale bar= 10 μm .

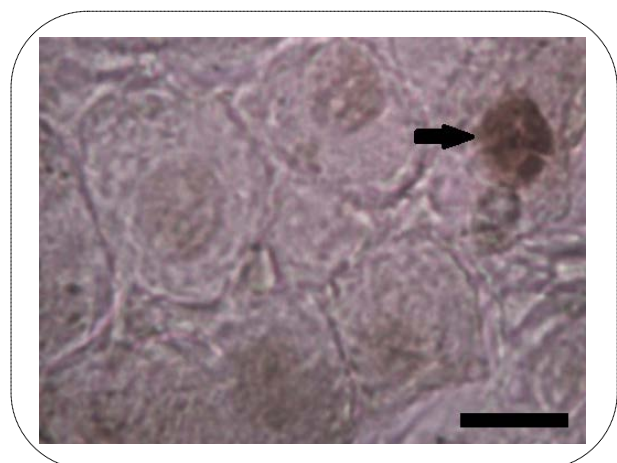


Figure 2- A. TUNEL assay-prepared micrograph of L5 dorsal root ganglion in the axotomized side of experimental group E1, treated with 250 mg/kg of *Nepeta menthoides*. The arrow demonstrates an apoptotic sensory neuron as a brown condensed feature. Scale bar= 10 μm .

Table 1. The results of morphometric cell count

	Control group	Experimental group 1	Experimental group 2
Intact side	1591.6± 160.63*	1565± 217.53*	1519± 240.66*
Axotomized side	978.6±168.43	1179.4± 176.17\$	1189.6± 166.2\$ i
Cell loss percentage	38.51%	24.64%	21.69%

The mean and standard deviation of sensory neurons in both L5-DRGs in different groups. The last row indicates the cell loss percentage of axotomized side compared to intact side in every group. *: indicates significant difference of values between axotomized and intact sides in each group ($P<0.001$). \$: indicates significant differences between axotomized side of each of the experimental groups and the control group ($P<0.05$). i: indicates insignificant difference between axotomized sides of both experimental groups.

In each of the three sections of every group, the percentage of apoptotic cells to the total number of sensory neurons in DRG was determined, and their means was calculated which will be referred to as Apoptotic Index (AI).

2.4. Statistical analysis

The findings of cell count and apoptosis assessment were given as mean \pm standard deviation and analyzed for statistical significance by one-way ANOVA and Tukey post hoc test, where the $P<0.05$ was assumed as significant.

3. Results

Our cell count study indicated that transection of sciatic nerve in control neonate rats induced an obvious reduction in the mean of related sensory neurons in the ipsilateral DRG, with a 38.5% cell loss of axotomized sensory neurons. Daily intraperitoneal administration of axotomized rats with 250 and 500 mg/kg of *Nepeta menthoides* for 3 days in experimental groups E1 and E2, decreased the cell loss percentage to 24.64% and 21.69%, respectively. In control as well as in both experimental groups, the difference between the means of sensory neurons in axotomized and

intact sides was significant. Comparison of means of spinal sensory neurons in axotomized side of control and experimental groups, indicated significant differences between control and each of the experimental groups E1 and E2 ($P<0.001$), but the difference between E1 and E2 was insignificant. The results of cell count are summarized in Table 1 as mean \pm standard deviation.

To further determine if axotomy-induced cell death was caused by apoptosis, we used TUNEL assay and DAB as chromogen and calculated the percentage of apoptotic cells to the total number of cells as Apoptotic Index (AI).

AI in the axotomized DRG of control, E1 and E2 groups was equal to 10.8, 8.65 and 7.76%, and in intact DRG of the same groups, it was 3.93, 3.66 and 3.27%, respectively (Figure 3). The difference between AI of axotomized and intact sides in control group was statistically significant, whereas in both experimental groups it was insignificant ($p<0.05$). Comparison of AI between axotomized sides of control and experimental groups also indicated insignificant differences.

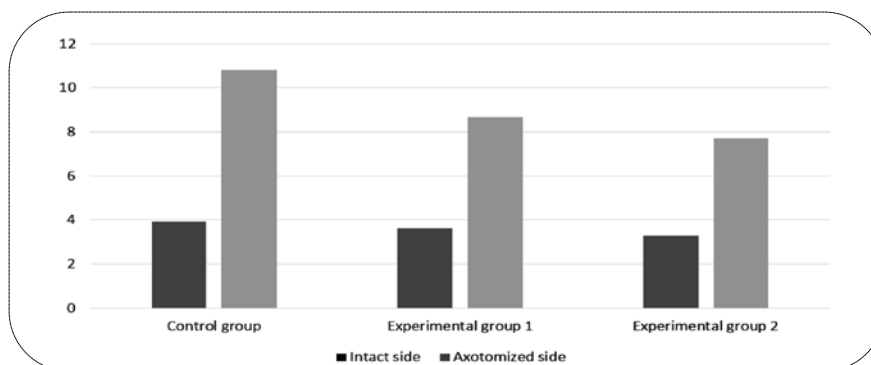


Figure 3. The bars indicate the percentage of apoptotic cells to the total number of sensory neurons (AI) in both DRGs in control and experimental groups. Comparing the AI of axotomized and intact sides indicated a significant difference in control group, and insignificant differences in both experimental groups. Also comparison between the values of axotomized sides of control and experimental groups indicated no significant differences.

4. Discussion

The significant difference between intact and axotomized sides in the control group approved the efficiency of our axotomy experimental model in inducing apoptotic cell death in sensory neurons of DRG, which has been reported repeatedly as a standard apoptosis-inducing model in motoneurons (17, 18). As presented in Table 1, in both experimental groups the difference between sensory neurons of intact and axotomized DRGs is significant, which implies that although *Nepeta Menthoides* can preserve some of the axotomized sensory neurons, still many of them will be lost. The difference between the means of sensory neurons of axotomized DRGs in control and each of the experimental groups E1 and E2 was significant whereas comparison of E1 and E2 showed no significant difference, which indicates that 250 mg/kg of *Nepeta Menthoides* can be taken into account as a proper neuroprotective dose.

The diminution of TUNEL-positive apoptotic cells in both experimental groups suggests that *Nepeta menthoides* may execute its neuroprotective effect through preventing apoptosis. Our findings demonstrated that in both groups the difference between the percentage of apoptotic features in intact and axotomized DRGs were considerably less than the difference between means of sensory neurons, in other words the percentage of apoptotic features was much less than the percentage of neural loss, which can be due to the fast phagocytosis and removal of apoptotic figures in the tissue by the neighboring cells.

Many reports suggest the neuroprotective capacity of many hormones such as steroids (19), androgens (20), estrogens and progesterone (21, 22) and some pharmacological agents such as isoflurane, Ketamine and lamotrigine (23, 24), anti-apoptotic antibiotic minocycline, neurotransmitter modulators such as remacemide, riluzole and paroxetine (7), neuroimmunophilin ligands such as tacrolimus, and dopamine agonists in Parkinson's disease (6). It has also been reported that providing physical and biochemical situations such Hypothermia and alkalization has significant neuroprotective effect (25).

It has been reported that androgens can prevent axotomy-induced cell death of facial

motoneurons in hamster neonates (26). Although estrogen has been considered as a potent neuroprotectant in acute stroke, its physiological levels couldn't exert any neuroprotection and only in pharmacological doses at the time around an ischemic event has been demonstrated to have neuroprotective effects (27). It has been documented that progesterone through reducing inflammation, swelling and apoptosis may promote neuroregeneration (21). It has been indicated that in cerebral ischaemia models, the pharmacologic agents isoflurane and lamotrigine can improve the neurologic function and reduce the histologic damage of hippocampal CA1 and CA2 neurons (23). It has been reported that after brain injury sedative and anesthetic doses of Ketamine through modulating the apoptosis regulating proteins and interfering the inflammatory responses, can be a neuroprotectant, whereas higher doses can induce neurotoxicity (24). Because of the well-known roll of oxidative stress in apoptosis, many antioxidant drugs such as coenzyme Q10, creatine, α -lipoic acid and dichloroacetate, have been suggested to be helpful in managing neurodegenerative diseases (7). In spite of the anti-apoptotic and neuroprotective properties of the above-mentioned hormones and drugs, on account of their extensive unwanted systemic effects on different parts of the body other than the nervous system, their clinical application as a medical neuroprotective strategy may be highly limited, so looking for new neuroprotectants must still be considered as necessary.

Recently, much attention has been paid to traditional and herbal medicine products such as green tea (28), *Verbena officinalis* Linn. (29), *Jacaranda caroba* (Vell.) (30), garlic (31), *Viburnum tinus* L. (32), soybean (33) and natural polyphenol antioxidants mangiferin and morin (34). Sutherland and his colleagues (2006) in a review on the neuroprotective mechanisms of green tea stated that the bioactive components catechins, can diminish oxidative stress and inflammatory responses and modulate apoptosis (28). Lai et al (2006) reported that aqueous extracts of *V. officinalis* significantly attenuated the β - amyloid peptide neurotoxicity on cortical neurons in vitro (29). Yu et al (2005) demonstrated that the oriental medicine *Lycium barbarum* through suppression of the c-Jun N-terminal signaling pathway can prevent β -amyloid-induced neurotoxicity and cell death on

cultured neurons (35). Li et al (2009) reported remarkable neuroprotective activities of several derivatives of a traditional Chinese medicinal plant which might prevent and slow down the neurodegeneration in Alzheimer's disease through concomitant inhibition of acetylcholinesterase, N-methyl-D-aspartate receptor, nitric oxide synthase, and amyloid precursor protein/ β -amyloid cascade (36). It has been reported that *Curcuma longo* can significantly ameliorate ethanol-induced memory deficits in mice by manipulating NOS/NO signaling pathway (37). Qian et al indicated the neuroprotective effect of genistein, one of the active ingredients of soybean, in transient focal ischemia which may involve regulation of mitochondria-dependent apoptosis pathways and suppression of ROS-induced NF- κ B activation (33). Cervantes and his colleagues reported that the present antioxidants in garlic extract may regulate ROS concentrations during ischemia, favor pro-survival pathways and attenuate mitochondrial dysfunction (31). In our last study, we indicated that daily intraperitoneal administration of axotomized rats with 500 mg/kg of *Nepeta menthoides* attenuates axotomy-induced apoptosis of spinal motoneurons (15). In the present study, administration of 250 mg/kg of *Nepeta menthoides* resulted in a significant protection of axotomized sensory DRG-neurons through prevention of apoptosis. Comparing the effective doses of *Nepeta menthoides* in these two studies indicated that the neuroprotective effect of *Nepeta menthoides* on sensory neurons needs a lower dose than the spinal motoneurons. In the peripheral sensory or mixed nerve injuries, which are the commonest form of nervous system trauma, the clinical sensory outcome remains very poor, and because sensory feedback is a vital component of the normal control loop, the fine motor functions will be impaired too. Although various factors are implicated in the poor sensory outcome, the single most important factor is probably the death of a relatively prominent percentage of relevant primary sensory neurons. Also because of the sufficient similarities between the mechanisms underlying axotomy-induced neuronal death within the peripheral and central nervous systems, any treatment which protects primary sensory neurons may also have therapeutic implications for the management of traumatic brain, spinal cord, or brachial plexus injury (38). Because of the above-mentioned facts which prove the

clinical importance of neuroprotective strategies in the sensory systems, we have designed the present study to investigate the neuroprotective capacity of *Nepeta menthoides* on the DRG sensory neurons. Although, the mechanism of the neuroprotective effects of *Nepeta menthoides* has not been identified thoroughly, it can be attributed to the antioxidant and anti-inflammatory properties of the included flavonoids which can reduce the axotomy-induced apoptotic cell death of sensory neurons and protect the cells from the insult. Miceli et al reported the anti-inflammatory effect and radical scavenging activity of methanol extract of *Nepeta sibthorpi* (9). Tepe et al have also shown antioxidant activity of essential oil and various extracts of *Nepeta flavida* (12). Sarahroodi et al have reported that aqueous extract of *Nepeta menthoides* has significant effect on memory retention and retrieval in mice (39). It has been reported that following a single intraperitoneal injection, fractions of flavonoids could be detected in the brains of rats, which indicates that the included flavonoids in the herb are able to cross the blood brain barrier in vivo (40). At the end, it can be concluded that the antioxidant and anti-inflammatory herb *Nepeta menthoides* through inhibiting apoptosis can act as a novel neuroprotectant in managing neurological conditions involving sensory neurons as well as motoneurons.

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