Journal of Medicinal Plants Research Vol. 5(18), pp. 4446-4451, 16 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

The Iranian traditional herbal medicine ostokhodus can prevent axotomy-induced apoptosis in spinal motoneurons in neonate rats

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Accepted 18 July, 2011

Ostokhodus (Nepeta Menthoides), is an herb widely used in Iranian Traditional Medicine to manage the neurologic disorders. Since apoptosis plays a crucial role in pathological situations of nervous system, in present study we investigated the putative anti-apoptotic effect of Ostokhodus in axotomy-induced apoptosis of spinal motoneurons in neonate rats. Following transecting the right sciatic nerve of 2 day old rat neonates, the experimental groups were treated with different doses of alcoholic extract of Ostokhodus intraperitoneally for 3 days starting at the day of axotomy. In all groups one day following the final injection, the motoneurons of sciatic pool were counted and apoptosis assessment was performed by TUNEL assay on ventral horns of both sides of the associated spinal cord segments, where the contralateral intact side served as internal control. Intraperitoneal administration of axotomized rats with Ostokhodus resulted in an increased survival of axotomized motoneurons and a decreased apoptotic rate compared to intact side, which was most prominent in the group treated with the dose of 500 mg/kg. These findings indicated a dose-dependent neuroprotective anti-apoptotic effect of Ostokhodus which can preserve the axotomized spinal motoneurons.

Key words: Ostokhodus, nepeta menthoides, neuroprotection, motoneuron, apoptosis, axotomy, TUNEL.

INTRODUCTION

Apoptosis is a cell death program central to cellular and tissue homeostasis and is involved in many physiological and pathological processes (Er et al., 2006), with the morphological features of chromatin condensation, Deoxyribonucleic acid (DNA) and nuclear fragmentation, cytoplasmic shrinkage and apoptotic body formation (Lossi and Merighi, 2003), which can be recognized by some specific methods including TUNEL (terminal deoxynucleotidyltransferase (TdT)-mediated deoxynucleotidyltransferase-(dUTP) nick end labeling) assay, an enzymatic in situ labeling of apoptosis-induced DNA strand breaks. One of the most studied extracellular induction protocols of apoptosis is the axotomy-induced withdrawal of target-derived trophic support of neurons (Becker and Bonni, 2004). Although, naturally occurring cell death in the spinal cord is completed before birth in rats and mice, both motoneurons and sensory neurons suffer apoptotic cell death following peripheral axotomy in immature animals (Kinugasa et al., 2002). Motoneurons and interneurons of lumbar enlargement of rats continue to be dependent on trophic inputs during the first week after birth and sciatic nerve transection will cause their apoptotic loss, when performed in this time period (Oliveira et al., 2002). More recent studies have indicated the roles of apoptosis versus necrosis in neuronal loss associated with the acute or chronic neurodegenerative disorders. These two types of cell death, which share certain common signal transduction pathways, may be induced by the same insult, with the magnitude of the insult determining the undergone cell death pathway (Yakovlev and Faden, 2004). Since apoptosis is a more delayed event, its prevention can be a putative neuroprotection strategy. The term neuroprotection can

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Abbreviations: DNA, Deoxyribonucleic acid; **TdT,** terminal deoxynucleotidyltransferase; **dUTP,** deoxynucleotidyltransferase; **AI,** apoptotic index.

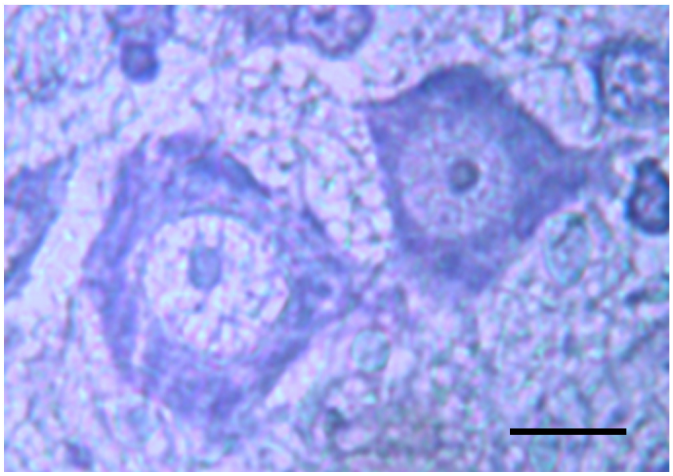

Figure 1. Photomicrograph of spinal ventral horn of intact side showing two Nissl-stained normal motoneurons with large nuclei and prominent nucleoli. Scale bar = 10 µm.

be applied to any treatment strategies preventing loss of neurons or axons, which are likely to be generic for all harmful factors and neurodegenerative disorders (Palace, 2008), and must be induced before initiation of neuronal loss and appearance of clinical signs (Muresanu, 2007).

Although, many natural and synthetic chemical compounds such as hormones and pharmacological drugs have been claimed to have neuroprotective capacity, because of their unwanted side effects there has been considerable interest in investigating the neuroprotective effects of new agents such as herbal medicines. Nepeta Menthoides Boiss and Bushe commonly known as Ostokhodus (Naghibi et al., 2005), has been used as an herbal medicine for many centuries in Irananian Traditional Medicine, to treat neural disorders such as epilepsy and melancholia (Aghili-Khorasani, 2004). The genus *Nepeta* (from family Lamiaceae) comprises about 400 species, most of which grow wild in Central and Southern Europe, North Africa and Central and Southern Asia, and are widely used in folk medicine because of their antispasmodic, diuretic, antiseptic, antitussive, antiasthmatic, and febrifuge activities (Miceli et al., 2005). About half of the existing species of Nepeta are recorded in Iran (Tepe et al., 2007), and some of them are used as medicinal herbs.

The medicinal properties of *Nepeta* species are usually attributed to their essential oils and flavonoids. The great majority of *Nepeta* species contain lipophilic flavonoids of the flavone group on the surface of their leaves (Jamzad et al., 2003), which due to their antioxidant and free radical scavenging capacity (Soobrattee et al., 2005) can be the effective components of these herbs exerting their therapeutic action. In the present study we investigated the putative neuroprotective and anti-apoptotic effects of Ostokhodus (Nepeta Menthoides) in axotomized spinal motoneurons of neonate rats.

MATERIALS AND METHODS

Preparation of ostokhodus

Dried aerial parts of Ostokhodus purchased from a local herbal medicine grocery in Mashhad, Iran was identified and confirmed by Professor Amin, the head of Herbarium of faculty of pharmacy Tehran Medical University, where a voucher specimen was deposited under the reference number PMP-302. A 20% solution of the herb in the 80% ethanol was evaporated to obtain the alcoholic extract of Ostokhodus, and 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 concentrations of 250, 500 and 1000 mg/kg.

Animal groups and surgery

The animal care and all experimental procedures were carried out according to ethical guidelines established by the Shahed University Tehran, Iran. 22 day old Sprague-Dawley rat neonates obtained from Razi Institute (Karaj, Iran) were housed under a 12 h light/dark cycle accompanied by their mothers. The neonates were subdivided into three experimental and one control groups, each consisting of 5 animals. In all groups, under hypothermia-induced anesthesia and sterile conditions the right sciatic nerve was transected at the mid-thigh level and to hinder innervation an approximately 2 mm piece of the distal stump was removed. After surgery the pups recovered from hypothermia and were returned to their mothers. The experimental groups (E1, E2 and E3) received 250, 500 and 1000 mg/kg of Ostokhodus, 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 Ostokhodus was administered intraperitoneally and the first injection was performed quickly after recovery from the surgery and repeated at the same hour on the next days. One day following the last injection 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 L4 and L6 spinal cord segments were dissected through laminectomy and transferred to the same fixative for 24 h. In all samples the contralateral intact side of the spinal cord was considered as an internal control.

Cell count and apoptosis assessment

The samples were processed and 8 µm transerve serial sections were obtained. Every fifth section was stained with cresyl violet and used for morphometry and counting of spinal motoneurons in ventral horns of both sides of the spinal cord, where cells with a large nucleus (>10 µm) and a distinct nucleolus were counted at 400 x magnification (Figure 1). In each group the mean of motoneurons in both ventral horns, and the mean percentage of motoneuron reduction of axotomized side compared to the intact side were calculated and the results were analyzed for statistical significance by one-way ANOVA and t-test, where the P value < 0.05 was assumed as significant. In all groups five 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

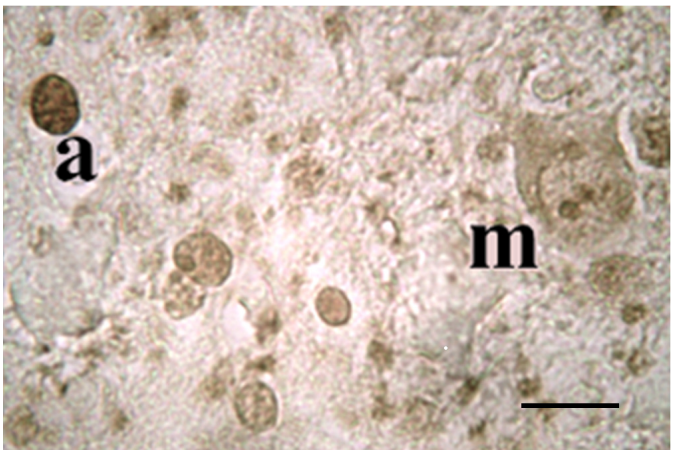

Figure 2. Photomicrograph of TUNEL- assay prepared spinal ventral horn of axotomized side showing a normal motoneuron (m) and an apoptotic cell (a) as a dark condensed figure in the upper left corner. Scale bar = 10 µm.

calf thymus in storage buffer, and 45 µl 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 µg/ml DNase I in 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$ and 1mg/ml BSA for 10 min at room temperature to induce DNA strand breaks artificially, served as positive control. Sections were washed with PBS and incubated for 30 min in a humidified chamber, at 37°C with 50 µl 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 DAB (Sigma-Germany) substrate at 20 to 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). In each of the five sections of every group the percentage of apoptotic cells to the total number of motoneurons in the ventral horn was determined, and their means was calculated which will be referred to as Apoptotic Index (AI).

RESULTS

Our cell count study indicated that transection of sciatic nerve in control neonate rats induced an obvious reduction in the mean of related spinal motoneurons in the ventral horn of ipsilateral side of spinal cord, specially on its lateral part, with a 28.3% cell loss of axotomized motoneurons. Daily intraperitoneal administration of axotomized rats with 250, 500 and 1000 mg/kg Ostokhodus for 3 days in experimental groups E1, E2 and E3, resulted in an increased mean of survived motoneurons and a decreased cell loss percentage compared to intact side, which was absolutely obvious in E2 group. In control as well as in all experimental groups, the difference between the means of motoneurons in axotomized and intact sides was significant. Comparison of means of spinal motoneurons in axotomized side of control and experimental groups, indicated insignificant differences between control and E1, E1 and E2 as well as E2 and E3 groups, and a prominent significant difference between control and E2, and control and E3 groups with *P<0.01*. The results of cell count are summarized in Table 1 as mean ± standard error. 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 as AI, which in axotomized side of spinal cord in control, E1, E2 and E3 groups was equal to 6.34, 5.86, 4.92 and 4.84%, and in intact side of the same groups it was 3.15, 2.87, 2.94 and 2.89%, respectively (Table 2).

Statistical analysis indicated no significant differences between four AI values in intact side of control and experimental groups, but in the axotomized side although the difference of AI between control and E1, E1 and E2 as well as E2 and E3 were insignificant, it was significant between control and E2 or E3 groups with $P<0.05$. Comparing the AI of two sides of the spinal cord in control group indicated a significant difference (*P<0.05*), whereas in the experimental groups it was insignificant. The last row indicates the cell loss percentage of axotomized side compared to intact side in every group. In each group (columns), the difference of values between axotomized and intact sides is significant (*P<0.05*). Comparing the axotomized sides of each of the three experimental groups with the control group (2^{nd} row), indicates an insignificant difference of E1 (*i*), and significant differences of E2 and E3 (*s*) compared to control group. The difference between E2 and E3 is also insignificant. The table indicates the percentage of apoptotic cells to total motoneurons in both ventral horns of spinal cord in different groups. Comparing the values of axotomized and intact sides in each group indicated a significant difference in control group (*), and insignificant differences in experimental groups. Comparing the values of intact sides of all groups with each other, as well as the values of axotomized sides indicated no significant differences except between the axotomized sides of each of the E2 and E3 groups with the control group (*s*).

DISCUSSION

The significant difference between the means of motoneurons of intact and axotomized sides of spinal cord in the control group approved the efficiency of our experimental model in inducing neuronal apoptosis, which has been reported repeatedly as a standard apoptosis-inducing model in motoneurons (Kinugasa et al., 2002; Rogerio et al., 2006). The attenuation of axotomy-induced neuronal loss following administration of different doses of Ostokhodus indicates that it could have a dose-dependent neuroprotective effect. As presented in Table 1, in all experimental groups the difference between motoneurons of intact and

Table 1. The means plus standard error of spinal motoneurons in ventral horn of spinal cord in different groups.

	Control group	Experimental group 1	Experimental group 2	Experimental group 3
Intact side	12.58 ± 0.429	12.34 ± 0.440	12.68 ± 0.369	12.45 ± 0.527
Axotomized side	9.02 ± 0.417	10.00 ± 0.423 *i*	11.20 ± 0.398 *s*	11.49 ± 0.628 *s*
Cell loss percentage	28.3 %	18.96 %	11.67%	8.03 %.

The last row indicates the cell loss percentage of axotomized side compared to intact side in every group. In each group (columns), the difference of values between axotomized and intact sides is significant ($P<0.05$). Comparing the axotomized sides of each of the three experimental groups with the control group (2nd row), indicates an insignificant difference of E1 (***i***), and significant differences of E2 and E3 (***s***) compared to control group. The difference between E2 and E3 is also insignificant.

Table 2. Apoptotic Index derived from the TUNEL assay study.

	Control group	Experimental group 1	Experimental group 2	Experimental group 3
Intact side	3.15% *	2.87%	2.94%	2.89%
Axotomized side	6.34% *	5.86%	4.92% *s*	4.84% *s*

The table indicates the percentage of apoptotic cells to total motoneurons in both ventral horns of spinal cord in different groups. Comparing the values of axotomized and intact sides in each group indicated a significant difference in control group (*), and insignificant differences in experimental groups. Comparing the values of intact sides of all groups with each other, as well as the values of axotomized sides indicated no significant differences except between the axotomized sides of each of the E2 and E3 groups with the control group (***s***).

axotomized sides is significant, which implies that although Ostokhodus can preserve some of the axotomized motoneurons still many of them will be lost. Furthermore the insignificant difference between axotomized side of control and E1 groups, and the significant difference between control and E2 indicate that 250 mg/kg of Ostokhodus cannot exert an effective neuroprotection whereas a 500 mg/kg intraperitoneal administration can be considered as a proper effective dose. In E3 group which got a 1000 mg/kg dose, despite the significant difference with the control group no more prominent changes compared to E2 group could be seen and there was a plateau which indicates that 500 mg/kg of Ostokhodus can be taken into account as a proper neuroprotective dose. Our findings of TUNEL assay study in all experimental groups indicated an associated reduction of TUNEL-positive apoptotic cells, which suggests that Ostokhodus may perform neuroprotection through inhibiting apoptosis. Comparing the Tables 1 and 2 demonstrates that in all groups the difference between the Apoptotic Index of intact and axotomized sides are considerably less than the difference of mean of motoneurons, which can be explained by the very short remaining of apoptotic figures in the tissue before being phagocytosed.

There are a lot of reports in the literature suggesting the neuroprotective properties of many hormones such as androgens (Fargo et al., 2009), estrogens and progestrones (DeNicola et al., 2009; Simpkins and Dykens, 2008), erythropoietin (King et al., 2007; McPherson and Juul, 2008), and some pharmacological agents such as isoflurane and lamotrigine (Weigl et al., 2005). It has been reported that androgen treatment following transection of facial nerve in neonatal hamsters can prevent axotomy-induced cell death of facial motoneurons (Huppenbauer et al., 2005). Although estrogens could be valuable candidates for brain protection during acute stroke in both males and females, no neuroprotection was afforded by the administration of physiological levels of estradiol at the onset of an ischemic event, but neuroprotective effects of pharmacological doses of estradiol were clearly demonstrated by the acute treatment at the time of or just before an ischemic event, as well as after its onset (Yang et al., 2003). The neuroprotective effects of progesterone and its metabolites have been documented in different lesion models, where progesterone may promote neuroregeneration by reducing inflammation, swelling and apoptosis (DeNicola et al., 2009).

King et al. (2007) reported that intravitreal administration of erythropoietin following transection of optic nerve in adult rats, significantly increases the survival of retinal ganglion cell somata and axons. The effective neuroprotective dose of erythropoietin is well above the range used to treat anemia, and this high dosing range has prompted concerns about its potential unwanted adverse consequences (McPherson and Juul, 2008). It has been indicated that in cerebral ischaemia models the anesthetic isoflurane and the anticonvulsive lamotrigine can improve the neurologic function and reduce the histologic damage of hippocampal CA1 and CA2 neurons (Weigl et al., 2005). Despite the reported antiapoptotic and neuroprotective properties of high doses of forementioned hormones and drugs, due to their extensive unwanted systemic effects on different parts of the body other than the nervous system, their use as a medical

neuroprotective treatment may be highly restricted, and still the need to new natural neuroprotectants must be taken into account.

In recent years many researchers are interested in traditional and herbal medicine and have reported a couple of neuroprotective agents, such as green tea (Sutherland et al., 2006), *Verbena Officinalis* Linn. (Lai et al., 2006) and natural polyphenol antioxidants mangiferin and morin (Campos-Esparza et al., 2009). Sutherland and his colleagues (2006) in a review on the neuroprotective mechanisms of green tea and its main bioactive components catechins, reported that catechins which have antioxidant and free radical scavenging effects, can attenuate oxidative stress and inflammatory responses and modulate apoptosis at various points in the sequence. Lai et al. (2006) reported that pre-treatment of cultures of cortical neurons with aqueous extracts of *V. officinalis*, significantly attenuated the β-amyloid peptide neurotoxicity. Yu et al. (2005) indicated that the water-soluble extracts of oriental medicine Lycium barbarum can protect cultured neurons from β-amyloid-induced neurotoxicity and cell death, by suppression of the c-Jun N-terminal signaling pathway. Li et al. (2009) studying the effects of several drugs derived from a traditional Chinese medicinal plant on Alzheimer's disease reported remarkable neuroprotective activities through concurrent inhibition of acetylcholinesterase, N-methyl-D-aspartate receptor, nitric oxide synthase, and amyloid precursor protein/β-amyloid cascade, which might serve as one of the most effective therapeutic strategies to prevent and slow down the neurodegeneration in Alzheimer's disease.

Our search in the existing literature has not found any publication investigating the putative effects of Ostokhodus on neurological conditions, and our findings indicating its prominent neuroprotective capacity has not been reported yet. Although, the mechanisms of this neuroprotective effect has not been identified throughly, 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 motoneurons and protect the cells from the insult. There can be a debate whether following intraperitoneal administration of the herb, the included flavonoids can traverse the blood brain barrier and reach sufficient levels in the central nervous system to affect neuronal function. However, it has been reported that following a single intraperitoneal injection, fractions of flavonoids could be detected in the brains of rats, which indicates that flavonoids are able to cross the blood brain barrier *in vivo* (Youdim et al., 2004). At the end it can be concluded that the Iranian Traditional herbal medicine Ostokhodus (*Nepeta Menthoides*) with the anticipated antioxidant and anti-inflammatory capacities because of its included flavonoids, can be taken into account as a novel neuroprotectant in managing neurological conditions such as neurodegenerative disorders through inhibiting apoptosis.

ACKNOWLEDGEMENT

This research was supported by the Shahed University grant number 4/263.

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