Original Article

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Neuroprotective Effect of Aqueous Extract of *Achillea millifolium* Against Retrograde Destruction of Neurons of Ventral Horn of the Spinal Cord After Sciatic Nerve Compression in Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: damage to peripheral nerves stimulates a series of morphological and biochemical changes in neurons, which are not concentrated only in the injured region, but are also observed in the cell body of neurons of the spinal cord and in the nerve ganglion. The aim of this study was evaluation of the protective effects of aqueous extract of Achillea millifolium on neuron density of ventral horn of the spinal cord after sciatic nerve compression.

METHODS: In this experimental study, 30 Wistar rats were divided randomly into 5 groups: group I (control), group II (compression), group III (compression + injection of the aqueous extract at a dose of 25 mg/kg), Group IV (compression+injection aqueous extract at a dose of 50 mg/kg), and group V (compression+aqueous plant extract at a dose of 75 mg/kg). Mice were anesthetized and the skin of the right thigh was incised, then the muscles were parted to expose the sciatic nerve. Using forceps, the sciatic nerve cord blood was severely compressed, then the muscles and skin were sutured. Administration of the aqueous extract with group specific doses was performed for three weeks, with one injection per week. 28 days after the surgery, the rats were fixed and the lumbar spinal cord (L4, L5) was removed. Tissue sections were prepared from the spinal cord sample and through toluidine blue staining and photography, the density of alpha motor neurons of the spinal ventral horn was investigated.

FINDINGS: The neuronal density in the compression group compared to the control group showed a significant decrease (943±59 vs. 1620±51.1, $p<0.001$). Neuronal density in the treatment groups showed a significant increase compared to the compression group. The 75 mg/kg dose showed the highest neuroprotective effect compared to the compression group (1421±139.7 vs. 943±59, $p<0.001$).

CONCLUSION: The aqueous extract of Achillea millifolium effectively protects motoneurons of the anterior horn of the spinal cord against retrograde injury during sciatic nerve compression.

KEY WORDS: *Sciatic nerve, Aqueous extract, Achillea millifolium, neuroprotection.*

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Introduction

Treatment and restoration of nervous damages is one of the fundamental goals of medicine and neuropharmacology. Accidents and injuries to the body, and diseases such as diabetes and infections can cause permanent damage to nerves and are followed by severe chronic pain, loss of sensation and motor control or paralysis. Anti-cancer chemotherapy may also irreversibly damage the nerves and affect the medical regimens and quality of life of cancer patients. Currently extensive research for identification of basic mechanisms of nerve damage and new approaches for neuroprotection and nerve repair are underway. Studies have shown that after peripheral nerves injury, a series of complex and highly regulated events are initiated in order to remove the damaged tissue and begin the healing process. Unlike cell regeneration in other parts of the body, peripheral nerve response to injury does not involve mitosis and cell proliferation. In fact, peripheral nerve response to damage is not limited to the injured area and does not happen locally, but affects the cell body of neurons located in the spinal cord or nerve ganglia (1,2).

Since the neuron cell body is the metabolic center of the neuron, in case of damage to the neuron, the cell body of the neuron is damaged and causes the death of neuron which is known as Valerian degeneration. In peripheral nervous system, after nervous damage, regeneration and repair would occur but in central nervous system, the restoration does not happen. Upon disconnection or nerve compression, Valerian degeneration begins in the posterior of axons. Blood-nerve barrier is composed of endothelial cells with no opening, which are connected by tight junctions. This barrier restricts movement of proteins, hormones, ions and toxins from blood to the nervous tissue (3,4).

After peripheral nerve injury the permeability of this barrier increases and allows the blood cells which facilitate the repair of the nerve to enter the nerve. One of the most important blood cells that enter the damaged area are the macrophages that bring out the remains of damaged myelin and axons. During this process, the basement membrane of axons, which surrounds the Schwann cells, remains intact. Schwann cells in the tubular part of the basement membrane line up and synthesize the growth factors. Axon branches can be absorbed by these factors in the proximal region. Tubes of basement membrane provide paths for regenerated axons to reach the target organs, such as muscle or skin, and rearrange neurogenically. Schwann cells then remyelinate the newly formed axons. The newly formed myelin is thinner than normal myelin and paths between the nodes are shorter than natural pathways. Immediately after restoration of the neuron cell body, it deflates and returns to normal (1,5,6).

The Achillea millifolium plant has about 130 permanent or perennial species which typically have fluffy and fragrant leaves and wide clusters of small flowers on the stem. Since these flowers have a variety of colors, some species are popular garden plants (7). Most species of Achillea are medicinal plants and have therapeutic applications. Native Americans and the first settlers, used it to heal wounds. Achillea species in Anatolia region of Turkey are the most important commercial plants, and are used as herbal tea for treatment of abdominal pain and flatulence (8 , 7). Many therapeutic uses of these plants have been confirmed by clinical and experimental studies. There are several reports on anti-inflammatory (9), antispasmodic (10), antioxidant (11), diaphoretic, diuretic, anti-thrombosis, antihypertensive, and menstruation inducing effects of the Achillea species (12). However, there are still many aspects of Achillea which should be investigated.

The aim of this study was to evaluate the neuroprotective effects of aqueous extract of Achillea millifolium on ventral horn neurons of the spinal cord after sciatic nerve compression in rats .

Methods

Preparation of the aqueous extract: Achillea millifolium was obtained in June 2012 from greenhouse complexes of Sistan and Baluchestan University and shade dried. Accurate identification of this plant species was performed by botanical specialists of Department of Biology, Sistan and Baluchestan University. The herbarium specimen (code 1238) is maintained in plant biology herbarium at the University of Sistan and Baluchestan. To prepare the extract, 20 g of stems, leaves and flowers was milled and with 150 ml of distilled water was poured into beaker and placed on magnet shaker for 24 hours. Then the solution was passed through filter paper and dried in incubator at 37C°.

The sciatic nerve compression: this experimental study was performed on 30 male, 3 month old Wistar rats weighing approximately 300–350 g. The experimental rats were purchased from the Medical University of Zahedan and transferred to the animal house of Sistan and Baluchestan University. Principles

of working with laboratory animals were observed. For adaptation, mice were kept for ten days under standard light conditions (12 hours light, 12 hours dark), temperature, nutrition and humidity in the animal house of University of Sistan and Baluchestan. Then they were randomly divided into 5 groups of 6 each: Group I (control), group II (compression group), Group III (compression + treatment at a dose of 25 mg / kg of aqueous extract), Group IV (compression + treatment at a dose of 50 mg / kg of aqueous extract) and Group V (compression + treatment at a dose of 75 mg / kg of aqueous extract). The mice were anesthetized by using ether and ketamine (0.16 cc per 300 g of body weight), the skin and muscles of the right thigh were cut using scalpel and scissors, and the sciatic nerve was compressed severely by using locking forceps for 60 seconds (13). Then muscles and skin were stitched and the operation site was disinfected with Betadine.

After the surgery, experimental groups III, IV, V, were injected with doses of 25, 50 and 75 milligrams (per kilogram of body weight), respectively, of the aqueous extract of Achillea millifolium, which was dissolved in normal saline, intraperitoneally. Groups I and II received only normal saline injections intraperitoneally. The first injection was performed after the surgery and two other injections were given, one week apart, on days 7 and 14 after the operation. On the 28th day, after compression, rats underwent perfusion for removal of the spinal cord. The spinal cord, to the end of the horse's tail, was removed from the spine, and the last 18 mm were cut; then 8 mm from the spinal cord was sampled. Since the sciatic nerve originates from the L4, L5 and first to third sacral nerves, the spinal cord sample incudes the cell body of neurons which form the sciatic nerve (13). Samples were fixed for one week (10% formalin).

Preparing the microscopic sections, staining them and counting the density of alpha motor neurons: in order to obtain histological sections, tissue preparation was performed by dehydrating with alcohol 70, 80, 90, absolute alcohol I and absolute alcohol II, clarifying with butanol 1 and 2, and soaking in paraffin 1 and 2. Then from the spinal cord tissue, paraffin blocks were prepared and then consecutive 7-micron sections were cut from them on a microtome. Sampling was performed by discarding the first 30 consecutive cuts from the first section and then sealing 3 consecutive sections on the slide. Likewise, of any 30 consecutive cuttings, three sections were sealed on glass slides and finally 30 cuts from each spinal cord section were prepared and stained with toluidine blue. In toluidine blue staining the nuclei of the nerve cells are stained purple and the background turns orange. The samples were then photographed and neuronal density of alpha motor neurons in the ventral horn in different groups were counted by dissector method (counting units in volume). The density of neurons is defined as the sum of counted neurons divided by the sampling frame and multiplied by the number of samplings. The area of the sampling frame was 2.5 by 2.5 cm. For actual calculation of this area in microns, a micro-meter slide was used (13). Obtained data were analyzed using SPSS 19 and one-way ANOVA and Student t-test, and $p<0.05$ was considered significant.

Result

The mean and standard deviation of density of motoneurons of ventral horn of the spinal cord in compression group (943±59), control (1620±51.1), group III (1117±188.1), group IV (1235 ± 83.85) and in group V (1421±139.7) were calculated (table 1 and fig. 1). Comparison of density of ventral horn motoneurons of spinal cord between the compression group and the control group showed a significant difference (943±59 vs. 1620±51.1, $p<0.001$). In fact, compression and damage to the sciatic nerve had caused significant reduction in neuron density in the compression group.

Table 1. The density of cell body of alpha motoneurons in ventral horn of the spinal cord in rats in different tested groups.

rat code	Compression	Control	Compression+dose۲۵	Compression +dose ۵۰	Compression +dose۷۵
C1	888	1611	1333	1250	1555
C2	1027	1694	927	1361	1555
C3	916	1583	1250	1377	1250
C4	888	1663	944	1222	1240
C5	934	1611	1277	1111	1444
C6	1000	1555	972	1194	1472

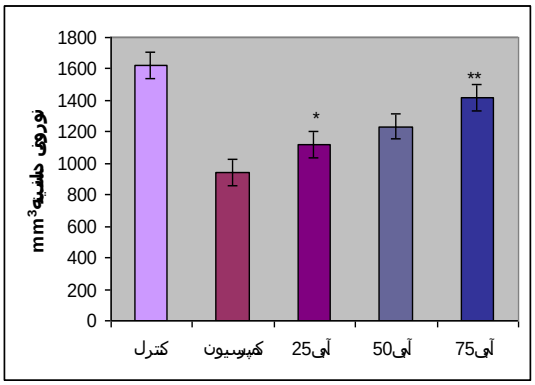

Figure 1. Comparison of average neuronal density between control, compression and treatment (n=6) groups.

But in the groups treated with aqueous extract of Achillea millifolium, the neurons were greatly protected against damage and destruction. Therefore, neuronal density in the groups treated with aqueous extract showed a significant increase compared to the compression group (fig 2-6).

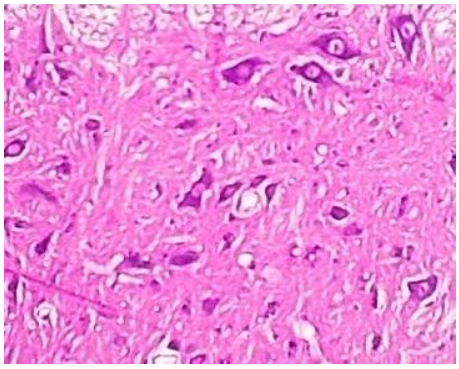

Figure 2. The neurons of the ventral horn in cross-section of the spinal cord in control group, toluidine blue-Erythrosine staining, nucleus is located in the center of perikaryon (magnification x1600)

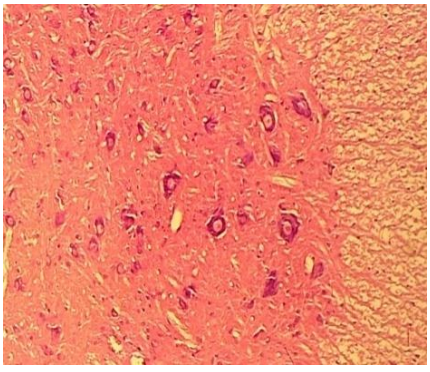

Figure 3. Section of ventral horn of the spinal cord in compression group, the nucleus is pulled aside of perikaryon and it is fading, toluidine blue-Erythrosine staining (magnification x1600)

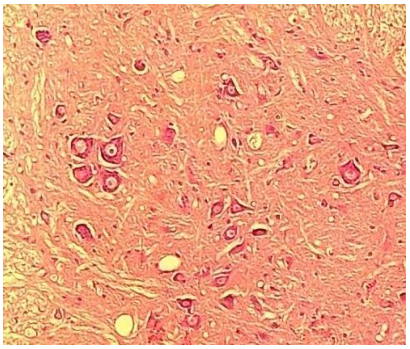

Figure 4. Alpha motor neurons treated with a dose of 25 mg/kg (magnification x1600)

Intraperitoneal administration of aqueous extract of Achillea millifolium with a dose 25 mg / kg in comparison with the compression group significantly increased the neuronal density and protected the neurons (1117±188.1 vs. 943± 59, $p < 0.05$). Increasing the dose of aqueous extract led to higher neuroprotective effect and therefore the density of neurons compared to the compression group was significantly higher, such that the difference between the neuronal density of the compression group and the treatment group increased with a dose of 50 mg / kg (1235±83.8 vs. 943±59, $p < 0.01$). The highest density of neurons was seen with a dose of 75 mg / kg of the aqueous extract in comparison with the compression group (1421±139.7 vs. 943±59, $p < 0.001$).

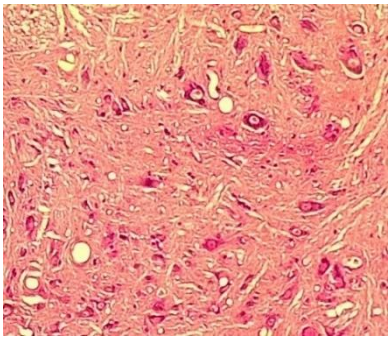

Figure 5. The alpha motor neurons treated with a dose of 50 mg/kg (magnification x1600)

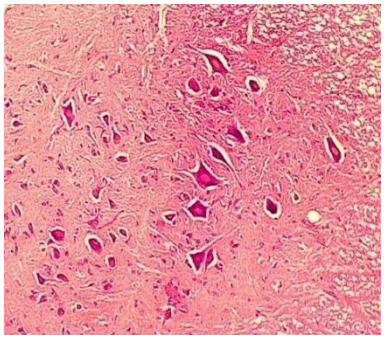

Figure 6. Alpha motor neurons treated with a dose of 75 mg/kg (magnification x1600)

Intraperitoneal administration of aqueous extract of Achillea millifolium with a dose of 25 mg/kg significantly increased the neuronal density as compared with the compression group, and protected the neurons (1117±188.1 vs. 943±59, $p<0.05$). Increasing the dose of the aqueous extract led to higher neuroprotective effect and therefore the density of neurons was significantly higher compared to the compression group, such that the difference of neuronal density between the compression group and the treatment group increased with a dose of 50 mg/kg (1235±83.83 vs. 943±59, $p<0.001$). The highest density of neurons was observed with a dose of 75 mg/kg of the aqueous extract in comparison with the compression group (1421±139.7 vs. 943± 59, $p <0.001$)

Discussion

In this study, injection of aqueous extract of Achillea millifolium (25, 50 and 75 milligrams per kilogram of body weight) could significantly protect the density of alpha neurons of ventral horn of the spinal cord compared to the compression group. The neuronal density in the compression group had significant reduction compared to the treatment groups. Studies have shown that A.millefolium is rich in phenolic compounds such as flavonoids and phenolic acids (14, 15). These antioxidants are natural compounds whose sweeping effects on free radicals have been proven. It seems that in the present study the significant neuroprotective effect of the aqueous extract compared to the compression group is due to these antioxidant effects of this plant.

Free radicals are produced in small quantities under physiological conditions and are immediately consumed or decompose. However, their excessive and prolonged production, destroys biological structures and causes DNA damage and cell death (16). Overproduction of reactive oxygen species and pre-inflammatory cytokines causes oxidative stress. Candal and colleagues demonstrated that A. Millefolium oil extract in vitro has high antioxidant activity and low antimicrobial activity (17).

Studies by Lopes and his colleagues clarified the mechanism of action of this plant extract. Mentioned studies showed that peritoneal macrophages in the presence of essential oil of A.millefolium produce an ordinary amount of H_2O_2, nitric oxide and TNF-α cytokines, without leading to the overproduction of these compounds. They concluded that this plant's compounds can regulate the activity of macrophages (16).

Other important factors in superior protection of neurons by aqueous extract of A.millefolium are the anti-inflammatory effects of this plant. In traditional Brazilian medicine, this plant has been used for treatment of respiratory infections, fever and rheumatic pain. The main compounds of this plant are azolen, cineole, borneol, pinene and camphor (16). Anti-inflammatory effects of this plant are mainly due to compounds such as azolen.

Research by Chou and colleagues showed that the anti-inflammatory activity of A.millefolium was due to the reduction of expression of enzymes such as cyclophosphamide oxygenase-2 and oxygenase-1, and also due to reduction of tumor necrosis factor -α and interleukin-6 (18). Borneol is a terpenes compound that easily crosses the blood-brain barrier and helps the absorption of compounds through the blood-brain barrier in the brain. Its neuroprotective effects are through reducing intracellular pathways regulating inflammatory factors iNOS / NO, decreasing the release of factor NF-κBP65, and decreasing apoptosis-related caspase (19).

Flavonoids, through phosphodiesterase inhibition, prevent the degradation of cAMP, and thus can prevent platelet adhesion, aggregation and discharge. In addition to blood clotting effects, platelets affect inflammation, by releasing many intermediaries involved in inflammation, such as thromboxane A2 and the platelet-activating factor. Many of the anti-thrombotic, anti-inflammatory and anti-spasmodic properties of the yarrow plant could be due to flavonoids. Of course, the kamazolen content of this plant has anti-inflammatory properties. The sesquiterpenes, highly oxygenated lactones, do not change to azolen by distillation, and they are more effective anti-inflammatories than the respective pro-azolens (20, 21). The third neuronal protective factor in A.millefolium maybe its estrogenic effects. In traditional medicine, this herb is prescribed to induce menses.

These effects could be due to the estrogenic characteristics of this plant, especially since studies showed that pure extract of aerial parts has estrogenic activity (22). Purification of extract compounds showed that luteolin 5 and epigine 6 were the most important estrogenic compounds among various tested components of this plant. Epigine can stimulate both α and β estrogen receptors, but it is less effective than

body's own estrogen. Luteolin does not stimulate α receptors at all and has little effect on β receptors (22). Ali mohammadi and colleagues showed that phytoestrogen compounds of the plant have neuroprotective effects after permanent occlusion of the middle cerebral artery in Syrian mice undergoing oophorectomy (23). The aqueous extract of A.millefolium, probably due to anti-antioxidant, anti-inflammatory and estrogenic properties, prevents the progression of inflammation, after peripheral nerve injury caused by compression and rebound reactions of these lesions to the alpha motoneurons, of ventral horn of the spinal cord.

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