Neuroprotective effects of *Cannabis sativa* leaves extracts on α-Motoneurons density after sciatic nerve injury in rats

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Abstract : *Cannabis Sativa* plant has many pharmacological properties. This study is aimed to investigate neuron-protective effects of extracts of this plant's leaves on α-Motor neurons in spinal cord of rats after sciatic nerve injury. Animals were divided into 6 groups (in each group N=8) ; A: control, B: compression, C: compression + treatment with a dose of 25 $mg(kg)^{-1}$ alcoholic extract, D: compression + treatment with a dose of 50 $mg(kg)^{-1}$ alcoholic extract, E: compression + treatment with a dose of 25 $mg(kg)^{-1}$ aquatic extract, and F: compression + treatment with a dose of 50 $mg(kg)^{-1}$ aquatic extract. After sciatic nerve compression, extract injection was done intra-peritoneal in treatment groups within 2 weeks (once a week). 28 days after compression, lumbar spinal cord was sampled and neuronal density of each group was compared with compression group. Neuronal density showed a significant difference in control and compression groups ($P<0.001$). Neuronal density had a significant increase in treatment groups compared with compression group ($P<0.001$). Aquatic and alcoholic extracts of *cannabis sativa* leaves have protective effects on α-Motor neurons which is probably due to antioxidant and anti-apoptotic factors in the plant extracts.[Bibi Zahra Javad Moosavi, Maryam Tehranipour, Mahtab Mollashahi , Homa Mahmoodzadeh**, Neuroprotective effects of *Cannabis sativa* leaves extracts on α-Motoneurons density after sciatic nerve injury in rats**. *Life Sci J* 2013;10(5s):644-648] (ISSN:1097-8135). http://www.lifesciencesite.com. 113

Key words: degeneration, neuron-protective, cannabis sativa, sciatic nerve, rat

Introduction

Neurons are injured under physical, chemical, and pathological conditions. Most neurons are amitotic (do not divide) and their injury is considered a permanent damage [1]. When a nerve fibre is cut or crushed, axon is separated from cell body and its distal segment starts degeneration from the lesion to the end. In addition, degeneration continues to the first Ranvier node and towards proximal. This process is called Wallerian degeneration [2]. Degeneration is accompanied by some symptoms such as lack of nerve conduction, axonal skeleton disintegration, and axolema breakage [3]. During this process myelin sheath sustains degenerative changes [4,5]. If proximal damage is severe, the effect of lesion develop toward neuron cell body and cause central (retrograde) degeneration [2]. Here Nissl body is disintegrated, nucleus changes its central position toward cell periphery and cell body is swollen [5]. Most axonal injuries are accompanied by the release of calcium signals [6]. In some axonal injuries which lead to acute axonal degeneration, separation of proximal and distal end occurs quickly and within 30 minutes after injury [7]. The nerve fibre neurolemma does not degenerate and remains as a hollow tube. During neuronal repair process, Schwann cells are reproduced and fill inside endoneural tubes. Then the proximal end of nerve fibre sends out sprouts towards these tubes. Bulbous shape buds are produced by growth factors and directed toward endoneural tubes by Schwann cells. They grow about 1-3 mm a day in these tubes [2]. Cannabis is an annual herbaceous plant belonging to Cannabaceae family. The leaves are palmate compound with serrate leaflets [8]. This plant produces a unique family of terpeno-phenolic compounds called cannabinoids. Two major cannabinoids are delts-9-tetra cannabinol (THC), and cannabidiol (CBD) which is psychoactive [9]. Today cannabis and its synthetic derivatives are used to treat a wide range of diseases [10]. This plant has different potential therapeutic uses, so that in the United States cannabis and cannabinoid receptor agonists are used for treatment of glaucoma, neurological diseases such as epilepsy, depression and bipolar disorders, anorexia, weight loss, movement disorders, bronchial asthma, inflection, inflammation and injuries of spinal cord, and reducing neuropathic pains [11,12]. Considering this plant's wide range of applications in medicine especially in nervous system treatment and given that researches conducted over the past few years have mostly focused on pharmacological properties of sativa extracts obtained from the leaves of flowering plants or synthesized derivatives and no comprehensive research has been done on the effects of aquatic and alcoholic extracts of cannabis leaves, this study is aimed to determine the effects of aquatic and alcoholic extracts of cannabis leaves on degeneration of alpha motor-neurons in anterior horn of spinal cord after sciatic compression in rats.

Materials and methods

Cannabis leaves were supplied commercially and confirmed by Dr Jafari at the herbarium centre of Islamic Azad University of Mashhad (IAUM) with herbarium number 2548. Cannabis leaves were completely ground by a grinder. Alcoholic and aquatic extracts were prepared using soxhlet extractor model H626. First 50 grams drained powder of cannabis leaves was put into filter paper and placed in the extractor. 450 cc of ethanol was used as alcoholic extract solvent and 450 cc of distilled water as aquatic extract solvent. At the end, solvents were removed from both extracts. Forty-eight albino wistar rats weighing 300-350 grams were included to the study. The rats were held in department of biology, Islamic Azad University of Mashhad. They were kept in a temperature of 21 degrees centigrade, humidity of 50%, and a cycle of 12 hours light- dark. Moreover, all of them had access to sufficient food and water. Moral protocols were also observed while working on animals. Animals were divided into 6 groups of eight. A: control, B: compression, C: compression + treatment with a dose of 25 mg alcoholic extract per kg body weight, D: compression + treatment with a dose of 50 mg alcoholic extract per kg body weight, E: compression + treatment with a dose of 25 mg aquatic extract per kg body weight, and F: compression + treatment with a dose of 50 mg aquatic extract per kg body weight. The rats of each group were anesthetized under intra-peritoneal injection of 60 mg/kg Rompun and 6 mg/kg Ketamine [13]. After removing animal hair on right femur, skin was cut for 2-3 cm and femoral muscle underwent surgery in order to find sciatic nerve. In the next stage, compression of right femur sciatic nerve was done for 60 seconds using locker pincers (second lock). After compression, the injured part was sterilized and stitched. In treatment group the first stage of extract injection was done immediately after compression. After consciousness, the rats were transferred into separate cages and kept under standard conditions. Second stage of extract injection was done one week after the first injection [14]. After 28 days of compression, using perfusion method animals tissues were fixed and then sampling was done from lumbar spinal cord [13]. Spinal was taken out of spinal column to cone medullary end and then after going 18 mm up cone medullary, 8 mm samples were provided. Samples were kept in fixator for two weeks and then entered tissue passage which included three steps: dehydration of tissue (using alcohol), transparency (by xylene), and soaking in paraffin. Cutting was done with microtome set, so that 7 micron cuts were transferred to glass slides out of each 30 cuts. The work continued until 30 slides were prepared. The slides were then stained with toluidine blue [15]. Some photos were provided from spinal anterior horn in the right part of glass slides. Dissector method was used to count alpha motor neurons of right anterior horn. In this method neurons are counted in a reference framework. When a particle is in the reference framework but is not present in the next frame (next consecutive cut), it is counted. However, if a neuron is present in both frameworks, it is not counted [16]. Neuron density was estimated as below:

$ND=\Sigma Q/\Sigma$ frame $\times V$ dissector

In which:

ΣQ: total neurons counted in one sample

Σ frame: Total number of sampled cases in a sample

V dissector: volume of sampling frame which equals:

V dissector= A frame × H

A frame: the area of sampling frame

H: distance between two consecutive slices, or depth of each cut

Data was analysed using Minitab13 software, Tukey and ANOVA statistical tests (for binary comparison). Tests significance level was considered less than 0.05 [15].

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Results

Based on the results of this study, average and standard deviation of alpha motor neurons in control and compression groups was respectively 1633.4 $\pm$ 30.7 and 611.5 $\pm$ 34.2. There was a significant difference in neuron density of two groups ($P<0.001$) (figure1).

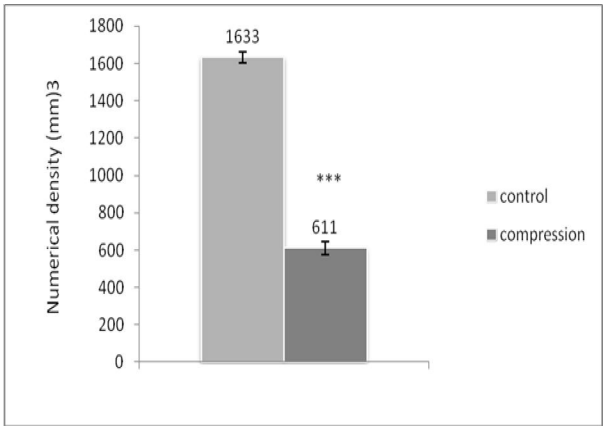

Figure 1. Comparing density of alpha motor neurons in spinal anterior horn between Group A: control and B: compression Average density of alpha motor neurons was 1179.5 $\pm$ 22.9 and 1060.9 $\pm$ 20.4 in treatment groups C and D.

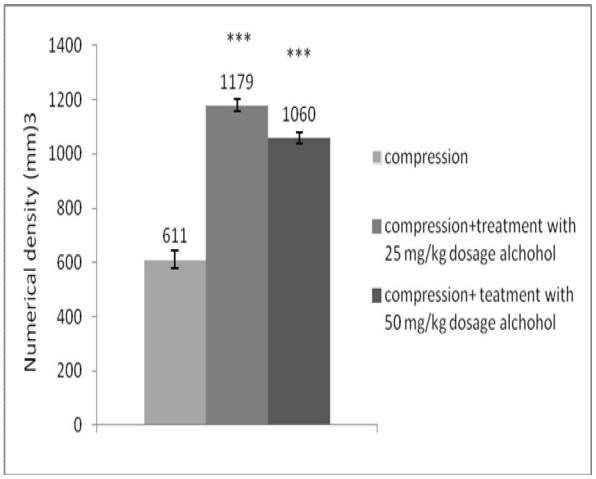

Figure 2. Comparing density of alpha motor neurons in spinal anterior horn in groups B: compression and C: compression + treatment with a dose of 25 mg alcoholic extract per kg body weight, groups B: compression and D: compression + treatment with a dose of 50 mg alcoholic extract per kg body weight

Comparing density of alpha motor neurons in these two groups and compression group showed a significant difference in each group ($P<0.001$) (figure2).

Average density of alpha motor neurons was respectively 1278.6 $\pm$ 28.1 and 1549.8 $\pm$ 28.7 in treatment groups E and F. These groups showed a significant difference compared with compression group ($P<0.001$) (figure3).

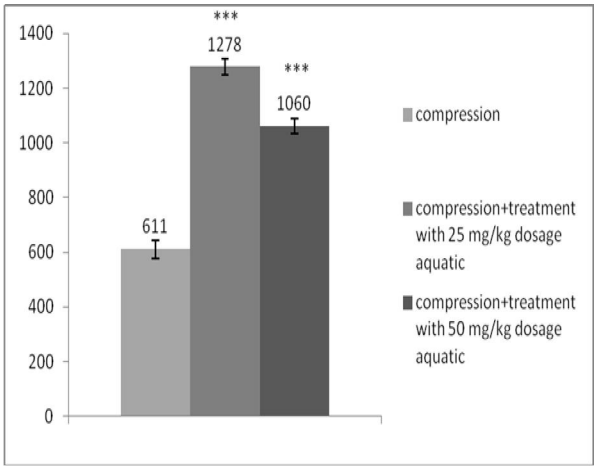

Figure 3. Comparing density of alpha motor neurons in spinal anterior horn in groups B: compression and E: compression + treatment with a dose of 25 mg aquatic extract per kg body weight, groups B: compression and F: compression + treatment with a dose of 50 mg aquatic extract per kg. body weight.

All photos are shown that in experimental groups the neurons have very normal shape in compare with compression group (Fig.4).

Discussion

For many centuries Cannabis sativa products (marijuana) have been used for different purposes like medicine and recreation [17].Research shows that spinal cord injuries lead to apoptosis and cause cell death [18]. Kinugasa and colleagues (2002) showed that it causes axotomy and induces apoptosis

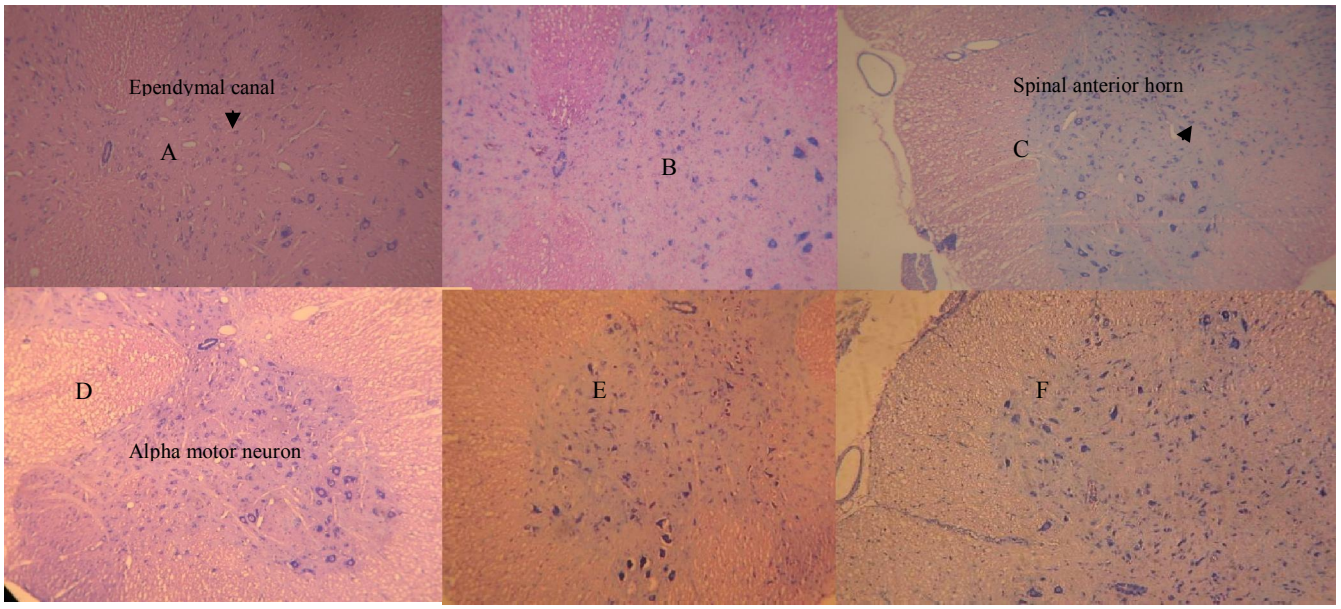

Figure 4. Cross-sectional representation of spinal and alpha motor neurons of spinal anterior horn in the right part of different groups (toluidine blue staining, zoom X 400) A: control, B: compression, C: compression + treatment with a dose of 25 mg alcoholic extract per kg body weight, D: compression + treatment with a dose of 50 mg alcoholic extract per kg body weight, E: compression + treatment with a dose of 25 mg aquatic extract per kg body weight, and F: compression + treatment with a dose of 50 mg aquatic extract per kg body weight

nerve crush [19]. Our study is also consistent with this fact. As it can be seen, neuron density had a significant decrease in compression group compared to control group. This means that animal's sciatic nerve compression leads to emergence of retrograde degeneration in the central cell body of motor neurons in anterior horn of spinal cord. Thus, neuronal density showed a significant decrease in compression group compared with control group (figure1). Data analysis also indicates that there is a significant difference between neuron density in compression group and treatment groups C and D (25 and 50 mg/kg alcohol) (figure2). Comparison of neuron density between compression and treatment groups E and F (25 and 50 mg/kg aquatic extract) also represents a significant increase in neuron density of treatment groups (figure3). This fact confirms neuron protective effect of alcoholic and aquatic extracts of cannabis sativa leaves. Recent evidence shows that endo-cannabinoid derivations are useful for reducing the effects of neuron degeneration in nervous system. In fact internal and external cannabinoids prevent calcium secretion into neurons, increase expression of neurotropic factors, raise blood supply to the affected areas and also increase antioxidant activities. In this way they can protect neurons [20,22]. Given above information, cannabinoids can be considered as neuron protective molecules. They have excellent antioxidant properties. In fact cannabinoids don't do their neuron protective function against glutamate toxicity through receptor-dependent pathways, induce their effects by their antioxidant properties. Antioxidant effects of these compounds are applied through ascorbate and alpha tocopherol which enable cannabinoids to deal with glutamate and oxidative damages [23]. Therefore it is likely that one of the main mechanisms of cannabis sativa extract (containing cannabinoid) is its antioxidant property which increases neuron density in treatment groups compared to compression group. Cannabinoids also reduce inflammation in affected areas so that in early stages after injury, secretion of inflammatory and main cytokines such as TNF-α necrosis factor, interlukin-1 beta, and interlukin-6 is prevented [23]. Thus another effective mechanism by which alcohol and aquatic extracts of cannabis leaves can prevent cell death after sciatic nerve compression is reducing inflammation in affected areas. Increasing blood flow to affected areas is another mechanism that may cause neuronal survival. According to researches cannabinoids prevent stimulated reactions of endothelin1 (which can cause vasoconstriction) and increase blood flow to injured areas by reducing vasoconstriction [24]. Therefore it can be said that clinically cannabinoids act as neuron protective molecules with mentioned mechanisms, and thus neuron density shows a significant increase in treatment groups with alcoholic and aquatic extracts of cannabis sativa leaves (containing cannabinoid) compared to compression group. According to other studies, cannabinoids use two main mechanisms to protect neurons:The first mechanism is independent of cannabinoid receptors and aims to reduce oxidative damage. The second mechanism involves stimulation of cannabinoid CB2 receptor expression which especially happens in response to microglial and is able to adjust the impact of glial cells on neuronal haemostasis [21]. As it was mentioned before, researches conducted over the past few years have mostly focused on pharmacological properties of sativa extracts obtained from the leaves of flowering plants or synthesized derivatives. In the present study which was done on extracts obtained from sative leaves, it was found that these extracts have neuron protective effects like similar compounds.

Conclusion

It seems that alcoholic and aquatic extracts of cannabis sativa leaves have effective substances that prevent calcium penetration into axons and ensure neurons survival by reducing inflammation and increasing blood flow to affected areas. In addition, this substance also increases neurotropic factors and may thereby cause restoration of damaged neurons. Results of this survey show that alcoholic and aquatic extracts of cannabis sativa leaves are neuron protective and increase the density of alpha motor neurons in anterior horn of spinal cord after sciatic nerve compression in rats. The increase correlates with the amount of extract injection. Therefore it can be used for treatment of neurodegenerative diseases.

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