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Aloe Vera's Effect on Acetyl Cholinesterase(AChE) and Ultra Structure of Motoneurons in Synaptic Zone After Spinal Cord Injury in Rats

Marjan Heshmati[1]*, Mohammad Reza Jalali[2], Nadoushan[3] and Maryam Sharayeli[3]

[1]Department of Anatomical Sciences and Patholog ,Faculty of Medicine, Shahed University , Opposite Holy Shrine of Imam Khomeini Khalij Fars Expressway Tehran, Iran.
[2]Mostafa Khomeyni Hospital,Faculty of Medicine,Shahed University,Tehran,Iran.
[3]Department of Anatomical Sciences and Patholog ,Faculty of Medicine ,Shahed University, Opposite Holy Shrine of Imam Khomeini Khalij Fars Expressway Tehran, Iran.

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Spinal cord injury(SCI) followed by transection, contusion or compression are considered to be similar to human SCI.Nowadays researchers demonstrated alternative therapies as pharmacological properties or herbs to repaire these damage.Aloe Vera is known as a plant with multi potential activity.We used this plant after SCI by Clips Aneurysm(30g /1 min). 32 female Sprague Dawley rats were divided randomly into four groups.Group 1: Sham(laminectomy) + gavage Distilled water, group2: Sham +gavage Aloe Vera gel powder(200mg/kg/d), group3: group 2+SCI, and group4:group 1+SCI (N=8).After 4 weeks they were sacrificed.Morphometric study and Acetyl cholinesterase(AChE) immune staining were done.Synaptic changes was analyzed in a blinded manner for qualitative ultra structural changes.The data analyzes with Tukey's test and one-way ANOVA in SPSS21 software and P≤0.05 was considered as significant level.Decreasing motoneurons ,reducing AChE immune activity with ultrastructure changes in mitochondria and synaptic zone were seen due to SCI.Usage of Aloe Vera gel powder demonstrated a reduction in death of motoneuron(Pd"0.05) with increasing AChE immunoreactivity after SCI(Pd"0.05).This herb affected on ultrastructure as seen pathological synaptic changes and mitochondria decreases.Aloe Vera gel powder effects might be due to it's antioxidant which has reduced neuronal cell death and preserved damaged neurons as intact neurons.

Key words: Aloe Vera, Spinal Cord Injury, Synapse, Rat.

Spinal Cord Injury (SCI) causes complex patterns of secondary destructive biochemical and pathophysiological processes that result in extensive tissue damage and often permanent loss of function[1]. Various procedures have been reported to reduce the effects of this process. SCI is one of the critical and devastating injuries and causes disability and dysfunction[2].As one of the gravest diseases affecting central nervous system (CNS)and ranked among the most costly disorders, because those who suffer from these disorders are affected not only with the sensory motor difficulties arising from the lesion proper, but are faced in their lifetime with numerous debilitating syndromes as well[3]. Nowadays achievements in neurobiology and mechanisms involving in the neuroregeneration with the idea that using chemical drugs and natural compounds of herbal origin with neurotherapeutic

* To whom all correspondence should be addressed.
Tel.: +98 21 88964792; Fax: +98 21 88966310;
E-mail: heshmatimarjan@hotmail.com
heshmati@shahed.ac.ir

properties.So far various procedures have been reported by the researches to treat the secondary spinal cord lesions[4].Further to clinical studies,pharmaceutical and non-pharmaceutical methods are note worthy. As a result of the increasing statistical frequency of the accidents, the workers have come up with the idea of presenting practical therapeutic patterns for those suffering from these lesions whose ultimate objective is to find an applied , simple and inexpensive pattern for materialization of this goal[5-6]. Along with new achievements involving in the neuroregeneration phenomen on based on biological models of spinal cord lesions, the researchers have come up with the idea that using chemical drugs and natural compounds of herbal origin with neurotherapeutic properties could be a break through to new research avenues. Given the new advancement in the recent years[7]. The transmission of nerve impulse at synapses is by chemical or electrical. In the chemical mechanisms the transmission takes place by means of neurotransmitters which induce the transmission of impulse from a neuron to the next. Several neurotransmitters have been identified among which acetylcholine, norepinephine, dopamine, glycine, serotonin, GABA, enkephalin, substance P and acid glutamic are note worthy[8]. Acetylcholine is one of the most important neurotransmitter ,it is transported by acetylcholine vesicle into the synaptic cleft.

Acetylcholine deficiency can cause any of these symptoms:

Alzheimerõs disease ,Anxiety, Arthritis, Autism, Multiple Sclerosis ,Involuntary movement… it is synthesized from acetyl co-enzyme A and choline by acetylcholine transferase. Once discharged into the synaptic cleft and binding to the ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. Acetyl cholinesterase(AChE), is localised in cholinergic cell bodies and axons it is synthesized in presynaptic neuron and stored in the axons. Myelinated axons contain less acetylcholine esterase. It is worth mentioning that acetylcholine esterase affects the growth of the axon[9].It is anchored in presynaptic membranes of the cholinergic neuron. Also, it is found in postsynaptic membranes and in the synaptic cleft [10]. However, the occurrence of AChE in non-cholinergic cells has been frequently reported[11-13].AChE, terminates the signal transmission by hydrolyzing ACh. The liberated choline is taken up again by the pre-synaptic nerve and ACh is synthetized by combining with acetyl-CoA through the action of choline acetyltransferase(ChAT). Relevant receptor, it is broken to acetate and choline by the AChE. AChE is found at mainly neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission. It belongs to carboxylesterase family of enzymes. It is the primary target of the nerve agent sarin[14]. In the study made by Manolov and Davidoff AChE was introduced as a marker to investigate the mode of action of cholinergic system at the ventral ramus[15]. In SCI, there is a marked reduction in expression of AChE[16].Study indicate following neuronal damage in midbrain the expression of AChE -receptor is increased which is due to the reduction AChE[17]. Cherian et al. demonstrated that in spinal cord injury, the rate of AChE is decreased whereas use of dexamethazone increases it[16].One of the frequently used methods nowadays is the use of chemical or herbal drugs.Among these drugs, Prednisolone[18] and Dexamethasone[19] are noteworthy .The chemical or natural compounds are used to prevent the apoptosis of neurons,on the other hand to prevent the glial reaction after neuronal damage.Recently, it is supposed that the use of natural compounds with herbal origin, instead of the chemical drugs,has simplicity and more benefit . Aloe Vera, one of the acient medicinal plants recently is being study because of itõs multiple effects[20]. Aloe Vera or A. barbadensis belongs to Liliaceae and has been extensively used as a medicinal plant and is usefull for wound healing[21].This plant has many medicinal and curing properties and is classified among plants which work wonders[22-23]. In the Iranian traditional medicine it is rated as a plant with warm and dry properties and is presently used to treat a plethora of diseases like migraine, indigestion, acne , internal and external wounds[24-25]. In addition, it stimulates dermal fibroblasts,to promote synthesis of collagen and elastin and to increase skin elasticity[7]. Gelatinuous material found in aloe leaves contains 96% water whereas the balance 4% contains many compounds of which 75 types have been identified[26].The compounds found in aloe gel are

mainly polysacharides which decrease inflammatory and induces skin growth and regeneration[27] responsible for regenerating mechanisms , antibacterial , antimicrobial , Anti oxidant[28-29] and Anti cancer[30]. Furthermore, aloe gel contains a glycoprotein which prevents swelling and pain while accelerating the recovery. Anthraquinone found in aloe has efficient purgative properties[29]. For demonstrate Aloe Vera effects on regeneration after nervous system injury, we damaged the neurons by the use of the method of Clips Aneurysm, which introduced by Dolan and Tator ,they proposed aneurysm clips for mechanical pressure on the spinal cord in order to damage neurons in the means of evaluate pathological effects[31].

MATERIALS AND METHODS

Animals and Surgical procedure:The animal care and all experimental procedure were done according to ethical guidelines established by the Shahed University. 32 young adult female Sprague-Dawley rats weighing 200-250 g, from Razi Institute (Karaj,Iran)were purchased. All rats were fed and kept, for a week, under the standard conditions of 12-hour light/ dark cycle , temperature of 22°-23° C with free access to food (Pars Animal Feed Co. -Iran) and water. Then they were randomly devided to 4 groups: group1: Sham + gavage Distilled water, group2: Sham +gavage Aloe Vera gel powder, group3: group 2+SCI, and group4:group 1+SCI (N=8) .SCI were done by Clips Aneurysm after anesthetizing the animals (ketamine 100mg/kg / xylazine 5mg/kg, ip) and exposure of the spinal cord through laminectomy, an extradural compression was applied at T6 level 30 g by clips aneurysm(Cadman, Johnson and Johnson Inc/ catalog No. 20-1264). Which was done by using clip applicator for 1 minute. Incision site of muscles,fascia and skin were separately sutured with 6-0 and 3-0 suture treads. Cephazoline and 5-8 ml Lactate serum was administered intraperitoneally if needed Animals were maintained at an ambient temperature of 25-27°C[31]. Manual compression of bladder was performed three times daily and all efforts were done to prevent bladder infection and any injury due to the sensory disorders. Daily administration of Aloe Vera gel powder (200mg/kg/d)or distilled water was given by gavage base on their groups for four weeks. The sham groups were only subjected to laminectomy without any compression applied on the cord. After 4 weeks (28 days)animals were sacrificed by lightly anesthetized with ketamine/ xylazinein ,cardiac perfusion was done. T9 vertebra extracted and transferred to fixative according to their protocol as: motoneuron counting and immunohistochemical technique (N=4), ultrastructural studies(N=4). Aloe Vera gel powder: Aloe gel powder was prepared by the procedure prescribed in the paper presented by Misawa *et al*.[32].We used fresh leaves of Aloe Vera from a single garden plant, identification of leaves was done by herbarium of the department of Acient Medicine of Shahed University Iran. Leaves were washed by water ,the inner gel was obtained by clean sharp knife.By hot air drying the gel were powdered and suspended in distilled water as a homogenized to 50 mg/ml ,sterility was cheked in Microbiology department and stored in dark container at -20°C for later use.

Preparation samples

For light microscope, spinal cord was removed and fixed in 10% formalin solution and the tissues were processed for paraffin embedding, by using microtome rotary(Leica 820)for 6 micrometer diameter slices. It is worth mentioning that to monitor modifications in neurons and their precise location in ventrolateral ramus, so the spinal cord sections were randomly colored by routine hematoxylin eosin technique.The serial sections were stained with Cresyl Violet (0.1%) (Merck/Germany) for cell counting.. Motor neurons in ventrolateral ramus with nucleus larger than 10 micrometer and with one distinct nucleolus were counted.Total motoneurons of both sides of spinal cord in each relevant segment were counted according to Bao and Liu[33]. Immunohistochemistry technique was done for demonstrating . AChE in ventrolateral motoneuron's. Totally 20 cross-sections were prepared for this step. They were labeled with , mouse anti- AChE (Chemicon International) as primary antibody and procceed by goat anti-mouse (Chemicon International) as the secondary antibody. Upon application of DAB on slice, DAB reacts with peroxidase in the secondary antibody and gives rise to a brownish color. As explained above, the rate and intensity of acetylcholine esterase is the main objective of this

study and, by the same taken, this enzyme depicts the modality of rate and hydrolysis of acetylcholine as a neurotransmitter. Based on the manifestation patterns, the assay is performed as a percentage in neurons counted. For electron microscope,in means of ultrastructure study,the spinal cord tissue were perfused by Karnovsky's fixative for 24 h. and immersed in 2.5% glutaraldehyde in phosphate buffer (0.1 M,PH 7.4) and post- fixed in 1%osmium tetroxide in phosphate buffer.Sections were cut,stained with uranyl acetate and lead citrate and examined under Zeiss EM 900(Germany).

Data analysis

The data were tested for normality using S-K test and analysed by using Student's t-test and one-way ANOVA with software SPSS Ver 21.The significance level ($p \leq 0.05$)for motoneuron counting and ($P \leq 0.05$) for AChE labeling.

RESULTS

The survey of motoneurons in the ventrolatral ramus indicated that in two groups which received mechanical pressure in addition to laminectomy, the decrease in motoneurons in the ventrolateral ramus is quite visible (Fig 1).The results of the motoneurons counting at the location of the pressure was exerted, in other words, Nissl bodies are greatly destroyed and cytoplasm is clear in the perimeter of nucleus with apoptotic bodies (Fig 2).

Whereas in the groups 1 and 2 which had been subjected to no pressure, the number of motoneurons differed [2306 ± 140 in the group 1 compared to 2451 ± 100 in the group 2] however it was not statistically significant. Furthermore, comparison of these two groups (1 and 2) with the other ones (3 and 4) which had received mechanical pressure with Aloe gel powder or distilled water, there was a significant difference (Table 1). The comparison results in terms of total cell count of motor neurons between the two groups 3 and 4 which had received mechanical pressure indicated that in the non-treated group, there is a significant decrease in motor neurons ($P < 0.05$).In other words, in the group which had received Aloe Vera gel powder for 4 weeks, motoneurons have been preserved ($P < 0.05$) .

Motoneurons in antrolateral horn of spinal cord in groups (Table 1), indicate in group 2 they are more than three other groups.Group 2 was used as a reference to evaluate differences. Percentage of reduction have been calculated (Tabel 2).

Table 1. Mean and SD of motoneurons in the anterolateral horn of spinal cord

Statistical Analysis	Mean & SD motoneurons located in lesion area	groups
$P < 0.05$	2306 ± 140	1: Sham + Distilled water
$P < 0.05$	2451 ± 100	2: Sham + Aloe Vera
$P < 0.05$	*1389 ± 137	3: SCI + Aloe Vera
$P < 0.05$	# * 893 ± 108	4: SCI + Distilled water

*Significant difference group 3 and 4 with two groups 1 and 2 ($P < 0.05$).
Significant difference group 4 with other groups($P < 0.05$).

Table 2. Percentage of reduction motoneurons in antrolateral horn of spinal cord relative to group 2

Study Groups	Percentage of decrease
Group 1	5.9%
Group 2	0
Group 3	*37%
Group 4	#*57%

*Significant difference with group 1 and 2 ($P < 0.05$).
Significant difference with group 1 , 2 and 3($P < 0.05$).

Table 3. Percentage of motor neurons responsive to immunohistochemical staining for AChE compared to the total motor neurons

	Group 1	Group 2	Group 3	Group 4
Acetylcholine esterase	85%	88%	*36%	*#21%

*Significant difference with group 1 and 2 ($P < 0.05$)
#Significant difference group 4 with other groups ($P < 0.05$) .

Results of Light Microscopy (Immunohistochemistry)

The effect of Aloe Vera gel powder on expression of AChE was assessed by immunohistochemical technique.To this end, the rate of it, was evaluated. By using this technique, the final reaction product of AChE was visualized as a brownish precipitate in the perikaryon of the neuron.By this procedure, those motor neurons in ventrolateral ramus which were positive in terms of AChE reaction were counted (Fig. 3) then the percentage of positive neurons calculated (Table 3).

Result of Electron Microscopy (ultrastructure study)

Induce of SCI synaptic changes were visible , reduction of electron density in active zone with irregularity in pre and post synaptic membrane ,collapsed synaptic cleft with low electron density in the synaptic active zone. Dispressed synaptic vesicle with mitochondria swollen from moderately to severely with

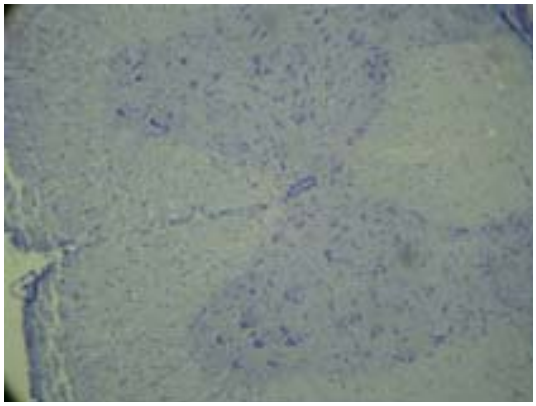

Fig. 1. Cross section of spinal cord tissue in rat ,Cresyl Violet staining. Gray and white matter are seen after mechanical pressure application,decline in number of motoneuron are visible (X 40)

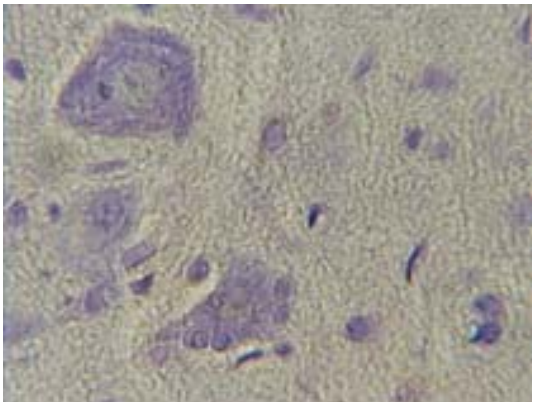

Fig. 2. Cresyl Violet staining.Chromatolysis of motoneuron with apoptotic bodies are visible (X 100)

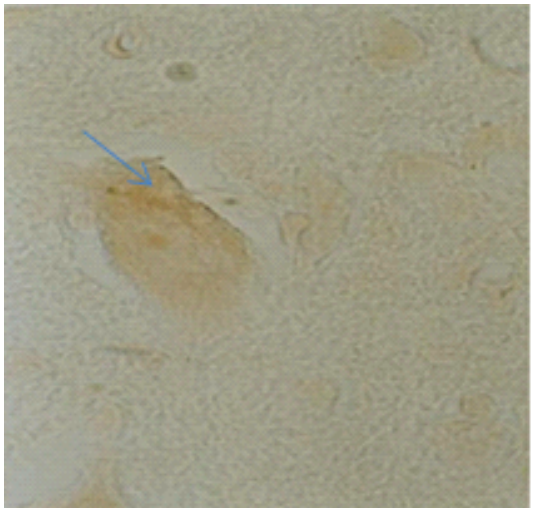

Fig. 3. Arrow indicated a motoneuron by immunohistochemical staining for AChE in Group 1 (X 100)

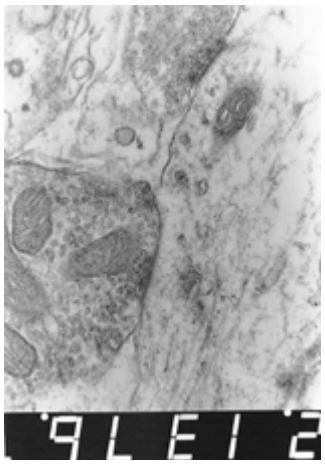

Fig. 4. An electron micrograph of synapse in a motoneuron from the spinal cord after SCI. which shows displacement of the synaptic vesicles ,collapsed synaptic cleft with low electron density in the synaptic active zone (X 35000)

irregularity in outer and inner membrane and poorly defined cristae(Fig 4).Increased mitochondria matrix density with some vesiculated body in it(Fig 5).These changes are most visible in group 4 which has received compression with out any treatment.Induce of Aloe Vera gel in group 3 these changes reduced visibly.

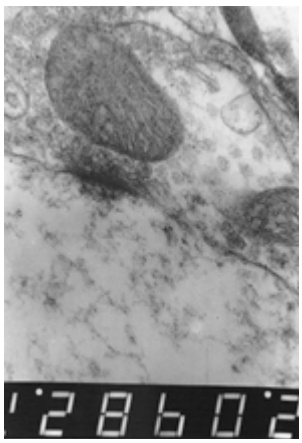

Fig. 5. An electron micrograph of synapse in a motoneuron from the spinal cord after SCI which shows irregularity in pre and post synaptic membrane,with mitochondria vesiculated and swelling ,with poorly defined cristae and larger than normal (X 50000)

DISCUSSION

We used SCI as described earlier by Krishna et al., in animal model training[34].Results in this study showed motoneuron reduced after SCI , which is consistent with the findings of other investigators who reported SCI causes motoneuron death (4 , 5 and 16).After SCI apotosis took place and reported it begin after 6 hours and continue to 3 weeks[35] in another study reported it reaches maximum second or even third day after injury[36]. Cherian *et al*. reported a decrease in phospholipid rate within the first 24 hours following the spinal cord lesion as well as a decrease in acetylcholine esterase within one week following it in adult rats[16]. In our study comparing third with fourth group, in group fourth motoneuron loss are significant. Group first and second which were as groups with no having any pressure ,total cell count was as the same as with no statistically significant. It means in group second which received Aloe Vera gel powder ,motoneuron were as intact group (first group)which did not received this herb,it seems Aloe Vera gel powder has not any significant effect on motoneuron cell number.The number of motoneuron in third group which received Aloe Vera gel powder in compare to fourth indicate the probably effect of Aole Vera gel powder as a preservative herb.Nowa days many researchers published the hypothesis and protocol as a cascade events after SCI, such as: free radical formation[37].Peroxynitrite,membrane lipids and protein cell homeostasis changes[38].Synaptic stripping or synaptic contact losses[39]. Neurotransmitter transferring deficiency[40]. Astrocyte activation and inflammatory signaling[41]. However, following SCI plasticity change is considered as the most important event following neuronal injury[42-43].A cascade of event accure after SCI and one of the most important seems accure in synaptic zone and nerve counduction.In neural pathway neurotransmitter releases in synaptic cleft and it act on post synaptic membrane for neuron surviving,any changes in this proccess causes neuron apoptosis[44-45]. As table 2 shows in third group inspite of spinal cord pressure more than 2/ 3 neuron has been preserved but in fourth group the vital neuron decreased and this deduction is more than 1/2 neurons. This herb seems has an un known potential to maintain motoneuron after SCI . Zhang and Tang reported Huperzine A (HupA), a novel alkaloid isolated from a Chinese herb has multi functional effect, include modification of beta-amyloid peptide processing, reduction of oxidative stress, neuronal protection against apoptosis, neuroprotective effects that go beyond the inhibition of AChE , regulation of the expression and secretion of nerve growth factor (NGF) and NGF signaling[46]. In our study after four weeks, the efficiency of this herb seems similar as preserving neurons and immunohistochemistry results for AChE demonstrates this effect .On the other hand comparing it's expression in group first and second indicates that Aloe Vera gel powder has not any significant effect. So it means aloe vera has a potencial efficiency which switch on after neuron damaged but not in normal coundition.As mentioned in table 3 after SCI ,AChE expression decreses as the same as reported earlier by Romeo demonstrated acetylcholine esterase receptor decreased[17], Nacimento et al., reported a transient reduction in expression of acetylcholine esterase was observed in the ventral ramus in adult rats

following spinal cord lesions[47].The results of Mis et al. which demonstrated localization of, cells expressing AChE mRNA in rat spinal cord[48] are in keeping with those achieved by Manolov and Davidoff., demonstrated AChE -activity was observed on the cellular membrane, cytoplasm of the perikarya , proximal processes of the motoneurons[15], Nacimento *et al.*,[47] and Romeo *et al.*,[17], demonstrated decreasing AChE following spinal cord injuried.This has raised the theory that decreasing the enzymatic expression could be served as a criterion to survey cholinergic system in ventral ramus[15,47,48] . In our study indicated Aloe Vera has significant effect on its expression.As it is showed in table 3 in third group it is expressed in more than 1/3 of neurons.But in fourth group this expression decreased significantly and it is not seen in 80% of vital neurons. This is the maximum in both group 1 and 2 which is responsible for keeping morphometric results, whereas that in group 4 is the minimum which reflects the decrease in motor neurons number. Comparing groups 3 and 4 where the enzymatic expression was significantly different, one could assert that Aloe Vera gel powder increases the expression rate and, put in other words, preserve the neurons. As mentioned ultra structural changes in this study has accurred.These changes are: reduction of electron density in active zone , irregularity in pre and post synaptic membrane ,collapsed synaptic cleft , low electron density in the synaptic active zone. Dispressed synaptic vesicle , mitochondria swollen with increasing matrix density ,irregularity in outer and inner membrane with poorly defined cristae with some vesiculated body in it (Fig 4 and 5).As other reported synaptic structure beside homeostasis of extra cellular matrix are the most important factor for cell survival[49-50]. In addition to mention cholinergic system, the presence of AChE in the cholinergic nervous system has neuroprotective properties due to two essential mechanisms, the extent that is said enzyme prevents the excessive stimulation of postsynaptic neuron , to boot, activates the ATP-mediated K channels which is endowed with neuroprotective effect however, the high rated of acetylcholine enzyme in the long run ends up with neurotoxicity as in ALS[51]. It is established mitochondria play an important role in intrinsic apoptotic pathway[52] by cell homeostasis maintaining[53], regulating signaling pathways[54] by cell signaling[55]. Interesting finding in this study is mitochondria swelling with changes in it's structure as an important organel in apoptosis[56-57]. At least ultra structure changes after SCI belongs to group 2 as compare with fourth group .This seems pararel with AChE expression as a factor for maintaining motoneurons.Many investigators show ,it has effect on intracellular events that leads to death or maintaining cells[58,59-60].On the whole results show, Aloe Vera may be has significant effect on neuron maintaining or preserving from death .Some other researcher show multi potential effect of this herb as indicated , anti inflammatory in human colorectal mucosa,changing gastric microcirculatory and cytokine levels ,causing down regulating interlukin 6 and 8 , dis attachment leukocytes, increasing level of interlukin 10,reduction TNFα and on the whole reduction inflammatory process achive[61-62]. Protective effect of Aloe Vera on mild damage caused by type-II diabetes on kidney tissue[63]. On the other hand Boudreau reported Aloe vera products contain toxicological activities as:diarrhea, electrolyte imbalance, kidney dysfunction[64].Nowadays anti oxidatnt potentiality of Aloe Vera is reported by Ozsoy which this effect could be related to the presence of phenolic compounds and antioxidant vitamins .Inhibiting peroxidation of liposomes and reduction malondialdehyde levels upon cellular pathways, it indicate Aloe Vera extract may useful for treating degenerative disorders[65]. Other reported pro-oxidant action of polyphenolics in Aloe emodinis is more than antioxidant activity,it seems is important mechanism for their anti cancer and apoptosis abilities[66] .Gupta reported Aloe Vera has multi activity such as inhibition histamine releasing,anti-inflammatory,anti viral,anti microbial,anti cancer and anti oxidant is depend on Barbaloin [67]which is in Aloe vera leaves[26] . In other word Aloe Vera causes bone growth and tibia length with body mass increasing in rat as a growth factor[59].On contrast Misawa reported it decreased body mass[32]. Friedman and Si demonstrated Aloe Vera affect in crayfish neuro muscular junction is as increasing nerve stimulation amplitude and change neuro transmission process which suggested analgesis and anti inflammatory effects[68].Lu et al. reported following cerebral ischemia Aloe Vera had a protective effect , that

may be due to the inhibition of neuronal cell apoptosis[69].By these finding and theories it seems Aloe Vera has some different effect as it is known multi potential herb and as an anti oxidant can be supposed make this herb act as a neuroprotective herb for maintaining motoneurons and their structure in SCI.

CONCLUSION

On the whole in this study ,seems Aloe Vera plant has neuroprotective properties which causes the neurons to be preserved.

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