

Full Length Research Paper

The effect of dried *Aloe vera* gel powder on Choline acetyl transferase and synaptic zone after spinal cord injury in adult rats

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Spinal cord injuries (SCI) cause disabilities. The design of alternative therapies like usage of herbs with pharmacological properties looks ideal. Aloe vera has been mentioned as a multi functional herb. This study was conducted to further research of this plant after SCI in rats.36 adult female rats were divided randomly into four groups, Group I:Sham + gavage distilled water. Group II: Sham +gavage Aloe vera gel powder (200mg/kg/d).Group III: Group II+SCI. Group IV: Group I+SCI. After 4 weeks, the rats were sacrificed. Morphometric study and choline acetyl transferase (ChAT) immune staining were done. Synaptic changes were analyzed in a blinded manner for qualitative ultra structural changes. The data analysed with Tukey's test and one-way ANOVA in SPSS 21software and $P \leq 0.05$ was considered as significant level. Decreasing survived motoneurons with synaptic changes and reducing ChAT immune reactivity was seen due to SCI($P \leq 0.05$).Usage of Aloe vera gel powder showed a reduction in death of motoneurons ($P \leq 0.05$) it also has increased the ChAT labeling after SCI($P \leq 0.001$), decreased pathological synaptic changes in synaptic zone and mitochondria. Aloe vera with neuro protective effects (might be due to its antioxidant) has reduced neuronal cell death and increased expression of the ChAT and kept synaptic ultra structure after SCI.

Key words: Spinal cord injury (SCI), Aloe vera, Choline acetyl transferase (ChAT), Synaptic zone, Rat.

INTRODUCTION

Spinal cord injuries are one of the most serious diseases of the central nervous system and are ranked as one the most costly diseases. In addition to their physical disabilities resulting from injury, they face life with a variety of syndromes that exacerbate their disabilities (Hall, 2001). This matter imposes an irreversible burden on communities and that is enough a reason for treatment planning. About 62% of patients with spinal cord injuries are young people aged 15-30 years, males make up about 80% of patients(Braddom,2007).Damage to the spinal cord result injury of local neurons, including sensory and motor neurons. Also the damage

to the neurons is followed by apoptotic pathways and it's affected by the deregulation in transmissions and cell signaling, (Lauri et al.,2003). Cell signaling, the presence of neurotransmitters are important as valuable markers in the assessment of rate and the manner neuron's behaviors. One of most importantly is Acetylcholine (Fernández-Chacón and Südhof, 1999) to investigate this mechanism, we use the enzyme ChAT, which synthesizes the neurotransmitter acetylcholine, and is significantly reduced in nerve injury (Kandel, 2000; González et al., 2002). Hence, by using immune histochemical techniques, neuronal function in the transmission of nerve messages can be examined. Today in most labs in the world, multiple basic science researches are being conducted on SCI. In this case, pharmacological approaches such as neurotrophic factors such as nerve growth factor NGF, BDNF, neurotrophin 3 and neurotrophin

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5/4(Priestley et al., 2002; Laramore et al., 2011)anti-oxidant compounds, and antiglutamatergic drugs have also been evaluated (De Nicola, 1993; Benedet al., 2004). It is demonstrated oxidative stress causes neurodegenerative disorder and synaptic plasticity (Maiese 2009). The goal is to utilize chemical compounds which act upon lowering or decreasing the rate of apoptosis in compressed nerves while preventing glial based tissues to form in the area of the injury (Gu et al., 1999). On the other hand, herbal drugs being used as such agents in promoting the quality of life and towards total or partial rehabilitation of the related patients are cheaper, easier to provide and have less side effects compared to synthetic drugs (Muller et al., 2003; Sosa et al., 2007). Aloe (often called Aloe vera) (Scientific name: *Aloe Barbadensis*, *Liliaceae*) have attempted to determine whether or not is toxic to animals or humans (Boudreau and Beland, 2006; Matsuda et al., 2008; Farahnejad et al., 2011; duPlessis and Hamman, 2013) beside these study recently, indicated was not genotoxic or toxic in vivo (Sehgal et al., 2013). So Aloe vera has been mentioned as a safety herbal plant and was chosen in this study. It's produces are two substances, gel and latex, which are used for medicines. Aloe gel is clear, jelly-like substance found in the inner part of the aloe plant leaf. Aloe latex comes from just under the plant's skin and is yellow in color(Abebe,2003). Some experimental products are from the whole leaf, so they contain both gel and latex. On the whole, kinds of Aloe vera has been demonstrated for properties such as wound healing (Khanet al., 2013), anti-inflammation (Langmead et al., 2004) anti-neo plastic (Chen et al., 2004; Beya et al., 2012) anti-diabetic (Can et al., 2004; Rajase karan et al., 2005), anti-oxidant (El-Shemy et al., 2010), anti-ulcer (Eamlamnam et al.,2006), anti-cancer in Hela cells (duPlessis and Hamman, 2013), increasing the bioavailability of vitamins (Lebitsaet al., 2012), anti-microbial against multi resistant organisms (Banu et al., 2012), against burning mouth syndrome (López-Jornet et al., 2013). Anti-diabetic which It may be useful in treating type I and type II diabetes mellitus because of anti-hyper glycaemic effects (Tanaka et al., 2006), by mannans and anthraquinones (Reynolds and Dweck, 1999), decreases gastric acid secretion and confers gastro-protection from hydrochloric acid injury (Yusuf et al., 2004).

On the whole it was concluded that the beneficial effects of Aloe vera could be attributed to its antioxidant activity and could be related to the presence of phenolic compounds and antioxidant vitamins (Ozsoy et al., 2009),which this ability is probably due to the opening of tight junctions to allow paracellular transport(Chen et al., 2009).

Aloe vera include lectins, arginine and β - carotene which are anti-tumor agents and immune modulators (Amusan et al., 2002), salicylates, phytosterols, γ -

linolenic acid, anthraquinones and resins (Esua and Rauwald, 2006). Aloe vera also contains mucopolysaccharides. Polysaccharides, consist mainly of linear chains of glucose and mannose molecules (Beneke et al., 2012). Aloe vera compounds may affect through modulating antioxidant and detoxification enzyme activity levels, as they are one of the indicators of tumorigenesis (El-Shemy et al., 2010). On the other hand Aloe vera causes adenomas and adenocarcinomas of the cecum, colon, and rectum in male Wistar Hannover rats which received Aloe barborescens whole-leaf powdered extract in the diet at the 4.0% level (Yokohira et al., 2009). In another report 1.5% Aloe vera whole-leaf extract causes adenomas, carcinoma and mucosal hyperplasia of the large intestine which is based to dose dependent, it increases by Aloe vera whole-leaf concentration (Boudreau et al., 2013).

It seems vice versa potential is proposed for Aloe vera. This multiple effects vary to different dosage, young or old and place of leaves. The dosage which is used both orally and through gavage are too varied, 25 mg (140 mg/kg/day) (Kosif and Aktas 2009), 20 -200 mg/kg/day(Misawa et al., 2012), 50mg/kg -500 mg/kg (Reynolds and Dweck 1999) or 4%-0.8%-0.16% (Matsuda et al., 2008) 5mg/ml/day (de Carvalho et al., 2009).

In this study any effect of Aloe vera gel powder 200 mg/kg/day for the late changes (4 weeks post-SCI) in injured motoneurons and the pattern of these changes in the expression of ChAT were evaluated by using immunohistochemical methods. Morphometric parameters were used to evaluate the trend of changes quantitatively. Ultra structure investigation of the injured motoneurons was used to characterize synaptic lesions.

MATERIALS AND METHODS

Animals: All experimental protocols of this study were approved by the animal care committee of Shahed University in accordance with the policies established in the guide to the care and use of experimental animals prepared by animal care and all efforts were made to minimize the number of animals and their suffering. 36 adult female rats (Razi Institute, Karaj/ Iran) weighing 250g (6-8 weeks old) were kept, in standard laboratory conditions of 12/12 light/dark cycle (22-23°C and 30-40 % humidity) and enough tap water and food plates, ad libitum (from Pars animal food company) for 1 week before experimental beginning. Then they were randomly divided into four groups ($n=9$) and separately kept in a cage. Each cage had labeled for groups, date of laminectomy.

Experimental Group

Group I: Sham + gavage distilled water. Group II: Sham +

gavage Aloe vera gel powder.

Group III: Group II +SCI. Group IV: Group I+SCI.

Spinal Cord Injury was done by the use of clips aneurysm based on Poon and et al. (2007). The sham groups were only subjected to laminectomy without any compression applied on the spinal cord.

Preparation of Aloe vera gel Powder

Fresh Aloe vera(L.) Burm .F. (Liliaceae) leaves (400g) were collected and processed from a single garden plant. The identification of the plant, was done by the herbarium of the Department of Acient Medicine of Shahed University Iran to obtain a fresh whole extract gel for experiment during this work. For this reason the protocol was considered by Misawa et al. (2012). Fresh leaves of Aloe vera washed by water carefully then it cut to species, the inner gel was obtained by clean sharp knife, then dried Aloevera gel powdered achieved by hot-air drying. For administration sample, the dried Aloe vera gel powder was suspended in distilled water and the dosage of homogenized suspension was adjusted to 50 mg/mL. (Misawa et al., 2012). They were stored in tightly sealed dark containers in a freezer at -20°C for later use. The sterility of Aloe vera was tested and confirmed in the Microbiology Department by inoculating a loopful of the undiluted Aloe vera on blood agar. No organism growth was observed even after 48h. The animals were administrated Aloe vera gel powder (200 mg/kg/d) once a day by gavage for 4 weeks in treated groups as the same as distilled water in non treated groups.

Surgical Procedure

Ninth thoracic vertebrae laminectomy was performed, slightly higher than the lumbar neural networks. For the creation of a spinal injury, aneurysm clips were used (Poon et al., 2007).First the rats were anesthetised by ketamine and xylazine.13th thoracic rib, then vertebra was counted and marked,T9 laminectomy was performed.

It should be noted that the spinal venous network and the overlying layers should be kept from any harm. After seeing the spine, aneurysm clips with pressure equivalent to 30 g (Joshi and Fehlings, 2002) were placed on either side of the spinal cord for 1 minute and then incision site of the muscles, fascia and skin, were respectively sutured with 6-0 and 3-0 suture threads. Serum lactate injection was performed to reduce bleeding. After 5-6 hours, applying low pressure to the bladder of the rat was begun and continued until 3 days post injury to prevent bladder infection. Also, daily administration of Aloe vera gel powder or distilled water was performed by gavage in their appropriate study groups.

Tissue Sampling

After 4 weeks (28 days) the rats in four groups were lightly anesthetized with ketamine/xylazinein each group (n=9)cardiac perfusion was performed for which, an average of 300 ml of fixative paraformaldehyde (%4) in 0.1 mol/L solution of buffer phosphate, then T9 vertebra, was excised and was then transferred to a formalin solution (10%) for light microscopic study(n=6)and for ultra structural studies(n=3). For light microscopic, spinal segment T9 was removed and the tissue was processed for paraffin embedding, by using microtome rotary (Leica 820), slides of 8 micrometer diameter were prepared and stained with Cresyl violet. Spinal motoneurons counting were done according to (Li et al.,1998).Five micrometer sections were taken from the spinal segment of the animals, labeled with mouse anti-ChAT antibody (Chemicon international) as primary antibody and followed by labeling with rabbit anti-mouse peroxidase conjugated antibody as secondary antibody (Chemicon international).The sections were treated with 10% hydrogen peroxide in order to inhibit endogenous peroxidase then treated with diaminobenzidinetetra hydrochloride in tris buffer (pH7.6) containing fresh hydrogen peroxide. ChAT immune labeled motoneurons in the ventral horn were classified into severe, moderate and mild intensities. All the parameters were tested for normality using nonparametric S-K test, and the results showed that all the parameters were not significantly different from normal ($p > 0.05$). The means were compared using Tukey's test, while the analysis of the variance was used to compare the difference among the groups. For ultra structural study, the tissue from spinal segment was sampled after perfusion with Karnovsky's fixative, immersed in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) and post-fixed in 1% osmium tetroxide in phosphate buffer. Thin sections were cut, stained with uranyl acetate and lead citrate and examined under Zeiss EM 900.

Data Analysis

After specifying the data distribution to compare the results of Tukey's test one-way ANOVA was performed with the software SPSS Ver 21. Furthermore, the significance level ($P \leq 0.05$) for motoneuron counting and ($P\leq 0.001$) for ChAT labeling.

RESULTS

Light microscopy studies (morphometry)

Light microscopic study of spinal motor neurons showed reduced number of motor neurons in the anterior horn of

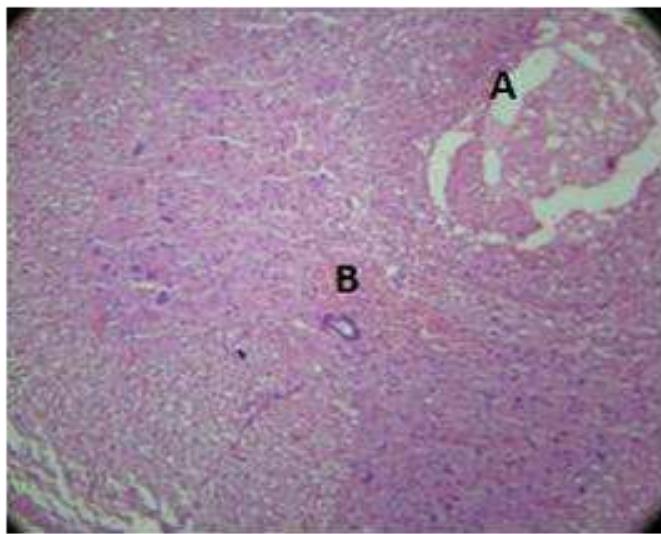


Figure 1. Cross section of spinal cord tissue in rat with H&E staining. In this picture the gray and white matter are seen. With mechanical pressure application, a decline in number of motor neuron in the ventral horn, hemorrhage (B) and hole(A) are evident (40X).

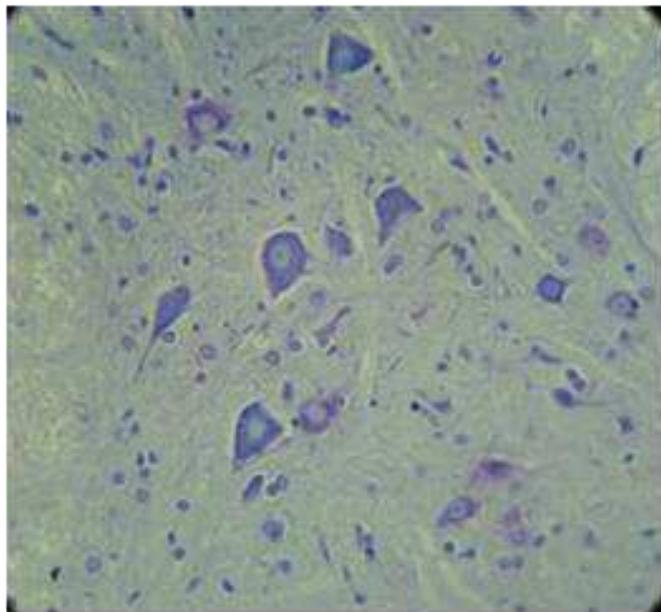


Figure 2. Cresyl Violet staining; chromatolysis of motor neurons (100X).

the spinal cord with holes and hemorrhage in the holes (Figure 1). Also Cresyl Violet staining showed chromatolysis of motor neurons after mechanical pressure on the spinal cord (Figure 2).

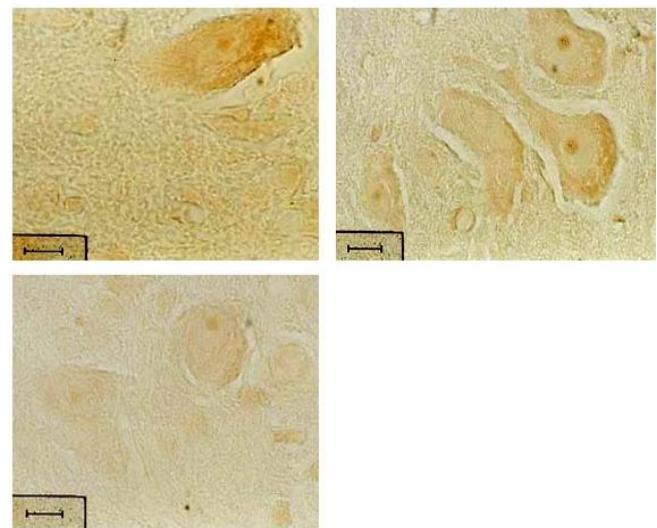


Figure 3. Immunohistochemical staining for ChAT. Upper left shows severe, upper right shows moderate and lower left shows mild (scale bar =25 μ m).

Light microscopy studies (immunohistochemistry)

The final product of this technique was a light brown cytoplasmic deposition which showed ChAT enzyme was present. ChAT immune labeling is shown in Figure 3 at mild, moderate and severe. Colored cells were counted across all groups.

As seen in the Table 1, the second group showed the highest number of motor neurons and the lowest number of motor neurons belongs to fourth group. Comparison between four groups, showed that the third and fourth group had the greatest decrease in the survived motoneurons (respectively 38.2 %, 63.71 %). The decrease in the proportion of each of these two groups compared to groups one and two, were significant ($P \leq 0.05$). Results showed that the number of motor neurons in the first group was less than the second group, but the difference was not statistically significant ($P \geq 0.05$). Comparing group 2 with 3 and 4, showed a significant increase in the number of motoneurons ($P \leq 0.05$). Also, the number of cells in group 4 compared to the other three groups were statistically significant.

Table 2 shows Mean and SD of stained cells in all groups according to the intensity of reaction. Figure 4 also shows percentage of any intensity (severity) pattern (mild, moderate, severe) in each group. The most percentage intensity belonged to fourth group. There was a statistically significant difference between the four groups studied regarding the mild response rate ($P \leq 0.001$) which the results of Tukey's test showed that this difference was due to the third group. The intensity of the moderate reaction was greatest in the second group and

Table 1. Mean and SD of motor neurons and percent reduction in the anterior horn of the spinal cord.

Groups*	Number of motor neurons (Mean ±SD)	Percent reduction in motor neurons versus 2nd group.
1	2012± 134	-1.71 %
2	2047± 101	0 %
3	1287 ±98	-38.2 % #
4	743 ± 75	-63.71 % #

Table 1. Shows the average number and standard deviation of the total motor neurons and the percentage reduction in motor neurons in the anterior horn of the spinal cord in each of the four groups*1: Sham +gavage distilled water.2: Sham +gavage Aloe vera gel powder (200mg/kg/d). 3: Group 2+SCI. 4: Group 1 +SCI. # Significant ($P \leq 0.05$).

Table 2. Mean and SD of stained cells in all groups according to the intensity of the reaction.

Groups*	1	2	3	4
Intensity of Reaction				
Mild Reaction	6.75±0.47	6.75±0.47	1.5±0.28	6.5±0.5
Moderate Reaction	8.75±0.47	10±0.4	8.25±0.47	2±0.0
Severe Reaction	7.25±0.25	10.25±0.25	5.25±0.94	1±0.4

Table 2. Shows mild reactions in the first and second and fourth groups are the highest, and the lowest mild response was in the third group. * Group1: Sham +gavage distilled water. Group2: Sham +gavage Aloe vera gel powder (200mg/kg/d). Group3: Group 2 +SCI. Group 4: Group 1 +SCI.

lowest in group fourth but percentage of intensity in third group was most. Also, regarding the moderate reaction, the statistical difference was significant among all four groups ($P \leq 0.001$), the results of Tukey's test revealed that this significance was due to the fourth group. Regarding the severe form of reaction, the second group had the highest. There was also a statistically significant difference between the four groups studied ($P \leq 0.001$), the results of Turkey's test showed that this difference was due to the fourth group. Percentage of reaction in second group was most and in fourth group at least (Figure 4).

Ultra structural study

SCI caused displaced synaptic vesicles in the synapses at the SCI group, while synaptic changes with low

electron density in the synaptic active zone and mitochondria swollen, which are moderately to severely swollen, and increased mitochondria matrix density with irregularity in outer and inner membrane and poorly defined cristae (Figure 5). Presynaptic whole comparing groups, indicated SCI causes synaptic changes, in group 4 are most visible and less visible in group3. The synaptic region and mitochondria in group 1 and 2(as sham) were intact.

DISCUSSION

This study showed the reduction of motor neurons in the third and fourth groups induce of SCI. This reduction was accompanied with mentioned cavitations between the cells and also, bleeding is present among both the interstitium and the cavities. Was not statistically significant

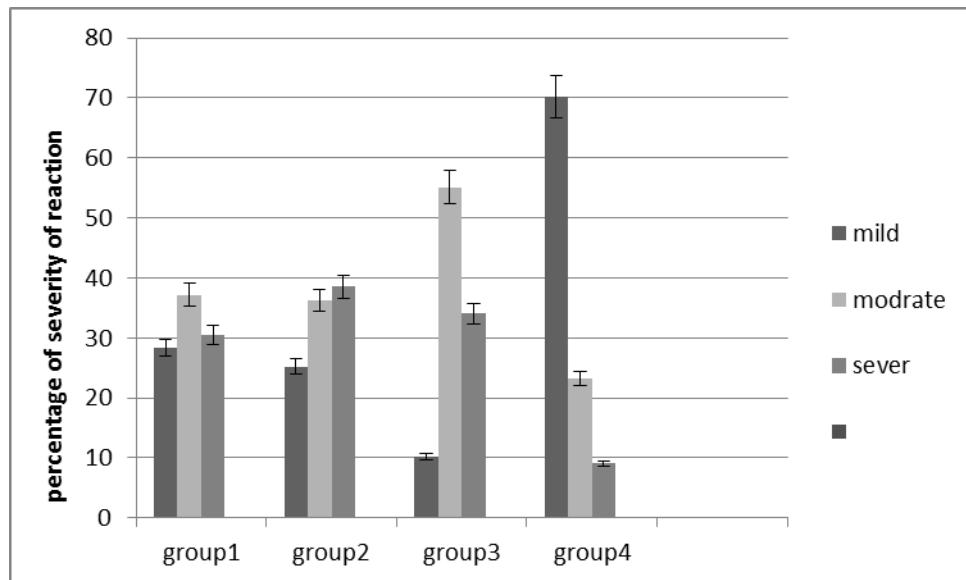


Figure 4. Percentage of intensity (severity) of reaction in each group.

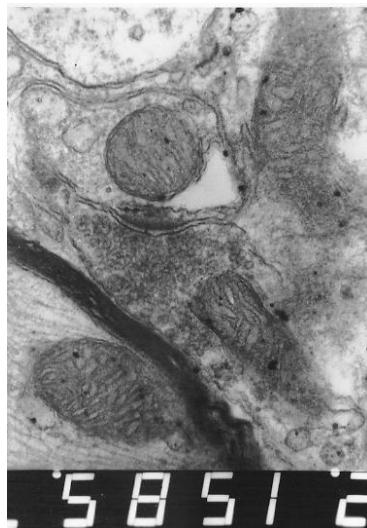


Figure 5. Electron micrograph of a synaptic region from spinal cord after SCI. Irregularities of synaptic membranes in an area with collapsed synaptic cleft. Low electron density in the synaptic active zone. Vesiculated mitochondria with increased mitochondria matrix density. Irregularity in mitochondria outer and inner membrane (5000X).

between the second and first group so it indicating Aloe vera has not any effect on intact or normal motoneurons; while discussing the number of neurons in the third and fourth group which had SCI due to mechanical pressure, the reduction in number of neurons was significantly

lower in the third group which had received Aloe Vera compared to the fourth group which had not received Aloe Vera so it seems Aloe vera has potentially to maintaining motoneuron or preserved them from death.

In this study, like other studies, reduction of

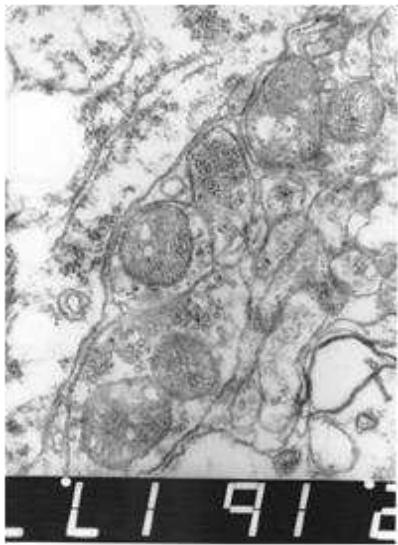


Figure 6. Electron micrograph of a synaptic region from spinal cord after SCI, which shows synapse with irregularity in pre and post synaptic membrane, with detachment .Mitochondria vesiculated and some round structures of the same size as intact mitochondria with poorly defined cristae. which shows a perforated synapse with low electron density presynaptic zone of the synapse(3000X).

motoneurons had happened following SCI. In one of the studies, it's mentioned that apoptosis took place 6 hours to 3 weeks after mechanical impact on spinal cord (Hubli and Dietz, 2013). In another study, apoptotic mortality of neuronal cells and glial cells have been reported following mechanical damage to the spinal cord (Liu et al., 1997). On the study of severity of the reaction of ChAT, the least amount of immune reactivity and difference between the groups was due to group 4 (spinal cord compression + distilled water). Comparing groups 3 and 4 on the subject of low grade of reaction, the third group has a lower average of low grade reactivity but regarding medium grade and severe grade reactivity. According to this significance between groups 3 and 4, maybe one can state that the higher production of ChAT enzyme can be linked to Aloe Vera parallel to maintaining motoneurons. Also, statistically significant difference was present between groups 2 and 3. Statistically significant difference between group 4 and groups 1 and 2 (no injury to the spinal cord) were expected due to compression of spinal cord injury and loss of nerve cells in the fourth group, but no statistically significant differences between the third group (compressive spinal cord injury + Aloe vera) and group 1 (without cord injury) were observed. Despite the destruction of nerve cells in the third group, moderate and severe-grade reactions are similar to the group 1 (without injury). Survived motoneuron which revealed ChAT in group 1 to 2 indicate after using Aloe

vera moderate intensity 14% and severity intensity 42.8% increased with no difference for mild intensity, it seems Aloe vera may act as an agent for ChAT producing. On the other hand comparing group 3 to 4 indicate Aloe vera increased moderate intensity (up to 3 times) and severe intensity (up to 4 times). Up regulating for moderate intensity in group 3 is as control (group 1) and up regulating for severity is 2/3 as control (group 1). ChAT expression after 4 weeks of SCI and remaining motoneurons with intense ChAT immune reactivity were less than 40%. This is consistent with reports from other investigators who reported a decline in the expression of ChAT in axotomized motoneurons (Yan et al., 1993). The decline in ChAT intensity was consistent with the progressive decline in the percentage of survived motoneurons, a finding reported also by other groups, which may reveal that the axotomized motoneurons had more tendencies to reverse the target deprivation to maintain neuronal structural integrity than function which is marked by neurotransmitter synthesis (Kreutzberg, 1996). The level of ChAT was reported to decline after 21 days of axotomy in the adult animal (Jacobsson et al., 1998; Goettl et al., 2003) while Chiu et al. (1993) reported no decline in ChAT level in axotomized neurons. Besides these, ultra structure synaptic zone demonstrated SCI caused synaptic changes as the same as mitochondria changes. The findings of this investigation were consistent with a previous communication which revealed that motoneuron losses as well as synaptic lesions were noticed in axotomized motoneurons in newborn rats (Tiraihi and Rezaie, 2004). After using Aloe vera synaptic ultra structure maintained as the same as intact or normal motoneuron, it was seemed due of unknown ability for Aloe vera. These finding along with the statistically significant difference between group 3 and 4 can be pertained to the neuro protective of Aloe vera which in turn, can result in the persistence and perseverance of the nerve cells post-injury. The mechanism in which Aloe vera promotes ChAT in the tissue is not yet known. In one study, the neuro trophic factor BDNF had a promoting effect on the mRNA of ChAT post-axotomy of the motoneurons in the medulla oblongata of adult rats (Wang et al., 1997). A pharmacological approach in the treatment of spinal cord injury which was currently being used named Methyl prednisolone, that in addition to having antioxidant properties by reducing production of Tumor Necrosis Factor (TNF- α) and decreasing activity of nuclear factor kappa-B, which appears to be caused by anti-inflammatory properties, results in inhibition of the second phase of inflammatory reaction in the SCI (Xu et al., 1998). In this study, it appears that Aloe vera plant has neuro protective properties; but Aloe vera's mechanism of action and what causes the nerve cells to be preserved, is not known. Investigations show that, it has many factors effect on intracellular events that leads to

death or maintaining cells (Can et al., 2004; Beya et al., 2012; Lebitsa et al., 2012). In other studies, it's shown that some herbal plants inhibiting inflammatory reactions by inhibiting the production of Interleukins 6 & 8, reducing the adhesiveness of the leukocytes, increasing IL-10 and decreasing TNF- α . Farahnejad et al. (2011) according to these studies, maybe one can state that the neuro protectivity of Aloe vera can be due to its Anti-Inflammatory properties. To establish our result, Ozsoy et al. (2009) demonstrated the antioxidant potential of Aloe vera, its influence upon cellular pathways by inhibiting the peroxidation of liposomes and reduce malondialdehyde levels, indicating that Aloe vera extract may provide treatment degenerative disorders. Other studies suggested pro-oxidant action of polyphenolics in aloe-emodin, rather than antioxidant activity, which may be important mechanism for their anti cancer and apoptosis abilities (Mahbubet al., 2013). Gupta and Malhotra (2012) reported multi activity such as strong inhibitory effect on histamine release, anti-inflammatory, cathartic, antiviral, antimicrobial, anticancer, antioxidant is depend on Barbaloin in Aloe vera which young leaves contain more than old one(Patel et al., 2012).

Another study reported Aloe vera 500 mg/kg/d (high dose) seems affected bone growth in rat 6 days old, as tibia length and body mass increased (Beya et al., 2012). On contrast Misawa et al. (2012), reported 20-200 mg/kg/d decreased body mass in rats. Increasing nerve stimulation amplitude with the latency increase and depression of action potential generation and conduction in crayfish neuro muscular junction indicated the effects of Aloe vera on the neuro transmission process, suggested partially account for Aloe's analgesic and anti inflammatory effects (Friedman and Si, 1999). Study on caspase-3 expression demonstrated Aloe polysaccharide has not any effect on it following cerebral ischemia and reperfusion injury in rats and seems has had a protective effect on cerebral ischemia that may be due to the inhibition of neuronal cell apoptosis(Lu et al., 2012).

CONCLUSION

Aloe vera with unknown neuroprotectively effect, which can be pertained to its anti-inflammatory and anti-oxidant properties, decrease motoneuron reduction and increase ChAT immunoreactivity besides maintaining ultra structures of synaptic zone.

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