Original Article

Histologic and Histomorphometric Study of Tibial Nerve treated by Hydro-alcoholic Aloe Vera Gel Extract in Diabetic Male Rats

Hamidreza Ghaffari, M.Sc.[1], Soghra Gholami, Ph.D.[1*], Majid Naghdi, Ph.D.[2]

1. Department of Anatomy,Faculty of Veterinary Medicine, University of Shiraz, Shiraz, Iran
2. Department of Anatomical Science, Faculty of Medicine, Fasa University of Medical Sciences, Fasa, Iran
***Corresponding author,** E-mail address:* gholami@shirazu.ac.ir

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Dr. Soghra Gholami is a Full Professor of Anatomy and Embryology in the Department of Anatomical Science at Shiraz University, Shiraz, Iran. She graduated with a DVM and PhD from the College of Veterinary Medicine at Shiraz University. Dr. Gholami has advisedDVM and PhD students at Shiraz University, in the fields of anatomy, embryology and histology, since 1993. Her special interests include neuroanatomy and embryology.

Abstract

Introduction: Diabetes mellitus impairs the metabolism of carbohydrates, lipids and proteins, which leads to the dysfunction of the central and peripheral nervous system. This study evaluates the effects of an aloe vera hydro-alcoholic extract on diabetic neuropathy.

Materials and Methods: For this study, 48 healthy male Sprague Dawley rats(weighed 250-200 g) were randomly divided in to three groups: control (n=16), diabetic + normal diet (n=16), and diabetic + aloe vera extract (n=16). To induce diabetes, an intraperitoneal injection of streptozotocin (50 mg/kg) was administered to rats. The control (normal diet) and diabetic + aloe vera group received 400 mg/kg of aloe vera extract once daily. All groups were maintained for 12 and 16 weeks. The tibial nerves was collected from anesthetized animals for tissue processing. Histomorphometric and histological results were analyzed by SPSS software.

Results: The diabetic rats showed a significant decrease in diameter of the myelinated fibers and myelin sheath thickness. axon diameter particularly after 16 weeks. In the untreated diabetic group endoneural edema, enfolding and irregularity of myelinated fibers was observed($P<0.05$). Long-term treatment with aloe vera prevented these abnormalities in the treated diabetic rats.

Conclusion: Our findings indicated that hydroalcoholic extract of aloe vera is a potential therapeutic agent that can prevent diabetic peripheral neuropathy, as well as histomorphometric and histological changes.

Keywords: Diabetes mellitus, Aloe vera, Neuropathy, Tibial nerve, Rats

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Introduction

Diabetes is a complex metabolic disease characterized by persistently elevated concentrations of glucose in the blood caused by the autoimmune destruction of the pancreatic cells (Type 1) or by insulin resistance coupled with relative insulin deficiency (Type 2). Type 2 cases comprise 90% to 95% of all diabetes whereas between 5% and 10% are Type 1 [1]. There are 21 million people with diabetes in the U.S.; approximately one-third are unaware of their disease [2]. Its microvascular complications include damage to the eyes, kidneys, and nerves, whereas macrovascular complications include atherosclerosis and other cardiovascular conditions [3]. The total cost of diabetes in the U.S. for 2002 was estimated at $132 billion [4].

Diabetic neuropathy occurs in 50% of diabetic patients who suffer from severe, unremitting pain. Diabetic neuropathy patients generally complain about persistent burning or tingling sensations, usually in the legs and feet. Other symptoms include the inability to detect heat and cold, cutaneous hyperesthesia, loss of vibration sensation and paradoxically, the loss of pain perception. The pathophysiology of the condition remains unclear, although it has been associated with peripheral demyelination, a decrease in peripheral nerve conduction and degeneration of myelinated and unmyelinated sensory fibers [5,6]. Hyperglycemia can induce oxidative stress via glucose auto-oxidation and the subsequent formation of advanced glycation end products, disruption of the polyol pathway, altered eicosanoid metabolism and decreased antioxidant defenses [7,8].

In diabetes mellitus, chronic hyperglycemia produces multiple biochemical sequelae Diabetes-induced oxidative stress could play a role in the symptoms and progression of the disease [9,10]. Oxidative stress in cells and tissues results from the increased generation of reactive oxygen species and/or from decreases in antioxidant defense potential [11]. Several hypotheses have been put forth to explain the genesis of free radicals in diabetes. These include autoxidation processes of glucose, the non-enzymatic and progressive glycation of proteins with the consequently increased formation of glucose-derived advanced glycosylation end products (AGEs) and enhanced glucose flux through the polyol pathway [11,12]. Elevated generation of free radicals resulting in the consumption of antioxidant defense components may lead to disruption of cellular functions and oxidative damage to membranes and may enhance susceptibility to lipid peroxidation [9].

Peripheral neuropathy is a progressive loss of nerve fibers that are susceptible to pain sensitivity in the lower limbs, resulting in neuropathic ulcers and amputations [13]. Morphological studies by Forrest et al. have indicated that diabetes leads to tissue damage in Tibial nerve such as endoneural edema accompanied by myelin destruction [14]. Sameni et al. have shown the protective effect of progesterone on the function and structure of rat sciatic nerve neuropathy [15]. Rajbhandari and colleagues have shown that neuropathy is a major problem of microvasculars. Control of blood sugar and duration of diabetes duration are important factors in controlling neuropathy [5]. Comparison of ultra-thin sections of the sciatic nerve of normal and diabetic rats by Nowickim et al. has shown reduction in a significant amount of myelin [16]. Skalsk et al. showed that oxidative stress caused by hyperglycemia was the most important factor in the development of diabetic neuropathy [17]. The use of antioxidant agents play an important role in reducing the consequences of diabetes

and the use of the compounds of plant origin that is usually associated with fewer side effects. [18]

There has been a resurgent in interest in traditional plant treatments for diabetes. Plants often contain substantial amounts of antioxidants including alpha tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids and tannins [19], and it has been suggested that antioxidant action may be an important property of plant medicines used in diabetes. Aloe vera is a perennial plant that belongs to the Liliaceae family, which includes about 360 species [20].

Aloe vera is the one of the few medicinal plants that has maintained its popularity for a long period of time. The plant has stiff gray-green lance-shaped leaves that contain clear gel in a central mucilaginous pulp. Clinical evaluations have revealed that the pharmacologically active ingredients are concentrated in both the gel and rind of aloe vera leaves.

In a previous study, we have shown highly encouraging results which revealed a significantly lower level of blood glucose after oral administration of ethanolic extract of aloe vera gel in a glucose load condition and in STZ-induced diabetes [21]. The aim of the present research was to perform histological and histomorphometric analyses of the effects of hydro-alcoholic aloe vera gel extracts of tibial nerve tissue from diabetic rats.

Materials and Methods

Animals

All studies were conducted on 48 adult male Sprague Dawley rats (Razi Vaccine and Serum Research Institute) that weighed 200 to 250 g. Animals were housed in an animal facility with standard 12-h light/dark cycles. The rats were fed standard food of rat ration, tap water and ad libitum. The Shiraz University Ethics Committee approved this study.

We divided the rats equally into three main groups: (A) healthy control, (B) diabetic + normal diet and (C) diabetic + aloe vera extract. For the experiments each of the three main groups were additionally subdivided into two age groups of eight animals each, as follows: groups 1 and 2 (healthy control) received food plus a once daily gavage of aloe vera extract (400 mg/kg) for 12 and 16 weeks. Groups 3 and 4 (diabetic) received just food for 12 and 16 weeks. Groups 4 and 5 (diabetic + aloe vera extract) received food plus a once daily gavage of aloe vera extract (400 mg/kg) for 12 and 16 weeks separatly. For all groups, animals were weighed at 12 and 16 weeks.

Experimental induction of diabetes in rats

Diabetes was induced by ab intraperitoneal administration of a single dose of streptozotocin (50 mg/kg body weight); Sigma Aldrich, USA) [22, 23]. Fasting blood glucose was assessed by glucometer (Accuchek® Active Roche, Germany) before and 24 h after the injection for confirmation of diabetes and at ten days after the injection for diabetes approval. Blood (1 ml) was collected from the rats' coccygeal veins. At the end of the experiment, the animals were anesthetized. Following dissection, the tibial nerve was exposed between the two heads of the gastrocnemius muscle. Finally, animals were euthanized by a sodium thiopental overdose [24].

Preparation of aloe vera gel extract

Aloe vera powder was prepared from aloe vera leaf gel according to a published procedure by Rajasekaran et al. with slight modifications [25]. Aloe vera leaves were collected from the Botanical Garden of Shiraz University. Mature, healthy, fresh aloe vera leaves that were

approximately 75 to 90 cm in length were washed with fresh water [26]. The leaves were longitudinally dissected and the fleshy mucilaginous pulp (parenchymatous tissues) were selectively scraped away, leaving behind the thick epidermis layers. The scraped pulp was homogenized, centrifuged at 10000 g for 25 min to remove the fibers and immediately lyophilized. The resultant samples were stored in a controlled environment at 4±1°C until further use. The known amount of lyophilized sample was subjected to methanolic solvent extraction. The recovered filtrates were evaporated until dry under reduced pressure (250 mmHg) in a rotary evaporator [19].

Surgical procedure

Rats were peritoneally anesthetized by administration of sodium thiopental (5mg/100g). The surgical procedure was performed under sterile conditions. The left tibial nerve was separated from between the two heads of the gastrocnemius muscle. The animals were easy to draw with an overdose of sodium thiopental administrated as a peritoneal injection.

Preparation of tissue for microscopy

The distal portion of the tibial nerve was biopsied at a constant site and immediately fixed by immersion in 2.5% glutaraldahyde in sodium cacodylate buffer (pH 7.3). After washing in the same buffer, the nerve was post-fixed in 1% cacodylate-buffered osmium tetroxide and following a second wash was dehydrated through ascending concentrations of ethanol before embedding in TAAB Spurr's resin using propylene oxide as an intermediary. Transverse semi-thin sections (0.5 μm) were stained with 1% toluidine blue and observed under a light microscope.

Morphometric evaluation

Morphometric evaluation of MF was performed by measuring 200 MF from the set of photographs chosen at a final magnification of 400x which corresponded to 1 semi-thin section of the tibial nerve of each animal. The outline of each axon and its myelin sheath in the sample was drawn with a digitizing pen and captured on the screen by means of a computer-linked digitizing tablet and specific software (Dino-Lite MSM1M) [27]. We compared the area, perimeter and diameter in order to determine the parameter that best characterized the size of the MF. Myelin sheath thickness was calculated as the difference between the fiber and axonal diameters, divided by 2 [27]. The index of circularity was calculated as the ratio between the measured axonal area and the area of a circle with the same circumference [15].

The following histomorphometric studies were performed for all groups: A) thickness of nerve fiber myelin sheath, B) diameter of myelinated nerve fibers, C) thickness of the whole tibial nerve, and D) thickness of the tibial nerve fascicle.

Statistical analysis

Grouped data were statistically evaluated with SPSS version 7.5 software. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by the post hoc Duncan test. P values less than 0.05 were indicative of statistical significance. Results were expressed as mean±SD for six animals in each group. Statistical comparison was performed at 12 and 16 weeks, separately.

Results

The changes in fasting blood glucose and body weight for the experimental groups at 12 and 16 weeks after diabetic induction are shown in Tables 1 and 2. Fasting blood glucose was significantly ($P<0.05$) increased at both 12 and 16 weeks post-diabetic induction. Treatment with aloe vera extract caused a remarkable

reduction in elevated fasting blood glucose.

Table 1: Effect of treatment with hydroalcoholic extract of aloe vera on fasting blood glucose (mg/dl) and body weight at 12 weeks (mean±SD)

Group	Body weight (g)	Fasting blood glucose concentration (mg/dl)
Control	262.4 ± 11.41	133 ± 8.36a
Diabetic	231.2 ± 21.2	429.6 ± 216b
Diabetic + aloe vera	241.4 ± 27.13	231.8 ± 84.38

a, b Mean±SD within each column at 12 weeks with different letters are significantly different (P<0.05).

Table 2: Effect of treatment with hydroalcoholic extract of aloe vera on fasting blood glucose (mg/dl) and body weight at 16 weeks (mean±SD)

Group	Body weight (g)	Fasting blood glucose concentration (mg/dl)
Control	214±19.49	108.6±10.31a
Diabetic	180.6±49.2	441±312b
Diabetic + aloe vera	198±34.92	199±97.8

a, b Mean±SD within each column at 16 weeks with different letters are significantly different (P=0.02).

Body weight at 12 and 16 weeks, compared to the control and treated groups, was not statistically significant. The thickness of whole nerve fibers and myelin thickness in different groups at 12 and 16 weeks post-diabetic induction was measured and is shown in Table 3.

As visualized by light microscopy, semi-thin sections of tibial nerves in diabetic rats compared with the untreated control group consisted of a large number of anomalies; such as endoneural swelling along with rupture of the nerves, destruction and degeneration of nerve, myelin sheath and irregularities in shape (infolding and out folding) (Figs. 1a,b and 2a,b). In treated animals(12 and 16 weeks with aloe vera) these abnormalitis of myelin sheath were reduced in a great extent(Figs. 1a,b and 2a,b). There was a significant decrease following treatment with aloe vera in the number of myelinated nerve fibers that had myelin abnormalities (Figures. 1b and 2b, Table 3).

Discussion

Diabetic neuropathy is a major problem for patients with diabetes mellitus and is the major cause of morbidity and mortality. Good glycemic control, with maintenance of normoglycemia, may be able to prevent chronic complications [5].

The pathophysiology of diabetic neuropathy involves a complex cascade mechanism that consists of several interrelated components and is not precisely known [28]. Diabetes is caused by increased oxidative stress in various tissues starting with a degenerative pathway in oxidative stress, such as reduced activity of antioxidant enzymes inside cells, vascular injury, increased production of free radicals in the mitochondria, reduced nitric oxide-induced hypoxia endoneural-induced programmed cell death process, degradation of proteins, lipids and nucleic acids, increased expression of inflammatory proteins and mitochondrial damage in neurons. This damages caused numerous injuries and lesions induced in nervous tissue such as axonal damage, neuronal death, and reduced blood flow to the nerves [28,29]. Wolff and Dean have shown for the first time in a study of auto-oxidation of glucose for hyperglycemia, that production of reactive oxygen radicals (ROS) induced chronic oxidative stress in diabetic rats and were causes for neuronal injury [30]. Mallon has shown that the main causes of neuronal injury and pain is ROS [31].

Table 3: The thicknesses (µm) of the whole tibial nerve, its fascicles and fibers in male rats (mean±SD) at 12 and 16 weeks.

	Groups					
	Control (a)		Diabetes (b)		Diabetes+ aloe vera (c)	
	12 weeks	16 weeks	12 weeks	16 weeks	12 weeks	16 weeks
N	1153.939±232.2b	1138.317±198.4b	493.1621±187.9a,c	605.0359±213.5a,c	973.2±176.8b	925.0759±165.9b
TF	139.7724±21.2b	148.269±19.7b	108.8103±31.2a,c	91.06138±45.7a,c	147.9241±28.9b	148.3879±32.7b
TFI	9.897241±2.3b	9.58±3.1b	6.506897±4.3a,c	6.422069±3.4a,c	8.93069±3.1b	8.716897±2.6b
MFT	3.400345±1.2b	3.470345±1.4b	2.202414±1.7a,c	2.334483±1.6a,c	3.005714±1.4b	3.318621±1.5b

Dissimilar letters a, b and c indicate significant differences between groups. N: Whole tibial nerve thickness, TF: Tibial fasicle, TFI: Tibial fiber, MFT: Fiber myelin thickness of tibial nerve.

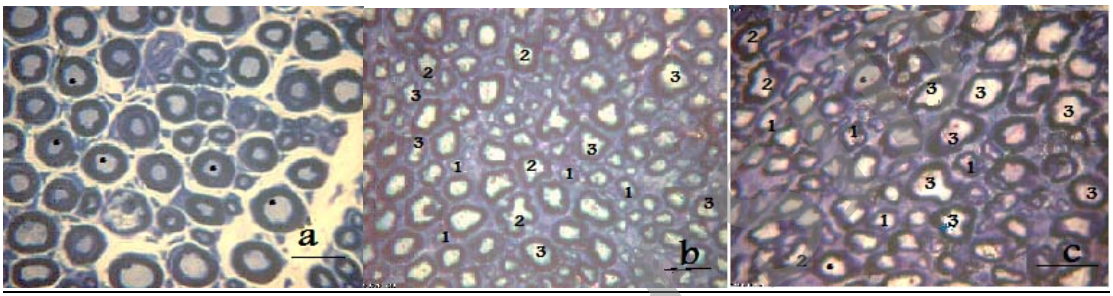

Figure 1: Light micrographs of toluidine blue stained transverse semi-thin section of the tibial nerve after 14 weeks at 400x magnification. Scale bar: 20 µm. (A) In the control group, myelinated nerve fibers are of normal morphology and structure (*). (B) In the untreated diabetic group, nerves revealed certain abnormalities, including: (1) degeneration, (2) myelin abnormalities including irregular fiber shapes, myelin infoldings and outfoldings and (3) alteration in myelin compaction. (C) In the aloe vera gel-treated diabetic group, a significantly reduced proportion of axons with myelin abnormalities was observed. The number of small myelinated nerve fibers increased in the diabetic group compared with the other two groups.

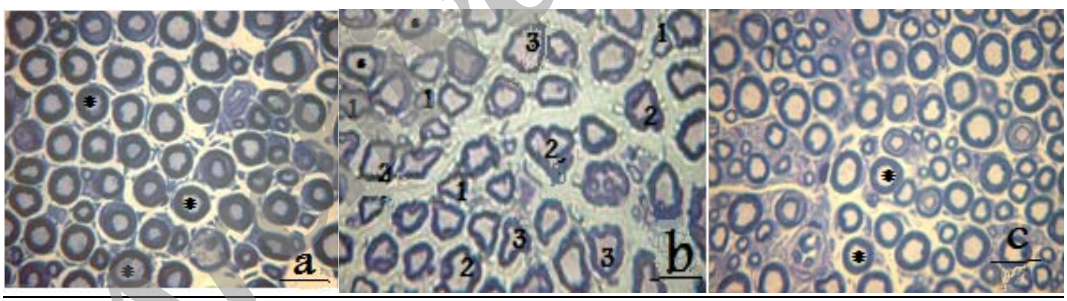

Figure 2: Light micrographs of toluidine blue stained transverse semi-thin section of the tibial nerve after 16 weeks at 400x magnification. Scale bar: 20 µm. (A) In the control group, myelinated nerve fibers show normal morphology and structure (*). (B) In the untreated diabetic group, nerves revealed certain abnormalities, including: (1) degeneration, (2) myelin abnormalities that included irregular fiber shapes, myelin infoldings and outfoldings and (3) alteration in myelin compaction. (C) In the aloe vera gel-treated diabetic group, a significantly reduced proportion of axons with myelin abnormality was observed. The number of small myelinated nerve fibers increased in the diabetic group compared with the other two groups.

Cooperative defense systems have been employed that protect the body from free radical damage and include antioxidant nutrients and enzymes [32,33]. Disease management includes lifestyle modifications, diet, exercise, and long term use of oral hypoglycemic agents or insulin therapy [34].

Although numerous drugs are used to treat diabetes, some have side effects. The main side effect is an increased risk of hypoglycemia. Hence many studies have been undertaken to investigate the hypoglycemic effect of some plants traditionally used to treat diabetes in addition to the identification of their active

ingredients, mode of action and safety. Herbal extracts have been confirmed for their hypoglycemic effects in humans and animals with type II diabetes [35]. Shane and Whorter have stated that aloe vera gel obtained from the inner portion of the aloe leaves contains glycomannan which may account for its hypoglycemic effect [36]. Ajabnoor reported that the hypoglycemic effect of aloe vera was mediated through the simulation and release of insulin from pancreatic beta-cells [37].

Tandon conducted a study that used a special recipe of aloe vera leaves administered to diabetic patients, twice daily for five years with no signs of abnormal blood sugar levels, serum total cholesterol and triglycerides reported [38]. The dried sap of the plant was used by Ghannam and Ajabnoor who observed that a hypoglycemic factor was shown to reduce blood glucose levels by long term used of Aloe Vera gel in diabetic rats [36].

Rajasekaran determined that consumption of aloe vera gel extract reduced blood sugar levels in diabetic rats [25]. The anti-diabetic activity of aloe vera has been associated with increased plasma insulin. Aloe vera extract stimulates secration of insulin from the remaining beta cells [39].

Hoseinifar et al. found that aloe vera decreased oxygen radicals and antioxidants which could reduce blood sugar and might exert a protective effect on vulnerable tissues [40]. Volgar and Emest noted that the use of aloe vera could increase hypoglycemic effect of glibenclamide [41].

Dey, in a study in Saudi Arabia, showed that the dried extract of aloe vera as a traditional medicine could be used to treat diabetes [42]. Extracts of aloe compounds that have antioxidants such as vitamins E and C can reduce blood lipids of weight gain in diabetes [43]. Rajasekaran et al. reported reduced blood glucose levels following the use of aloe vera extract [25].

Our findings demonstrated that administration of aloe vera extract provided beneficial effects on long term diabetic neuropathy via improving tibial nerve histomorphological alterations in its fibers. The most common abnormality observed in the present study was folding of the myelin sheath within the field of axoplasmic neurological disorders, which was directly related to increased age of the diabetics. Based on the findings of this study it could be concluded that diabetes causes considerable histologic and histometric changes in tibial nerve tissue.

The results of this study showed the effect of aloe vera extract on prevention of diabetic neuropathy in peripheral nerves. Both infolding and outfolding myelin sheath changes decreased and myelin thickness increased significantly. Fasting blood glucose levels and weight loss decreased in the treated animals. Our serologic and histomorphometric results clearly showed that aloe vera extract exerted a control effect on blood sugar and decreased neuropathy, one of the important side effects of diabetes. Although further studies should determine the functional implications and mechanisms of these protective effects of aloe vera extract, our findings suggest that the use of this compound may be considered to be a potential therapeutic approach for maintaining peripheral nerve integrity in diabetic peripheral neuropathy.

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