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Comparison of insulin and alpha lipoic acid treatment on laminin expression in sciatic nerve of diabetic rats

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Diabetic neuropathy is associated with changes in composition of extracellular matrix. Laminin is a major component of extracellular matrix. In this study, we investigated protective effects of insulin and alpha lipoic acid on improving of blood glucose levels, body weight and laminin expression of sciatic nerve in diabetic rats. Diabetes was induced in the rats by 55 mg/kg streptozotocin (STZ) via an intraperitoneal injection. 12 weeks after STZ treatment, laminin expression in sciatic nerves was evaluated by immunohistochemical study and real time polymerase chain reaction. Laminin expression increased in the perineurium, endoneurium and blood vessels walls in sciatic nerve of untreated diabetic rats compared to control rats. Insulin treatment significantly reduced overall laminin immunoreactivity but alpha lipoic acid treatment significantly decreased it only in the blood vessels walls. Laminin β1 mRNA level in sciatic nerve of untreated diabetic group increased 0.6 fold compared to control group. Although, alpha lipoic acid decreased this up regulation but unlike insulin, this reduction was not significant. These findings suggest that untreated diabetic neuropathy in rats might be associated with increased laminin expression and also it implies that insulin therapy is more effective than alpha lipoic acid treatment in improving hyperglycemia and laminin up-regulation in diabetic nerves.

Key words: Diabetic neuropathy, laminin, alpha lipoic acid, insulin.

INTRODUCTION

Persistent hyperglycemia in diabetes resulted in several complications, such as retinopathy, nephropathy and neuropathy. Neuropathy is a frequent complication in diabetic patients, but the exact pathogenic mechanism of diabetic neuropathy is not fully understood (Rajbhandari and Piya, 2005). Diabetic neuropathy is associated with changes in the Extracellular Matrix (ECM) and the basement membrane thickening in peripheral nerves (Bradley et al., 2000; Yasuda et al., 2003; Layton et al., 2004). The ECM provides physical support for cells and tissues and also has a major role in proliferation, differentiation, migration and regulating cell behavior (Yasuda et al., 2003; Layton et al., 2004; Chernousov et al., 2008; Miner, 2008). Basement membranes are structures of ECM that cover the basal side of epithelial and endothelial cells (Miner, 2008). Laminin is one of the major components of all basement membranes. It is a large glycoprotein (~ 800 kDa) distributed ubiquitously in basement membrane and links to other components of the basement membrane. Laminins are heterotrimeric glycoproteins composed of α, β and γ chains. There are currently five α, four β, and three γ chain genes that have

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been described in vertebrates and the chains can assemble into at least 15 different heterotrimers. Laminins give structure to the basement membrane, provide attachment sites for cells via cell surface proteins for example, dystroglycan and act as ligands for receptors on cells for example, integrins, initiating signals that influence cell behavior and survival (Chernousov et al., 2008; Miner, 2008; Hill, 2009; Hohenester and Yurchenco, 2013).

Laminin has an essential role in the basement membrane (Hill, 2009; Hohenester and Yurchenco, 2013) and support regeneration of the nerve (Yasuda et al., 2003). Long-lived proteins such as laminin are a potential target for glycation (Duran-Jimenez et al., 2009). Hyperglycemia leads to increased oxidative stress, polyol pathway activation, advanced glycation end products (AGEs) formation and reactive oxygen species (ROS) production (Duran-Jimenez et al., 2009; Vallianou et al., 2009). Polyol pathway converts glucose to fructose via sorbitol production which resulted in AGEs formation (Vallianou et al., 2009). AGEs accumulate in both intracellular and extracellular proteins, especially in those with poorly controlled diabetes (Duran-Jimenez et al., 2009). Hyperglycemia-induced oxidative stress contributes to the pathology of diabetic neuropathy (Vallianou et al., 2009). AGEs stimulate production of oxygen free radicals leading to oxidative stress (Duran-Jimenez et al., 2009; Vallianou et al., 2009; Han et al., 2012). Hyperglycemia and increased oxidative stress resulted in vascular damage and reduced peripheral nerve blood flow (Rajbhandari and Piya, 2005; Han et al., 2012). Oxygen free radicals activity increased in sciatic nerve in experimental diabetic neuropathy (Ziegler, 2004).

Alpha lipoic acid (ALA) is an antioxidant agent that acts via scavenging of oxygen free radicals (Vallianou et al., 2009; Han et al., 2012; Ziegler, 2004; Bhatti et al., 2005; Ranieri et al., 2010). Therapeutic approaches using ALA in both prevention and treatment of diabetes and oxidative stress have been reported in human and animal studies (Vallianou et al., 2009; Han et al., 2012; Ziegler, 2004; Bhatti et al., 2005; Ranieri et al., 2010; Melhem et al., 2002). ALA decreased oxidative stress and improved blood flow in diabetic nerves (Bhatti et al., 2005; Ranieri et al., 2010). In experimental models, ALA has been proved to reduce lipid peroxidation, correct deficits in neuropeptides, improve endoneurial blood flow and glucose uptake (Ziegler, 2004). ALA has been shown to improve motor-nerve conduction velocity and to protect peripheral nerves from ischemia in diabetic rats (Vallianou et al., 2009). In diabetic nephropathy, ALA has been proved to prevent renal insufficiency, glomerular mesangial matrix expansion and glomerulosclerosis (Melhem et al., 2002).

The aim of the present study was to investigate the effects of the ALA and insulin treatment on improving blood glucose levels, body weight and laminin expression in sciatic nerve of streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Animals

32 adult male Wistar rats (200 to 250 g body weight, eight to nine weeks old) were obtained from the animal centre at Mashhad University of Medical Sciences (MUMS). They were maintained under constant conditions with temperature (23 to 25°C) and 12 h light-dark cycles with free access to water and food. The study was approved by the MUMS animal ethics committee.

Experimental design and treatment

The rats were randomly divided into four groups as follows: Control (C) group, diabetic without treatment (D) group, diabetic with insulin treated (D + INS) group and diabetic with ALA treated (D + ALA) group. Diabetes was induced in the rats by a single intraperitoneal injection of STZ (Sigma, 55 mg/kg body weight) was dissolved in 0.1 mol/l citrate buffer, pH 4.5. The rats were fasting over night prior to STZ injection. Diabetes was verified by evaluating tail vein blood glucose levels by using a digital glucometer (Accua check, Germany).

The rats with a blood glucose level higher than 300 mg/dl were considered as diabetic. The group was treated with ALA (Sigma), the powder was mixed with saline and 100 mg/kg injected intraperitoneally five times a week and insulin received group, was treated daily with 4 to 6 units of NPH insulin (EXIR Co. Iran). The treatments of diabetic rats were conducted 12 weeks after the verification of diabetes. Finally all animals were anesthetized and an incision was made in the mid thigh and sciatic nerves were carefully removed, fixed in 10% neutral buffered formalin solution for 48 h, embedded in paraffin, sectioned at 5 µm thickness and were mounted on poly-L-lysine slides (Sigma) for immunohistochemical study.

Immunohistochemical study

For immunohistochemical study, sections were deparaffinized, rehydrated and rinsed in phosphate-buffered saline solution (PBS) (pH 7.4). Enzymatic antigen retrieval was carried out with Trypsin 0.05% in PBS and preincubated in 0.025% Triton X-100 in PBS for 10 min. To block non specific antibody, this was followed by 5% goat serum and bovine serum albumin (BSA) 2% in PBS for 1 h. Then sections were reacted with primary antibody (Laminin β1 chain antibody, Abcam 11575) diluted 1:250 in PBS with 1% BSA for overnight incubation at 4°C. The next day, sections were washed with 0.025% Triton X-100 in PBS for 10 min. Endogenous peroxidase activity was blocked using 0.03% H_2O_2 for 15 min. Then specimens incubated with goat poly clonal secondary antibody (Abcam 97051) diluted 1:800 in PBS with 1% BSA for 2 h in room temperature and reacted with 0.03% solution of 3,3-diaminobenzidine tetra hydrochloride (DAB) containing 0.3% H_2O_2 for 20 min. Specimens were washed and then were counter stained with hematoxylin. Finally, sections were washed, air-dried, dehydrated, cleared and mounted in glass slides. Sections stained only with secondary antibody were treated as the negative control.

The immunostaining sections photographed by a light microscope (Olympus DP12, Japan) and laminin β1 reaction in the sciatic nerves were evaluated. All nerve samples were graded blind by two observers. Quantitative analysis was performed with grading scores of 0, 1, 2, 3, and 4 representing no, weak, moderate, strong and very strong staining, respectively (Kohbanani et al., 2012;

Table 1. Characteristic parameters in the control and diabetic rats.

Group	Body Weight (g)		Blood glucose level (mg/dl) at the end of study
	Initial	Final	
Control	235.1±7.7	304.2±6.5	111.1±8.9
Diabetic	226.1±35.3	201.8±10.6*	480.5±32.0*
Diabetic+ insulin	218.0±31.6	253.6±20.8	128.0±10.0***
Diabetic+ ALA	218.8±32.6	214.8±11.7*	423.8±13.0**

Data are mean ± SEM. n = 8 *P<0.001: compared to control group. **P<0.05 and ***P<0.001: compared to untreated diabetic group.

Kranenburg et al., 2006; Ishiyama et al., 2009; Rajabzadeh et al., 2012).

Real time PCR study

RNA extraction and cDNA synthesis

Sciatic nerve samples were removed and collected to RNA stabilization reagent (RNA later, Qiagen, Germany). Samples were used fresh or kept at -70°C. Total RNA was isolated by the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's instructions. Briefly, 25 to 30 mg of sciatic nerves were lysed with buffer containing 1% mercaptoethanol and homogenized by using polytron homogenizer (PT 1200E, Switzerland) and subsequent ultrasonication. RNA extraction proceeded according to the manufacturer's instructions. The RNA integrity was checked by visualization of 18 and 28S ribosomal bands on 1% agarose gel. First strand cDNA were made by using a cDNA synthesis kit (Fermentas) according to the manufacturer instructions. 7 µl of total RNA with 1 µl of random primer and 3 µl of water were mixed and incubated at 65°C for 5 min and then add the filling component: 4 µl of 5x reaction buffer, 1 µl of ribolock RNase, 2 µl of 10 mm dNTP mix, 1 µl of reverse transcriptase and then incubated for 15 min at 25°C followed by 60 min at 42°C and terminated reaction by heating at 70°C for 5 min. cDNA samples were stored at -20°C.

Real-time polymerase chain reaction (PCR)

Real-time PCR was performed by using the Stratagene Max3000p (USA). The reaction mixture (total volume of 20 µl per well) consisted of :10 µl of SYBR Green PCR Master Mix (Pars Tous, Iran), 1 µl of each forward and reverse primer, 0.25 µl of Taq DNA polymerase, 6.75 µl of H_2O and 1 µl of cDNA template. The following primers were used:

Laminin β1, 5'-AAGGAGGCGTTGGAAGAAGCAG-3'(forward) 5'-GGGAGGCATTGGTCAGGGTTC-3'(reverse)
GAPDH, 5'-AACTCCCATTCTTCCACCTTTG-3'(forward) 5'-CTGTAGCCATATTCATTGTCATACCAG-3'(reverse)

The PCR amplification conditions were as follows: pre-denaturing at 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s. At the end of the runs, the temperature was 95°C to construct a melting curve. The relative amount of each mRNA was normalized to the housekeeping gene, GAPDH. The average of the relative amount of each mRNA in control group is defined as 1.0. Negative controls (cDNA-free solutions) were included in each reaction.

Statistical analysis

All values are expressed as mean ± SEM. Statistical evaluation was carried out by using one way ANOVA followed by Tukey test. The data were analyzed by using SPSS software. P-values <0.05 was considered significant.

RESULTS

General characteristic of animals

In this study, our data indicated that the blood glucose levels were significantly increased and body weights markedly reduced in untreated diabetic rats compared to control rats (P<0.001) (Table 1). Insulin treatment in D + INS group improved blood glucose levels significantly and corrected body weights towards a normal range. While the ALA treatment in D + ALA group significantly decreased blood glucose levels compared to untreated diabetic group (P<0.05), it has no significant effect on the weight gain in diabetic rats (Table 1).

Results of immunohistochemical study

In this study, we investigated the localization of laminin in the fascicles with similar sized sciatic nerves, according to the intensity of color darkness. Our findings revealed that reaction for laminin in normal sciatic nerves was present in the perineurim, around the Schwann cells and in the epineurial and endoneurial blood vessels' walls. In diabetic sciatic nerves, laminin was prominent especially in the inner layers of the perineurium, endoneurium immediately surrounding groups of Schwann cells (Figure 2b) and thickened layers of the epi- and endoneurial blood vessels' walls (Figure 1). Insulin treatment significantly decreased laminin expression in all sites (P<0.05) (Figure 2c), but ALA treatment significantly decreased it only in the epi- and endoneurial blood vessels' walls (P<0.05) (Figure 2d). In normal sciatic nerve, fibroblasts were seen in the epineurium and endoneurium and they were different in shape and size (Figure 2a). In untreated diabetic rats and in D+ALA group, fibroblasts were increased in number particularly around the epineurial and endoneurial blood vessels. Moreover, a stronger reaction was seen in association with endoneurial fibroblasts (Figure 2d).

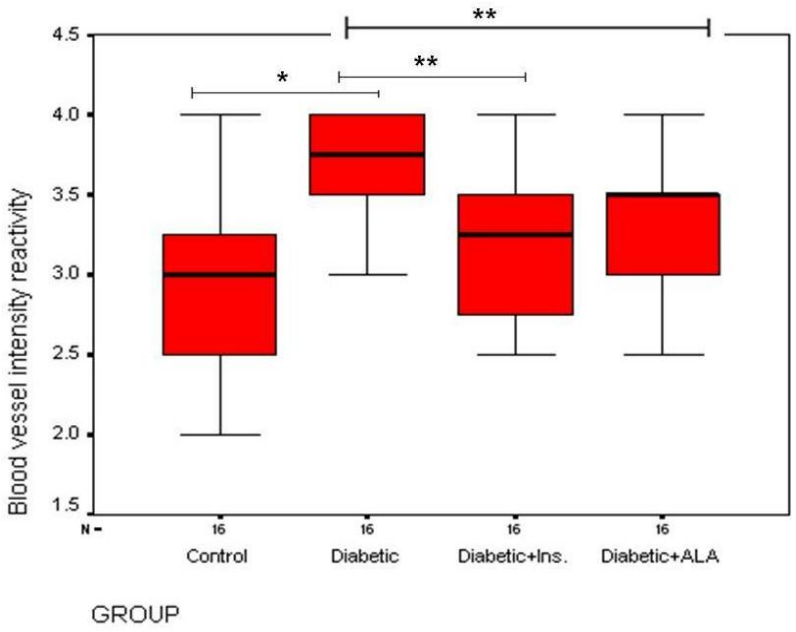

Figure 1. Boxplot shows the effect of diabetes on laminin reaction in the blood vessels walls of sciatic nerve. Data are presented median in the form of 50% (25 and 75%). N = 16. *P<0.05: compared to control group, **P<0.05: compared to diabetic group.

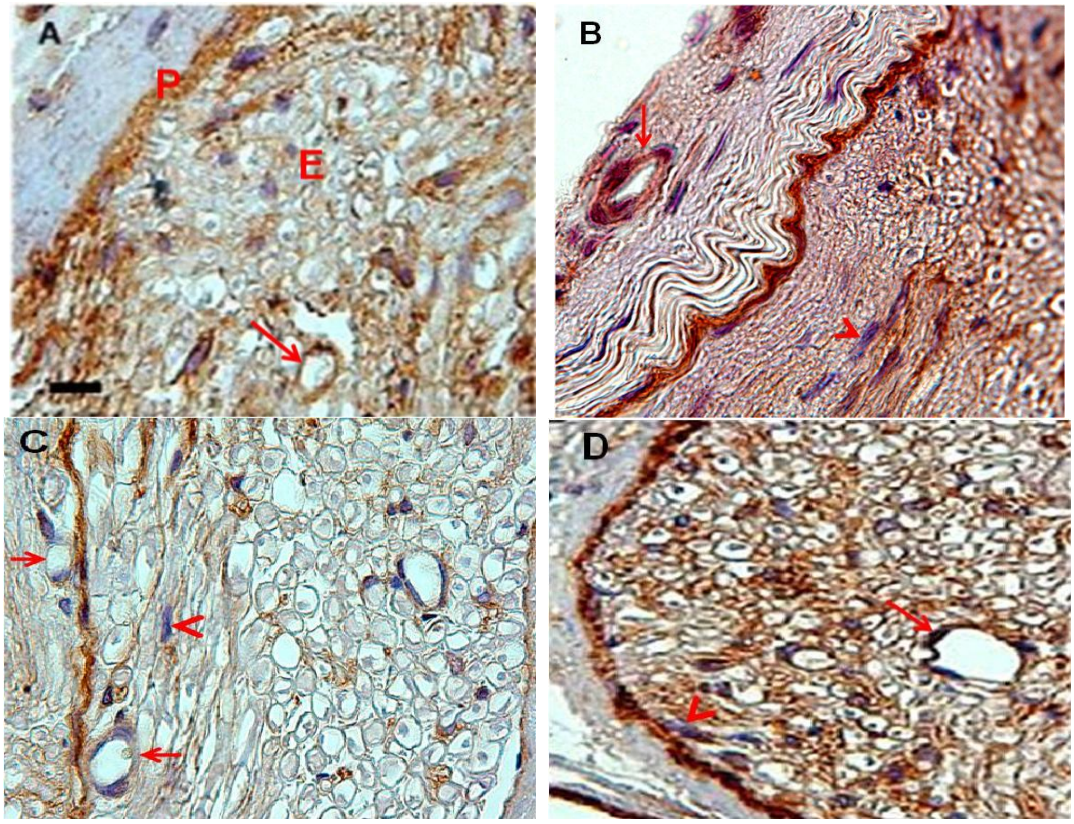

Figure 2. Transverse sections of control and diabetic nerve samples which were incubated with laminin antibody. The locations which positive expressed were brown. Control: (A), diabetic: (B), diabetic insulin treated: (C) and diabetic ALA treated: (D) groups. The arrows indicated epineurial and endoneurial blood vessels and arrow heads indicated fibroblasts. Perineurium (P), Endoneurium (E), Scale bar = 10 µm.

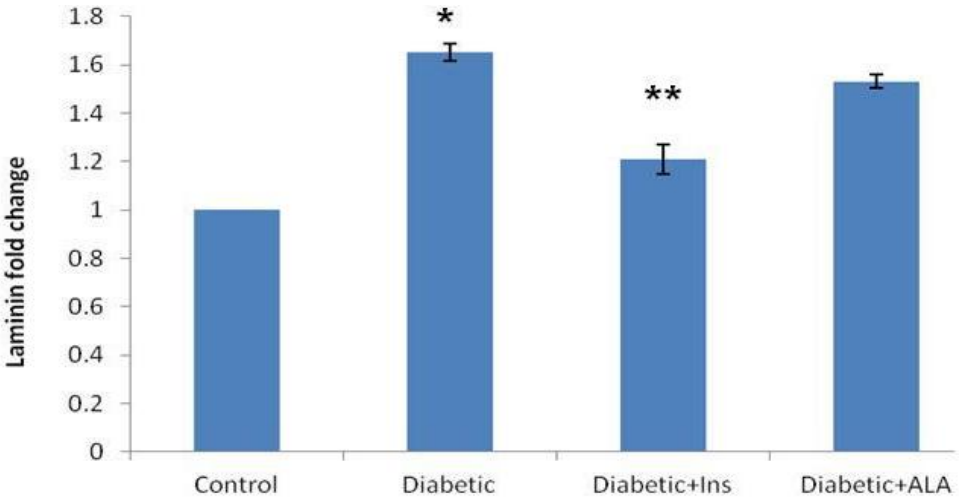

Figure 3. Comparative analysis of laminin β1 mRNA expression in sciatic nerve by using real-time PCR. Data are mean ± S.E.M. n = 6. *****P<0.05: compared to control group, ******P<0.05: compared to diabetic group.

Results of real-time PCR study

Laminin β1 mRNA expression in sciatic nerves was detected by real-time PCR. Data analysis revealed that laminin β1 mRNA level significantly increased (0.6 fold) in the untreated diabetic group compared to the control group (Figure 3). Insulin treatment significantly reduced laminin β1 mRNA over expression (P<0.05). Although, ALA reduced mRNA over expression, the results were not significant (Figure 3).

DISCUSSION

The present study was designed to investigate the therapeutic effects of ALA and also insulin on blood glucose levels, body weight and alteration in laminin expression of sciatic nerve in the STZ-induced diabetic rats. Laminin is described to be part of the normal adult sciatic nerve in the rat (Bannerman et al., 1986; Lorimier et al., 1992). Unsuccessful nerve regeneration in diabetic neuropathy suggested that it may be in part due to changes in extracellular matrix (ECM) composition (Yasuda et al., 2003). ECM proteins of peripheral nerve may cause alterations in the structure and function that is induced by hyperglycemia or advanced glycation end products (AGEs) (Duran-Jimenez et al., 2009). AGEs stimulate production of oxygen free radicals, which leads to oxidative stress. Alterations in the sorbitol, polyol pathway activation, decreased nitric oxide and impaired (Na+/K+) ATPase activities are some other pathogenetic mechanisms that have been implicated in diabetic neuropathy (Bhatti et al., 2005; Kathleen, 2006). ALA delays or reverses diabetic neuropathy through its multiple antioxidant properties (Vallianou et al., 2009).

Our findings reveal that ALA with 100 mg/kg, intraperitoneally injection has shown partial hypoglycemic effects in the diabetic rats. This may be due to increase glucose transport by ALA (Stevens et al., 2000). Sun et al. (2009) showed that rat sciatic nerves had rich laminin content. Glycation of laminin in ECM leads to impaired regenerative activity in diabetic neuropathy (Sugimoto et al., 2008). Our immunohistochemial results indicated that diabetes in rats resulted in significantly increased laminin expression in the perineurium, endoneurium, epineurial and endoneurial blood vessels. In an agreement with our results, some researchers have reported diabetic neuropathy resulted in abnormal amounts of laminin in human and animal models (Yasuda et al., 2003; Hill, 2009), but in a different way. Hill (2009) reported that, in human diabetic neuropathy laminin content in diabetic and control nerves was not significantly different. Also, Serafin et al. (2010) assessed laminin in peripheral nerves of STZ-induced diabetic mice, and reported that it did not differ in laminin expression in the diabetic and control nerves. These discrepancies are probably due to differences in experimental methods, in Serafin et al. (2010) study, mice was made diabetic with a low dose of STZ with a short-term study and in Hill (2009) study, duration of diabetes in diabetic patients were different. Duration of diabetes and long-term hyperglycemia are the most important reasons for polyneuropathy (Bureković et al., 2008). Beneficial effects of ALA on the neuropathic symptoms due to diabetic neuropathy had been reported (Vallianou et al., 2009; Han et al., 2012; Ziegler, 2004).

Our immunohistochemical results show that ALA treatment significantly decreased laminin expression only in the basement membrane of epineurial and endoneurial blood vessels. This may be due to the fact that the antioxidants reduce vascular impairment in diabetic rats

(Sotnikova et al., 2006). It is suggested that oxidative stress leads to defective nerve blood supply and ALA has been shown to protect peripheral nerves from ischemia in experimental diabetic neuropathy (Vallianou et al., 2009; Han et al., 2012). In agreement to our results, Okudan et al. (2011) revealed that ALA prevents the vascular complications in the STZ induced diabetic rats. Muona et al. (1991) investigated the effects of incubation of cell cultures, consisting of Schwann cells, perineurial cells, and fibroblasts, in high glucose concentrations and suggested that laminin B2 chain mRNA levels appeared unaltered. In contrast, Depto et al. (1993) have suggested that laminin B2 expression under basal conditions and during neuronal regeneration was decreased in diabetic animals. Previous studies have suggested that the level of laminin β1 mRNA in kidney or retina increased after diabetes induction (Fukui et al., 1992; Yang et al., 1995; Stitt et al., 2002; Winkler et al., 2012). Also, our findings show that, laminin β1 expression was increased in sciatic nerve of untreated diabetic rats at mRNA level 12 weeks after diabetes induction. Insulin was significantly reduced expression of laminin β1 upregulation at mRNA level but ALA only partially decreased it in sciatic nerves.

Diabetic neuropathy is a multi factorial disorder and our findings suggest that ALA with 100 mg/kg dose had no significant affect on improvement of laminin β1 mRNA regulation in diabetic nerves. This may also be due to inadequate amount or duration of ALA treatment.

Conclusions

This experiment showed that laminin glycoprotein is expressed in rat sciatic nerves, and that the expression of laminin is increased in sciatic nerves of STZ-induced diabetic rats. It implies that insulin therapy is more effective than ALA treatment in improving hyperglycemia and laminin up-regulation in diabetic nerves. It might be suggested that ALA treatment reduced the laminin up-regulation only in the epineurial and endoneurial blood vessels walls. Further investigation in this direction is warranted.

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