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REMEDIAL EFFECTS OF VITAMIN E AND L-ARGININE ON PERIPHERAL NEUROPATHY IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

It was shown that hyperglycemia in diabetic patients is the main factor of diabetic peripheral neuropathies. Various pathways related to oxidative stress, vascular defect and defective endothelium dependent relaxation have been implicated in the development of diabetic peripheral neuropathy. A substantial number of studies have shown that antioxidant treatment are promising therapeutics that can prevent or correct reduced motor nerve conduction in diabetic rats by acting on these mechanisms. This study was designed to investigate the possible role of insulin treatment along with or without vitamin E or L-arginine on diabetic neuropathy. This goal was accessed by examining nerve conduction, parameters of oxidative stress and lipid peroxidation as well as the expression level of endothelial nitric oxide synthase in the sciatic nerve of control and streptozotocine-induced diabetic rats. Data showed that diabetic rats showed increased levels of serum glucose (382.5%) and sciatic nerve lipid peroxidation Marker (MDA, 261.6%) with a concomitant decrease in the expression of sciatic nerve eNOS mRNA as compared to control rats. The nerve conduction studies of the sciatic nerves of these rats showed decrease conduction as evident by delayed NCV (63.6%) and low Amplitude of Muscle Contraction (AMC, 36.4%). Solitary insulin treatment (but not vitamin-E or L-arginine) corrected serum glucose to control values and corrected nerve conduction parameters in the sciatic nerve. However, treating diabetic rats with different doses of vitamin E (300 mg kg^{-1} and 600 mg kg^{-1}) significantly reduced oxidative stress by decreasing MDA and increasing GPx activity, corrected NCV by reducing the latency and improving AMC and increased eNOS mRNA expression in sciatic nerve with a more profound effect to seen with the high dose (600 mg kg^{-1}). However, the maximum potent ameliorating effect of the vitamin E on these parameters was seen when administered in combination with insulin. On the other hand, L-arginine treatment alone or in combination with insulin had no effect on the oxidative stress markers nor eNOS expression but significantly and maximally improved NCV through reducing the conduction latency and increasing AMC. This study supported the notion that diabetic peripheral neuropathy is a multifactorial complication, caused by hyperglycemia, oxidative stress and vascular impairment. It is concluded that conjugate treatment with vitamin-E, especially in higher doses, with insulin could be of great value. Moreover correction of impaired nerve blood flow by drugs that induce nitric oxide has proved to be efficient in the protection against and correction of experimental diabetic peripheral neuropathy.

Keywords: Antioxidants, Vitamin E, L-arginine, Diabetes, Neuropathy

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1. INTRODUCTION

Diabetes is one the major diseases with various complications. The prevalence rate is 8.9%-12.3% in human population (Zangiabadi *et al*., 2011). It leads to various complications in long term. Neuropathy is one of the most common diabetic complications (Zangiabadi *et al*., 2007) of the symmetrical diabetic neuropathies, distal symmetrical polyneuropathy, a predominantly axonal, length dependent neuropathy is most prevalent. The frequency of this complication in patients with long-term diabetes is approximately 50% which can observe in both insulin-dependent and noninsulin dependent patients (Quintanilha *et al*., 2011). The diagnosis is based on history taking, physical exam, Nerve Conduction Study (NCS), Electromyoghraphy (EMG) and finally nerve biopsy (Zangiabadi *et al*., 2007).

Evidences suggest that oxidative stress and free radicals play an important role in the pathogenesis of diabetes mellitus and diabetic complications including tissue damage and injury (Zangiabadi *et al*., 2011). Oxidative stress results from an imbalance between radical-generating and radical scavenging systems, that is, increased free radical production or reduced activity of antioxidant defense or both. In diabetes, protein glycation and glucose autoxidation may generate free radicals, which in turn catalyse lipid peroxidation and tissue injury (Zangiabadi *et al*., 2011). Moreover, disturbances of antioxidant defence systems in diabetes were shown to cause decreases in antioxidant enzymes (El-Nabarawy *et al*., 2010), impaired glutathione metabolism (El-Nabarawy *et al*., 2010) and decreased antioxidants levels, i.e., Ascorbic acid (Ambali *et al*., 2011).

However, it has been shown that hyperglycemia in diabetic patients is the main factor of diabetic neuropathies induces oxidative stress through various cellular pathways such as increasing aldose reductase activity (Srivastava *et al*., 2005), increasing glycation end-products (Sugimoto *et al*., 2008) and altering protein kinas C activity (Yamagishi *et al*., 2008). Additionally, longstanding hyperglycemia induced oxygen free radicals can damage mitochondrial DNA in dorsal root ganglia leading to peripheral nerves dysfunction (Schmeichel *et al*., 2003; Arora *et al*., 2008; Sharma *et al*., 2009). Moreover, it was shown that vascular defect and resistance following oxidative stress decreases blood flow and causes hypoxia and decrease of nerve function leading eventually to Nerve Conduction Velocity (NCV) reduction (Saini *et al*., 2007; Yorek *et al*., 2004) and treatment of established neuropathies with supplemental oxygen, vasodialtors or inhibitors of vasoconstriction improved nerve conduction by increasing blood flow (Low *et al*., 1984; Kihara *et al*., 1999; Hohman *et al*., 2000).

Interestingly, more attention was given now to the role of endothelial dysfunction in diabetic neuropathies. In sciatic nerve, it was shown that endothelial dysfunction and defective endothelium-dependent relaxation and develops before diabetic neuropathy (Coopey *et al*., 2000; Thipkaew *et al*., 2012), suggesting that impaired vasoreactivity may also contribute to decrease NCV. Some studies suggested that a deficiency of substrate or cofactors for nitric oxide synthase, increased quenching of nitric oxide by Advanced Glycosylation End products (AGE), decreased nitric oxide release and decrease nitric oxide availability may contribute to diabetic neuropathies and decreased NCV (Karasu, 2000). Indeed, the tendency towards increased vasoconstriction and decreased blood flow is exacerbated by reduced vasa nervorum NO synthesis or action (Kihara and Low, 1995), which further reinforces the argument that the effects on vasa nervorum endothelium are very important for antioxidant action and nerve function in experimental diabetes.

A substantial number of studies have shown that antioxidant treatment are promising therapeutics that can prevent or correct reduced motor and sensory NCV in diabetic rats (Cotter *et al*., 1995; Cameron *et al*., 1998). Where measured, there was accompanying improvement in nerve perfusion and prevention of defective NO-mediated endothelium dependent relaxation. Indeed, such effect was seen by treatment with butylated hydroxytoluene, a lipophilic scavenger (Archibald *et al*., 1996) and antioxidant effects to improve nerve blood flow and NCV in diabetic rats are blocked by co-treatment with a low dose of a NO synthase inhibitor (Cameron and Cotter, 1995).

Previously, it was shown that diabetic tissue injuries as a result of diabetic complications is not controllable only via inhibition of hyperglycemia (Liu *et al*., 2008) and monitoring of blood glucose levels solely is not sufficient in retarding diabetes complications. Thus, in treating of diabetes and its complications including neuropathies, a suitable treatment must contain agents that have both antioxidant and blood glucose decreasing properties (Ramesh and Pugalendi, 2006). Also, giving the important role of targeting the antioxidant on vasa nervorum endothelium and nitric oxide status, this study was designed to investigate the possible role of insulin treatment along with vitamin E, excellent antioxidant or with L-arginine, a substrate for nitric oxide production, on diabetic neuropathy. This goal was achieved by examining nerve conduction, parameters of oxidative

stress and lipid peroxidation as well as the expression level of endothelial nitric oxide synthase in the sciatic nerve of streptozotocine-induced-diabetic rats. This study is part of collaboration between Cairo University, Egypt and King Khalid University, Saudi Arabia and some preliminary results of it had been published previously in abstract form during the 2011 London themed meeting of the Physiological Society on cellular and integrative neuroscience (Bin-Jaliah *et al*., 2011).

2. MATERIALS AND METHODS

2.1. Animals

The experiments were performed on healthy male wistar rats of 10 weeks old and weighting 200-250 g. The rats were fed with standard laboratory diets, given water ad libitum and maintained under laboratory conditions of temperature 22°C (±3°C), with 12 h light and 12 h dark cycle. All experimental procedures involving the handling and treatment of animals were approved by the Ethical Committee of Physiology at the King Khalid University Medical School (Abha, KSA) and were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Induction of Diabetes

Diabetes was introduced to rats by a single i.v. dose of streptozotocin (Sigma, St Louis, Mo, USA) into the tail vein at a dose of 65 mg kg^{-1} body weight (Zeggwagh *et al*., 2007). STZ was extemporaneously dissolved in 0.1 M cold sodium citrate buffer, pH 4.5. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycaemia. After 3 days, animals with fasting blood glucose levels greater than 300 mg dL^{-1} were considered diabetic and then included in this study.

2.3. Experimental Design

After one week adaptations, the animal were classified into 9 groups (each of 10 rats) as follows:

Group1: Control rats received normal saline (10 mL kg^{-1}, i.p.)
Group2: Diabetic model group.
Group3: Diabetic rats received daily insulin injection (1IU/day, s.c., Lafci-Erol *et al*., 1994).
Group4: Diabetic rats received Vitamin E (300 mg kg^{-1}, i.m.), three times/week (Haidara *et al*., 2003).
Group5: Diabetic rats received Vitamin E (600 mg kg^{-1}, i.m), three times/week (Haidara *et al*., 2003).
Group6: Diabetic rats received combined doses of both insulin and Vitamin E (300 mg kg^{-1}) three times/week in same routs as above.
Group7: Diabetic rats received combined doses of both insulin and Vitamin E (600 mg kg^{-1}) three times/week in same rout as above.
Group8: Diabetic rats L-arginine (50 mg kg^{-1}, orally) on daily basis (Costa *et al*., 1998).
Group9: Diabetic rats received combined doses of both L-arginin and insulin in same rout as above

All treatments were continued for 4 weeks. At the end of day 28 and after overnight fasting, rats of all groups were anaesthetized using phenobarbitone (50 mg kg^{-1}). Blood samples were immediately taken from the heart and placed in plain tubes to clot at room temperature. Following centrifugation at 4000 rpm for 10 min, serum was collected and stored at -20°C until further biochemical analysis. Immediately after blood collection, all animals of the various groups were exposed for electrophysiogical study to measure nerve conduction latencies and muscle contractions after which they were killed by decapitation. Sciatic nerves from all groups were quickly collected, washed with Phosphatebuffered Saline (PBS), pH 7.4, containing 0.16 mg mL^{-1} of heparin to remove any red blood cells (erythrocytes). Some parts of the nerves were homogenized with an ultrasonic homogenizer in cold phosphate buffer, pH 7.0 with Ethylenediaminetetraacetic acid (EDTA), for Malondialdehyde (MDA) measurement and in cold buffer consists of 50 mM tris-HCl, pH 7.5, 5 mM ED-TA, 1 nM DTT for Glutathione Peroxidise (GPx) activity analysis. All supernatants were kept in separate tubes and stored at -20. Other parts of the nerves were used for RT-PCR reaction for detection of levels of mRNA expression of eNOS.

2.4. Electrophysiology

After induction of anaesthesia, the animals were exposed to NCV velocity study of sciatic nerve. The environment temperature was maintained at 25±1°C during all stages of study. The rats were prepared for nerve conduction by shaving the hind limbs and cleaning with alcohol. Motor conduction velocity was measured in the sciatic-tibial nerve using Nihon Kohden instrument (Japan). Stimulation was carried out per-cutaneously using a bipolar surface stimulating electrode to stimulate the nerve at the gluteal fold. Electrical square wave pulses were applied at 0.2 msec. duration were delivered at a rate of 10 Hz and the intensity was increased to be just supra-maximal. The provoked motor

response was recorded from the gastrocnemius muscle using concentric-needle electrodes and analyzed for its latency and Amplitude of Muscle Contraction (AMC). MNCV was calculated by dividing the distance between the two stimulated sites (mm) by the difference between proximal and distal latencies (ms).

2.5. Measurements of Serum Glucose

Serum glucose was determined by enzymatic colorimetric method, using kits provided by International Head Quarters Randox Laboratories Lts.

2.6. Measurement of Malondialdehyde (MDA) Levels

Lipid peroxidation levels in sciatic nerve homogenates were measured by the Thiobarbituric Acid (TBA) reaction according to Ohkawa *et al*. (1979). This method was used to measure spectrophotometrically the color produced by the reaction of TBA with Malondialdehyde (MDA) at 532 nm. For this purpose, TBARS levels were measured using a commercial assay as the Malondialdehyde Assay (Cat No. NWK-MDA01) supplied from NWLSS, USA. In breif, Tissue supernatant (50 µL) were added to test tubes containing 2 µL of Butylated Hydroxytoluene (BHT) in methanol. Next, 50 µL of acid reagent (1 M phosphoric acid) was added and finally 50 µL of TBA solution was added. The tubes were mixed vigorously and incubated for 60 min at 60°C. The mixture was centrifuged at 10,000×g for 3 min. The supernatant was put into wells on a microplate in aliquots of 75 µL and its absorbance was measured with a plate reader at 532 nm. TBARS (MDA) levels were expressed as nmol/mg protein.

2.7. Measurement of Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase activity in sciatic nerve homogenates was measured using the Glutathione Peroxidase Assay Kit (Cat NO.703102) provided by Caymen chemical as per manufacture instructions. Glutathione peroxidase catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. With the exception of phospholipid hydroperoxide GPX, a monomer, all of the GPX enzymes are tetramers of four identical subunits. Each subunit contains a selenocysteine in the active site, which participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimateelectron donor to regenerate the reduced form of the selenocysteine. The Cayman Chemical Glutathione Peroxidase Assay Kit measures GPX activity indirectly by a coupled reaction with Glutathione Reductase (GR). Oxidized Glutathione (GSSG) is produced upon reduction of hydroperoxide by GPX and is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPX activity is rate limiting, the rate of decrease in the A340 is directly proportional to the GPX activity in the sample. The results were presented as nmol/g protein. One unit is defined as the amount odf enzyme that causing the oxidation of 0.1nmol of NADPH to NADP+/min at 25°C.

2.8. RNA Extraction and RT-PCR

Oligonucleotide primer sequences of rat eNOS and and β-actin were designed according to published data sequence and are shown in **Table 1**. Total RNA was extracted from the sciatic nerves (30 mg) using the SV-total RNA isolation system (Promega-Madison, USA) according to manufacturer's directions. The concentration of total RNA was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan). The purity was estimated by the 260/280 nm absorbance ratio. Single strand cDNA synthesis was performed as follows: 30 µL of reverse transcription mixture contained 1 µg of DNase I pre-treated total RNA, 0.75 µg of oligo d(T) primer, 6 µL of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor and 240 U of reverse transcriptase (Invitrogen). The RT reaction was carried out at 42°C for 60 min followed by heat inactivation at 95°C for 5 min. The tested genes and that of the internal control (β-actin) were amplified by PCR using 2 µL RT products from each sample in a 20 µL reaction containing Taq polymerase (0.01 U mL^{-1}), dNTPs (100 mM), MgCl2 (1.5 mM) and buffer (50 mM Tris-HCl). PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification, defined by denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min. A final extension cycle of 72°C for 12 min was included. A control reaction without reverse transcriptase was included for every sample of RNA isolated to verify absence of contamination. PCR product (10 µL) were electrophoresed on 2% agarose gels containing 100 ng mL^{-1} ethidium bromide and photographed with a Polaroid camera under ultraviolet illumination. PCR products were semi quantitated by using gel documentation system (Bio Doc Analyze) supplied by Biometra. Data were presented as the mean density of the mRNA band in question relative to that of the b-actin band in the same sample.

Table 1. Primers and conditions used in PCR reactions

Target	Primer sequence (5' to 3')	AT (^{o}C)	Size (bp)
eNOS	5'-ATCCTGGCAGCCCTAAGACC-3'[c] 5'-TGGTAGCGTTGCTGATCCCG-3'[d]	57	229
β-actin	5'-TCACTATCGGCAATGTGCGG-3'[c] 5'-GCTCAGGAGGAGCAATGATG-3'[d]	57	260

AT: Annealing Temperature
[c]Sense.
[d]Antisense

2.9. Statistical Analysis

Values are measured as mean ± SD. Comparison of data was performed by using ANOVA test (analysis of variance test) using graph pad prism analysis software, version 5. Probability (P) values of <0.05 were considered to be significant.

3. RESULTS

3.1. Evaluation of Blood Glucose Level

Data in **Table 2** revealed that diabetic model rats exhibited significant increase in serum glucose levels (3.5 folds) as compared with control rats. Treating diabetic rats with insulin injection in a dose 1IU/day either alone or in combination with vitamin E (300 or 600 mg kg^{-1} BW) or with L-arginine (50 mg kg^{-1} BW) resulted in a significant reduction in serum glucose toward normal values when compared to diabetic model group. However, treating diabetic rats with both different doses of vitamin E alone (300 or 600 mg kg^{-1}) had no significant effect on serum glucose levels as compared with diabetic group of rats. Also treating diabetic rats with L-arginine alone (50 mg kg^{-1} BW) had no significant effect on serum glucose levels as compared with diabetic group of rats.

3.2. Sciatic Nerve MDA Levels and GPx Activity

Diabetic rats showed significant increase in MDA levels and significant decrease in the activity of GPx enzymes in their sciatic nerve homogenates as compared to corresponding values measured in control rats. Insulin treatment to diabetic rats alone was not able to ameliorate the changes in these parameters. On the other hand, all treatment included vitamin E resulted in significant decreases in MDA levels and significant increases in GPx activity in a dose dependent manner with the highest decrease in MDA and maximum activity of GPx to seen in the group of rats administered combined high vitamin E dose (600 mg kg^{-1}) and insulin. In this group of rats, The ANOVA test revealed that the levels of MDA were not significantly different with those obtained in the control group while Gpx activity was significantly higher than corresponding control levels reported in the control group. On the other hand, treatments with L-Arginine alone or in combination with insulin failed to restore the levels of MDA and activities of GPx in the treated rats and their levels remained not significantly different to those in diabetic model group (**Table 3**).

3.3. Nerve Electrophysiology

Studies on Nerve Conduction Velocity (NCV) of the sciatic nerve in this studies revealed significant increase latencies and decreased Amplitude of Muscles Contraction (AMC) in the nerves of diabetic group of rats as compared to those reported in the control group of rats. However, administration of insulin or vitamins of 300 mg kg^{-1} and 600 mg kg^{-1} alone, or combined administration of insulin and vitamin E at the dose of 300 resulted in a significant decreases in conduction as seen by decrease latencies recorded in all these group as compared to diabetic model group. The ANOVA analysis showed the the latancies recorded in all these three groups remained significantly higher than those recorded in the control group. Also, AMC remained reduced and not significantly changed to those recorded in the diabetic model in all of these treate groups. the On the other hand, Administration of L-arginine alone or incombination with insulin or the combined administration of insulin and vitamin E at its higher dose (600 mg) completely decreased the conduction latencies to their control levels as compared to diabetic model group. However, higher normal values of AMC were recorded in these groups of rats with the the maximum AMC to seen in L-arginine treated groups of either individual administration or in combination with insulin. The AMC in both of these groups were significantly higher than those recorded in the control group (**Table 4**).

Table 2. Fasting blood glucose levels (mg/dL) in the control and all experimental groups of rat

	FBS (mg/dL)	
Group	Mean	S.D.
Control	92.6[a]	8.69
Diabetic	354.2[b]	35.22
Diabetic+insulin 1 unit	111.3[a]	9.03
Diabetic+Vit E (300 mg kg^{-1})	352.5[b]	30.58
Diabetic+Vit E (600 mg kg^{-1})	343.8[b]	34.25
Diabetic+Vit E (300 mg kg^{-1}) + insulin (1U)	109.1[a]	11.30
Diabetic+Vit E (600 mg kg^{-1}) + insulin (1U)	104.33[a]	7.49
Diabetic+L-arginine (50 mg kg^{-1})	358.25[b]	28.65
Diabetic+L-arginine (50 mg kg^{-1}) + insulin (1U)	107.13[a]	10.52

Values are mean ±SD. Significance differences when $p<0.05$. Similar letters have the same significance and are significantly different from other letters within the same test column

Table 3. MDA levels and GPx activity in the sciatic nerves of the control and experimental groups of rat

	MDA (nmol/mg protein)		GPx Activity (nmol/g protein)	
Group	Mean	S.D.	Mean	S.D.
Control	0.0852[a]	0.02062	1.8900[a]	0.20187
Diabetic	0.2229[b]	0.04192	0.9086[b]	0.21075
Diabetic+insulin (1U)	0.1950[b]	0.01871	1.0520[b]	0.07259
Diabetic+Vit E (300 mg kg^{-1})	0.1249[a]	0.02251	1.7257[a]	0.42844
Diabetic+Vit E (600 mg kg^{-1})	0.1077[a]	0.02066	2.7867[c]	0.42283
Diabetic+Vit E (300 mg kg^{-1})+ insulin (1U)	0.1234[a]	0.01751	2.2333[c]	0.21602
Diabetic+Vit E (600 mg kg^{-1})+ insulin (1U)	0.0717[a]	0.01602	3.6340[s]	0.40924
Diabetic+L-arginine (50 mg kg^{-1})	0.2250[b]	0.04000	0.9200[b]	0.17470
Diabetic+L-arginine (50 mg kg^{-1})+ insulin (1U)	0.2300[b]	0.03162	1.0280[b]	0.17065

Values are mean ± SD. Significance differences when $p<0.05$ MDA: Malondialdehyde, GPX: Glutathioneperoxidase. Similar letters have the same significance and are significantly different from other letters within the same test column

Table 4. Sciatic nerve conduction velocities latency and Amplitude of Muscle Contraction (AMC) in the control and experimental groups of rat

	NCV Latency		AMC	
Group	Mean	S.D.	Mean	S.D.
Control	0.91[a]	0.24	22.44[a]	4.08
Diabetic	1.43[b]	0.22	8.17[b]	1.75
Diabetic+insulin 1 unit	1.21[c]	0.10	9.02[b]	0.81
Diabetic+Vit E (300 mg kg^{-1})	1.18[a]	0.11	11.94[b]	2.43
Diabetic+Vit E (600 mg kg^{-1})	1.03[a]	0.25	18.65[a]	1.93
Diabetic+Vit E (300 mg kg^{-1})+ insulin (1U)	1.12[a]	0.16	11.38[b]	2.95
Diabetic+Vit E (600 mg kg^{-1})+ insulin (1U)	1.07[a]	0.16	24.57[a]	5.10
Diabetic+L-arginine (50 mg kg^{-1})	1.14[a]	0.16	31.84[c]	9.65
Diabetic+L-arginine (50 mg kg^{-1}) + insulin (1U)	0.94[a]	0.19	32.25[c]	3.51

Values are mean±SD. Significance differences when $p<0.05$ Similar letters have the same significance and are significantly different from other letters within the same test column

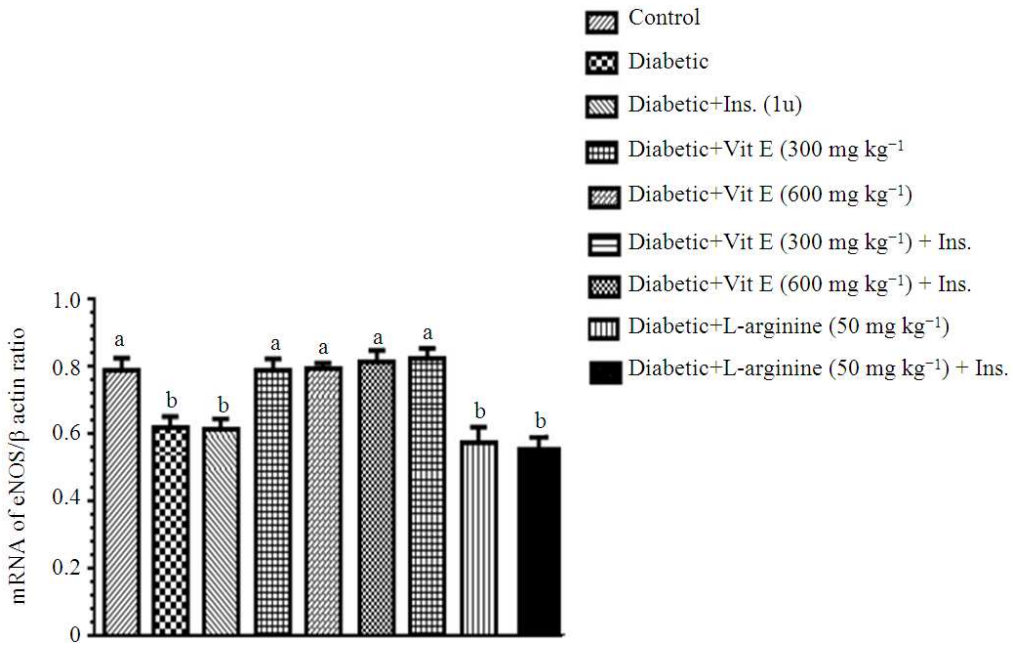

Fig. 1. Ratio of mRNA of eNOS/β actin in the control and experimental group. Values are mean±SD Significance differences when p<0.05. eNOS: Endothelial nitric oxide synthase. Similar letters have the same significance and are significantly different from other letters within the same test column

3.4. Evaluation of Sciatic Nerve mRNA Expression Levels

Shown in (**Fig. 1**) are the transcriptional changes of e NOS in the rat's sciatic nerve of all groups of rats. All tested transcripts were detected and RT-PCR resulted in fragments similar in size to those expected. The levels of the β-actin transcript remained relatively constant in the testes of all groups. The expression levels were presented as relative mRNA expression of eNOS to that of β-actin of same sample. In comparison to control groups. In the diabetic rats model, relative mRNA expression of eNOS/β-actin was suppressed (0.660+0.022 and 0.647±0.046%, respectively as compared to control group (0.805±0.013%). Similar suppressed ratios of eNOS relative expression were obtained in the diabetic groups treated with solitary insulin (0.647±0.046%) or L-Arginine (0.609±0.018%) or in combination with insulin (0.604±0.014%). On the other hand, vitamin E treatment of different doses in all groups as given alone or in combination to insulin resulted in significant increases in eNOS mRNA relative expression which were not significantly different to those obtaine in the control group but significantly higher than those calculated in the diabetic group. The ANOVA analysis showed that the relative levels of eNOS mRNA expression in all groups included vitamin treatment were not significantly different when compared to each other (**Fig. 1**).

4. DISCUSSION

Peripheral neuropathy is one of the major complains in both types I and II diabetic patients and it is associated with several problems such as cardiovascular defects, retinopathy and muscular pain or weakness (Yamagishi *et al*., 2008; Sima, 2003; Rajbhandari and Piya, 2005). Since these defects affect the quality and quantity of life, treatment of diabetic neuropathy or prevention of its accompanying symptoms has been considered as a major goal in the recent decades.

In our current study, administration of the insulin to diabetic rats improved glucose levels, improve nerve conduction as evident by decrease latencies and increased muscle contraction and increased levels in the sciatic nerve without any significant improvement in oxidative stress status nor eNOS mRNA expression in the sciatic nerve. The levels of MDA and GPx remained unchanged in the diabetic rats received insulin only. Such effect was reported previously by other investigators (Kihara and Low, 1995; Clark and Lee, 1995) who found that insulin therapy improved nerve blood flow and NCV in diabetic rats mainly by decreasing glucose levels. Since insulin was able to ameliorate nerve conduction function in diabetic rats, we could conclude that hyperglycemia suppresses the activity of eNOS and thus decreasing the availability of

Nitric Oxide (NO) in the sciatic nerve resulting in decrease nerve conduction. Previous studies have shown that decrease in No availability or action in the nerves associated with decrease conduction velocity by increasing vasoconstriction (Kihara and Low, 1995) leading to reduced peripheral nerve perfusion which in turns causes endoneurial hypoxia, which is a major factor in the etiology of diabetic neuropathy in patients and animal models (Low *et al*., 1989; Tesfaye *et al*., 1994; Cameron and Cotter, 1994).

However, Reactive Oxygen Species (ROS) are increased in diabetes and were implicated as the main cause of diabetic neuropathy. The main sources are metabolic including autoxidation of glucose and its metabolises, advanced glycation, altered prostanoid production and abnormal or inefficient mitochondrial function (Arora *et al*., 2008; Sharma *et al*., 2009). In patients, levels of ROS rise with poor metabolic control (Arora *et al*., 2008). For peripheral nerve, ROS can directly damage neurons and Schwann cells and in combination with diabetes, antioxidant protection mechanisms are compromised (Negi *et al*., 2010). Indeed, in our current study, Diabetes was associated with increased RSO generation as evident by increased sciatic nerve lipid peroxidation decrease activity of Glutathione Peroxidise (GPx). This in turns could lead to cumulative neurodegenerative changes such as axonopathy and demyelination, as well as deleterious effects on cell bodies and their mitochondria as has recently been observed in dorsal root ganglion (Nagamatsu *et al*., 1995; Low *et al*., 1997) resulting in decreased sensory and motor nerve conduction velocity. Increased conduction latencies and decrease muscle contraction reported in the diabetic rats of current study indicates decreased motor nerve conductivity velocity in those diabetic rats is an evidence of diabetic neuropathy development in these rats. These results are in accordance to Pascoe *et al*. (1997) who demonstrated that diabetes is invariably accompanied by concomitant axonal degeneration that resulted in proximal muscle weakness. Also, in the same line, Sima (2003) suggested that diabetic neuropathy was associated with axonal atrophy.

Independent of tissue injury, ROS also has effects on blood vessel function, which compromise perfusion of several organs including peripheral nerve. This is responsible for the earliest defects in nerve function in experimental models and will exacerbate nerve damage by causing further ROS-dependent ischemia-reperfusion effects (Cameron *et al*., 1991). Nitric Oxide (NO) is an important vascular target for ROS. Superoxide neutralizes NO and the peroxynitrite formed is a source of hydroxyl radicals that can cause endothelial damage (Beckman *et al*., 1990). Oxidative stress therefore diminishes vessel endothelium-dependent relaxation, which is apparent in some experimental preparations even after acute exposure to hyperglycemia (Tesfamariam and Cohen, 1992). Defective endothelium-dependent relaxation has been observed in chronic diabetic animals (Mayhan, 1992; Rosen *et al*., 1995) and also in type 1 and type 2 diabetic subjects (McVeigh *et al*., 1992) and is an important potential target for antioxidant treatment. Hence, Prevention or correction of ROS generation and/or enhancing blood flow deficit is considered promising treatments of diabetic neuropathy. Till now, no individual treatment was proven to show both antioxidant and blood flow enhancing effect. The only successful treatments to increase blood flow in diabetic neuropathies include chronic electrical nerve stimulation, vasodilators and drugs that compensate for some of the metabolic changes in diabetes such as n-6 essential fatty acids, aldose reductase and protein kinase C inhibitors, anti-advanced glycation agents and antioxidants (Cameron and Cotter, 1995). Thus searching for combination of treatment to ameliorate the diabetic neuropathy and decreased conduction velocities is highly favourable.

Also, it was observed in the present study that intramuscular injection of vitamin E at different doses (300 or 600 mg kg^{-1}) has protective effects against peripheral nerves injury and diabetic neuropathy due to hyperglycemia. The better protective effect of vitamin E is achieved when given at its high dose (600 mg kg^{-1}) as a combined treatment with insulin therapy. In the current study, the effect of vitamin E was shown to be due to its antioxidant potential, enhancement of eNOS expression in the sciatic nerves. To our knowledge, this is the firt report in literature that show enhancing effect of vitamin E on sciatic nerve eNOS expression. This action of vitamin E decreased conduction latencies in the sciatic nerve and increase muscle contraction in diabetic treated rats indicating a preventive effect of axonal degeneration and/or demyelination. In agreement to these findings, Haidara *et al*. (2003) reported that administration of vitamin E ameliorated the decrease in amplitude of muscle contraction in diabetic rats.

Also, the result of the current study showed that L-arginine, when given orally has the ability to restore nerve conduction in diabetic rats, an action that is independent of hypoglycemic effect and antioxidant potential but rather due to enhancement of NO levels. NO is normally produced from L-arginine by endothelial Nitric Oxide Synthase (eNOS) in the vasculature (Turko *et al*., 2001). NO mediates endothelium-dependent vasorelaxation by its action on guanylate cyclase in Vascular Smooth Muscle Cells (VSMC), initiating a cascade that leads to vasorelaxation (Vega-Lopez *et al*., 2004). Supporting to

these findings, supplying of L-arginine transdermally improved vascular function of the feet in patients with diabetes as indicated by flow and temperature (Fossel, 2004). Indeed, antioxidants-rich nutritional supplements could result in favorable effects on the life quality of diabetic patients and patients suffering from other chronic conditions. Such favorable effects may involve various mechanisms at different tissues (Dauqan *et al.*, 2011; Jalili *et al.*, 2011; Shittu *et al.*, 2012).

5. CONCLUSION

It could be concluded that Diabetic Neuropathy (DN) is a multi-factorial disease, caused by hyperglycemia, oxidative stress and vascular impairment. Administration of both insulin and or vitamin E especially in high dose could be of great value in amelioration of DN. Moreover correction of impaired nerve blood flow by drugs that induce NO has proved to be efficient in the correction of DN. Clinical study is required to prove the efficacy of combined administration of insulin and antioxidant in different doses and combinations on diabetic polyneuropathy.

6. DECLARATION OF INTEREST

The researchers report no conflict of interest.

7. REFERENCES

Ambali, S.F., M. Shittu, J.O. Ayo, K.A.N. Esievo and S.A. Ojo, 2011. Vitamin C alleviates chronic chlorpyrifos induced alterations in serum lipids and oxidative parameters in male wistar rats. Am. J. Pharmacol. Toxicol., 6: 109-118. DOI: 10.3844/ajptsp.2011.109.118

Archibald, V., M.A. Cotter, A. Keegan and N.E. Cameron, 1996. Contraction and relaxation of aortas from diabetic rats: Effects of chronic anti-oxidant and aminoguanidine treatments. Naunyn Schmiedeberg's Arch. Pharmacol., 353: 584-591. DOI: 10.1007/BF00169180

Arora, M., A. Kumar, R.K. Kaundal and S.S and Sharma, 2008. Amelioration of neurological and biochemical deficits by peroxynitrite decomposition catalysts in experimental diabetic neuropathy. Eur. J. Pharmacol., 596: 77-83. DOI: 10.1016/j.ejphar.2008.08.003

Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall and B.A. Freeman, 1990. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci., 87: 1620-1624. DOI: 10.1073/pnas.87.4.1620

Bin-Jaliah, I., E.F. Khaleel, S. El-Attar, L.A. El-Sayed and M.A, 2011. Effects of vitamin-E and L-arginine on experimental diabetic neuropathy in rats. Proc. Physiol. Soc., 22: PC03-PC03.

Cameron, N.E. and M.A. Cotter, 1994. The relationship of vascular changes to metabolic factors in diabetes mellitus and their role in the development of peripheral nerve complications. Diabetes Metab., 10: 189-224. DOI: 10.1002/dmr.5610100302

Cameron, N.E. and M.A. Cotter, 1995. Reversal of peripheral nerve conduction and perfusion deficits by the free radical scavenger, BM15.0639, in diabetic rats. Naunyn Schmiedeberg's Arch. Pharmacol., 321: 685-690. DOI: 10.1007/BF00171329

Cameron, N.E., M.A. Cotter and P.A. Low, 1991. Nerve blood flow in early experimental diabetes in rats: Relation to conduction deficits. Am. J. Physiol., 261 E1-E8. PMID: 1858864

Cameron, N.E., M.A. Cotter, D.H. Horrobin and H.J. Tritschler, 1998. Effects of a-lipoic acid on neurovascular function in diabetic rats: Interaction with essential fatty acids. Diabetologia, 41: 390-399. DOI: 10.1007/s001250050921

Clark, C.M. Jr. and D.A. Lee, 1995. Prevention and treatment of the complications of diabetes mellitus. N. Engl. J. Med., 332: 1210-1217. PMID: 7700316

Coopey, L.J., E.P. Davidson, J.A. Dunlap, D.D. Lund and M.A. Yorek, 2000. Slowing of motor nerve conduction velocity in streptozotocin-induced diabetic rats is preceded by impaired vasodilation in arterioles that overlie the sciatic nerve. Int. J. Exp. Diabetes Res., 1: 131-143. PMID: 11469397

Costa, M.A., A.M. Balaszczuk, A. Dominguez, O. Catanzaro and C. Arranz, 1998. Effects of L-NAME and L-Arg on arterial blood pressure in normotensive and hypertensive streptozotocin diabetic rats. Acta Physiol. Pharmacol. Ther. Latinoam., 48: 59-63. PMID: 9695876

Cotter, M.A., A. Love, M.J. Watt, N.E. Cameron and K.C. Dines, 1995. Effects of natural free radical scavengers on peripheral nerve and neurovascular function in diabetic rats. Diabetologia, 38: 1285-1294. DOI: 10.1007/BF00401760

Dauqan, E., H.A. Sani, A. Abdullah, H. Muhamad and A.G.M. Top, 2011. Vitamin E and beta carotene composition in four different vegetable oils. Am. J. Applied Sci., 8: 407-412. DOI: 10.3844/ajassp.2011.407.412

El-Nabarawy, S.K., M.A.E. Mohamed, M.M. Ahmed and G.H. El-Arabi, 2010. α-Lipoic acid therapy modulates serum levels of some trace elements and antioxidants in type 2 diabetic patients. Am. J. Pharmacol. Toxicol., 5: 152-158. DOI: 10.3844/ajptsp.2010.152.158

Fossel, E.T., 2004. Improvement of temperature and flow in feet of subjects with diabetes with use of a transdermal preparation of L-arginine: A pilot study. Diabetes Care, 27: 284-285. PMID: 14694013

Haidara, M.A., I.M. Ibrahim, A.S. Al-Tuwaijri, S.A. Awadalla and H. Yaseen, 2003. Effect of alpha-tocopherol on glucose uptake and contractility in rat skeletal muscle. Med Sci. Monit., 9: 174-177. PMID: 12761442

Hohman, T.C., M.A. Cotter and N.E. Cameron, 2000. ATP-sensitive K^+ channel effects on nerve function, Na^+, K^+ ATPase and glutathione in diabetic rats. Eur. J. Pharmacol., 397: 335-341. DOI: 10.1016/S0014-2999(00)00227-2

Jalili, M., S.R. Aref-Hosseini, S. Kolahi, M. Ebrahimi-Mamegani and S. Sabour, 2011. The Effect of antioxidants supplement on lipid peroxidation and serum aryl esterase enzyme in rheumatoid arthritis patients. Am. Med. J., 2: 119-124. DOI: 10.3844/amjsp.2011.119.124

Karasu, C., 2000. Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: Role of reactive oxygen species. Eur. J. Pharmacol., 392: 163-173. DOI: 10.1016/S0014-2999(00)00140-0

Kihara, M. and P.A. Low, 1995. Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. Exp. Neurol., 132: 180-185. DOI: 10.1016/0014-4886(95)90023-3

Kihara, M., M.K. Mitsui, Y. Mitsui, K. Okuda and Y. Nakasaka *et al*., 1999. Altered vasoreactivity to angiotensin II in experimental diabetic neuropathy: Role of nitric oxide. Muscle Nerve., 22: 920-925. DOI: 10.1002/(SICI)1097-4598

Lafci-Erol, D., V.M. Altan and Y. Oztürk, 1994. Increased alpha 1-adrenergic responsiveness of alloxan diabetic rat atria: Effects of insulin therapy and thyroidectomy. Gen Pharmacol., 25: 559-564. DOI: 10.1016/0306-3623(94)90214-3

Liu, H.R., X.Y. Tang, D.Z. Dai and Y. Dai, 2008. Ethanol extracts of Rehmannia complex (Di Huang) containing no Corni fructus improve early diabetic nephropathy by combining suppression on the ET-ROS axis with modulate hypoglycemic effect in rats. J. Ethnopharmacology, 118: 466-472. DOI: 10.1016/j.jep.2008.05.015

Low, P.A., Nickander K.K. and H.J. Tritschler, 1997. The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. Diabetes, 46: S38-S42. PMID: 9285497

Low, P.A., R.R. Tuck, P.J. Dyck, J.D. Schmelzer and J.K Yao, 1984. Prevention of some electrophysiologic and biochemical abnormalities with oxygen supplementation in experimental diabetic neuropathy. Proc. Natl. Acad. Sci., 81: 6894-6898. PMID: 6593734

Low, P.A., T.D. Lagerlund and P.G. McManis, 1989. Nerve blood flow and oxygen delivery in normal, diabetic and ischemic neuropathy. Int. Rev. Neurobiol., 31: 355-438. PMID: 2557297

Mayhan, W.G., 1992. Impairment of endothelium-dependent dilatation of the basilar artery during diabetes mellitus. Brain Res., 580: 297-302. DOI: 10.1016/0006-8993(92)90957-B

McVeigh, G.E., G.M. Brennan, G.D. Johnston, B.J. McDermott and L.T. McGrath *et al*., 1992. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia, 35: 771-776. PMID: 1511805

Nagamatsu, M., K.K. Nickander, J.D. and Schmelzer, 1995. Lipoic acid improves nerve blood flow, reduces oxidative stress and improves distal nerve conduction in experimental diabetic neuropathy. Diabetes Care, 18: 1160-1167. PMID: 7587852

Negi, G., A. Kumar, R.K. Kaundal, A. Gulati and S.S. Sharma, 2010. Functional and biochemical evidence indicating beneficial effect of Melatonin and Nicotinamide alone and in combination in experimental diabetic neuropathy. Neuropharmacology, 58: 585-592. PMID: 20005237

Ohkawa, H., N. Ohishi and K. Tagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95: 351-358. DOI: 10.1016/0003-2697(79)90738-3

Pascoe, M.K., P.A. Low, A.J. Windebank and W.J. Litchy, 1997. Subacute diabetic proximal neuropathy. Mayo Clin. Proc., 72: 1123-1132. PMID: 9413291

Quintanilha, G., O.J.M. Nascimento, M. Orsini and C. Pupe, 2011. Sensory neuropathy associated with glucose intolerance: A 35 patients study. Am. J. Neurosci., 2: 1-4. DOI: 10.3844/amjnsp.2011.1.4

Rajbhandari, S.M. and M.K. Piya, 2005. A brief review on the pathogenesis of human diabetic neuropathy: Observations and postulations. Int. J. Diabetes Metab., 13: 135-140. DOI: 10.4103/0973-3930.57344

Ramesh, B. and K.V. Pugalendi, 2006. Impact of umbelliferone (7-hydroxycoumarin) on hepatic marker enzymes in streptozotocin diabetic rats. Indian J. Pharmacol., 38: 209-210.

Rosen, P., T. Ballhausen, W. Bloch and K. Addicks, 1995. Endothelial relaxation is disturbed by oxidative stress in the diabetic rat heart: Influence of tocopherol as antioxidant. Diabetologia, 38: 1157-1168. DOI: 10.1007/BF00422364

Saini, A.K., K.H.S. Arun and S.S. Sharma, 2007. Preventive and curative effect of edaravone on nerve functions and oxidative stress in experimental diabetic neuropathy. Eur. J. Pharmacol., 568: 164-172. DOI:10.1016/j.ejphar.2007.04.016

Schmeichel, A.M., J.D. Schmelzer and P.A. Low, 2003. Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. Diabetes, 52: 165-171. DOI: 10.2337/diabetes.52.1.165

Sharma, S.S., A. Kumar, M. Arora and R.K. Kaundal, 2009. Neuroprotective potential of combination of resveratrol and 4-amino 1,8 naphthalimide in experimental diabetic neuropathy: Focus on functional, sensorimotor and biochemical changes. Free Radical Res., 43: 400-408. DOI: 10.1080/10715760902801509

Shittu, M., J.O. Ayo, S.F. Ambali, M.U. Kawu and S.O. Salami, 2012. Vitamin E mitigates chronic chlorpyrifos-induced oxidative changes in pituitary glands and testes in wistar rats. Am. J. Applied Sci., 9: 75-82. DOI: 10.3844/ajassp.2012.75.82

Sima, A.A., 2003. New insights into the metabolic and molecular basis for diabetic neuropathy. Cell. Mol. Life Sci., 60: 2445-2464. DOI: 10.1007/s00018-003-3084-x

Srivastava, S.K., K.V. Ramana and A. Bhatnagar, 2005. Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. Endocrine Rev., 26: 380-392. DOI: 10.1210/er.2004-0028

Sugimoto, K., M. Yasujima and S. Yagihashi, 2008. Role of advanced glycation end products in diabetic neuropathy. Curr. Pharm. Des., 14: 953-961. PMID: 18473845

Tesfamariam, B. and R.A. Cohen, 1992. Free radicals mediate endothelial cell dysfunction caused by elevated glucose, Am. J. Physiol., 263: H321-H326. PMID: 1510128

Tesfaye, S., R. Malik and J.D. Ward, 1994. Vascular factors in diabetic neuropathy. Diabetologia, 37: 847-854. PMID: 7806013

Thipkaew, C., J. Wattanathorn and S. Muchimapura, 2012. The beneficial effect of asiaticoside on experimental neuropathy in diabetic rats. Am. J. Applied Sci., 9: 1782-1788. DOI: 10.3844/ajassp.2012.1782.1788

Turko, I.V., S. Marcondes and F. Murad, 2001. Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase. Am. J. Physiol. Heart. Circ. Physiol., 281: H2289-2294. PMID: 11709394

Vega-Lopez, S., S. Devaraj and I. Jialal, 2004. Oxidative stress and antioxidant supplementation in the management of diabetic cardiovascular disease. J. Invest. Med., 52: 24-32. PMID: 14989367

Yamagishi, S.I., S. Ogasawara, H. Mizukami, N. Yajima and R. Wada *et al*., 2008. Correction of protein kinase C activity and macrophage migration in peripheral nerve by pioglitazone, peroxisome proliferator activated-γ-ligand, in insulin-deficient diabetic rats. J. Neurochem., 104: 491-499. DOI: 10.1111/j.1471-4159.2007.05050.x

Yorek, M.A., L.J. Coppey, J.S. Gellett and E.P. Davidson, 2004. Sensory nerve innervation of epineurial arterioles of the sciatic nerve containing calcitonin gene-related peptide: Effect of streptozotocin-induced diabetes. Exp. Diabesity Res., 5: 187-193. PMID: 15512786

Zangiabadi, N., M.N. Ahrari and N. Nakhaee, 2007. The effect of omega-3 fatty acids on Nerve Conduction Velocity (NCV) and f-wave latency in patients with diabetic polyneuropathy. Am. J. Pharmacol. Toxicol., 2: 1-3. DOI: 10.3844/ajptsp.2007.1.3

Zangiabadi, N., V. Sheibani, M. Asadi-Shekaari, M. Shabani and M. Jafari *et al*., 2011. Effects of melatonin in prevention of neuropathy in STZ-induced diabetic rats. Am. J. Pharmacol. Toxicol., 6: 59-67. DOI: 10.3844/ajptsp.2011.59.67

Zeggwagh, N.A., Sulpice and T.M. Eddouks, 2007. Anti-hyperglycaemic and hypolipidemic effects of ocimum basilicum aqueous extract in diabetic rats. Am. J. Pharmacol. Toxicol., 2: 123-129. DOI: 10.3844/ajptsp.2007.123.129