Molecular and Cellular Biochemistry **278:** 21–31, 2005. © Springer 2005

Restoration of ultrastructural and biochemical changes in alloxan-induced diabetic rat sciatic nerve on treatment with Na_3VO_4 and *Trigonella* – a promising antidiabetic agent

Anju Preet,[1,2] Bihari L. Gupta,[1] Mohamed R. Siddiqui,[1]
Pramod K. Yadava[2] and Nazma Zaheer Baquer[1]

[1]*Hormone and Drug Research Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India;* [2]*Applied Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India*

Received 21 September 2004; accepted 17 December 2004

Abstract

Vanadium has been reported to have broad pharmacological activity both *in vitro* and *in vivo*. Vanadium compound, sodium orthovanadate, Na_3VO_4, is well known for its hypoglycaemic effects. However, Na_3VO_4 exerts these effects at relatively high doses (0.6 mg/ml) and exhibit several toxic effects. In the present study lower doses of Na_3VO_4 (0.2 mg/ml) are combined with *Trigonella foenum graecum* seed powder (TSP), another hypoglycaemic agent, to reduce its toxicity without compromising its antidiabetic potential. The efficacy of the lower doses of Na_3VO_4 has been investigated in restoring the altered glucose metabolism and histological structure in the sciatic nerves in 21 and 60 days alloxan diabetic rats. A portion of the glucose was found to be channelled from the normal glycolytic route to polyol pathway, evident by the reduced hexokinase activity and increased polyol pathway enzymes aldose reductase and sorbitol dehydrogenase activity causing accumulation of sorbitol and fructose in diabetic conditions. Ultrastructural observation of the sciatic nerve showed extensive demylination and axonal loss after eight weeks of diabetes induction. Blood glucose levels increased in diabetic rats were normalized with the lower dose of vanadium and *Trigonella* treatment. The treatment of the diabetic rats with vanadium and *Trigonella* prevented the activation of the polyol pathway and sugar accumulations. The sciatic nerves were also protected against the structural abnormalities found in diabetes with *Trigonella foenum graecum* as well as Na_3VO_4. Results suggest that lower doses of Na_3VO_4 may be used in combination with TSP as an efficient antidiabetic agent to effectively control the long-term complications of diabetes in tissues like peripheral nerve. (Mol Cell Biochem **278:** 21–31, 2005)

Key words: diabetes, Na_3VO_4, nerve, polyol pathway, sodium orthovanadate, TSP *Trigonella foenum graecum* seed powder

Introduction

Diabetes mellitus is a chronic disease of metabolic dysregulation characterized by hyperglycemia and complications that include microvascular disease of eye, kidney and a variety of clinical neuropathies. Neuropathy is one of the major debilitating complications of long-term diabetes developing as a result of chronic structural and metabolic derangements in nerve associated with diabetes [1, 2]. Various glucose-related or "glucotoxic" pathogenetic mechanisms suggested [3] for

Address for offprints: Nazma Zaheer Baquer, Lab No. 106, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India (E-mail: nzbaquer@hotmail.com)

its development include nonenzymatic glycation of proteins [4], auto-oxidation of glucose [5], and dysfunction of various metabolic pathways such as activation of the polyol pathway [6, 7]. Aldose reductase (AR) is the rate-limiting enzyme of polyol pathway, which has been hypothesized as one of the major pathogenic factors of diabetic neuropathy [8].

Activation of the polyol pathway alters cellular redox couples, exacerbates oxidative stress and promotes intracellular sorbitol and fructose accumulation [9]. The excess sorbitol and fructose formation by increased aldose reductase and sorbitol dehydrogenase (SDH) in diabetes increases osmolarity, myoinositol depletion, reduction in cAMP [10, 11] and antioxidative capacity [12] that leads to the development of diabetic neuropathy [13]. Experimental diabetic neuropathy is associated with impaired nerve function [14] and neuroanatomical abnormalities [10]. It is characterized by the loss of myelinated and unmyelinated fibres, wallerian degeneration, blunted nerve-fibre reproduction, axonal degeneration and myelin wrinkling [15, 16].

Insulin, the most widely used antidiabetic to control insulin-dependent diabetes mellitus (IDDM), was able to bring about normoglycemia in experimental animals but as reported earlier failed to preserve the structural composition of peripheral nerves in long standing diabetes [17, 18]. May be the post-parandial fluctuations in the glucose levels even after intensive insulin therapy are causing severe damage to the sensitive tissues like nerve [19, 20]. Various therapeutic approaches developed to prevent the onset of neuropathy are still miles away from practical application [21]. Therefore there was a need for the development of a therapy, which could overcome the fluctuations in the blood glucose levels preventing the development of diabetic complications.

In the present study an effort has been made to find non-conventional methods of using transitional metal and plant products to have a better glycemic control in experimental diabetes and to visualize their effects on the development of peripheral neuropathy. Na_3VO_4, sodium orthovanadate, an insulin-mimetic agent was earlier found to be effective in controlling the elevated blood glucose levels in both type 1 and 2 diabetes [22]. Vanadium administration to diabetic animals has been found to restore most of the glucose metabolizing pathways including the polyol pathway, the pathway known to play the most important role in the development of neuropathy [11, 23]. However, the potential of Na_3VO_4 as a neuroprotective agent has not yet been explored. *Trigonella foenum graecum*, commonly called fenugreek has been extensively used in Ayurvedic system of medicine, known well for its hypoglycemic effects [24]. In the present work besides studying glucose metabolism in the sciatic nerve in alloxan-induced diabetic rats an attempt has been made to discover the efficacy of Na_3VO_4 and *Trigonella* as neuroprotectants in terms of restoration of the nerve ultrastructural changes. Glycolytic enzyme hexokinase (HK), polyol pathway enzymes AR and SDH, pentose phosphate enzyme glucose-6 phosphate dehydrogenase (G6PDH), and related metabolites in diabetic sciatic nerve were also measured. Due to the reported toxicity of vanadium [25], the present study besides using Na_3VO_4 alone, a combination of Na_3VO_4 at a lower dose was used with *Trigonella*. The combination of sodium orthovanadate and *Trigonella* was used in the present work for a possibility to develop a therapy showing better glycemic control and reduced toxicity as compared to giving vanadium alone.

Materials and methods

Animals

Adult female albino rats of Wistar strain, aged 11–12 weeks and weighing between 180–220 gms, were used for this study. The animals were maintained in the climate-controlled animal facility (Jawaharlal Nehru University, New Delhi) with a 12-h light/dark cycle at a stable temperature and relative humidity of 22 °C and 55%, respectively. The animals were fed with standard chow (Hindustan Lever Ltd.) and tap water *ad libitum* (unless otherwise stated). All institutional guidelines of the Institutional Animal Ethics Committee were strictly adhered to in the care and treatment of the animals used throughout the study.

Experimental design

About 120 rats were starved for 24 h and divided into control and experimental groups. Each experimental rat was injected alloxan monohydrate subcutaneously (15 mg per 100 gm body weight) freshly prepared in 0.154 M sodium acetate buffer (pH 4.5) using the method of Sochor *et al.* [26]. To stabilize and reduce the mortality rate of the experimental animals the alloxan-treated experimental rats were injected 2 IU of protamine-zinc-insulin i.p. daily, for the next seven days. The controls were given the vehicle only. The severity of diabetes was checked using urine glucose detection strips (Diastix, Bayer Diagnostic, India). The control rats and the experimental rats randomly assorted into different groups were treated as follows: Group-I: The non alloxan-treated animals were given standard food pellet and tap water until sacrifice, this group served as negative control for diabetes. (C), Group-II: These alloxan-induced diabetic animals were given standard food pellet and tap water and were frequently monitored for urine glucose to confirm the diabetes. (D), Group-III: The diabetic animals were given standard food pellet and tap water injected i.p. with 2 IU of protamine-zinc insulin daily until sacrifice. ($D + I$), Group-IV: The diabetic animals were given standard food pellet and administered

sodium orthovanadate orally at a dose of 0.2 mg/ml of 80 mM NaCl for initial three days followed by 0.4 mg/ml for next three days and after that a dose of 0.6 mg/ml was given until sacrifice. $(D + V)$, Group-V: The diabetic animals were given Trigonella seed powder (TSP) (5% w/w) mixed with their standard pellet food and normal tap water until sacrifice. $(D + T)$, Group-VI: The diabetic animals were given TSP (5% w/w) mixed with their standard pellet food and orally administered a reduced dose of Na_3VO_4 0.2 mg/ml of 80 mM sodium chloride until sacrifice. $(D + V + T)$

The experiments were carried out to see the effects of diabetes both short-term as well as long-term on the nerve glucose metabolism and ultrastructure. The animals were sacrificed for short-term after 21 days and for long-term after 60 days for studying the effect of diabetes on nerve histology.

Preparation of tissue extracts

The animals were sacrificed by cervical dislocation after starvation of 24 h at the specified time intervals and sciatic nerves carefully dissected out avoiding extraneous tissue, washed with normal saline, blotted dry and weighed. For metabolites the nerves were frozen immediately in liquid N_2 and kept at 80 °C to be used later. The nerve homogenates were made homogenate in 0.25 M sucrose, 0.02 M triethanolamine buffer, pH 7.4 containing 0.12 mM dithiothreitol using a Potter Elvehjem Homogenizer fitted with a Teflon plunger for enzyme assays. Homogenates were then centrifuged at $1000 \times g$ for 10 min to remove nuclei and cell debris. The pellet was discarded and the supernatant was further centrifuged at $12{,}000 \times g$ for 20 min at 4 °C. The supernatants were used for all the enzyme assays. For metabolite assays the frozen nerve tissue was homogenized in nine volumes of 1N perchloric acid. The homogenates were centrifuged at $6000 \times g$ for 10 min. The supernatants were neutralized with 2N KOH and centrifuged at $1200 \times g$ for 10 min to remove the $KClO_4$ resulting in clear extracts, these clear extracts were used for all metabolite determinations by coupling the reaction with purified enzymes using oxidation/reduction of NAD/NADP using a Beckman DU-68 spectrophotometer as described earlier [27].

Assay methods: Enzymes

Hexokinase (EC 2.7.1.1)
The enzyme activity was measured essentially by the method of Gumaa and McLean [28]. The total reaction mixture of 1 ml contained the following in final concentration of: 0.1 M Tris-HCl (pH 7.4), 8 mM $MgCl_2$ (pH 7.0), 0.4 mM $NADP^+$, 8 mM/2 mM of ATP/Mg^{2+} (pH 7.2), 0.5 mM glucose and one unit of purified glucose-6-phosphate dehydrogenase. The reaction was initiated by addition of 0.1 ml of cytosol, containing ~0.5–0.6 mg protein. The reduction of NADP at 340 nm was followed for 5 min at 25 °C as a measure of enzyme activity.

Aldose reductase (EC 1.1.1.21)
Activity of the enzyme in the cytosolic fraction was estimated in a Beckman DU-68 spectrophotometer essentially by the methods of Kinoshita *et al.* [29]. The reaction mixture contained the following in the final concentration: 50 mM phosphate buffer (pH 7.4), 300 mM glucose and 0.2 mM NADPH in the final volume of 1.0 ml. The reaction was initiated by adding 0.1 ml of cytosol, containing ~0.4–0.6 mg protein. The oxidation of NADPH at 340 nm was followed for 5 min at 25 °C as a measure of enzyme activity.

Sorbitol dehydrogenase (EC 1.1.1.14)
Activity of this enzyme was measured by the method of Gerlach and Hiby [30]. The reaction mixture contained the following in the final concentration of: 0.107 M Triethanolamine buffer (pH 7.4), 300 mM fructose and 0.2 mM NADH (alkaline pH) in the final reaction volume of 1.5 ml. The reaction was initiated by the addition of 0.05 ml of cytosol, containing ~0.3 mg protein.

The oxidation of NADH at 340 nm was followed for 5 min at 25 °C as a measure of enzyme activity.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
The enzyme was assayed by the method of Baquer *et al.* [31]. The reaction mixture contained the following in the final concentrations: 10 mM Tris-HCL (pH 7.8), 0.25 mM D-Glucose-6-phosphate, 6.5 μM $NADP^+$ and 0.25 mM $MgCl_2$ in a total reaction volume of 1.0 ml. The reaction was initiated by the addition of 0.05 ml of cytosol containing 0.2–0.5 mg of protein. The reduction of NADP at 340 nm was followed for 5 min at 25 °C as a measure of enzyme activity in a Beckman DU 68 Spectrophotometer.

Enzyme units

One enzyme unit is defined as the oxidation/reduction of one μ mole of NADH or NADPH per gram fresh wt/min at 25 °C.

Assay methods: Metabolites

Glucose
Glucose was measured as described by Bergmeyer [32] using a coupled assay system linked to hexokinase and glucose-6 phosphate dehydrogenase. The assay mixture of 3 ml contained the following in final concentration: 100 mM Tris HCl

pH 7.8, 0.26 mM $NADP^+$, 8.0 mM ATP, 5.0 mM $MgCl_2$ and appropriate sample volume. The reaction was initiated by adding one unit of purified yeast G-6PDH.

Fructose
Fructose was estimated as described by Bergmeyer [33] using coupled assay system linked to hexokinase, phosphoglucose isomerase and glucose-6 phosphate dehydrogenase on Beckman DU-68 spectrophotometer. The reaction mixture contained the following in final concentration of 0.25 M triethanolamine pH 7.5, 2.5 mM $MgSO_4$, 1.1 mM ATP, 1.5 unit of purified hexokinase, 0.8 Mm NADP and appropriate sample volume. The reaction was initiated by adding one unit of purified yeast G-6PDH followed by addition of 3 units of Phosphoglucose isomerase.

Sorbitol
Sorbitol was measured by modified enzymatic method of Malone *et al.* [34] on Varian Cary (Eclipse) Flourescence spectrophotometer. The reaction mixture contained the following in a final concentration of: 0.05 M glycine buffer pH 9.4, 0.2 mM NAD^+ and an appropriate amount of sample volume in a final volume of 3 ml. The reaction was initiated by adding 1 unit of sorbitol dehydrogenase.

For all the above metabolites measurement the reduction of NAD^+ and $NADP^+$ or oxidation of NADH or NADPH was measured at 340 nm.

Reduced glutathione
GSH was measured by the method of Griffith [35]. The reaction mixture contained the following in a final concentration of: 0.20 mM NADPH, 0.6 mM 5, 5′-dithiobis-(2-nitrobenzoic acid, 0.5 units of glutathione reductase in 125 mM sodium phosphate buffer (pH 7.5), 6.3 mM EDTA and appropriate amount of sample volume. The rate of reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) was measured at 412 nm.

Other estimations

Blood glucose was estimated in the plasma using glucose measuring kit from Ranbaxy laboratories Ltd., India utilizing glucose oxidase method. Protein contents in nerve extracts were determined by the method of Lowry *et al.* [36]. Bovine serum albumin was used as a standard. Glycosylated haemoglobin was measured by the method of Raheja *et al.* [37].

Histological processing

Small cylindrical pieces of sciatic nerves from the different experimental groups were taken and fixed in glutaraldehyde fixative (25% glutaraldehyde and 0.2 M phosphate buffer pH 7.0) overnight for electron microscopy [38]. Dehydration was done in graded series of ethanol followed by propylene oxide and the samples were embedded in araldite. Thin sections were cut (1 μm) and stained with 1% toluidine blue for light microscopic examination before processing for electron microscopy. Ultrathin sections (700–800Å) of the desired sectors were cut, mounted on copper grids and stained with uranyl acetate and lead acetate. The grids were observed on a Philips CM-10 Transmission Electron Microscope at 60 KeV.

Statistical analysis

Results are presented as mean ± S.E.M. of four or more separate experiments where in one experiment 36 rats were used, six in each group. Statistical difference between control and various groups was determined by one-way ANOVA followed by Dunnet multiple comparison test. p-values less than 0.05 were considered significant.

Chemicals

All purified enzymes; co-enzymes, substrates, standards and buffers were purchased from Sigma chemicals Company, USA. All other chemicals were of analytical grade and purchased from SRL & Qualigens, India.

Results

Physiological parameters

The physiological parameters like body weight, glycosylated Hb and neural proteins of all the experimental groups with short- and long-term diabetes are summarized in Table 1. Alloxan diabetes caused a significant (30%) decrease in the body weight in short-term diabetes which further shows a 35% decline in the long-term experiment in comparison to animals from the age-matched control group. There was no increase in the final body weight in the high dose treatment of Na_3VO_4 in both short-term as well as long-term study but combining a low dose of Na_3VO_4 with TSP maintained the body weight ($p < 0.05$) near to control levels in comparison to the diabetic animals. Alloxan-induced diabetes in rats caused a 4–5 fold increase in the blood glucose levels in comparison to the control group which were restored to normal levels in all the treatment groups. Levels of glycosylated haemoglobin (Hb A_{1c}), an important marker of the long-term glycemic state was found to be significantly elevated in both short-term ($p < 0.05$) and long-term ($p < 0.01$) diabetic animals when compared to age-matched control. The HbA_{1c}

Table 1. Restorative effects of the two different doses of sodium orthovanadate on general parameters from different experimental groups of: diabetic rats (C), alloxan-induced diabetic (D) and diabetic treated with insulin (I), Vanadate ($D + V$), *Trigonella* ($D + T$) and *Trigonella* and Vanadate ($D + V + T$)

	Body weight (gms)		Glycosylated Hb (%)		Nerve proteins (soluble) (mg/g tissue)	
Treatment groups	21 days	60 days	21 days	60 days	21 days	60 days
Control	217 ± 9	226 ± 5	4.5 ± 0.51	4.4 ± 0.07	63.1 ± 2.9	65.9 ± 1.98
Diabetic	$154 \pm 4^{**}$	$160 \pm 5^{**}$	$6.8 \pm 0.65^{**}$	$7.9 \pm 0.24^{**}$	$51.1 \pm 2.8^{*}$	$51.8 \pm 2.5^{*}$
Insulin-treated (2 IU/day)	$185 \pm 10^{\dagger}$	$190 \pm 4^{\dagger}$	5.6 ± 0.19	$5.6 \pm 0.35^{\dagger}$	53.4 ± 5.5	57.2 ± 4.2
Na_3VO_4 0.6 mg/ml	$175 \pm 8^{*}$	$188 \pm 4^{*\dagger}$	$5.2 \pm 0.2^{\dagger}$	$5.2 \pm 0.7^{\dagger}$	$44.6 \pm 4.1^{\dagger}$	59.4 ± 4.03
Trigonella	193 ± 11	$196 \pm 4^{\dagger}$	5.1 ± 0.16	$5.6 \pm 0.8^{\dagger}$	46.5 ± 3.1	$60.4 \pm 2.5^{\dagger}$
0.2 mg/ml Na_3VO_4 and *Trigonella*	$202 \pm 11^{\dagger}$	$205 \pm 6^{\dagger}$	$4.8 \pm 0.29^{\dagger}$	$4.6 \pm 0.56^{\ddagger}$	53.0 ± 4.8	57.4 ± 3.5

Values are expressed as mean ± S.E.M. of values from four to six separate experiments. ($^{*}p < 0.05$) and ($^{**}p < 0.01$) represent significant changes against control. ($^{\dagger}p < 0.05$) and ($^{\ddagger}p < 0.01$) represent significant changes against diabetic.

levels in the group $D + V + T$ was found significantly nearer ($p < 0.01$) to the control group in the long-term study. The total neural protein levels of sciatic nerve from diabetic group showed a significant decrease ($p < 0.05$) as compared to that of control and the treated groups in the long-term studies. The blood glucose levels significantly ($p < 0.01$) increased by about four fold in diabetic rats in comparison to controls and were normalized completely with the combined treatment of lower dose of vanadium and *Trigonella* both in short-term as well as long-term experimental groups, results are presented in Fig. 1. The increased water consumption of diabetic rats

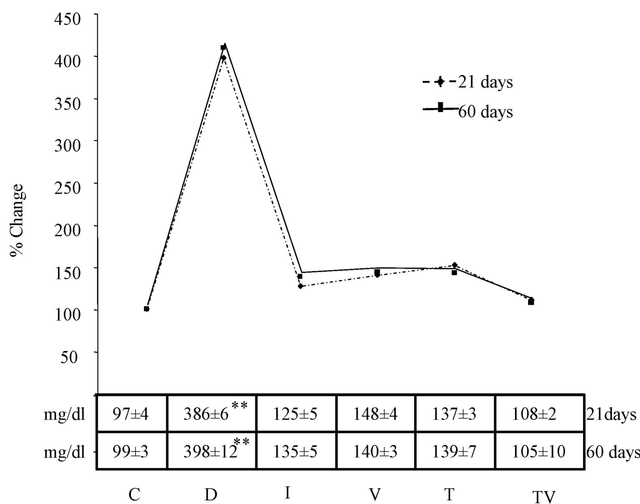

Fig. 1. Effect of different treatments on the percentage change in blood glucose levels from different treatment groups with control taken as 100%. x-Axis gives the blood glucose levels in mg/dl from the different groups over the two durations of experiments, 21 and 60 days. Values represent mean ± S.E.M. from five or more separate experiments. ($^{**}p < 0.01$) represent significant changes against control. C = control, D = diabetic, I = insulin treated, V = 0.6 mg/ml Na_3VO_4, T = 5% *Trigonella*, TV = 0.2 mg/ml Na_3VO_4 and 5% *Trigonella*.

was restored to normal consumption levels with SOV and TSP treatments alone as well as in combination over a period of continued treatment for 60 days results are shown in Table 2.

Changes in glucose metabolising enzymes

HK and G-6PDH activity

As glucose uptake in the nerves is non-insulin dependent, in alloxan diabetic rats there is increased flux of glucose into the nerves. The activity of hexokinase catalysing phosphorylaton of glucose to glucose-6-phosphate trapping glucose inside the cell, is decreased significantly by 30% in the diabetic animal after 21 days and 40% after 60 days of diabetes induction in comparison to the control animals. The activity of key enzyme of the pentose phosphate pathway G-6PDH is reduced significantly ($p < 0.05$) as compared to control group after 21 days of diabetes induction which then increases, with respect to control group, if the diabetic state is prolonged for 60 days. The changes in the activity of HK and G-6PD with short- and long-term are presented in Table 3. The different treatments used were found to be effective in maintaining the enzyme activity to normal levels. The $D + V + T$ group was found to show the maximum reversal in all the groups studied.

AR and SDH

Aldose reductase and sorbitol dehydrogenase are the constituent enzymes of sorbitol pathway, the major alternate route to glucose metabolism contributing significantly to the development of diabetic complications. Diabetes caused a significant increase in the activity of aldose reductase by 30%

Table 2. Total intake of fluid, sodium orthovanadate (SOV) and *Trogonella* seed powder in control (C), diabetic (D) and diabetic rats treated with insulin, Vanadate, *Trigonella* and *Trigonella* and Vanadate after 60 days of administration along with comparative changes in the blood glucose levels with two different doses of sodium orthovanadate

Treatment groups	Fluid intake (ml/rat/day)	SOV (mg/rat/day)	*Trigonella* seed powder (gram/rat/day)	Blood glucose mg/dl
Control	41 ± 6	–	–	99 ± 3
Diabetic	$220 \pm 10^{**}$	–	–	$398 \pm 12^{**}$
Insulin treated (2 IU/day)	51 ± 6	–	–	135 ± 5
0.6 mg/ml Na_3VO_4	39 ± 5	28.5 ± 2.3	–	140 ± 3
0.2 mg/ml Na_3VO_4	52 ± 7	16.5 ± 2.1	–	$355 \pm 18^{**}$
Trigonella	54 ± 8	–	1.21 ± 0.1	139 ± 7
0.2mg/ml Na_3VO_4 and *Trigonella*	49 ± 8	14.5 ± 1.8	1.20 ± 0.1	105 ± 10

Values are expressed as mean ± S.E.M. of values from four to six separate experiments. ($^{**}p < 0.01$) represent significant changes against control.

Table 3. Modulatory influence of insulin, *Trigonella* and different doses of sodium orthovanadate treatment on the glycolytic, polyol pathway and pentose phosphate pathway enzymes activities in the cytosolic fraction of sciatic nerves of rats after 21 and 60 days of diabetes induction

Enzymes	Treatment groups					
	Control	Diabetic	Diabetic + Insulin	Diabetic + Vanadate	Diabetic + *Trigonella*	Diabetic + Vanadate + *Trigonella*
Short-term (21 days)						
HK	0.87 ± 0.04	$0.60 \pm 0.04^{*}$	0.76 ± 0.01	0.86 ± 0.05	$0.70 \pm 0.04^{*}$	0.84 ± 0.03
G-6PDH	0.38 ± 0.08	$0.30 \pm 0.03^{*}$	0.36 ± 0.06	0.38 ± 0.04	0.38 ± 0.03	0.37 ± 0.04
AR	0.18 ± 0.002	$0.24 \pm 0.007^{*}$	0.20 ± 0.006	0.17 ± 0.004	0.20 ± 0.003	0.19 ± 0.002
SDH	0.59 ± 0.026	$0.69 \pm 0.025^{*}$	0.65 ± 0.016	$0.66 \pm 0.051^{*}$	0.62 ± 0.021	0.60 ± 0.018
Long-term (60 days)						
HK	1.13 ± 0.03	$0.69 \pm 0.02^{*}$	1.04 ± 0.02	$0.86 \pm 0.05^{*}$	0.94 ± 0.05	1.06 ± 0.03
G-6PDH	0.37 ± 0.05	0.42 ± 0.05	0.38 ± 0.07	0.39 ± 0.09	0.38 ± 0.06	0.38 ± 0.06
AR	0.18 ± 0.002	$0.23 \pm 0.007^{**}$	0.19 ± 0.008	$0.21 \pm 0.011^{*}$	0.21 ± 0.008	0.19 ± 0.004
SDH	0.62 ± 0.011	$0.74 \pm 0.016^{*}$	$0.70 \pm 0.014^{*}$	0.66 ± 0.006	$0.69 \pm 0.011^{*}$	0.61 ± 0.018

Values are expressed as mean ± S.E.M. of values from four to six separate experiments. HK: Hexokinase, G-6PDH: Glucose-6-phosphate dehydrogenase, AR: Aldose reductase, SDH: Sorbitol dehydrogenase. Enzyme units are expressed as units/g/min. ($^{*}p < 0.05$) and ($^{**}p < 0.01$) represent significant changes against control.

($p < 0.01$) in both short-term as well as long-term diabetic rats in comparison to the control group. Comparative changes in the enzyme activity over the two durations of study are presented in Table 3. Insulin and vanadate restored the altered activity to near control values. TSP alone also lowered the enzyme activity but it still showed an insignificant increase in the enzyme activity as compared to the control. The combination of TSP and Na_3VO_4 showed the reversal of the enzyme activity to control levels.

A significant increase of about 20% ($p < 0.05$) in the SDH activity was observed in the diabetic sciatic nerve in long-term diabetes as compared to a smaller (17%) increase in the activity in short-term diabetic animals with respect to their respective control groups. Insulin, Vanadate and TSP treatment lowered the enzyme activities in comparison to the diabetic animals but showed small increase in the $D + I$ and $D + V$ groups as compared to control both in short-term as well as long-term treatment. The $D + V + T$ group showed a complete reversal of the enzyme to control values ($p < 0.05$) lower than the diabetic animals at 60 days.

Changes in metabolite levels

Glucose, sorbitol and fructose

The levels of glucose were increased ($p < 0.01$) by about 5-fold in nerves from 60 days diabetic rats further reaffirming insulin-independent uptake of glucose by the nerve. The glucose levels could not be completely reversed to the level of

control normal rats with different treatments. Sixty days after antidiabetic treatment with insulin, Na_3VO_4 and TSP significant ($p < 0.01$) reduction in glucose levels was observed which was significantly higher than the non-diabetic control group by about 150% in $D + I$, 200% in $D + V$ and only 18% in the $D + V + T$ group in comparison to the normal rats.

The sorbitol levels were significantly ($p < 0.01$) high in the diabetic animals, showing a 7-fold increase in comparison to control. Significant decrease ($p < 0.01$) in sorbitol levels was observed by insulin, vanadate and TSP-treated groups in comparison to the diabetic animals. Complete reversal however was not achieved even in the $D+V+T$ group (Table 4) that otherwise had shown almost complete reversal in most of the enzyme changes (Table 3). The fructose levels also increased significantly by 7-fold in long-term diabetic nerve. A complete reversal of the increased fructose levels was observed in $D+V+T$ group. The other treatments of diabetic animals by insulin, vanadate and Trigonella decreased the fructose levels much below that of the diabetic but it was still significantly higher in different treatment groups as compared to the control group.

Reduced glutathione

The levels of GSH, the non-enzymatic antioxidant protein were reduced significantly in diabetes in both the short-term (21 days) as well as long-term (60 days) diabetic animals (Table 4). With low dose administration of Na_3VO_4 in combination with TSP, the levels were increased by about 37% ($p < 0.05$) in short-term treatment and by 74% ($p < 0.01$) in long-term treatment of the diabetic rats in comparison to the diabetic rats. The higher dose of Na_3VO_4 and TSP alone could not maintain the GSH levels to the basal levels as compared to the age-matched control animals.

Ultra-structural changes

Since the recovery of diseased nerve function in diabetes depends on both regeneration of the degenerated nerve fibres and reestablishment of their functional connections with the target tissue, it is important to examine how diabetic condition influences nerve regeneration and whether potential therapeutic agents can promote satisfactory regeneration of functioning nerve fibres. For this purpose the sciatic nerves from the different groups were observed under the transmission electron microscope. The visible morphological features of the sciatic nerve under TEM including axonal atrophy, demyelination, loss of nerve fibres, is consistent with earlier studies in human as well as experimental animals [39, 40]. This examination revealed segmental demyelination of nerve fibres Fig. 2B. increased extra-axonal space among nerve fibres, deposition of electron dense material within the axons. There was onion bulb formation towards axoplasm, unmyelinated nerve fibres including Schwann cell showed structural changes forming T-shaped structure in extra axonal space. The nucleus of Schwann cell in diabetes also showed irregular outer structure and mitochondrial swellings were also observed. The number of myelinated nerve fibres per fascicle decreased in untreated diabetic rats in comparison to control animals. Myelin segments form ovoid shapes and fold around the cavity occupied by the original axons. Thickening

Table 4. Changes in the level of metabolites in the sciatic nerve from control (C), Diabetic (D) and diabetic rats treated with insulin, Vanadate, *Trigonella* and *Trigonella* & Vanadate for 21 and 60 days

	Treatment groups					
Metabolites	Control	Diabetic	Diabetic + Insulin	Diabetic + Vanadate	Diabetic + *Trigonella*	Diabetic + Vanadate + *Trigonella*
Short-term (21 days)						
Glucose	1.5 ± 0.09	4.4 ± 0.1**	1.73 ± 0.09	2.03 ± 0.07	2.35 ± 0.05	1.68 ± 0.06
Sorbitol	6.9 ± 0.2	18.5 ± 0.5**	8.6 ± 0.4	9.0 ± 0.5*	9.65 ± 0.2*	7.2 ± 0.6
Fructose	0.78 ± 0.02	1.6 ± 0.01*	0.9 ± 0.03	1.03 ± 0.02*	0.96 ± 0.06	0.84 ± 0.02
GSH	1.06 ± 0.5	0.68 ± 0.09*	0.88 ± 0.4*	0.77 ± 0.3	0.85 ± 0.06	0.93 ± 0.6
Long-term (60 days)						
Glucose	1.6 ± 0.23	7.3 ± 0.8**	2.4 ± 0.2	3.1 ± 0.5*	3.3 ± 0.6*	1.95 ± 0.2
Sorbitol	6.9 ± 1.3	48.5 ± 3.9**	20.2 ± 2.6	18.4 ± 2.8*	18.5 ± 3.1*	9.4 ± 1.6
Fructose	0.75 ± 0.06	4.9 ± 0.23**	1.08 ± 0.21	1.2 ± 0.12	1.3 ± 0.23*	0.87 ± 0.60
GSH	0.8 ± 0.09	0.46 ± 0.05**	0.73 ± 0.08	0.56 ± 0.01*	0.75 ± 0.009	0.8 ± 0.08

Values are expressed as mean ± S.E.M. of values from four to six separate experiments. Metabolite levels are expressed as μ mol/g. ($^{*}p < 0.05$) and ($^{**}p < 0.01$) represent significant changes against control. GSH: Reduced glutathione.

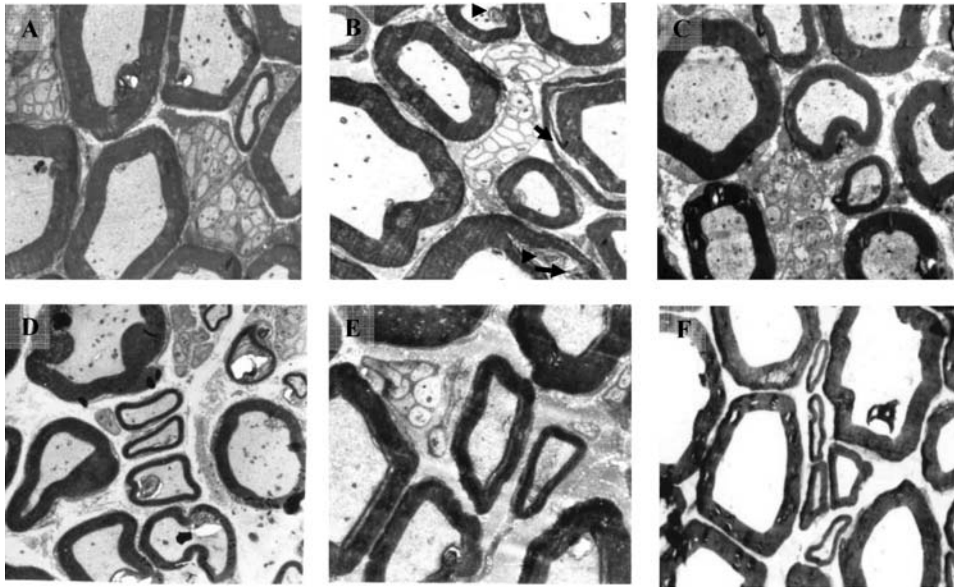

Fig. 2. Transmission electron micrographs (7750X) of rat sciatic nerve from control (A), diabetic (B, arrowhead showing onion bulb formation, arrow showing delamination) and diabetic rats after eight weeks of treatment with insulin (C), 0.6 mg/ml Na_3VO_4 (D), 5% *Trigonella* (E) and co-administration of 0.2 mg/ml Na_3VO_4 and 5% *Trigonella* (F).

was observed within the blood vessels leading to a reduced lumen which causes impaired blood flow within the nerves. In control animals no such structural abnormalities were observed in the myelinated or unmyelinated fibres, axons and Schwann cell shape and its nucleus.

Insulin treatment to diabetic animals showed a reversal of axonal loss with extra-axonal space reduction and Schwann cell size and its insertion in extra-axonal space. Demyelination of nerve fibres was also prevented and no onion bulb formation was observed. The myelin sheath appeared to be reduced in thickness in comparison to the diabetic nerve. The schwann cell nucleus still showed deformation consistent with earlier studies [18].

Vanadate treatment to diabetic animals restored most of the metabolic parameters; it did not however completely restore the ultra structural changes. Na_3VO_4 given in high 0.6 mg/ml and low doses 0.2 mg/ml showed a reduction in extra-axonal space in comparison to the diabetic animals. Demylination to a lesser extent when compared with the diabetic animals was observed in the $D + V$ (0.6 mg/ml) group and onion bulb formation was also seen. Basal lamina of capillary endothelia which was found thickened in the diabetic animal was thickened in this group, with rough endothelial apical membrane. *Trigonella* (5% TSP) treatment overcame the deposition of electron dense material within the axons which was seen with the Na_3VO_4 0.6 mg/ml treatment. Reducing the Na_3VO_4 dose from 0.6 mg/ml to 0.2 mg/ml and combining it with 5% TSP showed better results in this group, axonal loss was completely recovered, demyelination of nerve fibers also disappeared and no onion bulb formation was observed. Swelling of mitochondria was also not observed within the Schwann cells and a complete recovery of Schwann cell structure was observed. Regenerating fibres could be seen in this treatment group Fig. 2E.

Discussion

Diabetes is a multifaceted metabolic disorder affecting large number of metabolic pathways. An effective anti-diabetic agent should not only be able to restore but also to maintain normoglycemia and prevent the development of complications over a longer duration of diabetes. Vanadium compounds have been largely studied for their efficacy in controlling diabetes [41]. Na_3VO_4, sodium orthovanadate is one such proven hypoglycaemic compound of the transition metal vanadium shown to control blood glucose levels during diabetes [42]. However, a few toxic side effects of Na_3VO_4 have also been reported [25, 43]. It is becoming interestingly important to analyze natural products for their antidiabetic properties in the management of physiological and metabolic abnormalities associated with diabetes besides their hypoglycaemic effects. In this study the potential of *Trigonella foenum graecum* seed powder (TSP), already reported to have hypoglycaemic effects [24, 44], has been studied to see its effect in the management of long-term diabetic complications associated with diabetes. Besides using Na_3VO_4 and TSP alone, experiments were designed to see the metabolic and

structural efficacy of the combination of TSP and Na_3VO_4 in preventing the development of peripheral nerve complications. This combination was tried to reduce the toxicity associated with Na_3VO_4 by reducing its dose to 0.2 mg/ml from 0.6 mg/ml and to include TSP as an anti-diabetic by combining it with Na_3VO_4. Several physiological modifications such as decreased body weight, increased water uptake and elevated blood glucose levels characteristic of type I diabetes were observed in the diabetic rats in this study.

Persistent hyperglycemia is well known in initiating events in the development of diabetic complications. The duration of the controlled glycemic state is associated with the development and progression of this peripheral neuropathy [45]. The glycemic control in present study was not maintained in the diabetic animals as shown by the GHb values which were $>7\%$ throughout the experiment in both short- and long-term diabetes in which ultrastructural changes in the nerves were studied. The different antidiabetic treatments used for two months were able to improve the glycemic control in the diabetic animals, (Table 1). Persistent hyperglycemia also induced metabolic changes within the nerve (Tables 3 & 4). The hyperglycemia induced metabolic changes are very important for the initiation and progression of structural changes within the nerves leading to neuropathy [46].

The delamination of the myelin sheaths seen in Fig. 2B may be attributed to decreased protein synthesis in nerves in the diabetic state decreasing the synthesis of myelin protein in Schwann cells. The degradation of the cytoskeleton proteins may be responsible for axonal loss resulting in folding in of the myelin sheath [47]. Nerve dysfunction in diabetes is mostly related to glucose metabolism through other pathways thereby perturbing the normal glycolytic pathway and enhancing the sorbitol pathway [48]. Thereby a decreased utilization of glucose via glycolysis may be suggested in diabetic sciatic nerve. As glucose is freely permeable to nerve tissue [17, 48], there is accumulation of glucose in sciatic nerve which is metabolized, via the activated sorbitol pathway enzymes aldose reductase (AR) having high *K*m for glucose and sorbitol dehydrogenase (SDH), to sorbitol and fructose whose accumulation in the nerves is known to be implicated in the development of long-term diabetic complications. A decreased G-6PDH activity in short-term diabetes is also observed which will decrease the NADPH production which is used as a cofactor by the aldose reductase in the sorbitol pathway. In a persistent diabetic state over 60 days the G-6PDH activity was found to be increased significantly suggesting generation of more NADPH to overcome the building oxidative stress within the cells. This NADPH is competitively utilized by glutathione reductase with aldose reductase for the generation of reduced glutathione (GSH) an important antioxidant known to play a role in detoxification mechanisms and in the protection of cellular constituents against reactive oxygen species. Present results showing a seven fold increase in the accumulation of sorbitol and fructose in the nerves from diabetic animals were found to be in accordance with the earlier studies [23]. The sorbitol and fructose does not diffuse out of the cells and their accumulation may be responsible for the extension of extra-axonal space due to increase in osmolarity (Fig. 2B). The accumulation of these sugars also cause an increase in oxidative status of the cell, [49] causing stress within the Schwann cells leading to mitochondrial swelling, may be eventually leading to apoptotic loss of Schwann cells where this polyol pathway is known to be very active [50]. Diabetes induce the generation of free radicals and their products that manifest structural changes in the nerve fibres over its prolonged duration [4]. In this study the level of antioxidant reduced glutathione are also found to be decreased in the diabetic nerve, is in agreement with earlier studies [9].

Insulin treatment normalized all the morphological abnormalities observed in diabetic sciatic nerve but the Schwann cell recovery was not observed. May be its inability to maintain a good glycemic control resulted in oxidative stress causing structural changes. Earlier reports also confirmed that insulin might not be a neuroprotective agent [18]. The fluctuation in the blood glucose levels and the inability of exogenous insulin to have efficient glycemic control was evident in the glycosylated haemoglobin levels as shown in Table 1. In the present study insulin showed a very good reversal in the physiological parameters studied like body weight and fluid intake. The results showed that the combined treatment of Trigonella with Na_3VO_4 effectively controlled hyperglycemia (Fig. 1). Maintenance of normoglycemia with this treatment is evident in the restoration of the nerve morphology as good glycemic control is known to prevent the onset and delays the progression of complications in diabetes [45]. Vanadate, used in the concentration of 0.6 mg/ml of drinking water showed a good reversal in the biochemical parameters, could not restore the decrease in body weight in diabetes that was otherwise reversed in all the other treatment groups. It also could not completely restore the structural changes, as accumulation of electron dense material in the axons was still visible (Fig. 2D), may be because of its pro-oxidative activity and toxicity like with other transition elements [51].

The accumulated polyols may cause oxidative stress in diabetes and may inhibit glycolytic pathway enzymes [52] as evident in the reduced G6PDH activity which derives substrate from glycolysis. Fructose, whose accumulation in diabetic nerve is a slow process is a more effective glycating agent than glucose [53], generating AGEs, also plays an important role in its contribution to diabetic complications [54]. Insulin treatment to diabetic animals failed to limit the rising glucose, sorbitol and fructose levels in long-term diabetic nerve.

Complete reversal was seen in the hexokinase activity in the $D + V + T$ group. The administration of the different

antidiabetic treatments in diabetes could not however restore AR activity completely. TSP was able to restore the SDH activity resulting in comparatively reduced accumulation of fructose seen in diabetic animals though a much better reversal was found in the $D + V + T$ group wherein vanadate was combined with Trigonella, the results observed in $D + V + T$ group may be attributed to vanadate.

G-6PDH activity also showed the reversal in the different treated groups. The synergistic effect of the vanadate and TSP as reported in this study was found to be more pronounced after 60 days of treatment. Recent studies have suggested that loss of the neurotrophic effects of insulin and/or insulin-like growth factor-I (IGF-I) on nerves in diabetes rather than hyperglycemia *per se*, may be critical for development of long-term complications [55]. The restoration of structural alterations seen with the SOV and TSP treatment may be attributed to presence of certain neurotrophic factors/nerve growth factors in TSP besides the restoration of normal glucose metabolism with this treatment as the sugar accumulations even with this combination could not be maintained completely to the basal levels in comparison to the control. *Trigonella* is known to be rich in saponins and flavinoids the active principles of *Trigonella* [56]. Insulinotropic properties of 4-hydroxyisoleucine isolated from *Trigonella* have been reported which may also have neurotrophic properties [57, 58]. Also TSP may be sensitising the neurons to secrete its own insulin just like it said to do the pancreatic beta cells [57, 58], as has been suggested that the nervous system synthesizes its own insulin [59]. Here in this combination Na_3VO_4 in lower doses (0.2 mg/ml) also may be contributing in insulin-mimetic effect without causing prooxidative damages seen with higher dose (0.6 mg/ml) treatment with SOV alone.

Acknowledgments

A. Preet and B.L. Gupta are recipients of the Senior Research Fellowship from the Council of Scientific and Industrial Research (CSIR), India and Research Associateship from Indian Council of Medical Research (ICMR), India, respectively. P.K. Yadava and N.Z. Baquer acknowledge financial assistance received from CSIR and ICMR, Government of India.

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