EFFECTS OF TROLOX ON NERVE DYSFUNCTION, THERMAL HYPERALGESIA AND OXIDATIVE STRESS IN EXPERIMENTAL DIABETIC NEUROPATHY

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SUMMARY

1. Diabetic neuropathy is one of the most common complications of diabetes and oxidative stress has been implicated to play a major role in its pathophysiology.

2. In the present study, we targeted oxidative stress using trolox, an anti-oxidant, in streptozotocin-induced diabetic neuropathy in rats.

3. Compared with control rats, diabetic rats showed significant deficits in motor nerve conduction velocity (MNCV; 49.91 ± 1.94 vs 42.77 ± 1.39 m/s, respectively) and nerve blood flow (NBF; 107.98 ± 8.22 vs 38.9 ± 2.7 arbitrary perfusion units, respectively) after 8 weeks of diabetes. Tail flick latencies for cold and hot immersion tests were also significantly reduced in diabetic rats, indicating thermal hyperalgesia. These observations indicate development of diabetic neuropathy.

4. A significant decrease in the activity of anti-oxidant enzymes (superoxide dismutase and catalase) and an increase in lipid peroxidation were observed in sciatic nerves from diabetic rats compared with age-matched control rats. Alterations in the activity of anti-oxidant enzymes and lipid peroxidation in diabetic rats indicate oxidative stress in diabetic neuropathy.

5. Two weeks treatment with trolox (10 and 30 mg/kg, i.p.) started on completion of the 6th week of diabetes significantly improved MNCV, NBF and inhibited thermal hyperalgesia. Trolox treatment also improved the activity of anti-oxidant enzymes and inhibited lipid peroxidation in sciatic nerves of diabetic rats.

6. The results of the present study suggest the beneficial effects of trolox in experimental diabetic neuropathy.

Key words: diabetic neuropathy, nerve function, nociception, oxidative stress, trolox.

INTRODUCTION

The increasing incidence of diabetes mellitus has made it one of the major health concerns in developing as well as developed countries. In the year 2000, the world-wide diabetic population was 171 million; this has been projected recently to become 366 million by the year 2030.1 Diabetes-associated chronic hyperglycaemia results in several microvascular and macrovascular complications in diabetic patients, including retinopathy, nephropathy, autonomic dysfunction and neuropathy. Diabetic neuropathy is the most common complication of diabetes mellitus and affects more than 50% of the diabetic population.

The major pathophysiological mechanisms involved in the development of diabetic microvascular complications include increased polyol pathway activity, the formation of advanced glycation end-products (AGE), the activation of protein kinase C (PKC) and increased hexosamine pathway flux. All these pathways share oxidative stress as a mechanism of tissue damage, resulting in further complications of disease. Diabetes-induced deficits in nerve conduction velocities and other manifestations of peripheral diabetic neuropathy (PDN) have been correlated with oxidative stress.2-4 Reactive oxygen species (ROS), such as superoxide (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (OH•), and reactive nitrogen species (RNS), such as peroxynitrite (ONOO−), have been implicated in diabetic complications.5 Increased oxidative stress is responsible for vascular impairments,6,7 leading to endoneurial hypoxia resulting in impaired neuronal function, reduced nerve conduction velocity and loss of neurotrophic support. In addition to the excessive production of free radicals, anti-oxidant enzyme levels are markedly reduced in the peripheral nerves of diabetic animals, indicating a vital role of oxidative stress in diabetic neuropathy.8 Long-term oxidative stress can mediate apoptosis of neurons and Schwann cells, leading to neuronal cell damage.9 Anti-oxidants have been shown to prevent or reverse hyperglycaemia-induced nerve dysfunction.10 Several studies with anti-oxidants, such as α-lipoic acid, taurine, N-acetylcysteine and vitamin E, have demonstrated that the anti-oxidants ameliorate the nerve function deficit and vascular impairments in diabetic rats.3,11-17 In the present study, we investigated the effect of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a cell-permeable and water-soluble derivative of vitamin E, in streptozotocin (STZ)-induced experimental diabetic neuropathy in rats.

METHODS

Chemicals and preparation of drug solutions

Trolox was purchased from Calbiochem (Darmstadt, Germany). Atropine sulphate and STZ were procured from Sigma (St Louis, MO, USA). Thiopentone...
sodium was obtained from Neon (Mumbai, India). The glucose oxidase peroxidase (GOD/POD) kit was purchased from Accurex (Mumbai, India). All other chemicals of analytical grade were purchased locally. Streptozotocin was dissolved in citrate buffer (pH 4.4), whereas trolox was dissolved in phosphate buffer (0.1 mol/L, pH 7.4).

Animals
Healthy male Sprague-Dawley rats (250–270 g) were obtained from the Central Animal Facility (CAF), National Institute of Pharmaceutical Education and Research (NIPER). Animals were provided with standard diet and water ad libitum. They were housed in plastic cages (three in each) at a controlled temperature (24 ± 1°C) and humidity (55 ± 5%) on a 12 h light–dark cycle. All animals were acclimatized for a minimum period of 1 week prior to the beginning of the study. The experimental protocols were approved by the Institutional Animal Ethics Committee of NIPER.

Induction of diabetes
A single dose of STZ (55 mg/kg, i.p.) was used for the induction of diabetes in rats. Age-matched control rats received an equal volume of vehicle (citrate buffer). Diabetes was confirmed 48 h after STZ injection. Plasma glucose levels were estimated using the GOD/POD kit and rats with plasma glucose levels > 250 mg/dL were considered for further studies.

Motor nerve conduction velocity
The Power Laboratory 8sp (ADInstruments, Bella Vista, NSW, Australia) was used for the measurement of motor nerve conduction velocity (MNCV). Rats were anaesthetized with 1.5% thiopentone sodium (30 mg/kg, i.p.) and the MNCV was measured by stimulating the sciatic (proximal to sciatic notch) and tibial (distal to the ankle) nerves using bipolar needle (262 gauge) electrodes with a 3 V single stimulus, as described previously.18,19 At the time of MNCV measurement, the body temperature of the rats was maintained at 37°C using a homeothermic blanket system (Harvard, Kent, UK). The MNCV was calculated using the following formula:

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\text{MNCV} = \frac{\text{distance between sciatic and tibial nerve stimulation point}}{\text{sciatic M wave latency} - \text{tibial M wave latency}}
\]

Composite nerve blood flow
Nerve blood flow (NBF) was measured using a laser Doppler flowmeter (Perimed, Jarfalla, Sweden), as described previously.19 Animals were anaesthetized with 1.5% thiopentone sodium (30 mg/kg, i.p.) and were placed on a stereotaxic apparatus to achieve uniform positioning of the laser Doppler probe each time. The sciatic nerve of the left flank was exposed and the laser probe was placed just above the nerve. The NBF was recorded for 10 min, followed by a 15 min stabilization period, as described previously.18,19 The average of continuous 10 min recordings is given in arbitrary perfusion units (PU). During the study, the body temperature of the rats was maintained at 37°C using a homeothermic blanket system (Harvard) and the temperature of the sciatic nerve was monitored using a digital thermometer (Century, Ambala, India). The laser Doppler technique is considered to be an appropriate method for the measurement of composite sciatic NBF,20 even though it cannot discriminate between endoneurial and epineurial blood flow. Data obtained from the laser Doppler flow meter have been reported to have a significant correlation with conventional techniques, such as the hydrogen clearance method21 and the [14C]-iodoantipyrine method.22

Thermal hyperalgesia
Hyperalgesia was assessed using tail immersion tests.23 Tails were immersed in cold (10°C) or warm (45°C) water and the tail flick response latency (withdrawal of the tail) was taken as the end-point response.

Lipid peroxidation
Lipid peroxidation was estimated by measuring the formation of malonaldehyde (MDA) using the method described by Zhang et al.24 Briefly, the sciatic nerve was isolated bilaterally from the inguinal ligament to its trifurcation and homogenized in 100 mmol/L phosphate buffer solution (PBS, pH 7.4, using a Polytron homogenizer (Littau, Switzerland). An aliquot (100 μL) of homogenate was added to reaction mixture containing 50 μL of 8.1% sodium dodecyl sulphate, 300 μL of 20% acetic acid, 300 μL of 0.8% thiobarbituric acid and 60 μL distilled water in tightly closed capped tubes. Samples were then incubated at 95°C for 1 h, incubation cooled to room temperature and then centrifuged at 4000 g for 10 min. Absorbance of supernatant was measured at 532 nm and quantification was performed using the standard curve generated with authentic MDA following similar conditions. The MDA content was expressed as μmol/mg tissue protein.24 Protein estimation was performed according to the method of Lowry et al.25

Anti-oxidant enzyme activity
Superoxide dismutase activity
Superoxide dismutase (SOD) activity was measured using the NADH oxidation method described by Paoletti et al.26 Briefly, 800 μL triethanolamine-diethanolamine buffer (pH 7.4), 40 μL of 7.5 mmol/L NADH, 25 μL of 100–50 EDTA-MnCl_2 (pH 7) and 100 μL homogenate (sample) or phosphate buffer (blank) were added to a cuvette. After thorough mixing, the reaction was initiated by the addition of 100 μL of 10 mmol/L mercaptoethanol and the reaction was followed at 340 nm using a Perkin Elmer (Norwalk, CT, USA) spectrophotometer. The rate of nucleotide oxidation (in the blank) and inhibition of the same by homogenate was calculated.26

Catalase activity
The sciatic nerve was isolated bilaterally and homogenized in 100 mmol/L PBS, pH 7.4, using a Polytron homogenizer. The homogenate was then centrifuged at 20 584 g for 10 min at 4°C. Then supernatant was used for the measurement of catalase activity according to the H_2O_2 degradation method.27

Treatment schedule
The 2 week treatment with trolox (10 and 30 mg/kg per day, i.p.) was started 6 weeks after the induction of diabetes and continued until the 8th week. All parameters (MNCV, NBF, nociception and biochemical parameters) were measured on completion of treatment. The effect of trolox treatment was also investigated in normal rats. Five to seven animals were used for determination of MNCV, NBF and thermal hyperalgesia, whereas between four and six were used in the biochemical studies (lipid peroxidation and anti-oxidant enzymes).

Statistical analysis
Results are expressed as the mean±SEM. Jandel SigmaStat Version 2 software (Jandel, San Rafael, CA, USA) was used for statistical analysis. The significance of differences between the two groups was evaluated using Student’s t-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When the ANOVA showed a significant difference, post hoc analysis was performed with Tukey’s test. P < 0.05 was considered statistically significant.

RESULTS
Plasma glucose levels
Diabetic rats showed a significant increase in blood glucose levels compared with control and increased levels were consistent throughout the study period (581.54 ± 17.48 vs 106.96 ± 3.15 mg/dL at 8 weeks
in diabetic and control rats, respectively). Trolox treatment had no effect on plasma glucose levels in diabetic rats compared with vehicle-treated diabetic rats.

**Motor nerve conduction velocity**

Diabetic rats 8 weeks after the induction of diabetes showed a significant (14%) decrease in MNCV compared with control rats (42.77 ± 1.39 vs 49.91 ± 1.94 m/s, respectively). This deficit in MNCV was improved following 2 weeks treatment with trolox. Trolox, at 10 and 30 mg/kg i.p., inhibited the MNCV deficit by 76 and 97%, respectively, compared with vehicle-treated diabetic rats (Fig. 1; P < 0.05 for both).

Trolox treatment had no significant effect on MNCV in non-diabetic control rats, with the MNCV in the 10 mg/kg trolox-treated and untreated control groups being 50.85 ± 0.84 and 49.91 ± 1.94 m/s, respectively. The MNCV in the 30 mg/kg trolox-treated group was 51.02 ± 0.80 m/s.

**Nerve blood flow**

At 8 weeks, diabetic rats showed a 64% reduction in NBF compared with control rats (38.9 ± 2.7 vs 107.98 ± 8.22 PU, respectively). This deficit was reversed by 23 and 29% following treatment with 10 and 30 mg/kg i.p., trolox, respectively (Fig. 2; both P < 0.01).

Trolox treatment had no significant effect on NBF in non-diabetic control rats. The NBF in the 10 mg/kg trolox-treated and untreated control groups was 101.30 ± 2.70 and 107.98 ± 8.22 PU, respectively. The NBF in the 30 mg/kg trolox-treated group was 102.47 ± 2.36 PU.

**Nociception**

The tail flick latency for the cold and hot immersion tests in diabetic rats at 8 weeks was significantly decreased (P < 0.01) compared with age-matched control rats. This deficit in tail flick latency for the cold and hot immersion tests was improved significantly following treatment with 10 and 30 mg/kg trolox (Fig. 3a,b).
Trolox protects against diabetic neuropathy

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Lipid peroxidation

The MDA levels in sciatic nerves from diabetic rats at 8 weeks were significantly (P < 0.05) increased compared with those in age-matched control rats (12.86 ± 0.56 vs 15.06 ± 0.70 μmol/mg protein, respectively). Trolox (10 and 30 mg/kg) treatment significantly decreased MDA levels compared with those in vehicle-treated diabetic rats (Fig. 4).

Anti-oxidant enzyme activity

The activity of SOD and catalase was significantly decreased in diabetic rats at 8 weeks compared with age-matched control rats (Figs 5, 6). Two weeks treatment with trolox (10 and 30 mg/kg) significantly (P < 0.05) reversed the decrease in SOD and catalase activity in diabetic rats (Figs 5, 6).

DISCUSSION

Diabetic neuropathy in STZ-induced diabetic rats was evident from the significant decreases in MNCV, NBF and tail flick latencies. We observed 14 and 64% reductions in MNCV and NBF, respectively,
8 weeks after the induction of diabetes in rats. These results are in accordance with other reports, wherein similar reductions in MNCV and NBF have been reported in STZ-induced diabetic rats.19,20,29

The role of oxidative stress in diabetes and diabetic neuropathy has been strongly suggested. In diabetes, high glucose levels have been shown to stimulate ROS production in cultured vascular cells through PKC-dependent activation of NAD(P)H oxidase,30 which has also been linked to the increased production of AGE.31 Increased formation of O$_2^•$ in diabetes is also associated with the activation of xanthine oxidase in the liver and plasma of diabetic animals.32 In diabetes, the bioactivity and/or generation of nitric oxide (NO) by endothelial NO synthase is reduced33 and this may be due, in part, to the quenching of NO by O$_2^•$ to form ONOO$^–$. Altered anti-oxidant enzyme levels have been reported in the diabetic condition.3 In the present study, we observed increased lipid peroxidation and decrease SOD and catalase activity in diabetic nerves.

In the present study, we observed a 14% reduction in the MNCV of diabetic rats. Decreased nerve conduction velocity associated with diabetic neuropathy has been well correlated with oxidative stress in several studies.3,15,17,34,35 Hence, it is plausible to assume that a suitable anti-oxidant would be beneficial in the treatment of diabetic neuropathy. In the present study, we used trolox, a cell-permeable, water-soluble derivative of vitamin E, as the anti-oxidant. Trolox has already been reported to reduce intracellular ROS.36 At 10 and 30 mg/kg, trolox produced a significant improvement in MNCV (76 and 97%, respectively) in diabetic rats. Trolox treatment per se did not alter MNCV in non-diabetic rats. Vitamin E has already been reported to ameliorate diabetic neuropathy in animals, but the dose at which vitamin E is able to produce its beneficial effects is high (up to 12 g/kg per day as a dietary supplement in rats).37 The beneficial effects of trolox (a water-soluble derivative of vitamin E) in the present study were observed at lower doses (10 and 30 mg/kg) and the use of lower doses of trolox is further supported by a recent report in which trolox inhibited lipid peroxidation much more effectively than vitamin E and some of its other derivatives.38

Reduced nerve perfusion is a contributing factor in the aetiology of diabetic neuropathy. A number of reports have indicated that PDN is a hypoxic neuropathy and that a decrease in NBF, with resulting endoneurial hypoxia, is a key mechanism responsible for reduced nerve conduction in the diabetic nerve.3,39,40 Impaired endothelium-dependent vasodilatation has been demonstrated in various vascular beds of animal models of diabetes and in humans with type 1 and type 2 diabetes.31,42 Free radicals, such as O$_2^•$ and OH$^–$, cause vascular endothelial damage and reduced NO-mediated vasodilatation.59 Studies have provided evidence that the generation of O$_2^•$ and peroxynitrite impairs vascular function and endothelium-dependent vascular relaxation of epineural arterioles of the sciatic nerve from diabetic rats, which precedes the slowing of nerve conduction velocity.4,39,40,43,44 Several reports have ascribed free radical-induced oxidative stress under diabetic conditions to hyperglycaemia45 and have observed decreased NBF as a consequence of free radical-induced vascular dysfunction.45–49 In the present study, we observed a 64% decrease in sciatic NBF in diabetic rats compared with control rats. Treatment with 10 and 30 mg/kg trolox improved NBF in diabetic rats by 19 and 23%, respectively. Deficits in NBF were not completely reversed in diabetic rats following trolox treatment. It has been reported that complete reversal of NBF deficits is not required for the correction of MNCV in diabetic rats.50 Trolox treatment did not produce significant changes in NBF in non-diabetic rats.

We observed a significant increase in MDA levels and a reduction in the activity of anti-oxidant enzymes in diabetic rats. Trolox treatment significantly reduced MDA levels and increased the activity of anti-oxidant enzymes (SOD and catalase) in rats with diabetic neuropathy. The beneficial effects of trolox are further supported by its usefulness in diabetic retinopathy. Its beneficial effects in diabetic retinopathy are attributed to its anti-oxidant activity and have been correlated with a decrease in oxidized glutathione levels, ameliora-
tion of SOD and catalase activity, inhibition of lipid peroxidation and activation of PKC.51–54 The anti-oxidant property of trolox may also increase energy metabolism and reduce the formation of AGE and inflammation, as reported for other anti-oxidants (α-lipoic acid, vitamin E, vitamin C and N-acetyl-l-cysteine). α-Lipoic acid has been shown to increase energy metabolism and myo-inositol levels in diabetic nerves.10,55 Vitamin C and vitamin E have been reported to decrease the formation of AGE.56 N-Acetyl-l-cysteine and α-tocopherol have been shown to reduce levels of pro-inflammatory cytokines (interleukin, tumour necrosis factor-α), chemokines and C-reactive proteins in diabetic rats.14,57 In addition, trolox treatment reduces apoptotic cell death, which is usually a consequence of oxidative stress.58

Neuropathic pain is commonly associated with diabetic neuropathy; thus, in the present study, we evaluated thermal hyperalgesia in STZ-diabetic rats. A decreased tail flick response latency was observed in diabetic rats, which is in accordance with several other reports.59,60 Several mechanisms, such as tissue injury as a result of ischaemia, sensitization of peripheral receptors, ectopic activity in sprouting fibres and alterations in dorsal root ganglion cells, have been reported to contribute to changes in nociception.61 In the present study, we observed a significant increase in tail flick response latency for hot as well as cold immersion tests following trolox treatment, suggesting a role for oxidative stress in the nociceptive changes in diabetic rats.

In conclusion, trolox treatment resulted in significant protection against diabetic neuropathy, as evidenced by improvements in

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**Fig. 6** Effect of 2 weeks treatment with trolox on catalase activity, determined in rats 8 weeks after the induction of diabetes, with or without trolox treatment. Data are the mean±SEM (n = 4–6). *P < 0.01 compared with age-matched control rats (○); P < 0.05 compared with vehicle-treated diabetic rats (▲). (□), diabetic rats treated with 10 mg/kg trolox; (△), diabetic rats treated with 30 mg/kg trolox.
MNCV and NBF and a reduction in thermal hyperalgesia. The protective effects of trolox were associated with a decrease in lipid peroxidation and an improvement in the activity of anti-oxidant enzymes. The present study suggests the beneficial effects of trolox in diabetic neuropathy.

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