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PREVENTIVE AND INTERVENTIONARY EFFECTS OF PERINDOPRIL IN STREPTOZOTOCIN INDUCED DIABETIC NEUROPATHY IN RATS

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ABSTRACT

Objective: The present study was planned to evaluate the effects of perindopril on Streptozotocin (STZ) induced diabetic neuropathy by comparing preventive and interventionary treatment groups.

Material and methods: STZ induced diabetic neuropathy in rats was monitored by measuring blood sugar levels, motor nerve conduction velocity (MNCV) and nociception. Fifty rats were divided in to five groups of 10 rats each. Group I: Control (vehicle). Group II: STZ (50mg/ kg, iv, single injection). Group III: perindopril [1 mg/kg,per oral (po), daily + STZ]. Group IV: STZ+ perindopril (1 mg/kg, po, daily) 10 weeks after STZ. Group V: STZ + insulin (4 unit/kg, sc, bid). Similar protocol was used for other parameters also.

Results: Perindopril (1 mg/kg, po, daily + STZ) pre-treatment reduced blood sugar levels in diabetic rats and prevented deterioration of motor nerve conduction velocity as compared to STZ diabetic rats. Hyperalgesia induced by STZ was antagonized by perindopril.

Conclusion: The study shows that perindopril ameliorates some neuropathic changes in STZ diabetic rats and preventive treatment is more effective than interventionary treatment.

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INTRODUCTION

Diabetic neuropathy (DN) is the most common symptomatic chronic complication in diabetic patients accounting for substantial morbidity. The prevalence of neuropathy is more than 50% in those who have been diabetic for 20 years. The natural history of diabetic neuropathy is progressive and irreversible loss of sensibility in the feet leading to ulceration and or amputation in 15% of patients. [1] The different types of pain that afflict the patients range from superficial dysesthetic or allodynic to deep-seated gnawing,

and lightning pains that compromise the quality of life of patients. Even with standard therapeutic treatment, responders rarely exceed 50% pain relief, making it one of the most challenging pain syndromes.

The pathophysiology of diabetic neuropathy is complex and the etiology is multifactorial; causations include metabolic, immunological, neuronal and vasomediated defects. Microangiopathy is deemed as the root cause of both nephropathy, and retinopathy and mounting evidence provides support for a vascular basis of diabetic neuropathy. Metabolic events that have been implicated in the pathogenesis of DN are impaired Na^+ K^+ ATPase, increased aldose reductase activity, nonenzymatic glycation/glycoxidation, activation of protein kinase C, impaired neurotrophic support, enhanced oxidative-nitrosative stress and, recently, downstream effectors of free radical and oxidant-induced injury, i.e., mitogen-activated protein kinase (MAPK) activation, poly(ADP ribose) polymerase [PARP] activation, and impaired calcium signaling. [2, 3]All these mechanisms have been demonstrated to contribute to early DN and cause motor and sensory nerve conduction deficits, neurovascular dysfunction, altered sensation, and diabetic neuropathic pain.

Anti-depressants, anti-convulsants, α-Lipoic acid and an aldose reductase inhibitor are available for the treatment of diabetic neuropathy but more powerful therapies are needed for the treatment of painful diabetic neuropathy not only for relief from pain but also to improve nerve functions.

Angiotensin converting enzyme (ACE) inhibitors are first line treatment for hypertension as well as diabetes related nephropathy and cardiovascular diseases. Benefits of ACE inhibitors have been established in diabetic retinopathy [4] and current studies are evaluating them in preventing recurrence of stroke. [5] Increased plasma levels of ACE have been reported in streptozotocin (STZ) diabetic rats. Elevated angiotensin II levels have been associated with hypertension, diabetes, and polyneuropathy. [6] European trial on reduction of cardiac events with perindopril in stable coronary artery disease [EUROPA]trial demonstrated improvement in abnormal endothelial function in patients with coronary artery disease.[7] ACE inhibitors improve the function of endothelium by several mechanisms including anti-oxidative properties, beneficial effects on fibrinolysis, lowering of angiotensin II and an increase in bradykinin. [8]. However, little is known about the potential benefits of these drugs in diabetic neuropathy. Though few studies have studied the effects of different ACE inhibitors in diabetic neuropathy and results have been encouraging, more investigation is required before clinical practice can be advocated. [9, 10] Animal lines such as STZ diabetic rats and mice, BB/Wor rats, Zucker diabetic fatty rats, and ob/ob mice show pain-related behaviors that mimic symptoms of painful diabetic neuropathy in humans. The present study was undertaken to compare pretreatment or preventive effects with treatment effects (after neuropathy is established) of the ACE inhibitor perindopril in STZ induced diabetic neuropathy.

MATERIAL AND METHODS:

Animals:

Albino rats of either sex weighing 250–300g were maintained under standard conditions with food and water *ad libitum*. Streptozotocin was purchased from Sigma Inc. (USA). Perindopril (1 mg/kg, po, daily + STZ) was purchased (Coversyl tablets, 4mg).A stock solution of perindopril was prepared using distilled water. Solution was made in a concentration of 1mg/ml and animals were given the drug by gavage. Ethical clearance was taken from Institutional Animal Ethical committee.

Induction of diabetes:

Rats were made diabetic with a single intravenous injection of streptozotocin (50 mg/kg) in 0.05 mol/l citrate buffer, pH 4.5. Samples for blood sugar were obtained after 48 h of STZ and every 2 weeks thereafter. Blood glucose levels were determined by the glucose oxidase method. [11] Rats showing serum glucose levels greater than 250 mg/dl after 48 h of STZ were considered diabetic.

Electrophysiological study:

Electromyograph (Medicor, Budapest) was used to measure motor nerve conduction velocity (MNCV). [12] Rats were anaesthetized with ether to give light anesthesia and ensure minimum interference with nerve conduction. To minimize effects of differences in body temperature on MNCV, animals were placed under a 40 W light bulb. Left leg of rat was shaved and cleaned. MNCV was measured in the sciatic-tibial conducting motor system at weeks 0, 4, 6, 8, 10 and 12. The left sciatic nerve was stimulated with square pulses (2Hz) at the sciatic notch and tibial nerve at the ankle by supramaximal (8 V) stimulation. Responses were recorded from the small muscles of the foot by surface electrodes. MNCV was calculated by substracting distal from proximal latency (milliseconds) and the difference was divided by the distance (millimetre) between the two stimulating electrodes giving the MNCV in meter/second.

MNCV (m/s) = Distance between two points of stimulation (in mm) / Latency (from proximal-distal point) to onset of muscle action potential

Nociceptive test:

Nociceptive response was evaluated using the tail flick test to thermal stimulation by an analgesiometer. [13] The heat intensity was adjusted so that the rats had control (pre-drug) tail flick latencies of 4 to 5 sec. A cut off latency of 10 sec was used to prevent damage to the tail. Tail withdrawn from heat source was taken as end point. The initial (control) reaction time was recorded in all animals and compared with drug treated groups every 2 weeks for 12 weeks.

Experimental design:

Rats were divided into five groups of 10 each. Group I (A1) served as control (vehicle). Group II (A2) were given STZ (50 mg/ kg, i.v, single injection). Group III(A3) received perindopril (1 mg/kg, po, daily by gavage) 5 days prior to STZ and continued for 12 weeks after STZ. Group IV (A4) were administered perindopril (1 mg/kg, po, daily by gavage) 10 weeks after administration of STZ. Group V (A5) were injected STZ+ regular insulin (4 unit/kg, sc, twice daily for 12 weeks) to make rats euglycaemic.

Statistical analysis:

Results were statistically analysed by comparing with control using Student's t test (unpaired). Probability values less than 0.05 were considered significant. Values were expressed as mean ± SE.

RESULTS:

Effect on body weight:

Table 1 provides data on the final weight for the rats used in these studies from all five groups. Rats in control study group gained weight. In contrast, diabetic rats showed a marked and gradual reduction in their body weight during the course of the study period. Perindopril pre-treatment as well as treatment group failed to improve the body weight significantly. The increase in body weight in insulin treated group was significant ($p<0.05$) when compared to STZ diabetic group.

Table 1 Effect of perindopril (1 mg/kg, po) pretreatment and treatment on body weight in rats with STZ induced diabetic neuropathy.

Data represented as mean ± SE

Body Weight (grams)							
Drugs/Groups	**0 weeks**	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**	**12 weeks**
Control (A1)	268.8±4.19	273.5±3.77	275.7±3.7	279.0±3.69	281.8±3.56	283.4±3.43	285.4±3.1
STZ (A2)	262.0±3.06	257.3±2.9*	249.0±2.53*	242.5±2.17*	238.6±2.1*	235.4±2.3*	232.0±1.9*
Perindopril + STZ (A3)	253.7±0.35	249.7±0.31*++	244.8±0.28*++	240.8±0.9*++	240.8±0.9*++	238.2±1.24*++	238.0±0.61*++
Perindopril +STZ	252.4±0.58	252.6±2.5*++	244.6±1.8*++	242.6±2.7*++	242.6±2.4*++	240.0±2.25*++	234.8±2.03*++

(A4)							
Insulin + STZ (A5)	260.2±0.55	262.4±0.58** ++	259.6±0.77** +	261.6±0.77* *+	261.6±0.77* *+	261.2±0.70* *+	263.6±0.82*+

* p<0.05, when compared with control (initial);** p>0.05, when compared with control (initial);+ p<0.05, when compared with STZ (group A2) (Student's 't' test);++ p>0.05, when compared with STZ.

Blood sugar:

As shown in Table 2 and Figure1, blood sugar level in the control group ranged from mean 89.5 ± 1.45 to 91.2± 0.95 mg/dL and remained almost same at weeks 2, 4, 6, 8, 10 and 12. A significant ($p < 0.001$) and sustained increase in blood sugar levels was observed in animals injected STZ. Perindopril pre-treatment and treatment significantly decreased blood sugar levels in STZ diabetic rats. Insulin treatment prevented the development of STZ induced hyperglycaemia in rats.

Table 2 Effect of perindopril (1 mg/kg, po) pretreatment and treatment on blood sugar levels *in rats with STZ induced diabetic neuropathy.*

Data represented as mean ± SE

	Blood Sugar (mg/dl)						
Drugs/Groups	**0 weeks**	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**	**12 weeks**
Control (A1)	89.5±1.45	90.4±1.09	90.1±1.41	89.9±1.1	91.2±0.95	90.7±1.09	89.0±1.28
STZ (A2)	91.4±1.4	257.3±1.31*	259.0±1.1*	261.4±1.97*	259.4±2.43*	266.4±2.43*	266.8±2.31*
Perindopril + STZ (A3)	93.7±0.79	196.7±0.79* +	194.2±1.18* +	188.0±0.96* +	187.6±0.79* +	183.4±0.76* +	180.2±0.78* +
STZ + Perindopril (A4)	90.7±0.95**	252.4±2.58* ++	258.4±1.18* ++	261.4±2.24* ++	254.4±0.53* ++	267.1±2.29* ++	220.2±0.78* +
Insulin + STZ (A5)	89.0±0.83	88.4±0.79** +	89.9±1.1**+	90.7±0.95** +	90.2±1.41** +	90.9±1.3**+	90.1±1.41** +

* p<0.01, when compared with control;** p>0.005, when compared with control; + p<0.01, when compared with STZ (Student's 't' test);++ p>0.01, when compared with STZ

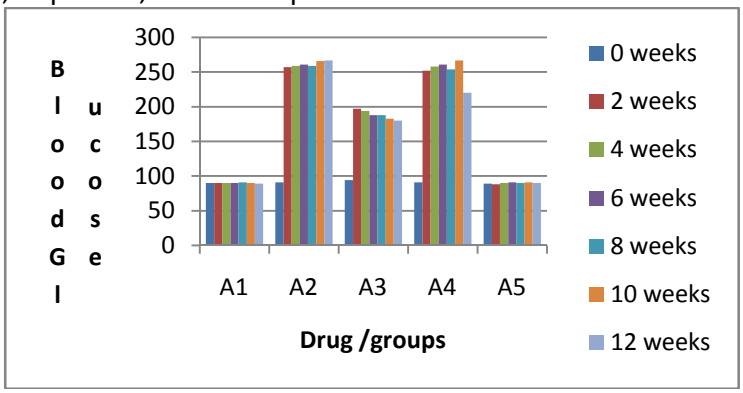

Fig.1 Effect of pretreatment and treatment with perindopril on blood sugar levels in STZ-diabetic rats.

MNCV:

As shown in Table 3, age matched control rats showed statistically non-significant increase in MNCV recorded at 4, 6, 8, 10 and 12 weeks as compared with initial value (0 week). STZ diabetic rats showed a gradual decrease in MNCV. The MNCV was not significantly different from the initial value up to week 8.

However, 8 weeks onward the decrease in MNCV was highly significant ($p < 0.01$) compared with initial value. Perindopril pre- treatment resulted in an increase in MNCV compared with diabetic animals. The increase at weeks 10 and 12 was highly significant compared with animals receiving STZ alone but remained less compared with control. Perindopril treatment did not significantly alter the MNCV. Insulin pre-treatment prevented the reduction in MNCV in diabetic rats. The increase in MNCV was significant compared with diabetic animals but remained lower than that of the control group.

Table 3 Effect of perindopril (1 mg/kg, po) pretreatment and treatment on MNCV in rats with STZ induced diabetic neuropathy.

Data represented as mean ± SE

Motor nerve conduction velocity (m/s)						
Drugs/Groups	**0 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**	**12 weeks**
Control (A1)	51.2±1.2	52.0±0.65	52.1±0.81	53.1±0.76	53.8±0.61	52.2±0.73
STZ (A2)	52.4±0.47**	52.8±0.51**	52.3±0.55**	50.2±0.46*	48.0±1.03*	46.5±1.24*
Perindopril + STZ (A3)	50.0±0.49**+	50.6±0.49**+	50.9±0.31**++	51.4±0.49**++	51.8±0.36**+	52.4±0.37**+
Perindopril +STZ (A4)	54.6±0.65**	53.8±0.54**++	52.8±0.26**++	50.4±0.24**++	45.6±0.47*++	46.2±0.44*++
Insulin + STZ (A5)	50.4±0.45**+	50.8±0.25**+	50.3±0.42**++	52.8±0.25**+	52.6±0.26**+	52.2±0.41**+

* $p<0.05$, when compared with control;** $p>0.05$, when compared with control;+ $p<0.05$, when compared with STZ (group A2) (Student's 't' test);++ $p>0.05$, when compared with STZ.

Nociception:

As shown in Table 4, administration of STZ resulted in hyperalgesic response in rats, observed only after 8 weeks onward. Perindopril pre-treatment antagonized the STZ induced hyperalgesia in diabetic rats. The increase in reaction time was significant ($p < 0.05$) at weeks 8, 10 and 12, compared with STZ diabetic animals, though it was less when compared with the control group. Perindopril treatment also improved the tail flick reaction time in diabetic animals. Tail flick reaction time in insulin+ STZ treated animals was not significantly different when compared with the control group. However, there was significant difference in reaction time when compared with animals receiving only STZ, i.e. diabetic control.

Table 4 Effect of perindopril (1 mg/kg, po) pretreatment and treatment on tail flick reaction time to thermal stimulation in rats with STZ induced diabetic neuropathy.

Data represented as mean ± SE

Tail flick reaction time (seconds)							
Drugs/Groups	**0 weeks**	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**	**12 weeks**
Control (A1)	4.5±0.22	4.6±0.15	4.4±0.15	4.4±0.15	4.7±0.20	4.6±0.16	4.3±0.11

STZ (A2)	4.6±0.16***	4.6±0.16**	4.4±0.15**	4.2±0.80**	3.2±0.13*	2.5±0.16*	2.2±0.08*
Perindopril +STZ (A3)	4.6±0.16***++	4.5±0.22**++	4.3±0.20**++	3.7±0.20**++	3.3±0.21*+	3.3±0.21*+	3.0±0.21*+
Perindopril +STZ (A4)	4.5±0.22***	4.1±0.22**++	3.7±1.02**++	3.5±0.87**++	3.2±0.22**++	2.8±0.21*++	4.0±0.21**+
Insulin + STZ (A5)	4.8±0.17***++	4.7±0.15**++	4.4±0.16**++	4.4±0.16**++	4.3±0.15**+	4.3±0.15**+	4.4±0.16**+

* p<0.05, when compared with control;** p>0.05, when compared with control;+ p<0.05, when compared with STZ (group A2) (Student's 't' test);++ p>0.05, when compared with STZ.

DISCUSSION:

Data from the present study revealed marked hyperglycemia, reduced MNCV and hyperalgesia after STZ administration in rats.STZ acts through a common pathway dependent on the formation of single strand breaks in β cell DNA, leading to selective destruction of pancreatic β cells. These breaks then activate nuclear enzyme poly (ADP-ribose) synthetase in β cell to such an extent that the stores of its substrate nicotinamide adenine nucleotide (NAD) become critically depleted. [14] Additionally, increased catabolism leading to activation of glycogeonolysis, gluconeogenesis, proteolysis, lipolysis and ketogenesis is responsible for hyperglycemia.

Both pre-treatment and treatment groups showed lowering of blood glucose levels in STZ diabetic rats. The mechanisms proposed for the improvement in glucose tolerance with ACE inhibitors in experimental studies are increased energy expenditure, liver and adipose tissue metabolic modulation, lower concentration of leptin, improved insulin signaling, and increased glucose and fatty acid utilization by muscle. [15] Perindopril has been shown to improve insulin sensitivity and increase HDL. [16, 17]ACE inhibition by captopril improved glucose transport in insulin-resistant muscle of the obese Zucker rat. [18] This improvement in glucose uptake has been attributed to modulation of insulin action by bradykinin mediated through B_2 receptors and by an increase in nitric oxide production. [19]

Diabetic rats showed thermal hyperalgesia, as determined by decrease in latency of withdrawal threshold of tail. Hyperalgesia observed did not follow the acute changes in blood glucose levels. Elevated blood glucose levels were seen as early as on third day whereas no change in nociception threshold was observed at this point of time. Significant hyperalgesia was observed only after 8 weeks of STZ administration; implying that acute hyperglycemia is not responsible for hyperalgesia. The anti-nociceptive effect of perindopril may be due to modulation of neuropeptides and/or endogenous opioid peptides.

In a study by Takai et al, antinociceptive effects of ACE inhibitors were opposed by naloxone implying that brain endogenous angiotensin II is involved in central nociceptive mechanisms and that it has an antagonistic interaction with the endogenous opioid system. By reducing the degradation of opioids, ACE inhibitors increase their levels in brain, producing antinociceptive action. [20]

Insufficient nerve-derived mediators such as substance P may contribute to the impaired response to injury, predisposing the patient to foot ulcer. [21] Plasma concentrations of neuropeptides [calcitonin gene related peptide, substance P (SP), neuropeptide Y(NPY)] have been found to be raised but levels are decreased in the diabetic tissues [22]Apart from angiotensin II, ACE also hydrolyzes neuropeptides; consequently it can be hypothesized that, ACE inhibitor perindopril led to an increased bioavailability of SP and NPY in the nerves-producing antinociceptive action.

Singh *et al* demonstrated activation of the renin-angiotensin system (RAS) as a major event in

hyperglycemia as a result of increased oxidative stress, increased protein kinase C (PKC) levels, and/or increased activity of the hexosamine biosynthesis pathway. [23, 24] Hyperactivation of the RAS pathway can cause neural dysfunction by inducing accelerated degradation of some neuronal proteins such as synaptophysin and by activating pathological glial responses. [25] The generation of oxidative stress through the production of superoxide and peroxynitrite leads to endoneurial hypoxia that impairs vascular function of epineurial arterioles of the sciatic nerve, and this precedes slowing of MNCV. [26] It can be deduced that microcirculatory defects lead to impaired blood flow in vasa nervorum, decreasing the blood flow to the sciatic nerve; this initiates neuropathic changes.Beneficial effect of perindopril in improving MNCV may be due to improved neurovascular function, increased endoneurial blood flow, mediated through a reduction in peripheral resistance, by antagonizing vasoconstrictor angiotensin II and augmenting synthesis of vasodilatory prostaglandins via reduced degradation of bradykinin. [27] In the vascular wall, AT_1receptor activation leads to unfavorable cellular responses including oxidative stress, lipid peroxidation, nitric oxide (NO) inactivation, and activation of redox-sensitive genes, which collectively participate in the initiation of vascular dysfunction. [23, 28] By opposing these changes, ACE inhibitors appear to correct many of the abnormalities associated with the vascular dysfunction found in diabetes. [29] Additionally, by preventing degradation of vasoactive peptides such as calcitonin gene-related peptide and C-type natriuretic peptide, ACE inhibitors help to maintain endoneurial blood flow and prevent ischemia. [30]Moreover, ACE inhibitors have also been shown to promote angiogenesis, leading to an increase in capillary density in the sciatic nerve. [31]

Hyperglycemia and oxidative stress lead to elevated levels of advanced glycation end products (AGEs) that have been documented in the peripheral nerves of subjects with diabetes. Accumulation o AGEs reduce the vessel compliance and increase vascular stiffness, contributing to progression of diabetic complications; thus linking microangiopathy and neuropathy. ACE inhibitors decrease the formation of AGEs, as assessed in animal models of diabetes and this may contribute to improvement in vascular dysfunction in diabetics. [32]

Significant evidence points to increased oxidative stress in diabetic neuropathy-either due to increased production of reactive oxygen species (ROS) or defective scavenging of free radicals. [23] NADPH oxidases and flavin oxidases that convert atmospheric oxygen (O_2) to superoxide radical ($O_2^{\bullet-}$), are at the center of these events.

Hyperglycemia generates ROS and attenuates antioxidative mechanisms through glycation of scavenging enzymes. Angiotensin II increases the activity of NADPH oxidase and production of superoxide radical. [33] By preventing angiotensin-II induced NADPH oxidase activation and upregulating endothelial nitric oxide synthase, ACE inhibitors suppress intracellular ROS production and exert profound anti oxidant effect. [7, 34]

Therefore, perindopril exerts beneficial effects in ameliorating peripheral nerve dysfunction in diabetes through its anti-oxidative action, improvement in nerve blood flow leading to amelioration of microcirculatory defects, and metabolic effects. Compared to treatment arm, the pretreatment arm was more effective in improving the parameters of nerve dysfunction.

CONCLUSION:

Perindopril improves peripheral nerve function in experimental diabetic rats. The pretreatment arm showed greater improvement compared to treatment arm. Therefore, starting perindopril at an early stage of disease may help ameliorate neuronal dysfunction to a greater extent and prove to be a valuable approach to treat DN in the future.

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