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Concurrent targeting of nitrosative stress–PARP pathway corrects functional, behavioral and biochemical deficits in experimental diabetic neuropathy

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ABSTRACT

Peroxynitrite mediated nitrosative stress, an indisputable initiator of DNA damage and overactivation of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme activated after sensing DNA damage, are two crucial pathogenetic mechanisms in diabetic neuropathy. The intent of the present study was to investigate the effect of combination of a peroxynitrite decomposition catalyst (PDC), FeTMPyP and a PARP inhibitor, 4-ANI against diabetic peripheral neuropathy. The end points of evaluation of the study included motor nerve conduction velocity (MNCV) and nerve blood flow (NBF) for evaluating nerve functions; thermal hyperalgesia and mechanical allodynia for assessing nociceptive alterations, malondialdehyde and peroxynitrite levels to detect oxidative stress–nitrosative stress; NAD concentration in sciatic nerve to assess overactivation of PARP. Additionally immunohistochemical studies for nitrotyrosine and Poly(ADP-ribose) (PAR) was also performed. Treatment with the combination of FeTMPyP and 4-ANI led to significant improvement in nerve functions and pain parameters and also attenuated the oxidative–nitrosative stress markers. Further, the combination also reduced the overactivation of PARP as evident from increased NAD levels and decreased PAR immunopositivity in sciatic nerve microsections. Thus, it can be concluded that treatment with the combination of a PDC and PARP inhibitor attenuates alteration in peripheral nerves in diabetic neuropathy (DN).

Introduction

The pathomechanisms of diabetic neuropathy (DN) are complex and involve the activation of numerous pathways involving nerve growth factors [1], inflammatory mediators [2] and reactive oxygen and nitrogen species [3]. As nitrosative stress is emerging as a concept of various pathological conditions, dysregulation of nitric oxide (NO) and increased levels of reactive oxygen and nitrogen species have been implicated in the pathogenesis of DN [3–5]. NO may react with oxidants specially superoxide to form peroxynitrite, a potent oxidant that can exert deleterious effects on biological molecules. All the downstream targets of peroxynitrite are crucial for the maintenance and normal functioning of the peripheral nerves. Tyrosine nitration mediated by peroxynitrite can result in dysfunctional nitrosylated proteins whereas oxidation of sulfydryl groups can lead to the inhibition of important enzymes in the mitochondrial respiratory chain and enzymes involved in the antioxidants defense machinery. Moreover, peroxynitrite inhibits a variety of ion pumps and peroxynitrite-modified cellular proteins are put through accelerated proteosomal degradation [6,7]. Peroxynitrite is also known to trigger pro-inflammatory responses via enhancement of nuclear factor kB activation. Studies with various peroxynitrite decomposition catalysts have provided a more direct approach to judge the role played by peroxynitrite in DN [8]. In one of the recent studies with two novel PDCs, FeTPPS and FeTMPyP, our group implicated peroxynitrite in sciatic nerve conduction deficits, impaired nociception and increased lipid peroxidation, known to be the key features of peripheral neuropathy in STZ induced diabetes in rats [9].

In addition to its toxic effects by direct interaction with biomolecules, peroxynitrite also exerts its cytotoxic effects via numerous downstream mechanisms. Along with other reactive oxidants particularly hydrogen peroxide it causes DNA strand breaks which activates the DNA repair machinery, poly(ADP-ribose) polymerase (PARP) activation being one of the most important among them. Activation of PARP, a nuclear enzyme, represents a forefront

Abbreviations: DN, diabetic neuropathy; MNCV, motor nerve conduction velocity; NBF, nerve blood flow; ND, non-diabetic; PARP, poly (ADP-ribose) polymerase; PDC, peroxynitrite decomposition catalyst; STZ, streptozotocin

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cellular response to DNA damage working towards maintaining genome integrity. However when overactivated by massive DNA damage caused by peroxynitrite, PARP initiates an energy-consuming cycle by catalyzing the transfer of ADP-ribose units from NAD^+ to nuclear proteins. This leads to exhaustion of the cellular NAD^+ and ATP pools, affecting mitochondrial respiration and ultimately resulting in cellular dysfunction and death [10,11]. Several strategies have been applied to uncover the role of PARP in physiological and pathological conditions such as antisense oligonucleotides, gene deletion [12] and RNA interference [13]. Additionally, several classes of competitive PARP inhibitors have been reported. Relevant examples include 3-aminobenzamide [3], nicotinamide [14], 1,5-isoquinolinediol [15,16] and GPI-15427 [17].

In the present study, the role of nitrosative stress and downstream mechanisms, including activation of PARP and novel emerging therapeutic strategies offered by concurrent neutralization of peroxynitrite and inhibition of PARP in experimental DN has been investigated employing FeTMPyP (5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrinato iron(III)), a PDC and 4-ANI (4-amino-1,8-napthalimide), a PARP inhibitor.

Materials and methods

Induction of diabetes and experimental design

The experiments were performed in accordance with regulations specified by the Institute Animal Ethics Committee (IAEC), NIPER. Male Sprague–Dawley rats (250–270 g) were used for the study and were fed on standard rat diet and water *ad libitum*. Diabetes was induced by streptozotocin (STZ) at a dose of 55 mg/kg (i.p.). Blood samples were collected from tail vein ~48 h after STZ administration. The rats with blood glucose more than 250 mg/dl were considered as diabetics and were further considered for study. The experimental groups were comprised of non-diabetic control group (ND), diabetic control rats (STZ-D), and diabetic rats treated with FeTMPyP (STZ-D/FM, FeTMPyP 1 mg/kg, i.p.), 4-ANI (STZ-D/ANI, 4-ANI 3 mg/kg, i.p.) and combination of FeTMPyP 1 mg/kg and 4-ANI 3 mg/kg (STZ-D/C). The treatment was started 6 weeks after diabetes induction and continued for two weeks. The behavioral and biochemical experiments were performed 24 h after administration of last dose.

Functional studies

Motor nerve conduction velocity (MNCV). MNCV was determined in the sciatic-posterior tibial conducting system using Power Lab 8sp system (AD Instruments, Bellaviata, NSW, Australia) as previously described [9]. Sciatic nerve was stimulated with 3 V proximally at sciatic notch and distally at ankle via bipolar electrodes. Receiving electrodes were placed on the muscle of foot. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. MNCV was calculated by subtracting the distal latency from the proximal latency, and then dividing by the distance between the stimulating and recording electrode. MNCV was expressed in m/s.

Sciatic nerve blood flow. NBF was measured using Laser Doppler system (Perimed, Jarfalla, Sweden) [9,18]. Briefly, animals were anesthetized and body temperature was monitored using a rectal probe and maintained with the help of homeothermic blanket throughout the experiment. Sciatic nerve was exposed by giving incision on the left flank and the Laser Doppler probe (tip diameter 0.85 mm) was applied just in contact with an area of sciatic trunk free from epi or perineurial blood vessels. Flux measurement was obtained from the same part of nerve and for the same time period (over a 10 min period). The blood flow was reported in arbitrary perfusion units (PU).

Behavioral studies

Thermal hyperalgesia. The thermal hyperalgesia to both hot (45 °C) and cold (10 °C) immersion test was studied. The tail flick latency was taken as end point in the tail immersion test. The 15 s cutoff time was kept for both tests. Three consecutive readings were taken at an interval of ~30 min [19].

Mechanical allodynia. Sensitivity to noxious mechanical stimuli was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using a von Frey anesthesiometer (model 2290-4; IITC Life Science, USA) and rigid von Frey filaments. The rats were placed in individual plexiglass boxes on a stainless steel mesh floor and were allowed to acclimatize for at least 20 min. A 0.5-mm diameter polypropylene rigid tip was used to apply a force to the plantar surface of the hindpaw. The force causing the withdrawal response was recorded by the anesthesiometer. The anesthesiometer was calibrated before each recording. The test was repeated four to five times at ~5-min intervals on each animal, and the mean value was calculated [9].

Biochemical parameters

Plasma glucose levels. Blood was collected from tail vein in microcentrifuge tubes containing heparin. Plasma was separated and blood glucose was estimated from GOD–POD kit from Accurex, India as per manufacturer's instructions.

Lipid peroxidation. For estimation of lipid peroxidation sciatic nerve was homogenized in phosphate buffer saline (PBS, pH 7.4). The thiobarbituric acid reactive substances (TBARS) were measured as per method described by Ohkawa et al. [20].

Peroxynitrite levels. Peroxynitrite formation was estimated by using a fluorescent dye dihydrorhodamine123 as reported earlier [21]. Dihydrorhodamine123 get oxidized to rhodamine123 in a peroxynitrite dependent manner. Rats were injected dihydrorhodamine123 through the jugular vein (2×10^{-6} M 1 ml/kg in saline). After two hours of dihydrorhodamine123 injections blood samples were collected and plasma was separated for peroxynitrite estimation. The fluorescence in the plasma was measured using the spectrofluorometer (Perkin Elmer, Norwalk, CT) at an excitation wavelength of 500 nm and emission wavelength of 536 nm. The plasma level of rhodamine123 was calculated from the standard curve obtained from authentic rhodamine123.

Measurement of NAD levels. NAD content in nerve homogenate was measured using enzyme cycling assay as described earlier [22]. In brief, sciatic nerve was isolated bilaterally and homogenized in nine volume potassium phosphate buffer and kept in boiling water bath for 5 min. Then the homogenate was centrifuged at 1000*g* on 4 °C and supernatant stored at −20 °C until further used. The NAD content was analyzed using enzyme cycling mixture containing alcohol dehydrogenase and the absorbance was measured at 556 nm using spectrophotometer (Beckmen DU 7400).

Immunohistochemical studies

NT and PAR immunoreactivity were assessed as described earlier [9]. After hydration the 6 μm sciatic nerve sections were washed with TBS (Tris-buffered saline, 20 mM, pH 7.4), followed by incubation with proteinase K for 20 min. Following a rinse with TBS, the sections were incubated with blocking buffer (5% goat serum in TBS) for 120 min. Endogenous biotin-binding sites were

blocked by sequential incubation with avidin and biotin for 30 min each. The sections were then incubated with primary antibody, mouse anti-nitrotyrosine for nitrotyrosine and anti-PAR mouse IgG for PAR in blocking buffer at 4 °C overnight. The sections were washed three times with TBS and then incubated with biotin-conjugated anti-mouse IgG (Sigma–Aldrich, Inc.) in blocking buffer for 2 h at 37 °C. The specific labeling was detected using avidin-conjugated HRP system and its substrate diaminobenzidine. The sections were observed under light microscope (Leica, Germany). The immuno histochemical scores were given as per the following scheme (1: very low intensity; 2: low intensity; 3: moderate intensity and 4: high intensity).

Statistical analysis

Data are expressed as means ± SEM. For comparing the differences between the two groups Student *t*-test was used. For multiple comparisons analysis of variance was used. If the ANOVA test showed significant difference further post hoc Tukey or Dunnet test was applied. Significant was defined as $p < 0.05$. All statistical analysis was performed using Jandel Sigma Stat 2, statistical software.

Results

Effect of FeTMPyP and 4-ANI alone and in combination on nerve function parameters in treated rats

Eight weeks of persistent hyperglycemia resulted in the development of significant deficits in motor conduction velocity and nerve blood flow in diabetic (STZ-D) rats as compared to non-diabetic (ND) rats ($p < 0.001$). Monotherapy with FeTMPyP and 4-ANI failed to demonstrate significant reversal of conduction deficits but produced a significant improvement in nerve blood flow. The combination of FeTMPyP and 4-ANI showed much superior results as compared to monotherapy as NBF was significantly ($p < 0.001$) improved and MNCV was restored to a level comparable to that of non-diabetic (ND) group (Table 1).

Effect of FeTMPyP and 4-ANI alone or in combination on diabetic pain parameters

Tail flick latency in hot and cold immersion tests and paw withdrawal pressure in Electro-von Frey test were significantly decreased in STZ-D rats as compared to ND animals ($p < 0.001$). Two week treatment with FeTMPyP 1 mg/kg alone did not reversed hyperalgesia significantly and 4-ANI 3 mg/kg produced significant correction in pain parameters. The combination however, produced a significant improvement in both thermal and mechanical pain perception ($p < 0.001$) (Table 1).

Effect of FeTMPyP and 4-ANI alone and in combination on oxidative stress induced increased MDA and peroxynitrite levels

The MDA and peroxynitrite levels in eight week diabetic rats were increased significantly ($p < 0.001$) in STZ-D rats as compared to age matched normal rats, ND. A significant reduction in both these oxidative stress parameters was observed with FeTMPyP 1 mg/kg, but with 4-ANI reduction was significant only in MDA levels. The combination regimen showed decreased oxidative stress as manifested by decreased MDA and peroxynitrite levels ($p < 0.001$) (Table 2).

Effect of FeTMPyP and 4-ANI alone and in combination on NAD Levels

PARP overactivation caused excessive utilization of NAD pools in the nerve cells which coincides well with reduced nerve NAD levels in diabetic animals as compared to normal animals ($p < 0.001$). FeTMPyP and 4-ANI treatment alone as well as in combination showed a significant protection against NAD depletion ($p < 0.001$) (Table 2).

Effect of FeTMPyP and 4-ANI alone and in combination on PAR and NT immunohistochemistry

Diabetic animals showed an increased NT and PAR immunoreactivity as compared to age matched control animals ($p < 0.001$).

Table 1
Effect of FeTMPyP and 4-ANI alone or in combination on nerve function parameters and diabetic pain parameters. ND: non-diabetic; STZ-D: diabetic; STZ-D/FM: diabetic group treated with FeTMPyP 1 mg/kg; STZ-D/ANI: diabetic group treated with 4-ANI 3 mg/kg; STZ-D/C: diabetic group treated with combination.

Parameter	ND	STZ-D	STZ-D/FM	STZ-D/ANI	STZ-D/C
Nerve function parameters					
MNCV (m/s)	55.2 ± 1.8	39.2 ± 2.2$^{###}$	43.3 ± 1.4	45.2 ± 2.4	53.9 ± 2.2***
NBF (PU)	105.9 ± 2.3	39.3 ± 1.5$^{###}$	57.5 ± 2.1***	63.4 ± 2.0***	82.4 ± 3.3***
Diabetic pain parameters					
Tail flick latency, hot immersion (s)	13.8 ± 0.5	5.2 ± 0.5$^{###}$	7.1 ± 1.0	9.9 ± 0.8**	12.7 ± 0.8***
Tail flick latency, cold immersion (s)	14.5 ± 0.3	4.2 ± 0.8$^{###}$	6.0 ± 1.2	10.1 ± 0.8***	12.4 ± 1.2***
Mechanical allodynia	70.5 ± 1.4	38.1 ± 2.5$^{###}$	50.1 ± 1.8*	53.9 ± 1.6***	65.6 ± 1.2***

$^{###}$ $p < 0.001$ vs ND ($n = 8$).
* $p < 0.05$ vs STZ-D ($n = 8$).
** $p < 0.01$ vs STZ-D ($n = 8$).
*** $p < 0.001$ vs STZ-D ($n = 8$).

Table 2
Effect of FeTMPyP and 4-ANI alone or in combination on MDA, peroxynitrite and NAD levels. ND: non-diabetic; STZ-D: diabetic; STZ-D/FM: diabetic group treated with FeTMPyP 1 mg/kg; STZ-D/ANI: diabetic group treated with 4-ANI 3 mg/kg; STZ-D/C: diabetic group treated with combination.

Parameters	ND	STZ-D	STZ-D/FM	STZ-D/ANI	STZ-D/C
MDA (μM/mg of protein)	7.5 ± 0.2	29.9 ± 0.3$^{###}$	15.8 ± 2.8*	17.2 ± 2.1*	11.4 ± 1.8***
Peroxynitrite (fluorescence of rhodamine123)	1.1 ± 0.3	6.0 ± 0.4$^{###}$	2.2 ± 0.1**	4.2 ± 0.5	1.2 ± 0.6***
NAD (ng/mg of protein)	384.4 ± 10.9	52.1 ± 10.4$^{###}$	94.6 ± 9.2**	184.3 ± 15.2	290.6 ± 13.3***

$^{###}$ $p < 0.001$ vs ND ($n = 8$).
* $p < 0.05$ ($n = 8$).
** $p < 0.01$ vs STZ-D ($n = 8$).
*** $p < 0.001$ vs STZ-D ($n = 8$).

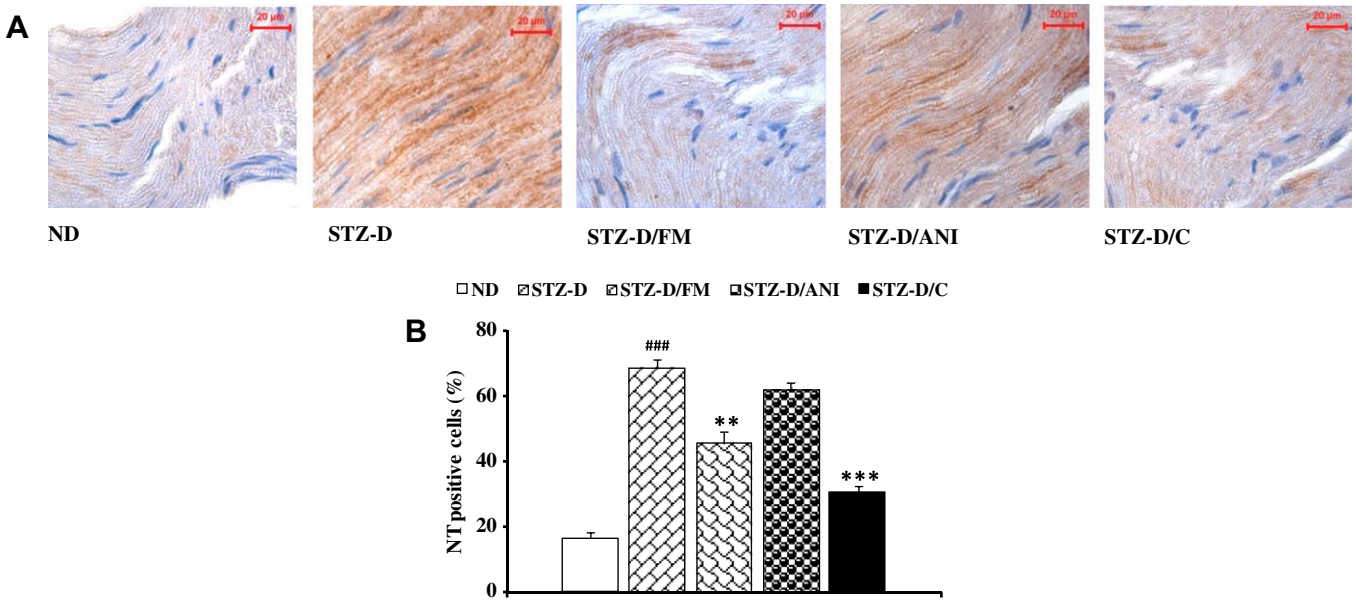

Fig. 1. FeTMPyP and 4-ANI in combination protects peripheral nerves against nitrosative stress induced damage. (A) Immunohistochemistry of sciatic nerve microsections showed that the combination reduced nitrotyrosine staining in the nerve (magnification 400×). Micron bar shows a length of 20 μm (B) Nerve cells were counted as described previously. Combination significantly decreased the percentage of the cells showing nitrotyrosine immunoreactivity in sciatic nerve sections. ND: non-diabetic; STZ-D: diabetic; STZ-D/FM: diabetic group treated with FeTMPyP 1 mg/kg; STZ-D/ANI: diabetic group treated with 4-ANI 3 mg/kg; STZ-D/C: diabetic group treated with combination). $^{###}p<0.001$ vs ND and $^{**}p<0.01$ and $^{***}p<0.001$ vs STZ-D ($n = 6$).

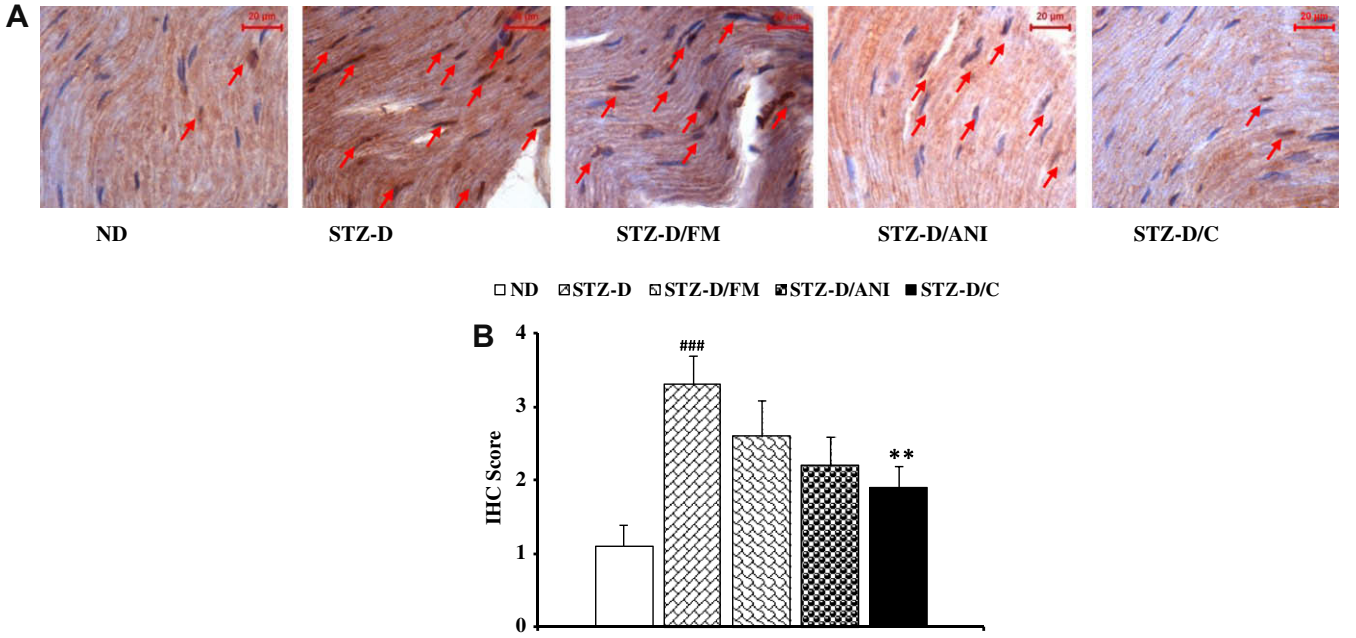

Fig. 2. Effect of two week treatment with FeTMPyP and 4-ANI, alone or in combination on PAR immunoreactivity. (A) Monoclonal anti-PAR antibody detects the PAR in nerve nuclei (blue) magnification 400×. Immunohistochemistry indicates a PAR (brown) strong positive stain, scattered in sciatic nerve microsection, in the STZ-D group; no/insignificant positive staining was found in the ND group. Arrows indicate PAR positive staining corresponding to the magnification images 400×; (B) graph represents PAR immunohistochemical score of various groups. ND: non-diabetic; STZ-D: diabetic; STZ-D/FM: diabetic group treated with FeTMPyP 1 mg/kg; STZ-D/ANI: diabetic group treated with 4-ANI 3 mg/kg; STZ-D/C: diabetic group treated with combination). $^{###}p < 0.001$ vs ND and $^{**}p < 0.01$ vs STZ-D ($n = 6$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FeTMPyP 1 mg/kg displayed reduction in NT positive cells but no significant reduction in PAR positive nuclei was observed. With 4-ANI 3 mg/kg treatment decrease in both, NT and PAR immunopositivity was not significant. The combination therapy showed statistically significant protection against the NT (Fig 1A and B) and PAR (Fig 2A and B) accumulation in the nerve cell ($p < 0.001$ and $p < 0.01$, respectively).

Discussion

Increased oxidative–nitrosative stress and poly(ADP-ribosyl)ation are early responses of peripheral nervous system to hyperglycemia, which suggests the importance of this cascade in the pathogenesis of human DN. Combination therapy containing PDCs with low-dose PARP inhibitor used in the present study

was significantly effective against multiple manifestations of DN. The combination regimen thus may constitute a perspective approach to attain beneficial properties of both the agents at significantly low-doses.

Increased reactive oxygen and nitrogen species, PARP overactivation and subsequent damage to endothelium and ATP sensitive ion channels in neurons are the major causes of perfusion and conduction deficits developing in DN [23,24]. Both the individual agents (FeTMPyP and 4-ANI) failed to overturn the MNCV deficits, but there was restoration of nerve blood flow. When combination of FeTMPyP and 4-ANI was administered to diabetic group, NBF and MNCV deficits were significantly improved which were associated with effective reduction in the levels of peroxynitrite and MDA and improvement in energy metabolism.

Alterations in pain perception as evidenced by thermal hyperalgesia and mechanical allodynia were in conformity with the previous studies with diabetic rats [18,25,26]. FeTMPyP monotherapy corrected the allodynia but did not essentially show effects on hyperalgesia whereas 4-ANI was effective in reversing these deficits. This could be due to the multiple etiology of pain perception in DN. Combination of the two, however corrected these deficits comparable to that of non-diabetic animals. The possible mechanisms that can be attributed to this are inhibition of oxidative damage [9], NF-kB and expression of inflammatory cytokines [27].

Various experimental evidence place oxidative and nitrosative stress in the axis of the pathogenic mechanism that leads to degeneration of neurons under hyperglycemic conditions [5,23,28]. Recent data have also recognized nitric oxide-derived reactive nitrogen species as significant contributor of protein modification and cell injury in diverse pathological conditions [23,24]. FeTMPyP significantly reduced elevated plasma peroxynitrite levels, however monotherapy with PARP inhibitor, 4-ANI failed to show an essential decrement. Combination therapy dampened the amplified nitrosative stress. NT immunoreactivity, a marker of peroxynitrite mediated protein modification was also reduced in sciatic nerves of animals treated with the combination.

PARP activation executes an imperative role in peripheral sensory nerve fiber dysfunction and degeneration in DN. Previous studies with PARP inhibitors have shown to counteract small sensory nerve fiber dysfunction and degeneration [3,17]. ROS generation and the activation of the NF-kB involved in various upstream and downstream pathways of nerve damage are blocked by PARP inhibition [2]. Combination treatment resulted in the reduction in PAR immunopositive nuclei in sciatic nerve microsections which indicates an essential PARP inhibition. Depleted nerve NAD levels were also significantly replenished. The combination thus subsequently provided protection against loss of nerve fibers in peripheral nervous system.

In conclusion, we have demonstrated that nitrosative stress followed by overactivation of PARP is associated with the nerve damage evoked by persistent hyperglycemia. Employing PDCs and PARP inhibitors as pharmacological tools in animal models may guide to a better insight into the role played by nitrosative stress–PARP cascade in the development of various biochemical deficits and nerve dysfunction in DN. Thus, combining PDC and PARP inhibitor may offer a novel approach for manipulating the pathological sequel that are associated with DN.

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