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Neuroprotective potential of combination of resveratrol and 4-amino 1,8 naphthalimide in experimental diabetic neuropathy: Focus on functional, sensorimotor and biochemical changes

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Abstract
The present study investigated whether combination of resveratrol and 4-amino 1,8 naphthalimide (4-ANI) is effective in the development of diabetic neuropathy (DN). After 6 weeks of diabetes induction, rats were treated for 2 weeks with resveratrol and 4-amino 1,8 naphthalimide (4-ANI) either alone or in combination. Experimental end points included functional, behavioural and biochemical parameters along with PAR immunohistochemistry and were performed at the end of treatment. Combination of resveratrol (10 mg/kg) and 4-ANI (3 mg/kg) attenuated conduction and nerve blood flow deficits and resulted in amelioration of diabetic neuropathic pain. Significant reversal of biochemical alterations (peroxynitrite, MDA and NAD levels) were also observed, as well as PAR accumulation in the sciatic nerve. This study suggests the beneficial effect of combining resveratrol and 4-ANI in experimental diabetic neuropathy.

Keywords: *Reactive oxygen species (ROS), resveratrol, diabetes*

Abbreviations: *DN, Diabetic neuropathy; MNCV, motor nerve conduction velocity; NBF, nerve blood flow; 4-ANI, 4 Amino 1,8 naphthalimide, STZ, streptozotocin; ND, non-diabetic; PARP, poly (ADP-ribose) polymerase.*

Introduction

Diabetes mellitus is one of the serious problems in developing as well as developed countries. Between 1995–2025 the number of adult population affected by diabetes is projected to grow by 170%. India is projected to have the largest number (57 million) of people with diabetes in 2025 [1,2]. Diabetic neuropathy (DN) is one of the most common complications affecting more than 50–60% of diabetic patients and it is a common cause of non-traumatic amputation and autonomic failure. Diabetic neuropathy is characterized by complex changes in functional and sensorimotor parameters in diabetic subjects, which leads to tremendous ramifications in the quality-of-life of a person. The DN is one of the major causes of morbidity and mortality associated with diabetes. To manage the diabetic complications successfully we need to clarify the role of many interwoven pathogenic pathways that are known to be involved in aetiology of disease. We have tried to enlighten the involvement of two of the important, inter-related pathways that forms the crux of pathophysiology of DN. The underlying biochemical mechanisms of diabetic neuropathy remain poorly understood and, due to the unavailability of treatments for DN, the quest for a promising agent is still there.

DN has several complex pathophysiological factors igniting its onset. Oxidative stress and associated

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damage is one of the widely studied subjects in pathophysiology of DN [3,4]. Oxidative stress [3], PARP activation [5], Polyol pathway [6], AGE formation [7] and PKC activation [8] are amongst the major contributors in the pathophysiology of DN. Oxidative stress is one of the major contributor of the damage in diabetic condition [9]. The level of free radicals generated in the body tends to increase drastically in diabetic subjects [4,10]. The radicals exert diminishing effects by directly damaging cellular proteins, lipids and DNA and indirectly by affecting normal cellular signalling and gene regulation. Oxidative stress causes vascular impairment leading to endoneurial hypoxia resulting in impaired neural function, reduced conduction velocity and loss of neuroptrophic support [5,11]. We have shown beneficial effects of various antioxidants in experimental DN [12–16]. In this study, we have taken resveratrol (3, 5, 4′-trihydroxystilbene) as one of the agents for combination strategy. It not only prevents free radical formation but also attenuates their toxicity by inhibiting the lipid peroxidation [17]. Apart from these effects it also has a modulatory effect on COX-2 [18,19] and Nrf-2 pathways [20,21]. Protective effects of resveratrol have been reported in various disease conditions [22].

PARP over-activation has been proven to play a crucial role in the pathophysiology of diabetic neuropathy [5,23,24]. Experimental evidences also suggests the beneficial effect of PARP inhibitors in diabetic complications [5,23,25,26]. Excessive DNA damage is a major cause for PARP over-activation. PARP is present in the nucleus in an inactive form, waiting for DNA damage to activate it [27]. In DN elevated glucose level increases the ROS production and these free radicals induce DNA strand breaks, thereby activating PARP. PARP repairs damaged DNA, in lieu of that it consumes NAD and indirectly ATP. Beside cytotoxic mediators, PARP is also known to play a role in transcription regulation AP-1 and NF-kB [28,29]. So overactivation of PARP also leads to NF-kB activation, thereby playing an important role in the consequences of inflammation-led tissue injuries [29]. Considering the importance of PARP in DN, in the present study we have targeted PARP over-activation in experimental DN by using 4-ANI (4 amino 1, 8 naphthalimide), a PARP inhibitor. 4-ANI has IC50 = 180 nM [30] and has shown neuroprotection in animal models of acute cerebral ischemia and diabetic neuropathy [26,31].

The pathophysiological mechanisms leading to DN are so complex that a single neuroprotective approach has never been shown to be sufficient in alleviating deficits associated with DN. Still there is no single therapy available which promises to either cure or mitigate the various deficits that are associated with DN. We propose that combination of resveratrol and 4 amino 1,8-naphthalimide would produce substantial beneficial effects in experimental diabetic neuropathy.

Materials and methods

Unless otherwise stated, all chemicals were of reagent grade and were purchased from Sigma (St Louis, MO). 4-ANI was purchased from Calbiochem (Germany). PAR antibody was from Alexis biochemicals (USA). NAD & MTT was obtained from HiMedia laboratories (India). Halothane was obtained from Nicholas Piramal (India). Glucose oxidase-peroxidase [32] glucose kit was purchased from Accurex (India).

Induction of diabetes and experimental design

The experiments were performed in accordance with regulations specified by the institutional animal ethics committee (IAEC), NIPER. Male Sprague Dawley rats (250–270 g) were used and were fed on standard rat diet and had free access to water *ad libitum*. Diabetes was induced by Streptozotocin (STZ) at a dose of 55 mg/kg. 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 diabetic and were further considered for study. The experimental groups were comprised of non-diabetic control group (ND), non-diabetic rats treated with resveratrol (ND/R) and 4-ANI (ND/A), diabetic control rats (STZ-D) and diabetic rats treated with resveratrol (STZ-D/R) (10 mg/kg, i.p.), 4-ANI (3 mg/kg, p.o.) (STZ-D/A) and their combination (STZ-D/C). The treatment was started 6 weeks after diabetes induction and was continued for 2 weeks (Figure 1). The behavioural and biochemical experiments were done 24 h after last drug administration and were performed in the following order: thermal hyperalgesia followed by Vonfrey Anaesthesiometer.

Anaesthesia, euthanasia and tissue sampling

Animals were anaesthetized by 4% halothane in a mixture of nitrous oxide and O_2 and anaesthesia was maintained with 1% halothane, using a gaseous anaesthesia system (Harvard apparatus, UK). Core temperature was monitored and maintained ($37 \pm 1^{\circ}C$) using a rectal probe with help of a homoeothermic blanket. Animals were sacrificed by high dose of anaesthesia. Both nerves were dissected and were collected until trifurcation. The nerves which were to be used for immunohistochemistry were fixed in formalin. For biochemical estimations, the nerve was homogenized in phosphate buffer and was used for various estimations.

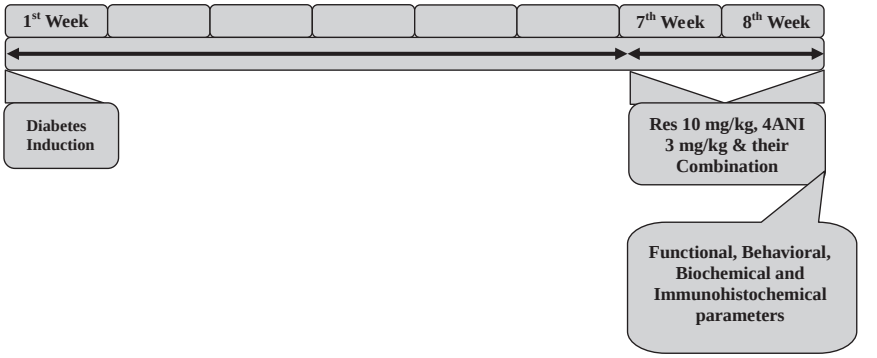

Figure 1. Schematic representation of experimental design. Res: resveratrol, 4-ANI: 4 Amino 1,8 naphthalimide.

Functional studies

Sciatic MNCV. MNCV was determined in the sciatic-posterior tibial conducting system using a Power Lab 8sp system (ADInstuments, Bellaviata, NSW, Australia) as previously described [13]. Sciatic nerve was stimulated with 3V proximally at sciatic notch and distally at ankle via bipolar electrodes. Receiving electrodes were placed on the muscle of the foot. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hindpaw 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 the result was divided into the distance between the stimulating and recording electrode. MNCV was expressed in m/s.

Sciatic NBF. Immediately after nerve MNCV determination, NBF was measured using a LASER Doppler system (Perimed, Jarfalla, Sweden) [26, 33]. Briefly, animals were anaesthetized and body temperature was monitored using a rectal probe and maintained with the help of a 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 as free as possible from epi or perineurial blood vessels. Flux measurement was obtained from the same part of the nerve and for the same time period (over a 10-min period). The blood flow was reported in arbitrary perfusion units (PU).

Behavioural 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 the end point in the tail immersion test. The flicking of the tail or the symptoms of struggling were taken as a positive response. The 15 s cut-off time was kept for both tests. Three consecutive readings were taken at an interval of ~30 min [15].

Mechanical hyperalgesia. 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 anaesthesiometer (model 2290-4; IITC Life Science) 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 anaesthesiometer. The anaesthesiometer 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 [12].

Biochemical parameters

Plasma glucose levels. Blood was collected from the tail vein in microcentrifuge tubes containing heparin. Plasma was separated and blood glucose was estimated from a 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 the method of Ohkawa et al. [34] with slight modification. The reaction was initialized by adding 100 µl of plasma/buffer/standard to a reaction mixture that consist of 750 µl of 0.8 w/v thiobarbituric acid, 750 µl of 20% acetic acid pH 3.4 and 100 µl of 10% Sodium dodecyl sulphate. The mixture was heated for 60 min in boiling water. Then the contents were centrifuged and the absorbance at 532 nm was taken.

Peroxynitrite levels. Peroxynitrite formation was estimated by using a fluorescent dye dihydrorhodamine123 as reported earlier [13]. Dihydrorhodamine123 get oxidized to rhodamine123 in a peroxynitrite-dependent manner. Rats were injected

dihydrorhodamine123 through the jugular vein (2×10^{-6} M/ml/kg in saline). 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 an enzyme cycling assay as described by Nisselbaum and Green [35]. In brief, the sciatic nerve was isolated bilaterally from the inguinal ligament to its trifurcation. The isolated nerve was then homogenized using Polytron PT 3100, in nine volume potassium phosphate buffer and kept in a boiling water bath for 5 min. Then the homogenate was centrifuged at 1000X g on 4°C and supernatant stored at −20°C until further used. The NAD content was analysed using an enzyme cycling mixture containing alcohol dehydrogenase and the absorbance was measured at 556 nm using a spectrophotometer (Beckmen DU 7400).

Immunohistochemical studies

PAR immunoreactivity was assessed as described by Sharma et al. [26]. 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. Significance was defined as $p < 0.05$. All statistical analyses were performed using Jandel Sigma Stat 2, statistical software.

Results

Effect of resveratrol and 4-ANI alone and in combination on body weight and plasma glucose level

The final body weights were comparably lower in untreated and treated diabetic rats than in the control group (Table I). The final blood glucose concentrations were similarly elevated in untreated and treated diabetic rats compared with the control rats (Table I).

Effect of resveratrol and 4-ANI alone and in combination on functional parameters

Motor nerve conduction velocity. Sciatic-tibial MNCV was reduced in untreated diabetic (STZ-D) rats compared with those of ND rats. Treatment with resveratrol (10 mg/kg), 4-ANI (3 mg/kg) alone and in combination significantly improved the deficit in conduction velocity. The combination group (STZ-D/C) showed remission from the conduction deficits and the combination group more or less resembled the ND group. The combination showed much better results as compared to monotherapy (Figure 2A). There was no effect of treatment on non-diabetic animals.

Table I. Body weight and plasma glucose characteristic of experimental groups.

	Body weight (g)		Plasma glucose level (mg/dl)	
	Initial	Final	Initial	Final
ND	243±8	415±15	104±9	105±8
STZ-D	245±9	210±12[a,b]	441±13	450±16[a,b]
STZ-D/R	246±7	210±12[a,b]	446±15	440±12
STZ-D/A	242±8	200±10	437±15	446±11
STZ-D/C	245±8	225±14	444±15	430±10
ND/R	243±10	426±15	102±11	101±12
ND/A	246±8	410±18	104±8	103±8

Data are expressed as mean ± SEM ($n = 6$–8).
[a]ND vs STZ-D; [b]$p < 0.001$.

Nerve blood flow. Composite nerve blood flow which was measured using laser doppler was markedly reduced in diabetic (STZ-D) vs non-diabetic (ND) group. The drugs used as monotherapy as well as combination therapy increased the composite nerve blood flow in diabetic animals as compared with the STZ-D group (Figure 2B). Non-diabetic animals showed no effect on nerve blood flow after treatment with either agent.

Effect of resveratrol and 4-ANI alone or in combination on Nociceptive behaviour

Thermal hyperalgesia. The 6 week diabetic rats clearly manifested thermal hyperalgesia detected from measuring tail flick latency in response to thermal stimuli. The treated rats showed improved tail flick latency in both hot and cold immersion tests as compared to STZ-D rats. The combination showed the improvement that was comparable to that of ND rats (Figure 3A and B).

Mechanical allodynia. This was measured using a Vonfrey anaesthesiometer in unrestrained animals. There was marked decrease, $p < 0.001$, in the paw withdrawal pressure as compared to ND animals. The reduction in paw withdrawal pressure in diabetic animals was ~50% that of ND animals. The paw withdrawal latency by combination therapy showed improvement in the treated groups as compared with the STZ-D group, $p < 0.001$ (Figure 3C).

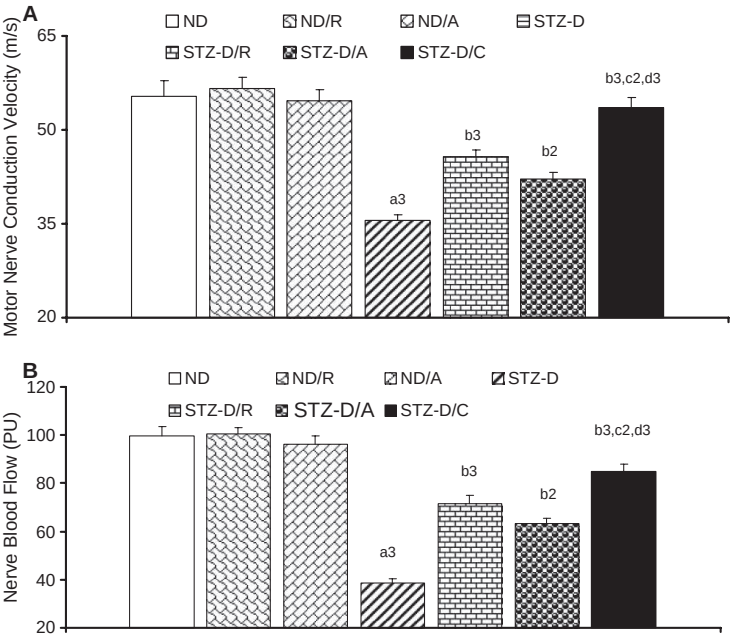

Figure 2. Effect of resveratrol and 4-amino 1,8 naphthalimide alone or in combination on MNCV (A) and Composite Nerve Blood Flow (B). (a) control vs diabetic; (b) treatment (resveratrol and 4-ANI and combination) vs diabetic; (c) combination vs resveratrol alone, (d) combination group vs 4-ANI alone; (1) $p < 0.05$; (2) $p < 0.01$; (3) $p < 0.001$. ND: non-diabetic; STZ-D: Diabetic; STZ-D/R: Diabetic group treated with resveratrol; STZ-D/A: Diabetic group treated with 4-ANI; SIZ-D/C: Diabetic group treated with combination.

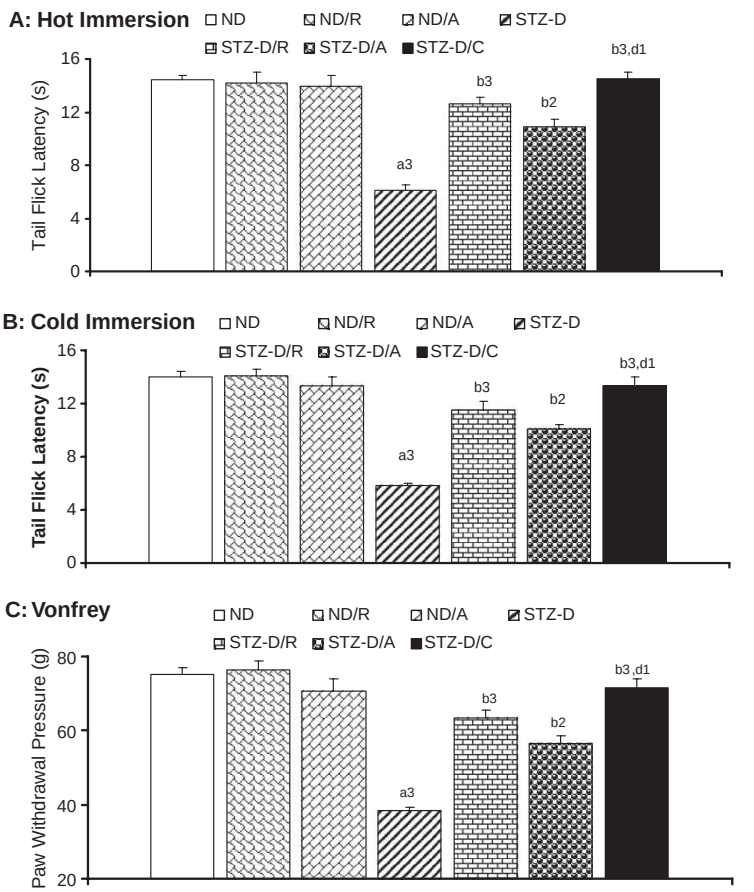

Figure 3. Effect of resveratrol and 4-amino 1,8 naphthalimide alone or in combination on Thermal hyperalgesia: (A) Hot immersion test and (B) Cold immersion test; and Paw withdrawal pressure (C). (a) control vs diabetic; (b) treatment (resveratrol and 4-ANI and combination) vs diabetic; (c) combination vs resveratrol alone, (d) vombination vs 4-ANI alone; (1) $p < 0.05$; (2) $p < 0.01$; (3) $p < 0.001$.

Effect of resveratrol and 4-ANI alone and in combination on oxidative stress induced altered MDA and Peroxynitrite levels

Diabetic animals showed an increased oxidative stress which was evident from increased levels of MDA and peroxynitrite levels. The hyperglycemia in the animals was the root cause of the generation of free radicals which resulted in increased lipid peroxidation. The increased peroxynitrite levels were also seen in diabetic animals as compared with control animals (Figure 4A–C). The combination regimen showed decreased oxidative stress as manifested by decreased MDA and peroxynitrite levels.

Effect of resveratrol and 4-ANI alone and in combination on NAD levels

The nerve NAD levels were significantly decreased in the nerve of diabetic animals. This result correlates with excessive DNA damage followed by PARP over-activation that finally caused the NAD depletion in the nerve cells. The resveratrol treatment did not show much improvement in NAD levels of sciatic nerves. 4-ANI showed protection against NAD depletion. Combination therapy showed enhanced protection against NAD depletion as evident from NAD levels of rats treated with combination regimen (Figure 5).

Effect of resveratrol and 4-ANI alone and in combination on PAR immuno-flouroscence

STZ treated rats showed increased PAR levels in the nuclei of nerve cells which was significantly different from the age-matched non-diabetic rats. The resveratrol treatment and 4-ANI treatment decreased the PAR immunopositivity in sciatic nerve sections. The combination therapy showed statistically significant protection against the PAR accumulation in the nerve cell nuclei (Figure 6A and B).

Discussion

The present study demonstrates the neuroprotective potential of combination of resveratrol and 4 Amino 1,8-naphthalimide in diabetic neuropathy. DN was exemplified by the slowing of nerve conduction, augmented change in pain sensation, nerve blood flow deficits and other biochemical and immunohistological changes in diabetic rats. Growing evidence implicates the activation of PARP and oxidative stress as a major contributor to development of diabetic neuropathy [4,32,36]. In addition to activation of PARP by oxidative-nitrosative stress there are some alternative pathways like inflammatory cascade and NF-kB activation and MAPK activation [36,37] responsible for PARP over-activation.

Hyperglycemia-mediated PARP activation has shown to increase ROS and nitrogen species, which again contribute to PARP activation by causing single strand break, thus imitating a chain reaction [32]. We need to target oxidative stress as it is more or less involved in all the pathways in pathogenesis of DN along with the PARP activation pathway. Therefore combining an inhibitor of PARP and inhibitor of oxidative stress can not only tone down oxidative stress and PARP pathways but also can target other

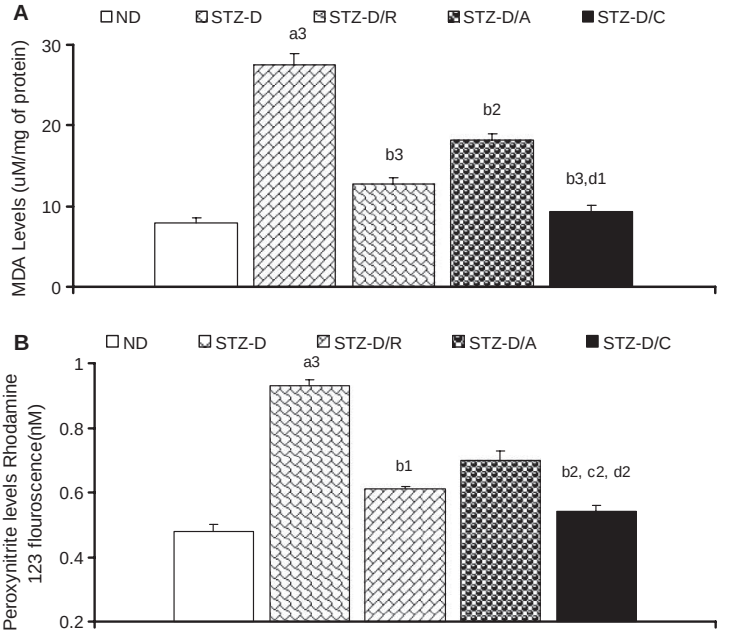

Figure 4. Effect of resveratrol and 4-amino 1,8 naphthalimide alone or in combination on MDA levels (A), Peroxynitrite levels (B) (a) control vs diabetic; (b) treatment (resveratrol and 4-ANI and combination) vs diabetic; (c) combination vs resveratrol alone; (d) combination vs 4-ANI alone; (1) $p < 0.05$; (2) $p < 0.01$; (3) $p < 0.001$.

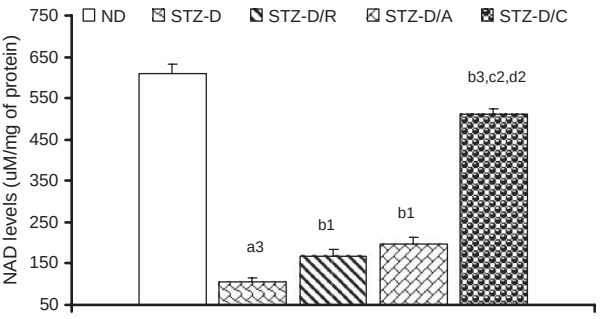

Figure 5. Effect of resveratrol and 4-amino 1,8 naphthalimide alone or in combination on NAD levels in Sciatic nerve homogenate. (a) control vs diabetic; (b) treatment (resveratrol and 4-ANI and combination) vs diabetic; (c) combination vs resveratrol alone; (d) combination vs 4-ANI alone; (1) $p < 0.05$; (2) $p < 0.01$; (3) $p < 0.001$.

pathways like MAPK, NF-κB, PKC activation, AGE formation, all of which forms an army of pathogenetic demons causing DN.

We have used resveratrol and 4-ANI to treat developed DN in STZ-treated rats. These two agents when administered in monotherapy were able to alter the deficits significantly but failed in complete reversal. The combination therapy reversed the conduction deficits that developed in STZ-treated rats. The improvement in conduction deficits may be due to improved nerve perfusion as indicated by increase in NBF in treated animals. One reason that could be cited for nerve perfusion deficit is impaired endothelial function. Both *in vivo* and *in vitro* reports suggest that endothelial dysfunction is associated with increased oxidative stress and PARP activity [38,39].

Neuropathic pain is one of the major unmet clinical needs in DN patients, which seriously affects the quality-of-life of diabetic patients. The tail-flick latency test where the time to withdraw or movement of tail or the symptoms of struggle are considered as end point from a noxious heat/cold source were measured. This test gives information on peripheral nerve and spinal function apart from higher nociceptive processing centres [40]. We also measured mechanical hyperalgesia, by studying the paw withdrawal thresholds in two different tests using a Vonfrey anaesthesiometer. The monotherapy with both agents partially corrected the altered mechanical hyperalgesia evidenced by increase in paw withdrawal pressure in treated animals. Both these behavioural tests provide information about acute sensory stimuli and threshold to nociceptive pain which are transduced by myelinated and unmyelinated fibres, respectively [25]. Recently Ilnytska et al. [25] has postulated involvement of PARP pathways and beneficial effect of PARP inhibitor in ameliorating

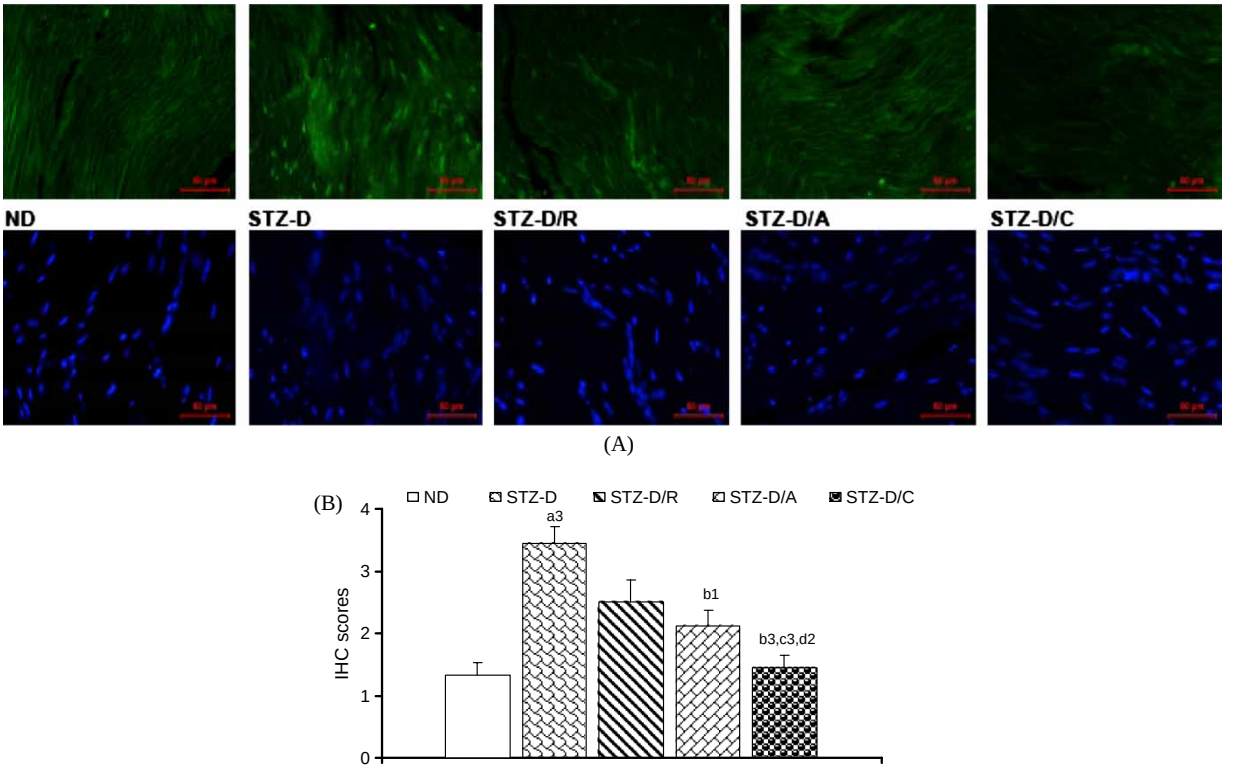

Figure 6. Effect of resveratrol and 4-amino 1,8 naphthalimide alone or in combination on PAR levels in nerve microsection. Upper panel (FITC) showing PAR positive cells and Lower panel (DAPI) showing total number of cells in the corresponding micro section (A). Immunohistochemical score of various groups (B); (a) control vs diabetic; (b) treatment vs diabetic; (c) combination group vs resveratrol monotherapy; (d) combination group vs 4-ANI; (1) $p < 0.05$; (2) $p < 0.01$; (3) $p < 0.001$.

diabetic neuropathic pain, which prompted us to investigate the effect of 4-ANI and resveratrol alone or in combination therapy on DN pain. The possible mechanisms that may be responsible for protection against neuropathic pain are inhibition of oxidative damage due to ROS, inhibition of peroxynitrite mediated apoptotic neuronal damage, calcium regulating activity as shown by few antioxidant like taurine in the DRG neurons [18], PARP inhibition [25], inhibition of NF-kB [28,41] and inhibition of expression of inflammatory cytokines (e.g. TNF-α).

We estimated peroxynitrite level in plasma of diabetic animals using dihydrorhodamine123 assay. We have observed an increase in rhodamine fluorescence in plasma, which is an index of increased peroxynitrite levels in diabetic rats. resveratrol treatment significantly reduced the elevated plasma peroxynitrite levels as evident from decreased rhodamine fluorescence in diabetic animals. The monotherapy with resveratrol partially attenuated the elevated MDA and peroxynitrite levels. The combination therapy has checked the increased oxidative stress in rats. There is a possibility that it may have also inhibited activation of ROS mediated pathways like AGE formation, PKC activation, MAPK activation, apoptotic neuronal death, etc. Peroxynitrite along with other ROS is also known to cause massive DNA fragmentation [42]. 4-ANI and combination therapy improved the diminished NAD levels in nerves of diabetic animals; thereby protecting the neuronal cells from energy crisis-led necrotic death.

PARP over-activation leads to a deleterious effects, as it can cause cellular death due to energy crisis in nerve cells. An alternative pathway in development of DN is via regulation of transcription factors by PARP. PAR accumulation, an index of increased PARP activation, was studied in nerve microsections of treated animals. PAR immunopositivity was significantly increased in diabetic nerves. The combination therapy was able to reduce PARP over-activation as evident from reduced PAR accumulation in nuclei of diabetic-treated animals.

We have also studied NAD levels to check whether the combination therapy can combat the oxidative stress-PARP over-activation mediated NAD depletion in diabetic animals. We found that the combination regimen substantially restored the deleted levels of NAD in diabetic nerves. Restoration of NAD levels in nerves of diabetic animals narrates the ability of combination therapy in protecting diabetic nerves from energy crisis and the aftermath of PARP over-activation.

This study demonstrates the protective effect of a combination of resveratrol (antioxidant) and 4-ANI (PARP inhibitor) in experimental diabetic neuropathy. The combination of resveratrol and 4-ANI produced enhanced neuroprotection in DN as compared with either agent alone. Enhanced neuroprotection may be attributed to inhibition of oxidative stress-PARP cascade in DN.

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***Declaration of interest*:** The authors report no conflicts of interest.

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