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Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by *N*-acetyl-L-cysteine in rats

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Abstract

Objectives: The objectives of this study were to examine the role of reactive oxygen species and oxidative stress in peripheral neuropathy and behavioural pain responses in experimentally induced chronic constriction injury (CCI) of sciatic nerve of rat. Effect of *N*-acetyl-L-cysteine (NAC) administered intraperitoneally, was also investigated on CCI-induced neuropathic pain in rats.
Methods: Neuropathy was induced by CCI of the right sciatic nerve in ketamine anaesthetized rats. Effect of intraperitoneally administered NAC in rats was also investigated using nociceptive behavioural tests. Malondialdehyde, an index of oxidative stress and antioxidant enzymes was also estimated in ligated sciatic nerve.
Results: Behavioural tests, mechanical, thermal and cold stimuli confirmed the development of neuropathic pain after the CCI. The malondialdehyde levels of ligated sciatic nerves were significantly increased compared to non-ligated sciatic nerves (sham operated). The antioxidant enzyme reduced, glutathione was inhibited, while superoxide dismutase increased. However, catalase remained unaffected in the injured sciatic nerves. Intraperitoneal administration of NAC resulted in significant reduction of hyperalgesia in CCI-induced neuropathic rats.
Conclusions: This study identifies antioxidants superoxide dismutase and reduced glutathione, and oxidative stress as important determinants of neuropathological and behavioural consequences of CCI-induced neuropathy, and NAC may be a potential candidate for alleviation of neuropathic pain.

Keywords: Neuropathy; Oxidative stress; *N*-acetyl-L-cysteine; Chronic constriction injury; Rats

1. Introduction

Oxidative stress and reactive oxygen species (ROS) have been implicated in brain disease states, such as Alzheimer's disease, Parkinson's disease, brain dysfunction due to injury or ageing (Balazs and Leon, 1994; Lewen et al., 2000). Oxidative stress is imposed on the cells as a result of one of the three factors (i) an increase in oxidant generation (ii) decrease in antioxidant protection and (iii) a failure to repair oxidative damage. Cell damage is induced by ROS which are either free radicals, reactive anions containing oxygen atoms or molecules containing oxygen atoms that can either produce free radicals or chemically activated by them. Examples are

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hydroxyl radicals, superoxide, hydrogen peroxide and peroxinitrite. The main source of ROS in vivo is aerobic respiration, although ROS is also produced by peroxisomal-β oxidation of fatty acids, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism and tissue specific enzymes. Unchecked, ROS attack many key molecules including enzymes, membranes, lipids and DNA. Under normal conditions, ROS is cleared from cells by the action of superoxide dismutase (SOD), catalase or glutathione, as well as antioxidant vitamin C and E. In pathological conditions, however, intracellular ROS level is elevated due to increased production or impaired removal and ROS causes cell damage ranging from cytoplasmic swelling to death. Obviously, removal of excessive ROS is often important for restoring normal conditions.

In spite of much work on oxidative stress and ROS, studies showing the involvement of ROS in chronic pain are limited. In chronic constriction injury (CCI) model of rat neuropathic pain, heat hyperalgesia was reduced by systemically injected antioxidants (Tal, 1996; Khalil et al., 1999). Recently, in spinal nerve ligation (SNL) model of neuropathic pain, systemic injection of ROS scavenger phenyl-*N*-*tert*-butylnitrone (PBN) relieved mechanical allodynia (Kim et al., 2004). Although, these studies suggest the involvement of free radicals/oxidative stress in neuropathic pain, little attention has been paid to critical role of antioxidant enzymes in neuropathic pain. The present study, therefore, examines the involvement of various antioxidant enzymes in CCI-induced neuropathic pain besides free radical activity.

In neurons, glutathione acts as free radical scavenger (Cooper and Kristal, 1997). By acting as cysteine donor, *N*-acetyl-L-cysteine (NAC) maintains intracellular glutathione level and is neuroprotective for range of neuronal cell type against variety of stimuli in vitro (Mayer and Noble, 1994; Seaton et al., 1997). The present study, therefore, investigates the effect of NAC on pain threshold in CCI-induced neuropathy in rats.

2. Materials and methods

2.1. Animals

Adult male albino rats (175–225 g) of Wistar strain obtained from Laboratory Animal Resource Section of the Institute were used in the present study. The animals were housed in groups of 5–6 in colony cages for one week till CCI of sciatic nerve was done. After surgery, the rats were kept in individual cages. The rats were kept at room temperature of 25 ± 2 °C. During this period, the animals were repeatedly and gently handled to minimize the stress and to get them acclimatized to the laboratory environment. A balanced rat feed obtained from the Feed Technology Unit of this Institute and clean drinking water were provided ad libitum. The experimental procedures were approved by Institute Animal Ethics Committee.

2.2. Surgery

Selected rats were kept off feed for 12 h prior to surgery. The rats were anaesthetized with ketamine hydrochloride (100 mg/kg) i.m. After induction of anaesthesia, the hair around the mid-thigh were clipped and then shaved. CCI was induced as described by Bennett and Xie (1988). The common sciatic nerve of the right hind limb was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatic's trifurcation, about 7 mm of the nerve was freed of the adhering tissue and four ligatures (4.0 silk) were tied loosely around it with about 1 mm spacing, with the length of the affected nerve being 4–5 mm long. The desired degree of constriction was such that it could retard, but not arrest circulation through the superficial epineural vasculature. Similar dissection was performed on opposite side (left) without ligating the sciatic nerve (sham procedure). The incision was closed in layers. For estimation of free radical and antioxidant enzyme activities, separate animals were used as sham-exposed controls.

After suturing the skin, povidone iodine solution was applied externally by cotton swab and prophylactically oxytetracycline (Terramycin, Pfizer, India) was injected i.m. at a dose of 50 mg/kg body weight for three consecutive days to prevent any infection. The operated animals were caged individually and feed and water were given ad libitum. They were allowed to recover for two weeks before estimation of free radical/antioxidant enzyme activity or drug administration (NAC) was undertaken i.e. on 15th day of CCI surgery. A total of 60 rats were used for various studies and each group consisted of 6 rats.

2.3. Effect of NAC on pain threshold

NAC, dissolved in luke-warm normal saline (300 mg/ml, which was further diluted to 30 and 100 mg/ml) was administered intraperitoneally to rats with CCI at 30, 100 and 300 mg/kg doses. The doses of NAC were based on a pilot study conducted in our laboratory. Rats having CCI were administered with normal saline and served as vehicle-treated control. Each group consisted of 6 animals and a total of 24 animals were used to study the effect of NAC on pain threshold/paw withdrawal latency. Pain threshold in rats was recorded by mechanical and paw withdrawal latencies in thermal and cold allodynia tests at different time intervals. Pain withdrawal latencies/threshold were also determined before surgery. These tests were conducted blindly in the manner that

experimenter did not know the nature of experimental manipulation. Pain threshold/paw withdrawal latency tests were done on the same day with the sequence of mechanical hyperalgesia followed by thermal hyperalgesia and cold allodynia tests.

2.3.1. Mechanical hyperalgesia

The pressure was recorded by Randall-Selitto assay method (1957), using Randall-Selitto analgesiometer (UGO Basile, Varese, Italy), immediately prior to the administration of NAC (0 h) in CCI-induced rats, and at 1, 3, 5 and 7 h after drug administration. The change in pain threshold in test group was compared with that of vehicle-treated control group. Results are expressed as mean pressure in g ± SE.

2.3.2. Thermal hyperalgesia

The latency to radiant heat was measured by radiant heat apparatus (UGO Basile, Varese, Italy), immediately prior to (0 h) and at 1, 3, 5 and 7 h after drug administration. The paw was placed on the heat radiator in a manner that planter surface of the affected paw was touching the heat radiator without any apparent stress to the animal under test and the withdrawal time (s) of paw was recorded. The change in paw withdrawal latency of test group was compared with that of vehicle-treated control group. Results are expressed as mean time in s ± SE. A cut of latency of 20 s was used to avoid tissue damage.

2.3.3. Cold allodynia

Ice-cold water (4 ± 1 °C) was taken in a beaker. The paws of rats with CCI of control and test group were submerged gently in water and the withdrawal time was measured, just prior to (0 h) and at 1, 3, 5 and 7 h after drug administration. The change in withdrawal latency of treated group and vehicle-treated control groups were compared. Results are expressed as mean time in s ± SE.

2.3.4. Behaviour analysis

Motor function was evaluated at each time point by observation of two specific behaviours: (i) Placing/stepping reflex – this response was evoked by drawing the dorsum of either hind paw across the edge of the table. This stimulus elicits an upward lifting of paw from the surface of the table (stepping). (ii) Righting reflex – a rat placed horizontally with its back on the table will normally show an immediate coordinated twisting of body to an upright position to regain its normal posture.

2.4. Effect of NAC on non-ligated control rats

Effect of NAC treatment (30, 100 and 300 mg/kg intraperitoneal) on pain threshold in non-CCI rats was determined by mechanical, radiant heat and cold allodynia tests as described above. Pain threshold in different procedures was determined immediately prior to NAC administration and at 1, 3, 5 and 7 h after drug administration. The change in pain threshold in test groups was compared with that of vehicle-treated control rats. Each group consisted of 6 rats and a total of 24 animals were employed in this part of the experiment.

2.5. Estimation of free radical and antioxidant enzyme activities

Sciatic nerves from CCI-induced and sham operated rats were obtained on 15th day of surgery. A segment of sciatic nerve, approximately 1.5 cm in length, 5 mm proximal and 5 mm distal to the injured site was used for preparing the homogenate for biochemical estimation. Homogenates of equal concentration, i.e. 1:100 (w:v) were prepared to control variability among different samples. These tissues were then analysed for free radical *and* antioxidant enzyme activities.

2.5.1. Estimation of lipid peroxidation

Sciatic nerve homogenates (1:100 w/v) were prepared in 0.15 M NaCl solution in thick glass tube of Potter–Elvehjen homogeniser taking sterilized sea sand under cold conditions. The homogenate thus obtained was used for measurement of lipid peroxidation (Shafiq-Ur-Rehman, 1984). The absorbance was read at 535 nm using extinction coefficient of malondialdehyde. The results are expressed in nmol of malondialdehyde formed per 30 min/g of tissue.

2.5.2. Estimation of reduced glutathione

To estimate reduced glutathione, sciatic nerve homogenate was prepared with 0.02 M chilled EDTA (1% homogenate). Reduced glutathione estimation was carried out immediately after preparing the homogenate without storing it, by following the method as described by Sedlak and Lindsay (1968).

2.5.3. Estimation of superoxide dismutase

Tissue homogenate (1%) was prepared with 0.05 M Tris–HCl buffer. Superoxide dismutase was estimated following the method, as described by Madesh and Balsubramanium (1998). Amount of superoxide formed was calculated using molar extinction coefficient of (4,5-dimethylthiazole-2-4) 2,5-diphenytetrazolium bromide (MTT) formazan. Per cent inhibition by the presence of superoxide dismutase was calculated from the reduction of MTT colour formation as compared to MTT colour formation, in the absence of superoxide dismutase which was taken as 100%. One unit (U) of superoxide dismutase was defined as the amount of protein required to inhibit the MTT reduction by 50%.

2.5.4. Estimation of catalase

Catalase was estimated according to the method of Maehl and Chance (1954). Tissue homogenate (1%) was prepared in 0.15 M NaCl solution. Change in optical density was read at 230 nm at 0, 10, 20, 30, 40 and 50 s.

2.6. Statistical analysis

Data of free radical were analysed by unpaired *t*-test. Results of NAC were analysed by analysis of variance followed by Dunnet '*t*' test. *P* values <0.05 and 0.01 were considered significant.

3. Results

3.1. Behavioural observations

Preoperatively, there were no differences between right and left paw withdrawal latencies in each group. At no time, there were differences among the left paw withdrawal latencies after sham surgery in each group.

The rats with CCI developed abnormal gait, posture, guarding and protecting behaviour and licking of the hind paw of the ipsilateral side of sciatic ligation after 1–2 days of operation. The rats did not put weight on the affected side and the hind leg of the affected side was drawn close to the body with distinctive guarding posture. The foot was ventroflexed and the toes were held together tightly. The abnormal behaviour was observed even after 2 weeks of operation. But, the sham operated rats behaved normally throughout the postoperative days.

3.2. Effect of NAC on CCI rats

Pain behaviour was attenuated significantly in dose-dependant manner in CCI-induced rats, administered with antioxidant NAC. In mechanical stimulation, the antihyperalgesic effect of NAC was observed and the results are presented in Fig. 1. The pain threshold increased significantly at 3 and 5 h with 30 mg/kg dose. With 100 and 300 mg/kg doses of NAC, the pain threshold increased significantly at 1, 3, 5 and 7 h.

In radiant heat assay of pain, NAC at 30 mg/kg dose showed significant antihyperalgesic effect only at 3 h, but, at 100 mg/kg dose, the effect was significant at 1, 3 and 5 h. At 300 mg/kg dose of NAC the antihyperalgesic effect was observed at 1, 3, 5 and 7 h (Fig. 2).

The ameliorative effect of NAC on cold allodynia at 30 mg/kg dose was significant only at 3 h post-NAC administration. But, at 100 mg/kg dose, the analgesic effect was significant at 1, 3 and 5 h, while at 300 mg/kg dose, the effect was observed at 1, 3, 5 and 7 h (Fig. 3).

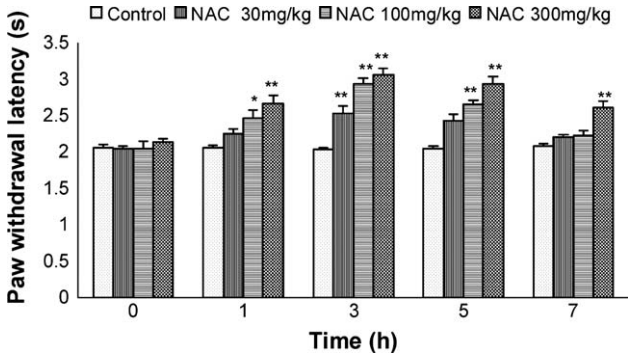

Fig. 2. Time course of the effects of i.p. administered NAC on the development of thermal hyperalgesia induced by CCI in rats. ($n = 6$; data represent the mean ± SE; $*p < 0.05$, $**p < 0.01$ when compared with that of control).

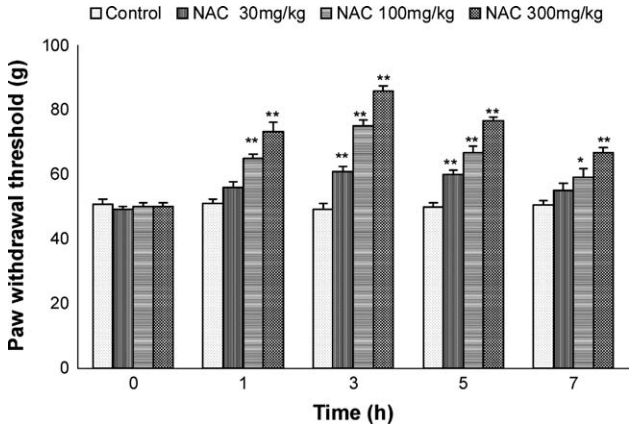

Fig. 1. Time course of the effects of i.p. administered NAC on the development of mechanical hyperalgesia induced by CCI in rats. ($n = 6$; data represent the mean ± SE; $*p < 0.05$, $**p < 0.01$ when compared with that of control).

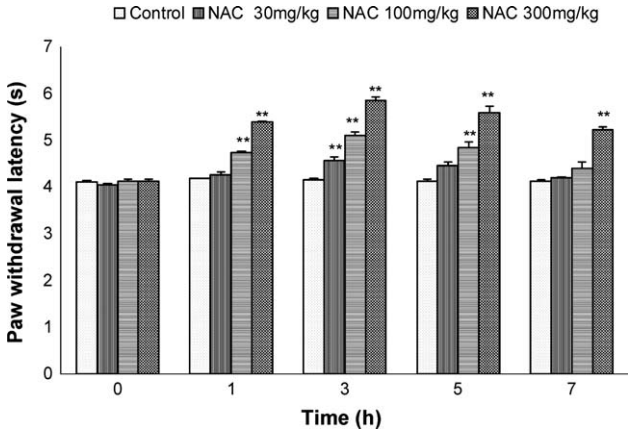

Fig. 3. Time course of the effects of i.p. administered NAC on the development of cold allodynia induced by CCI in rats. ($n = 6$; data represent the mean ± SE; $**p < 0.01$ when compared with that of control).

Table 1
Effect of CCI on lipid peroxidation and antioxidant enzymes in sciatic nerve of rats[n]

	Lipid peroxidation (mole $\times 10^{-5}$malondialdehyde/g tissue)	Reduced glutathione (mole $\times 10^{-3}$/mg of tissue)	Superoxide dismutase (U)	Catalase (K/g of tissue)
Control	56.57 ± 2.04	0.40 ± 0.03	19.10 ± 0.01	0.56 ± 0.01
Ligated nerve	68.58 ± 2.18[a]	0.30 ± 0.01[b]	44.10 ± 0.04[a]	0.57 ± 0.01

$n = 6$.
[a] $P < 0.01$.
[b] $P < 0.05$.

3.3. Effect of NAC on non-CCI rats

NAC treatment (30, 100 and 300 mg/kg doses intraperitoneal) did not produce any significant effect on mechanical, radian heat and cold allodynia test at 1, 3, 5 and 7 h. Subjective observations of rats injected with NAC (30, 100 and 300 mg/kg) revealed no obvious change in animal behaviour during a period of 7 h when compared to control animals. Neither sedative nor toxic effect was observed after intraperitoneal administration of NAC in this study.

3.4. Assessment of free radical activity and antioxidant enzymes in CCI

Lipid peroxidation measured two weeks after surgery was increased significantly ($P < 0.01$) in ligated nerve, as compared to sham-operated controls (Table 1).

Reduced glutathione was inhibited significantly ($P < 0.05$) in ligated nerve when compared to sham operated controls.

The amount of superoxide dismutase increased significantly ($P < 0.01$) in ligated nerve than the sham-operated nerves. But, the catalase activity did not differ significantly between ligated nerves and in sham-operated controls.

4. Discussion

The major findings from this work on neuropathic pain in rats were (i) sciatic nerve malondialdehyde levels (reflective of neuronal oxidative damage) were significantly high in rats with CCI and (ii) treatment with NAC significantly attenuated hyperalgesic effect in rat.

Among neuropathic pain models, CCI of sciatic nerve in rats has been widely used as it produces reliable and sustained tactile allodynia which resembles the condition observed in patients with neuropathic pain (Bennett and Xie, 1988). In the present study, rats with CCI showed a significant reduction in thermal, mechanical and cold thresholds in hind limb, suggesting hyperalgesic effect.

Sciatic nerve malondialdehyde level was significantly elevated in rats with CCI in the present study. An increase in lipid hydroperoxide (LPO) has been reported earlier in injured sciatic nerve of old rats (Khalil and Khodr, 2001). In a related study in rat sciatic nerve, 2 h of ischemia followed by 3 h reperfusion resulted in significant increase in lipid peroxidation (Sayan et al., 2004). These findings suggest that endoneural lipid peroxidation is increased as a consequence of CCI in sciatic nerve. The increased lipid peroxidation in the present study could be due to free radical generation (reactive oxygen species or nitric oxide) in sciatic nerve of rat.

Oxidative injury has been implicated in pathophysiology of neural injury and neurodegenerative disease. Antioxidant proteins provide an endogenous defense against such oxidative injury and may yield important clues to mechanisms of cytoprotection and neuronal recovery. Nervous tissue is poor in antioxidant defense enzymes catalase and superoxide dismutase. In the present study, superoxide dismutase was significantly increased, but not catalase in sciatic nerve of CCI-induced rats. An increase in expression of superoxide dismutase was demonstrated in sciatic nerve following sciatic axotomy (Rosenfeld et al., 1997). The data suggests that changes in endoneural oxidative stress lead to nerve dysfunction in rats with CCI (Kim et al., 2004).

Reduced glutathione is a major low molecular weight scavenger of free radicals in cytoplasm. We examined whether reduced glutathione, one of whose many functions is an important endogenous antioxidant is influenced by CCI resulting in neuropathology and hyperalgesia. Depletion of glutathione, particularly in mitochondria (Cooper and Kristal, 1997; Wullner et al., 1999) increases the susceptibility of neurons to a variety of toxic stimuli and oxidative stress (Ratan et al., 1994; Wullner et al., 1999). Since glutathione is one of the principle defences against ROS and oxidative stress within the neurons, increasing level may be protective (Ratan et al., 1994; Cooper and Kristal, 1997). In this study, reduced glutathione levels were significantly decreased suggesting increased susceptibility of neurons to oxidative stress and hyperalgesia.

NAC is currently in clinical use as mucolytic agent is respiratory disease, and as a treatment for paracetamol (acetaminophen) poisoning where it acts to maintain glutathione levels in hepatocytes. Treatment with NAC, a rate-limiting component of glutathione

production, significantly produced antihyperalgesic effect in mechanical, thermal and cold allodynia tests in rats with CCI in this study. Based on the test for motor function and righting reflex, NAC is not sedative at the doses we used. So, the behavioural changes are interpreted as analgesia. In a related study, NAC *po* over six days attenuated CCI-caused thermal hyperalgesia (Wagner et al., 1998). Besides this, NAC (150 mg/kg/day) almost totally eliminated extensive neuronal loss found in controls, both in 2 week and 2 months after sciatic nerve axotomy (Hart et al., 2004). However, in the present study, we observed antihyperalgesic effect 1 h after NAC treatment.

Since glutathione is one of the principle defences against ROS and oxidative stress within neurons, increase in its levels may be protective (Cooper and Kristal, 1997). NAC treatment might have raised the nerve glutathione levels, compared to untreated nerve because of its capacity to donate cysteine amino acid, a component of glutathione (Wagner et al., 1998). Further, at high doses NAC has direct reductant (Yan et al., 1995; Kamata et al., 1996) and antioxidant effect and can block lipid peroxidation by peroxinitrate (Han et al., 1997). It might have also scavenged reactive oxygen species and nitric oxide due to free sulphydryl group in NAC. The present study suggested a significant reduction in hyperalgesia by NAC. A detailed analysis into the mechanism of NAC is further needed.

If NAC in the present study produces antihyperalgesia by scavenging ROS, this implies that excessive ROS is important in the generation of pain in CCI model. This raises number of issues namely, (i) which specific ROS are important for neuropathic pain, (ii) what are the sources of excessive ROS and (iii) by what mechanism does ROS produce pain. There are number of types of ROS that can damage neuronal function. Mitochondria normally produce superoxide and it is readily converted to hydrogen peroxide by superoxide dismutase and then to highly toxic hydroxyl radical. In addition, superoxide and nitric oxide are also produced in the cytoplasm by enzymatic reactions, which are activated by increased cytoplasmic calcium. Superoxide and nitric oxide can readily be converted to peroxinitrite which is highly toxic. Primary injury afferent discharges at the time of nerve injury, followed by ectopic discharges in the spinal cord in neuropathic conditions, may increase mitochondrial respiration as well as intracellular calcium, and consequently lead to increased ROS production. It is also possible that excess ROS build up in glial cells which then leak to produce neuronal damage and dysfunction.

Excessive ROS produce pain. It is well established that sensitization of dorsal horn cells in spinal cord (central sensitization) plays a fundamentally important role in neuropathic pain. ROS affects central sesitization. ROS initiates factors already known to be involved in central sensitization rather than triggering an independent additional mechanism. It is likely that ROS triggers second messengers involved in central sensitization of dorsal horn cells (Ali and Salter, 2001; Zhang et al., 2003) or ROS activates spinal glial cells which in turn plays an important role in chronic pain (Raghavendra et al., 2003).

It has been demonstrated that ROS reduction mildly relieved neuropathic pain behaviour. Antioxidant, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) caused significant reduction of thermal hyperalgesia in CCI rats by mimicking the activity of SOD (Tal, 1996). In addition, systemic injection of tirilazad in CCI rats also significantly alleviated thermal hyperalgesia by improving peripheral vascular blood flow in the area innervated by injured nerve (Khalil et al., 1999). Trilazad is not a typical antioxidant but has antilipid peroxidation, thereby, stabilizing the membrane (Kavanagh and Kam, 2001). Action of systemically injected compound to act on spinal cord requires passing through blood brain barrier. NAC is low molecular weight compound and readily passes through the blood brain barrier. Further, systemically injected NAC can be detected in brain (Farr et al., 2003), thus, reducing central sensitization in spinal cord.

In conclusion, the present study suggests that endoneural oxidative stress is probably responsible for nerve dysfunction unlike systemic oxidative stress in diabetes. The study identifies glutathione levels and presumably oxidative stress, as important determinants of neuropathological and behavioural consequences of chronic constriction nerve injury and suggests that NAC may be potential candidate for the treatment of neuropathic pain.

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