Ameliorative Potential of Pralidoxime in Tibial and Sural Nerve Transection-Induced Neuropathic Pain in Rats

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The present study was designed to investigate the ameliorative potential of pralidoxime in tibial and sural nerve transection-induced neuropathy in rats. Tibial and sural nerve transection was performed by sectioning tibial and sural nerve portions (2 mm) of the sciatic nerve, and leaving the common peroneal nerve intact. The pinprick, acetone, hot and cold tail immersion tests were performed to assess the degree of motor functions, mechanical hyperalgesia, cold allodynia, heat and cold hyperalgesia respectively. Biochemically, the tissue thio-barbituric acid reactive species (TBARS), super-oxide anion contents (the markers of oxidative stress) and total calcium levels were measured. Tibial sural nerve transection resulted in the development of mechanical hyperalgesia, cold allodynia, heat and cold hyperalgesia along with the rise in oxidative stress and calcium levels. However, administration of pralidoxime (10, 20 mg/kg intraperitoneally (i.p.)) for 14 d attenuated tibial and sural nerve transection-induced cold allodynia, mechanical, hot and cold hyperalgesia. Furthermore, pralidoxime also attenuated tibial and sural nerve transection induced increase in oxidative stress and calcium levels. It may be concluded that pralidoxime has ameliorative potential in attenuating the painful neuropathic state associated with tibial and sural nerve transection, which may possibly be attributed to decrease in oxidative stress and calcium levels.

Key words tibial sural nerve transection; calcium; oxidative stress; pralidoxime

Neuropathic pain has been described as “the most terrible of all tortures which a nerve wound may inflict.”1) Despite progress in the understanding of this syndrome, the mechanistic details underlying the disease remain elusive. Neuropathic pain is generally characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia).2) Peripheral neuropathic pain is frequently observed in patients with cancer, AIDS, long standing diabetes, lumbar disc syndrome, herpes infection, traumatic spinal cord injury, multiple sclerosis and stroke.3,4) Moreover, post-thoracotomy, post-herniorrhaphy, post-mastectomy and post-sternotomy are some other conditions often associated with peripheral neuropathic pain.5,6)

Tibial and sural nerve transection is a novel model of neuropathic pain developed by Lee et al.7) characterized by vigorous mechanical allodynia, cold allodynia, mechanical hyperalgesia and spontaneous pain.7–9) The animal model possesses number of advantageous over the other models such as easy surgical procedure and hence, high reproducibility. Furthermore, the number of injured fibers remain constant each time as compared to other conventional models (based on the ligation mediated nerve compression i.e., chronic constriction and partial sciatic nerve ligation), in which there is a significant variability regarding the number of injured and uninjured fibers due to variability in degree of tightness of the ligating suture around the nerve.9,10) Currently, the clinically used drugs for neuropathic pain have limited efficacy and are associated with number of intolerable side effects. Thus, neuropathic pain represents a substantial unmet challenge to medical treatment and scientific research.

Pralidoxime, acetylcholinesterase reactivator, has been used to treat overdose of medicines such as ambenonium, neostigmine, and pyridostigmine, used for treating myasthenia gravis. Recently, it has been reported from our own laboratory that pralidoxime has a potential to attenuate the neuropathic pain state in chronic constriction and vincristine-induced neuropathy in rats.11) However, it ameliorative potential in other types of neuropathies is yet to be explored. Therefore, the present study was designed to investigate the role of pralidoxime in tibial sural nerve transection induced painful neuropathy in rats.

MATERIALS AND METHODS

Experimental Animals Wistar albino rats weighing 250—300 g (procured from Punjab Agriculture University, Ludhiana, India) of either sex were employed in present study. They were housed in animal house with free access of water and standard laboratory pellet chow diet (Kisan Feeds Ltd., Mumbai, India). The rats were exposed to 12 h light and 12 h dark cycle. The experimental protocol was duly approved by the Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.-37/1999/CPCSEA).

Drugs and Reagents Pralidoxime (VHB Pharmaceuticals Ltd., India), 1,1,3,3 tetra methoxy propene (Sigma Aldrich, U.S.A.), Bovine Serum Albumin (BSA), (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), thio-barbituric acid, nitroblue tetrazolium (NBT), were purchased from Loba Chem, Mumbai, India. (Loba Chem, Mumbai), Folin-Ciocalteu’s phenol reagent (Merck Ltd., Mumbai, India) were procured for the present study. All the chemicals used in the present study were of analytical grade.

Induction of Neuropathy by Tibial and Sural Nerve Transection Peripheral neuropathy was induced by tibial and sural nerve transection as described earlier.7,12) In brief, rat was deeply anesthetized with ketamine (60 mg/kg in-
traperitoneally (i.p.). The skin of its lateral surface of the left thigh was incised and a cut made directly through the biceps femoris muscle to expose the sciatic nerve and its three terminal branches (the sural, common peroneal and tibial nerves). Thereafter, the tibial and sural nerve sections of 2 mm (distal to the trifurcation) were ligated and cut. The common peroneal nerve was left intact and no contact was made with it. The muscle and the skin were closed in two layers. Sham controls were performed by exposing the sciatic nerve and its branches without inducing any lesion. Due to a distinct development of postural defect in the paw of tibial and sural nerve transected animals, the behavioral studies could not be conducted blind for comparing normal control; sham control, and tibial and sural nerve transection control groups. However, the behavioral studies were blind for comparing the other groups including tibial and sural nerve transection control, and tibial and sural nerve transection treated groups.

Behavioral Examination. Tail Heat Hyperalgesia Test

In peripheral nerve injury, initial burst from peripheral site and later sustained activation of peripheral nociceptors leads to a central sensitization of the dorsal horn neurons in the spinal cord. This central sensitization is an important feature in inducing long-term thermal allodynia and hyperalgesia, which may be assessed by noting down the changes in thermal sensitivity in the tail.11,13,14 Thermal heat hyperalgesia was assessed by the tail immersion test as described by Necker and Hellon.15 Tail heat-hyperalgesia was noted with the immersion of terminal part of the tail (1 cm) in water, maintained at a temperature of 52±1.0 °C. The tail withdrawal latency was recorded, as a response of heat thermal sensation, and a cut-off time of 20 s was maintained.

Tail Cold Hyperalgesia (Tail Immersion Test) Thermal cold sensitivity was assessed by the tail immersion method as described by Necker and Hellon.15 Tail cold-hyperalgesia was noted with the immersion of terminal part of the tail (1 cm) in water maintained at a temperature of 0–4 °C. The tail withdrawal latency was recorded as a response of cold thermal sensation and a cut-off time of 20 s was maintained.

Cold Allodynia (Acetone Test) Cold allodynia was assessed by spraying a 100 µl of acetone onto the medial planter surface of the paw, using a blunt needle connected to a syringe without touching the skin. The duration of withdrawal response was recorded with an arbitrary minimum value of 0.5 s and a maximum value of 20 s.16,17

Mechanical Hyperalgesia (Pin Prick Test) Mechanical hyperalgesia was assessed by the pinprick test as described by Decosterd and Woolf.17 The medial planter surface of injured hind paw was touched with the point of the safety pin at intensity sufficient to produce a reflex withdrawal response in normal non-operated animals, but at an intensity which was insufficient to penetrate the skin. The duration of the paw withdrawal was recorded in seconds with an arbitrary minimum value of 0.5 s. A cut-off time of 10 s was maintained.

Biochemical Estimations All the animals were sacrificed after 14th day of surgery by high dose anesthesia (diethyl ether). The sciatic nerve and tissue beneath the sciatic nerve was isolated immediately. The sciatic nerve portion, proximal to the point of transection up-to its point of emergence from the spinal cord, and distal to the point of transection up-to its ending, was excised. The tissue, 1 cm diameter, exactly beneath the point of the sciatic nerve transection was taken. The uniformity among the different nerve and the tissue samples was maintained by taking the constant weight of the respective samples. Freshly excised sciatic nerve homogenate (10%) was prepared with 0.1 M Tris–HCl buffer (pH 7.4). The tubes with homogenate were kept in ice water for 30 min and centrifuged at 4 °C (2000 g, 10 min). The supernatant of homogenate was separated, and employed to estimate total protein content, thio-barbituric acid reactive substances, superoxide dismutase content and total calcium content.

Estimation of Total Protein Content The protein concentration was estimated according to the method of Lowry et al.18 using bovine serum albumin as a standard. The absorbance was determined spectrophotometrically at 750 nm.

Estimation of Thio-Barbituric Acid Reactive Substances The estimation of lipid peroxidation was done by measuring the thio-barbituric acid reactive substances by the method of Ohkawa et al.19 The absorbance was measured spectrophotometrically at 532 nm. The concentration was expressed in terms of nmol of thio-barbituric acid reactive substances/mg protein.

Estimation of Superoxide Anion Generation The superoxide anion generation in the sciatic nerve was estimated in terms of measuring reduced nitroblue tetrazolium (NBT).20 The absorbance of formazan was determined spectrophotometrically at 540 nm.

Estimation of Total Calcium Total calcium levels were estimated in the sciatic nerve as described earlier.11,12,21 Briefly, the sciatic nerve homogenate was mixed with 1 ml of trichloroacetic acid (4%) in ice cold conditions and centrifuged at 1500 g for 10 min. The clear supernatant was used for the estimation of total calcium ion by atomic emission spectroscopy at 556 nm.

Experimental Protocol Ten groups, each comprising six Wistar albino rats, were employed in the present study.

Group I (Normal Control) Rats were not subjected to surgical procedure and were kept for 2 weeks. The behavioral tests were performed on different days i.e., day 2nd, 4th, 6th, 8th, 11th and 14th. Thereafter, all the animals were sacrificed and the biochemical analysis was done for estimation of total protein content, thio-barbituric acid reactive substances, total calcium and superoxide anion generation.

Group II (Sham Control) Rats were subjected to surgical procedure (on day 0) to expose tibial and sural nerve branches without any transection. The behavioral tests were employed before surgery (day 0) and after surgery (from day two) on different days as described in group I. The biochemical analysis was also done as described in group I.

Group III (Tibial and Sural Nerve Transection) Rats were subjected to surgical procedure to expose and transect tibial and sural nerve branches of the sciatic nerve. The behavioral tests and the biochemical parameters were assessed as mentioned in group I.

Group IV (Vehicle in Tibial and Sural Nerve Transection) Normal saline (0.9% w/v, i.p.) was administered for 14 d (starting from day 1), in rats subjected to tibial and sural nerve transection. The behavioral tests and the biochemical parameters were assessed as mentioned in group I.
Group V (Pralidoxime 20 mg/kg *per se*) Pralidoxime (20 mg/kg i.p.) was administered to normal rats for 14 consecutive days, starting from the day 1. The behavioral tests and the biochemical parameters were assessed as mentioned in group I.

Group VI and VII, (Pralidoxime 10, 20 mg/kg in Tibial and Sural Nerve Transection) Pralidoxime (10, 20 mg/kg; i.p.) was administered for 14 d in rats subjected to tibial and sural nerve transection, starting from the day 1. The behavioral tests and the biochemical parameters were assessed as mentioned in group I.

Statistical Analysis All the results were expressed as mean ± standard error of means (S.E.M.). The data of behavioral results were statistically analyzed by two-way analysis of variance followed by Bonferonni’s *post hoc* test by using Graph pad prism Version-5.0 software. The data of biochemical results was statistically analyzed by one-way analysis of variance followed by Tukey’s multiple range tests by using Graph pad prism Version-5.0 software. The *p*-value <0.05 was considered to be statistically significant.

RESULTS

**Effect of Pralidoxime on Hot and Cold Tail Hyperalgesia in Tibial and Sural Nerve Transection Induced Neuropathy** Tibial and sural nerve transection resulted in significant development of hot tail and cold tail hyperalgesia. Administration of pralidoxime (10, 20 mg/kg) significantly attenuated tibial and sural nerve transection induced decrease in nociceptive threshold for hot and cold hyperalgesia in a dose dependent manner. Moreover, vehicle administration did not modulate tibial and sural nerve transection induced thermal hyperalgesia. *Per se* administration of pralidoxime did not alter the responsiveness of normal rat’s hind paw to the pain threshold for thermal stimuli (Figs. 1, 2).

**Effect of Pralidoxime on Cold Allodynia in Tibial and Sural Nerve Transection Induced Neuropathy** Tibial sural nerve transection resulted in significant development of cold allodynia as reflected by increase in the duration of hind paw withdrawal, when compared to sham group. However, administration of pralidoxime (10, 20 mg/kg i.p.) attenuated tibial and sural nerve transaction-induced increase in the withdrawal duration in a significant manner. Moreover, there was significant statistical difference between anti-allodynic effects of 10 and 20 mg/kg dose of pralidoxime in tibial and sural nerve transected rats. Vehicle administration did not modulate tibial and sural nerve transection induced alteration in cold allodynia. *Per se* administration of pralidoxime also did not alter the responsiveness of normal rat’s hind paw to thermal stimuli (Figs. 3).

![Fig. 1. Effect of Pralidoxime on Heat Hyperalgesia, Assessed by Hot Tail Immersion Test, in Tibial and Sural Nerve Transection-Induced Neuropathic Pain](image1)

Data were expressed as mean±S.E.M., *n*=6 rats per group. *a* *p*<0.05 vs. sham control group. *b* *p*<0.05 vs. TST control group. *c* *p*<0.05 vs. pralidoxime 10 mg/kg group.

![Fig. 2. Effect of Pralidoxime on Tail Cold Hyperalgesia, Assessed by Cold Tail Immersion Test, in Tibial and Sural Nerve Transection-Induced Neuropathic Pain](image2)

Data were expressed as mean±S.E.M., *n*=6 rats per group. *a* *p*<0.05 vs. sham control group. *b* *p*<0.05 vs. TST control group. *c* *p*<0.05 vs. pralidoxime 10 mg/kg group.

![Fig. 3. Effect of Pralidoxime on Paw Cold Allodynia, Assessed by Acetone Test, in Tibial and Sural Nerve Transection-Induced Neuropathic Pain](image3)

Data were expressed as mean±S.E.M., *n*=6 rats per group. *a* *p*<0.05 vs. sham control group. *b* *p*<0.05 vs. TST control group. *c* *p*<0.05 vs. pralidoxime 10 mg/kg group.
Effect of Pralidoxime on Mechanical Hyperalgesia in Tibial and Sural Nerve Transection Induced Neuropathy

Tibial and sural nerve transection resulted in development of mechanical hyperalgesia as reflected by a significant increase in withdrawal duration of the hind paw in the pin-prick test as compared to sham group. However, administration of pralidoxime (10, 20 mg/kg i.p.) significantly attenuated tibial and sural nerve transection-induced increase in withdrawal duration in a dose dependent manner. Vehicle administration did not modulate tibial and sural nerve transection-induced alteration in mechanical nociception. Per se administration of pralidoxime also did not alter the responsiveness of normal rat’s hind paw to mechanical nociception in a significant manner (Fig. 4).

Effect of Pralidoxime on Oxidative Stress Markers and Total Calcium in Tibial and Sural Nerve Transection Induced Neuropathy

Tibial and sural nerve transection resulted in significant increase in the levels of oxidant anion, increase in the levels of thio-barbituric acid reactive substances and total calcium, when compared to sham control. Administration of pralidoxime (10, 20 mg/kg i.p.) attenuated tibial and sural nerve transection induced increase in the levels of thio-barbituric acid reactive substances superoxide anion content and total calcium, in sciatic nerve, in a dose dependent manner. On the other hand, vehicle administration did not modulate the tibial and sural nerve transection induced alteration in oxidative stress markers and calcium levels. Per se administration of pralidoxime did not alter the levels of oxidative stress markers and calcium level (Table 1).

DISCUSSION

In the present study, tibial and sural nerve transection led to significant development of cold allodynia, mechanical, cold and heat hyperalgesia. These behavioral changes were evident from the 2nd–3rd day of surgery and were at peak after 12—14 d of surgery. Tibial and sural nerve transection is a novel model of neuropathy in which two (the tibial and sural nerves) of the three sciatic nerve branches are transected and remaining peroneal nerve is left intact.7) The tibial and sural nerves innervate the lateral and central part of hind paw in an overlapping fashion and transection of these has been documented to produce sensory deficits in the corresponding areas of hind paw. On the other hand, the medial plantar region of hind paw has been demonstrated to exhibit hypersensitivity in response to mechanical, chemical, cold and heat noxious as well as non-noxious stimuli suggesting the induction of hyperalgesia and allodynia.22) Because of the nerve transection, there is loss of peripheral nerve continuity along with Wallerian degeneration followed by sprouting of axons from proximal stump to contact the distal stump to restore the peripheral nerve continuity. However, the process of sprouting of axons and endeavor to restore the nerve continuity is associated with neuroma formation (regenerating axons, proliferating Schwann cells and fibroblasts). The induction of hyperalgesia and allodynia has been attributed to generation of spontaneous ectopic activity in the neuroma and associated neurons in the dorsal root ganglia.10,23—26)

In the present study, administration of pralidoxime (10, 20 mg/kg) significantly attenuated tibial and sural nerve transection-induced mechanical hyperalgesia, cold allodynia, heat and cold hyperalgesia. Pralidoxime is reactivator of inhibited form of acetyl–cholinesterase and has been employed clinically for organophosphate poisoning and overdosing of anti-cholinesterase inhibitors in the treatment of myasthenia gravis. Recently, from our laboratory it had been documented that pralidoxime attenuates the painful state of neuropathy in rats associated with chronic constriction injury and vincristine administration.11)

In the present investigation, tibial and sural nerve transection was also associated with the rise in calcium levels and non noxious cold stimuli in a significant manner (Fig. 3).

Table 1. Effect of Pralidoxime on Thio-Barbituric Acid Reactive Substances, Superoxide Anion Content and Total Calcium in Tibial and Sural Nerve Transection-Induced Neuropathic Pain

<table>
<thead>
<tr>
<th>Group</th>
<th>Total protein (mg/ml)</th>
<th>TBARS (nmol/mg of protein)</th>
<th>Superoxide anion content (pmol/min/mg)</th>
<th>Total calcium (ppm/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5.27 ± 0.27</td>
<td>6.07 ± 0.26</td>
<td>0.14 ± 0.01</td>
<td>4.81 ± 0.20</td>
</tr>
<tr>
<td>Sham control</td>
<td>5.31 ± 0.24</td>
<td>6.12 ± 0.21</td>
<td>0.17 ± 0.02</td>
<td>4.73 ± 0.23</td>
</tr>
<tr>
<td>TST control</td>
<td>5.52 ± 0.20</td>
<td>8.13 ± 0.13</td>
<td>0.72 ± 0.04</td>
<td>35.76 ± 0.21</td>
</tr>
<tr>
<td>Vehicle in TST control</td>
<td>5.39 ± 0.22</td>
<td>8.01 ± 0.18</td>
<td>0.77 ± 0.06</td>
<td>35.54 ± 0.22</td>
</tr>
<tr>
<td>Pralidoxime per se</td>
<td>5.32 ± 0.26</td>
<td>6.16 ± 0.21</td>
<td>0.12 ± 0.02</td>
<td>4.85 ± 0.16</td>
</tr>
<tr>
<td>Pralidoxime 10 mg/kg</td>
<td>5.27 ± 0.17</td>
<td>7.53 ± 0.19</td>
<td>0.61 ± 0.05</td>
<td>25.82 ± 0.21</td>
</tr>
<tr>
<td>Pralidoxime 20 mg/kg</td>
<td>5.41 ± 0.15</td>
<td>6.61 ± 0.12</td>
<td>0.46 ± 0.02</td>
<td>18.21 ± 0.26</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± S.E.M., n=6 rats per group. a) p<0.05 vs. sham control group. b) p<0.05 vs. TST control group. c) p<0.05 vs. pralidoxime 10 mg/kg group.
oxidative stress. Calcium ion accumulation has been presumed to trigger an auto-destructive cascade of secondary biochemical changes including electrical hyper-excitability, ATP depletion, activation of phospholipases, proteases and calpains. 27,28 Calcium induced activation of calpains has been reported to degrade axonal cytoskeleton and thus resulting in axonal degeneration. 29 Calcium accumulation has been well documented to play an important role in formalin, post-traumatic, axotomy, chronic constriction injury, anti-human immunodeficiency virus (HIV) drugs, vincristine and tibial sural nerve transection induced neuropathy. 11,12,30—32 Further, free radicals also been documented to induce tissue injury and pain in chronic constriction injury, vincristine, diabetes and tibial sural nerve transection induced neuropathy. 11,12,33,34

However, administration of pralidoxime attenuated tibial and sural nerve transection induced increase in oxidative stress and calcium levels, indicating that pralidoxime mediated reduction in calcium levels and oxidative stress is contributing significantly for its noted effects in neuropathic pain. Oximes have also been documented to decrease oxidation stress. 35 Pralidoxime has been reported to inhibit Na+ /Ca2+ type I (cardiac type) exchanger and to decrease the intracellular calcium accumulation. 36,37 Na+ /Ca2+ exchanger, a bi-directional ion transporter is an important regulator of intracellular calcium levels. 38

In peripheral nerve injury, a key role of primary afferent inputs (sensitization of primary afferents) in the pathogenesis of neuropathic pain has been strongly suggested by several pharmacological studies. The nerve injury is associated with altered expression of different ion channels in peripheral myelinated mammalian axons and dorsal root ganglia. The high density of sodium-calcium exchanger (the canine cardiac type 1) has been demonstrated in the peripheral myelinated mammalian axons with glial and axonal localization. 39,40 However, the detailed characterization of Na+/Ca2+ exchanger including its various subtypes on the peripheral nerves has not been documented. It has been demonstrated using in-vitro optic nerve, tibial nerve myelinated axons and dorsal roots that axonic injury to the nerve is associated with Ca2+ loading via reverse operation of the Na+/Ca2+ exchanger due to axon-axia-induced depolarization and Na+ influx. 39,41,42 Furthermore, Na+/Ca2+ exchange inhibitors such as bepridil, benzamil and dichlorobenzamil significantly protected the optic nerve from axonic injury. Acrylamide-induced distal axon degeneration has also been linked to activation of reverse mode operation of the Na+/Ca2+ exchanger with axonal Ca2+ entry in exchange for Na+. 43 Based on these, it may be proposed that pralidoxime reduced the axoplasmic Ca2+ loading by inhibiting the reverse mode operation of the Na+/Ca2+ exchanger on peripheral myelinated axons and dorsal roots, which in turn may be responsible for attenuated peripheral sensitization in tibial and sural nerve transection (TST)-induced neuropathic pain.

Furthermore, neuropathy associated hyper-responsive to painful as well as non-painful stimuli may be attributed to decrease in peripheral as well as central sensitization on account of inhibition of Na+/Ca2+ exchanger on peripheral demyelinated fibers, dorsal ganglia and on central dorsal horn neurons in spinal cord. The dose selection was based on our previous report with pralidoxime in chronic constriction injury (CCI) and vincristine-induced neuropathy. 35 In this study, pralidoxime at the dose of 20 mg/kg produced significant dose dependent effect. Nevertheless, further studies with higher dose (40 mg/kg) are required to substantiate the dose dependent effect of pralidoxime in TST-induced neuropathic pain. Though the role of Na+/Ca2+ exchanger has been documented in nerve injury-induced changes, yet the potential of Na+/Ca2+ exchange inhibitors have not been investigated in attenuating the neuropathic pain. Therefore, it may be concluded that pralidoxime has ameliorative potential in tibial and sural nerve transection-induced neuropathic pain and thus, Na+/Ca2+ exchange inhibitors may be employed in attenuating neuropathic pain.

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