Effect of nitric oxide in protective effect of melatonin against chronic constriction sciatic nerve injury induced neuropathic pain in rats

Anil Kumar*, Seema Meena, Harikesh Kalonia, Amit Gupta & Puneet Kumar
Pharmacology Division, University Institute of Pharmaceutical Sciences,
UGC Centre of Advanced Studies, Panjab University, Chandigarh 160 014, India

Received 9 August 2010; revised 24 May 2011

Neuropathic pain is one of the chronic painful and debilitating conditions which affect large population worldwide with approximately 4 million people in India. Therapeutic management of neuropathic pain is still one of the challenges among researchers and clinicians. Trauma or injury to central or peripheral nervous system and unrelated to ongoing tissue damage or inflammation generally results in the development of severe neuropathic pain. Nerve degeneration accompanied by the up-regulation of inflammatory mediators such as macrophages, the production of cytokines/interleukins and nerve growth factors is thought to play a role in development of neuropathic pain. Neurotrophins have also been suggested to play a role in the genesis of neuropathic pain.

Various in vivo models have been developed to understand the mechanism of neuropathic pain. Different drugs such as tricyclic antidepressants, anticonvulsants, antioxidants and neuroprotectants have been tried with limited success in this condition. There is a need to understand the exact mechanism and cellular event involved in its pathophysiology in order to develop and design of suitable neuroprotective strategy against neuropathic pain. Various studies have implicated the involvement of nitric oxide in the pathophysiology of neuropathic pain. Studies have also been indicated that inhibition of nitric oxide synthase could be effective drug strategy to enhance the clinical efficacy of therapeutic agents against neuropathic pain.

Melatonin (N-acetyl-5-methoxytryptamine), chief neurosecretory product of the pineal gland, is a well known antioxidant. Melatonin has a free radical scavenging activity that explains its potent antioxidant property. Melatonin also inhibits post traumatic polymorph nuclear infiltration and stimulates superoxide dismutase and glutathione peroxidase reductase suggesting its therapeutic potential against peripheral nerve injuries. Melatonin has also been demonstrated for its anti-inflammatory and antinociceptive actions, suggesting that it could be a potent drug to treat inflammation, hyperalgesia and neuropathic pain.
Therefore, the present study has been designed to explore the possible nitric oxide mechanism in the protective effect of melatonin against chronic constriction injury of sciatic nerve in rats.

Materials and Methods

Animals—Male Wistar rats (180-200 g) bred in the Central Animal House facility of Panjab University were used. Total (88) animal were used and divided into eleven group (11) eight (8) animal in each. The animals were housed under standard laboratory conditions, maintained on a normal light–dark cycle and free access of food and water. Animals were acclimatized to laboratory conditions before the test. All the experiments were carried out between 0900 and 1700 hrs. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and animal experiments were conducted according to the guidelines of Indian National Science Academy for the use and care of experimental animals. Following surgery, the animals were kept in group of two rats in one plastic cage with soft bedding under standard conditions of 12:12 h L/D.

Induction of peripheral neuropathy by sciatic nerve ligation—The unilateral peripheral neuropathy in rats was induced by chronic constriction injury as per modified method of Bennet and Xie. In brief, rats were deeply anesthetized with thiopental sodium (40 mg/kg, ip). The hair of lower back and thigh were shaved. The skin of the lateral surface of the right thigh was incised and a cut was made directly through the biceps. After exposing the sciatic nerve four ligatures were applied. After performing the ligation, muscular and skin layer was immediately sutured with thread and topical antibiotic was applied. Nociceptive threshold was assessed at weekly intervals on day 7, 14 and 21 after the surgery.

Drugs and treatment schedule—The experimental protocol was divided into two phases: In the entire study, animals were sub divided into eleven groups, 8 animals each.

Study I—To investigate the protective effect of melatonin in chronic constriction induced sciatic nerve injury in rats.

Group I - vehicle treated group; Group II – sham group in which sciatic nerve was exposed but not ligated; Group III – Chronic constriction injury (CCI) group; Group IV to V received melatonin (2.5 and 5 mg/kg, ip) + CCI; Group VI to VII received gabapentin (50 and 100 mg/kg, ip) + CCI.

Study 2—to explore the role of nitric oxide mechanism in the protective effect of melatonin

Group VIII received L-arginine (100 mg/kg, ip) + CCI; Group IX received L-NNAME (5 mg/kg, ip) + CCI; Group X received L-arginine (100 mg/kg, ip) + melatonin (2.5 mg/kg, ip) + CCI; Group XI received L-NNAME (L-NG-Nitroarginine methyl ester) (5 mg/kg, ip) + melatonin (2.5 mg/kg, ip) + CCI.

In order to compare the effect with test drug gabapentin was used as a standard drug. L-arginine and L-NNAME were freshly prepared in distilled water and administered intraperitoneally 15 minutes before melatonin treatment. Melatonin was mixed with one drop of DMSO and then diluted with distilled water. All the drugs were given, once daily for a total 21 days. Doses were selected on the basis of earlier studies.

Behavioral examinations

Hot plate test—Thermal hyperalgesia was assessed on weekly intervals (i.e. on 7th, 14th and 21st day) by placing individual animal on a hot plate (Eddy’s hot plate) maintained at 55°C after CCI. The time latency of either of the first signs of paw licking or jumping response was recorded as an index of pain threshold. A cut off time of 15 sec was maintained throughout the experimental protocol.

Cold allodynia—Cold allodynia was assessed after 2 h of assessment of hyperalgesia by measuring paw (ipsilateral) withdrawal latency (PWL). Ice-cold water (4°C±2°C) was taken in a beaker. The paws of rats were submerged gently in water and the withdrawal time was measured, on weekly intervals on (7th, 14th and 21st day) after chronic constriction injury. A cut off 15 sec was maintained throughout the experimental protocol.

Biochemical estimations for oxidative stress determination

Dissection and homogenization—On day 21, animals were sacrificed by decapitation immediately after behavioral assessments. 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 homogenates for biochemical estimation 10% (w/v). Tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). Homogenate were then centrifuged for 20 min at
15000 rpm and supernatants were used for estimation of lipid peroxidation and reduced glutathione levels. For catalase assay the post nuclear fractions were obtained by centrifugation of the homogenates at 1000 g for 20 min, at 4°C and for other enzyme assays centrifuged at 12,000 g for 60 min at 4°C.

**Lipid peroxidation assay**—The quantitative measurement of lipid peroxidation was performed according to the method of Wills. The amount of malondialdehyde (MDA), a measure of lipid peroxidation was assayed in the form of thiobarbituric acid reacting substances (TBARS). Briefly, 0.5 ml of post mitochondrial supernatants and 0.5 ml of Tris HCl were incubated at 37°C for 2 h. After incubation, 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000 g for 10 min.

To 1 ml of supernatant, 1 ml of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling 1 ml double distilled water was added and absorbance was measured at 532 nm using a Perkin-Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). Thiobarbituric acid reactive substances were quantified using an extinction coefficient of 1.56×10^5 M-1cm-1 and expressed as nmol of malondialdehyde per mg protein.

**Estimation of reduced glutathione**—Reduced glutathione was estimated according to the method described by Ellman. Results were calculated using molar extinction coefficient of chromophore (1.36×10^5 M-1cm-1) and expressed as percentage of control.

**Estimation of nitrite**—The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO) was determined with a colorimetric assay with Greiss reagent ([0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid])[^35]. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as percentage of control.

**Estimation of catalase**—Catalase activity was assayed by method of Luck.[^36] where in the breakdown of H₂O₂ was measured at 240 nm.

**Protein estimation**—Tissue protein content was measured according to the Biuret method of protein assay using bovine serum albumin as standard.[^37]

**Statistical analysis**—All data are expressed as mean±SE. Data were analyzed by two way analysis of variance (ANOVA) for thermal hyperalgesia and cold allodynia; one way of analysis of variance (ANOVA) followed by Tukey’s test for oxidative stress measurement. Value of P<0.05 was considered to be significant.

**Results**

**Effects of melatonin and its modulation by nitric oxide modulators on thermal hyperalgesia in CCI rats**—Chronic constriction injury (CCI) significantly caused hyperalgesia, demonstrated by decreased pain threshold (jump or licking time latency), assessed on 7th, 14th and 21st day as compared to control group. Gabapentin (50 and 100 mg/kg) treatment significantly attenuated CCI induced hyperalgesia as compared to control (CCI) group observed on 7th, 14th and 21st day. Chronic melatonin (5 mg/kg, ip) treatment significantly attenuated CCI induced hyperalgesia as compared to control (CCI) after 14th and 21st day (Fig. 1) whereas lower dose of melatonin (2.5 mg/kg, ip) did not show any significant effect on hyperalgesia as compared to control (CCI).

Further, L-NAME (5 mg/kg) pretreatment with lower dose of melatonin (2.5 mg/kg) significantly potentiated the protective effects of melatonin on 14th and 21st day as compared to their effect per se (Fig. 1). However, L-arginine (100 mg/kg) pretreatment with lower dose of melatonin (2.5 mg/kg) significantly reversed its effect which was significant as compared to their effect per se. L-arginine (100 mg/kg) and L-NAME (5 mg/kg) per se treatment did not produce any significant effect on hyperalgesic response even after 3rd week as compared sham treated animals.

**Effects of melatonin and its modulation by nitric oxide modulators on cold allodynia in CCI rats**—Chronic constriction injury significantly caused cold allodynia as reflected by decrease in paw withdrawal latency after 7th, 14th and 21st day as compared to sham group. Gabapentin (50 and 100 mg/kg) treatment significantly delayed CCI induced paw withdrawal threshold as compared to control (CCI) as observed on 7th, 14th and 21st day. Chronic melatonin (5 mg/kg, ip) treatment significantly delayed withdrawal latency as compared to control (CCI) after 14th and 21st day (Fig. 2) whereas lower dose of melatonin (2.5 mg/kg, i.p.) did not show any significant effect on allodynia as compared to control (CCI).

L-NAME (5 mg/kg) pretreatment with lower dose of melatonin (2.5 mg/kg) potentiated its antiallodynic effect after 14th and 21st day, which was significant as compared their effects per se. However, L-arginine
pretreatment with lower dose of melatonin (2.5 mg/kg) significantly reversed the antiallodynic effect of melatonin after day 21 (Fig. 2). L-arginine (100 mg/kg) and L-NAME (5 mg/kg) treatment per se did not produce significant antiallodynic effect even after day 21.
**Effects of melatonin and its modulation by nitric oxide modulators on oxidative damage in CCI rats**—Chronic constriction injury significantly caused an oxidative damage as indicated by increase in lipid peroxidation level and nitrite concentration, depleted reduced glutathione level and catalase activity in sciatic nerve as compared to sham treated animals (Table 1). Gabapentin (50 and 100 mg/kg) and melatonin (5.0 mg/kg, ip) treatments significantly attenuated the oxidative damage (as evidenced by decrease in lipid peroxidation levels and nitrite concentration; restoration of depleted reduced glutathione and catalase activity) as compared to the control (CCI) in sciatic nerve ($P<0.05$) (Table 1). However, lower dose of melatonin (2.5 mg/kg, ip) did not show any significant antioxidant effects as compared to control (CCI).

Further, L-NAME (5 mg/kg) pretreatment with melatonin (2.5 mg/kg, ip) significantly potentiated its antioxidant effect as compared to their effect per se. However, L-arginine (100 mg/kg) pretreatment with melatonin (2.5 mg/kg, ip) significantly reversed the antioxidant effect of melatonin (Table 1). L-arginine (100 mg/kg) and L-NAME (5 mg/kg) treatment per se did not produce any significant effect on oxidative stress parameter as compared to CCI group in sciatic nerve.

**Discussion**

Peripheral nerve injury produces a persistent neuropathic painful state characterized by spontaneous pain, allodynia and hyperalgesia. Studies demonstrated that unilateral sciatic nerve ligation induces an ipsilateral cold alldynia, thermal hyperalgesia and oxidative damage in sciatic nerves. These behavioral modifications are always present and last over 2 weeks, it must be noted that their time-course can vary depending upon the model and species. Ligation of sciatic nerve induced neuropathic pain causes hyperalgesia and cold allodynia-like behaviors in animals. Gabapentin is standard drug for neuropathic pain. Therefore, gabapentin was used to compare the protective effect of melatonin in experimental model of neuropathic pain. Similar observations have also been documented in different models and species which may be related to the fact that noxious heat or mechanical information involves partially independent neural pathways. Sensitization of the primary afferent nerves has been suggested as one of the mechanism involved in hyperalgesic action.

In the present study, CCI caused thermal hyperalgesia and cold allodynia (in response to non-noxious cold stimulus) in animals that was significant after 2nd and 3rd week post CCI. Melatonin treatment at higher dose significantly improved the pain threshold and attenuated the cold allodynia, suggesting its therapeutic potential against neuropathic states. However, melatonin (2.5 mg/kg) treatment did not show any significant on paw withdrawal latency on first week; it could be due to its weak protective effect on first week. Potential usefulness of melatonergic drugs in diverse experimental models of neuropathic pain have been demonstrated. Chronic constriction injury also produced significant oxidative damage in sciatic nerve.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>LPO (nM of MDA/mgpr)</th>
<th>Nitrite (µg/ml)</th>
<th>GSH (µg/mg of protein)</th>
<th>Catalase (µ moles of H$_2$O$_2$/min/mgpr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.2±0.025 (97.5)</td>
<td>28±0.29 (93.3)</td>
<td>22±0.22 (+110)</td>
<td>3.02±0.4 (-96.44)</td>
</tr>
<tr>
<td>Sham</td>
<td>1.23±0.024 (100)</td>
<td>30±0.2 (100)</td>
<td>20±1.9 (100)</td>
<td>3.12±0.71 (100)</td>
</tr>
<tr>
<td>CCI</td>
<td>2.09±0.024* (+169.91)</td>
<td>40±0.2* (+133.3)</td>
<td>11±0.27* (-55)</td>
<td>1.56±0.42* (-50)</td>
</tr>
<tr>
<td>GP (50)</td>
<td>1.68±0.051* (+136.5)</td>
<td>35.5±0.11* (+118.3)</td>
<td>14.6±0.33* (-73)</td>
<td>1.9±11* (-60.89)</td>
</tr>
<tr>
<td>GP (100)</td>
<td>1.41±0.012* (+114.63)</td>
<td>22.1±0.3* (-73.66)</td>
<td>21±0.11* (+105)</td>
<td>2.89±0.33* (-92.62)</td>
</tr>
<tr>
<td>Mel (2.5)</td>
<td>1.9±0.013* (+154.44)</td>
<td>36.3±0.11* (+121)</td>
<td>12.1±0.33* (-60.5)</td>
<td>1.8±011* (-58.33)</td>
</tr>
<tr>
<td>Mel (5)</td>
<td>1.7±0.04* (+121.95)</td>
<td>28.±0.3* (+121.95)</td>
<td>18.1±0.11* (-90.5)</td>
<td>2.45±0.33* (-78.52)</td>
</tr>
<tr>
<td>L-NAME (5)</td>
<td>1.9±0.012* (+169.9)</td>
<td>39±0.36* (+130)</td>
<td>12±0.21* (-60)</td>
<td>1.7±0.06* (-54.4)</td>
</tr>
<tr>
<td>L-NAME (5) +Mel (2.5)</td>
<td>1.6±0.08* (+130.08)</td>
<td>22.3±0.36* (-73.3)</td>
<td>18±0.26* (-90)</td>
<td>2.1±0.21* (-67.30)</td>
</tr>
<tr>
<td>L-Arg (100)</td>
<td>2.2±0.002 (+170.73)</td>
<td>41.5±0.42* (+138.3)</td>
<td>10.5±0.12* (-53)</td>
<td>1.5±0.045 (-48.0)</td>
</tr>
<tr>
<td>L-Arg (100) +Mel (2.5)</td>
<td>2.1±0.034 (+170.7)</td>
<td>43±0.66* (+143.19)</td>
<td>10.62±0.01* (-53)</td>
<td>1.45±0.64* (-46.47)</td>
</tr>
</tbody>
</table>

*P<0.05 as compared to ‘sham,’ **CCI,’ ‘GP (50),’ ‘Mel (2.5),’ ‘L-NAME (5) (one-way ANOVA followed by Tukey’s test). CCI: Chronic constriction injury; GP: Gabapentin; L-Arg: L-arginine; L-NAME: L-NG-Nitroarginine methyl ester; Mel: Melatonin.

**Table 1—Effects of melatonin and its interaction with L-NAME and L-arginine on oxidative damage in SNL nerves**

[Values are expressed as mean ± SE from 8 animals in each group. Figures in parentheses are % increase (+) or decrease over sham values.]}
nerve as indicated by rise in lipid peroxidation and nitric concentration as well as depletion of reduced glutathione and catalase activity. Reactive oxidant species (ROS) are critically involved in the development and maintenance of neuropathic pain, while administration of non-toxic doses of free radical scavengers could be useful for treatment of neuropathic pain. Increasing evidences suggest a key role of oxidative stress in neuropathic pain and other neurological diseases, but the precise mechanisms that underlie pain and oxidative stress still remains unclear. Increased production of ROS leads to marked changes in cellular structure and function by damage to cellular protein and DNA damage. Increased lipid peroxide and nitrite levels were reported in injured sciatic nerve of old rats. These findings suggest that endoneurial lipid peroxidation increases as a consequence of CCI in sciatic nerve. However, melatonin treatment significantly reduced oxidative damage by attenuating the increased lipid peroxides, nitrite concentration and restoration of depleted antioxidant enzymes. It seems that beneficial effects of melatonin could be because of its antioxidant property.

Since nitric oxide plays a crucial role in the normal physiology and pathophysiology of various neurodegenerative conditions, NO modulators play an important role in neuropathic pain conditions, however its exact role is still not clear. In the present study, L-NAME and L-arginine pretreatment significantly modified the protective effect of melatonin, indicating the involvement of NO pathways in its protective effect. Inhibition of NO pathway by L-NAME, a non-specific inhibitor of nitric oxide synthase, significantly potentiated the protective effect of melatonin (anti-hyperalgesic, anti-allodynic and antioxidant effect). However, administration of nitric oxide precursor, L-arginine along with melatonin significantly deteriorated the sciatic nerve injury as compared to administration of melatonin alone. These results suggest the possible involvement of NO pathway in the protective action of melatonin. Similar findings have been observed in other models of neuropathic pain where L-NAME administration reversed the thermal or mechanical hyperalgesia.

Melatonin is known to reduce the harmful effects of free radicals in the central nervous system either by free radical scavenging or decreasing nitric oxide synthase activity. Melatonin has been reported to have anti-inflammatory and antioxidant property experimental animal models of acute and chronic inflammation. However, melatonin’s protective mechanism in reversing hyperalgesia that could be secondary to the inflammation is not known yet. In the present study, higher dose of melatonin (5 mg/kg) significantly attenuated thermal hyperalgesia, cold allodynia and oxidative stress in sciatic nerve ligated animals. The antinociceptive activity of melatonin has been demonstrated earlier which further supports to the present hypothesis. It seems that melatonin plays an important role in pain regulation at central and peripheral level. Activation of the endogenous melatonin system in the spinal cord can reduce the generation, development and maintenance of central sensitization, with a resultant inhibition of capsaicin-induced secondary mechanical allodynia and hyperalgesia.

Melatonin has been shown to enter the nucleus of the cell, thereby providing protection to DNA. The diffusible property of melatonin makes it available to the subcellular component. Although, pathogenesis of neuropathic pain is not clear so far, yet free radical-induced lipid peroxidation along neural fibers and nitric oxide-induced damage have been reported to be involved in neuropathic pain. Therefore, in the present study efforts were made to explore the antioxidant mechanism of melatonin l-arginine-NO pathway. Activation of NMDA receptor is associated with increased intracellular Ca concentration and activation of Ca sensitive protein kinase C, resulting in the production of NO, which produces persistent enhancement of pain. It is now believed that the mechanism responsible for neuropathic pain in chronic pain may involve not only NO itself, but also the product of its reaction with superoxide radicals, the peroxynitrite.

The finding of the present study suggests that the nitric oxide pathway may be involved in the protective action of melatonin against the CCI induced behavioral and biochemical alterations in rats.

References
1 Attal N & Bshassria D. Can pain be more or less neuropathic? Pain, 110 (2004) 5101.


Dowdall T, Robinson I & Meert TF, Comparison of five different rat models of peripheral nerve injury, Pharmacol Biochem Behav, 80 (2005) 93.


Nam E, Lee SM, Koh SE, Joo WS, Maeng S, Im HI & Kim YS, Melatonin protects against neuronal damage induced by 3-nitropipionic acid in rat striatum, Brain Res, 1046 (2005) 90.


Ellman GL, Tissue sulfhydryl groups, Arch Biochem Biophys, 82 (1959) 70.


48 Naik AK, Tandan SK, Dudhgaonkar SP, Jadhav SH, Katara M, Prakash VR & Kumar D, Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats, Eur J Pain, 10 (2006) 573.