Standardization and Validation of Spinal Nerve Ligation Model of Neuropathic Pain in Rats

1Piyush Patel, 2Bhavesh Vyas, 2Adarshjit Singh and 3Praful Talaviya
1Intas Pharmaceuticals Ltd, Ahmedabad, India
2Department of Pharmacology, AMC-MET Medical College, Ahmedabad, India
3Department of Pharmaceutical Sciences, Pacific University, Udaipur, India

ABSTRACT

Background and Objective: Models based on peripheral nerve injury ligation are commonly employed models for evaluation of neuropathic pain. Variation in the experimental outcomes is reported between laboratories with animal models of neuropathic pain. This study was aimed to standardize and to validate (using pregabalin) the L5 and L6 Spinal Nerve Ligation (SNL) model of neuropathic pain in male Sprague-Dawley rats. Materials and Methods: Male Sprague-dawley rats were used to standardize and validate the animal model by observing behavioral assessment using spontaneous test, cold allodynia, pinprick test, thermal hyperalgesia and mechanical allodynia. Model was validated using pregabalin at dose of 30 mg kg⁻¹ (i.p.) against vehicle treated groups using different behavioral parameters. Results: The observational data obtained according to schedule testing, which produced consistent results in term of development of different symptoms associated with neuropathic pain in the L5 and L6 Spinal Nerve Ligation (SNL) model of neuropathic pain in male Sprague-Dawley rats. Conclusion: Animal models play a pivotal role in the preclinical study and in the development of effective therapeutic agents. Various types of animal models are developed to meet the diverse manifestations of neuropathy pain that includes central and peripheral models of neuropathy. Further study is required for the effective and better development of animal model for evaluation of therapeutic agents in preclinical studies.

Key words: Neuropathic pain, pregabalin, spinal nerve ligation, cold allodynia, thermal hyperalgesia, mechanical allodynia


INTRODUCTION

Neuropathic pain, primary lesion or dysfunction in the nervous system¹, is an area of largely unmet therapeutic need. Pain is the primary reason for patients seeking healthcare and it has been estimated to result in more than $100 billion per year in both direct and indirect medical costs due to pain². According to the American Pain Society, prevalence of chronic pain in the United States is estimated to be 35.5% or 105 million people and employment status is affected in approximately 43% of neuropathic pain patients³. The community based Indian study reported 2.8% of prevalence of pain among Parsi adults⁴. Neuropathic pain alone has been associated with an approximately 3-fold increase in use of healthcare resources which is responsible for poor quality of life in patients⁵. Animal models are the mainstay of neuropathic pain research and aspects of these models should be reviewed in detail. Animal models provide pivotal system for preclinical study of neuropathic pain. A need exists for validated and easily reproducible animal model of neuropathic pain symptoms to evaluate the analgesic potential of novel pharmacotherapies to enhance treatment outcomes.

The most widely used neuropathic pain models are the nerve injury models, principally the Chronic Constriction Injury (CCI) model⁶, the partial sciatic nerve ligation model⁷ and the Spinal Nerve Ligation (SNL) model⁸. Various animal models are available for evaluation of neuropathic pain symptoms, in which spinal nerve ligation has been widely used model for evaluation of peripheral neuropathic pain. This model is associated with various neuropathic pain symptoms such as spontaneous pain, allodynia (mechanical/cold) and hyperalgesia (mechanical/thermal)⁹.
The SNL model is based on the ligation of the L5 and L6 spinal nerves. A variety of pain-related phenomena have been associated with peripheral nerve injury. These include ectopic discharges ofafferent neurons linked to the injury site, collateral sprouting of primary afferent terminals, coupling between the sympathetic and the sensory nervous system, anatomical reorganization of the spinal cord where central terminals of the large myelinated primary afferent neurons sprout into lamina II of the superficial horn, resulting in second order neurons receiving inputs from low-threshold mechanoreceptors, hyper excitability of dorsal horn neurons and supra spinal influences.

However, between laboratories, variation in the experimental outcomes is reported with animal models of neuropathic pain. Henceforth, the present study was aimed to standardize and to validate (using pregabalin) the L5 and L6 Spinal Nerve Ligation (SNL) model of neuropathic pain in male Sprague-dawley rats.

**MATERIALS AND METHODS**

**Animals:** Male Sprague-Dawley rats of around seven weeks of age, body weight between 160-250 g were procured from animal facility of Smt NHL Municipal Medical College, Ahmedabad, India. The ethics approval was obtained from Animal ethics committee of Smt NHL Municipal Medical College, Ahmedabad, India. Each rat was housed in transparent plastic box cage individually with well controlled supplied air, humidity (<70%) and had free access to autoclaved, untreated tap water and standard rat chow. The environment was maintained at 22±2°C with a 12 h light/dark cycle. After surgery, rats were inspected daily for activity and healing of surgical wounds. All tests were performed during the light phase. The protocol used was approved by the institutional ethical committee for animal research.

**Drugs and chemicals:** Pentobarbitone sodium (Sigma Ltd) and Isoflurane (Abott chemicals Ltd) were used as anaesthetics. Benzyl Penicillin (injection ip.) is used as antibiotic in this experiment that was procured from Alembic Pharma Ltd, India. Framycetin was obtained from Aventis Pharma Ltd, India. Pregabalin was obtained from Symed Labs, India. All other chemicals used in the experiments were of analytical grade.

**Sutures:** A 3-0 and 6-0 black braided silk sutures were obtained from Suture India Pvt Ltd, India.

**Instruments:**
- Plantar test instrument for thermal hyperalgesia (UGO BASILE, Italy)
- Dynamic plantar aesthesiometer for mechanical alldynia (UGO BASILE, Italy)
- Homeothermic blanket control unit (TSE System, Axiom Biotech Inc, India)
- Bone rongeur (3 mm Jaw-width) (Prime Biosciences Pte Ltd, India)
- Observation cages with mesh floor (Abbot and Abbot, India)

**Induction of peripheral neuropathy (Spinal Nerve Ligation model (SNL)):** Surgery was performed according to method explained by Kim and Chung11 and Bharucha et al. Surgery was performed using Pentobarbitone sodium 50 mg kg^-1 as an anesthetic by intraperitoneal route. After checking righting reflex, the large area of back was shaved and then put the anesthetized animal on its ventral surface on heating pad of Homeothermic blanket control unit. After applying Isopropyl alcohol as an antiseptic on shaved area, around 3 cm incision was made from L4-S2 level on the back around 5 mm lateral to midline in left side (preferred according to literature and convenience). After removing the subcutaneous tissue, cut the lumbar fascia. Then, the paraspinal muscles were separated from spinal process toward left side. After identifying the L6 transverse process and it was removed with bone rongeur as completely as possible. The L4 and L5 nerves run just beneath the transverse process. After identification, they were separated from each other and tightly ligated the L5 nerve which is situated more medially with silk 6-0 thread as like lesioning all the axons within it. As the L4 nerve mainly contains motor nerve fibers, it is very important to avoid damage to the L4 nerve during L5 nerve ligation and all other procedures. Then by inserting a small hook beneath the medial end of sacrum in 11-5 direction of clock, the L6 nerve was gently pulled outwards until it could be visualized, where it was then tightly ligated with silk 6-0 thread. Wound was cleaned by removing the muscle bits and blood clots and it was closed in layers. Lumbar fascia and muscles were sutured with use of 3-0 black braided silk suture and skin was sutured with cotton thread. The animals were then transferred to their home cage and left to recover. All surgical procedure was carried out under normal sterile conditions. Sham surgeries were also done by using same technique except ligation of nerves.
Post operative care has been taken with administration of sophramycin skin cream as an antibiotic and benzyl penicillin in dose of 20,000 IU kg\(^{-1}\) intramuscularly, as an antibiotic twice daily for 5 days.

**Behavioral assessment:** Total five behavioral tests were aimed to carry out standardization of SNL model. Initially, only semiquantitative methods were used to check behavioral symptoms. The Ugo Basil instrument was used to evaluate neuropathic pain symptoms using quantitative methods.

**Semiquantitative methods**

**Spontaneous pain:** Spontaneous pain \(n = 6\) was assessed for a total time period of 15 min as described by Yoon et al.\(^5\). The operated rats were placed individually inside an observation cage. An initial acclimatization period of 10 min was given to each of the rat. Then the cumulative duration during which the rat held its ipsilateral paw off the floor within 5 min was noted. The paw lifts associated with locomotion or body repositioning were not counted. It has been suggested that those paw lifts in the absence of any overt external stimuli are associated with spontaneous pain and are correlative of ongoing pain. For each measurement, three successive readings were taken without any gap period and the mean was calculated.

**Cold allodynia (acetone test):** The rats demonstrating unilateral mononeuropathy were assessed for cold allodynia using the acetone application technique \(n = 6\)\(^6\). The operated rats were placed individually inside an observation cage. After 5 min of acclimatization period, a few drops (100 \(\mu\)L) of freshly prepared acetone were sprayed on to the midplantar region of left paw by syringe with 90° bent needle\(^7\). A cold allodynia response was assessed by noting the cumulative duration of the left paw withdrawal response for 2 min. For each measurement, test was repeated three times and the mean was calculated. Three min gap was maintained between each observation.

**Mechanical hyperalgesia (pinprick test):** The rats demonstrating unilateral mononeuropathy were assessed for mechanical hyperalgesia sensitivity using the pinprick application technique \(n = 8\)\(^8\). Initial 5 min acclimatization period was given to each rat. Then a pinprick test was performed using a bent needle (at 90° to the syringe) attached to a syringe to measure mechanical hyperalgesia. Hind paw withdrawal duration was measured for 1 min after a mild pin-prick stimulus to the rear part of the left hind paw. The mean duration of withdrawal was taken from a set of three responses. Three min gap was maintained between each observation.

**Quantitative methods**

**Mechanical allodynia:** The rats demonstrating unilateral mononeuropathy were assessed for mechanical allodynia by using the dynamic plantar aesthesiometer (UGO BASILE, Comerio, Italy)\(^9\). The operated rats were placed individually inside an observation cage of instrument (SNL, \(n = 14\); Sham, \(n = 8\)). The Dynamic Plantar Aesthesiometer (DPA) instrument is positioned beneath the animal. After 10 min of acclimatization period, a pin with gradually increased force was applied to operated side paw and withdrawal of the paw was recorded automatically by the instrument and the threshold was taken as a reading. Mechanical allodynia was checked with applying 50 g force and 20 sec ramp. Total 3 readings were taken with 3 min gap in between them and mean was calculated. Withdrawals related to walking or movements were not taken as a reading. Cleaning was carefully taken care of during observation.

**Thermal hyperalgesia:** The rats demonstrating unilateral mononeuropathy were assessed for thermal hyperalgesia by using the plantar test\(^10\). The operated rats were placed individually inside an observation cage of instrument (SNL, \(n = 14\); Sham, \(n = 8\)). After 10 min of acclimatization period, radiant heat as an infrared beam was applied to operated side paw and withdrawal of the paw is recorded automatically by the instrument and the reaction time (paw withdrawal latency) is taken as a reading. Thermal hyperalgesia at 30 intensity of infrared beam was checked. Total 3 readings were taken everytime with 3 min gap in between and mean was calculated. Withdrawal related to walking or movement is not taken as a reading. Cleaning was carefully taken care of during observation.

**Validation of Spinal Nerve Ligation model (SNL):**

Surgery was performed according to method explained by Kim and Chung\(^11\). Postoperative care was taken in same way. On postoperative day 1st, animals were checked for any gross motor impairment like dragging or inability to lift their paw during walking. Animals with motor impairment were excluded from validation study.

On postoperative day, 12 animals were selected out of total 16 animals in both groups and randomized into treatment (pregabalin treated) and control (vehicle treated) groups, for each mechanical allodynia and thermal hyperalgesia group \(n = 6\).
Drug preparation and administration: Pregabalin was obtained from Symed Labs. Pregabalin solution was prepared freshly in sterile water at 10 mg mL\(^{-1}\) concentrated solution and given at dose of 30 mg kg\(^{-1}\) intraperitoneally twice a day 12 h apart. Vehicle group was given sterile water at 3 mL kg\(^{-1}\) volume in same schedule as treatment group intraperitoneally.

On postoperative day 7th, 9th, 11th and 13th, the observation was taken. Maximum effect of Intraperitoneal administration of pregabalin reached after 30-120 min\(^{17}\). So, after 45 and 90 min of drug administration, effects were checked.

Three behavioral tests aiming to validation of spinal nerve ligation model with pregabalin were carried out. Mechanical allodynia and thermal hyperalgesia were performed on two separate groups and spontaneous pain was recorded in both groups. Each group had total 6 animals (treatment group n = 6, vehicle control group n = 6). All animals were tested according to the experimental protocol. The procedure for each behavioral test was similar as performed during the standardization. Spontaneous pain was recorded by taking both paw withdrawal duration and paw withdrawal frequency.

Statistical analysis: Results were expressed as Mean±SEM in graph. Results from each group were compared with that day respective value of control group. All comparison was done on software PRISM version 3.02 using two tail unpaired t-test. Data was considered statistically significant according to p value. In graph, degree of significant were marked as *p<0.05, **p<0.01, ***p<0.001.

RESULTS
Standardization of spinal nerve ligation model of neuropathic pain:

- **Semi quantitative methods:** Spontaneous pain: It is assumed that foot lifting in the absence of any overt external stimuli, is a form of paw withdrawal reflex that is associated with spontaneous pain, although it reflects the amount of pain is uncertain. Therefore, spontaneous pain was recorded as a cumulative foot lifting duration in 5 min.

Spontaneous pain as paw withdrawal was started from 3rd day and remained upto 14th day as shown in Fig. 1. But quantity of spontaneous pain in SNL rats was very less (<5 sec). The reproducibility of spontaneous pain was also very less. There was a wide variation among animals.

- **Cold allodynia:** Cold allodynia was measured by acetone spray and plotted in Fig. 2. Cold allodynia was assessed by taking cumulative foot duration after acetone spray. Cold allodynia was started to increase from 7th day and steadily increased up to 14th day. Because the intensity and reproducibility was somewhat less as like with spontaneous pain and manual errors were also high, this parameter was not chosen for validation purpose.

- **Mechanical hyperalgesia:** Mechanical hyperalgesia was measured by pinprick method and plotted in Fig. 3. Cumulative foot duration after pinprick was taken into consideration as mechanical hyperalgesia.
Quantitative methods: Mechanical allodynia and thermal hyperalgesia started from the 3rd day and remained up to the day 14th. There was a large reduction in threshold in spinal nerve ligation group in comparison to sham operated animal group.

There wasn't a large difference in both groups in day 3 but from the day 5 onwards, the difference was clearly seen. Similar to mechanical allodynia, thermal hyperalgesia results also showed very high reproducibility. So, thermal hyperalgesia parameter was also taken for the validation purpose.

Mechanical hyperalgesia steadily increased after three days. But, as like in cold allodynia, manual errors were large with this parameter which was also not selected for validation.

Initially, animals were observed for developing mechanical allodynia as shown in Fig. 4. So, only the basal observation (-1 day) included six SNL animals and six Sham observed postoperatively upto 2 batch with SNL (n = 8) and sham (n = 6). In last batch, with SNL (n = 6) and sham (n = 6), Preoperative day (-1) reading was also taken for comparison as shown in animals and after that, SNL (n = 14) and sham (n = 12) animals data has been shown in Fig. 5.

As shown in Fig. 6, the spontaneous pain was almost similar in both groups till the day of treatment. But afterwards, the spontaneous pain (paw withdrawal duration) was decreased in pregabalin groups in comparison to vehicle group. The spontaneous pain remained decreased throughout study period upto 14th day. The paw withdrawal frequency parameter (Fig. 7) showed few frequency and large variation among animals as like paw withdrawal duration. But after 7th day treatment, there was a uniform decrease in paw withdrawal frequency among pregabalin treated rats.

Effect of pregabalin: On the post operative day 5 after randomization of animals into drug and vehicle group, pregabalin treatment was given to animals (30 mg kg⁻¹, b.i.d., i.p.) for 7 days. Treatment was stopped at post operative day 13th. Animals were observed according to the predetermined schedule.

There was a significant decrease in mechanical allodynia in pregabalin treatment group in comparison to vehicle group as shown in Fig. 8 by increasing the threshold for paw withdrawal. The result shows that the increase in threshold is higher at 90 min than at 45 min throughout on treatment day. The basal observation was also increased on 9th day onwards which was taken before the treatment. It suggests the effect of treatment given on the previous days.

Figure 9 shows the significant decline in thermal hyperalgesia among pregabalin treated animals in
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**Fig. 8:** Effect of pregabalin on mechanical allodynia in SNL rats (n = 6), *p<0.05, **p<0.01, ***p<0.001

**Fig. 9:** Effect of pregabalin on thermal hyperalgesia in SNL rats (n = 12), *p<0.05, **p<0.01, ***p<0.001

**DISCUSSION**

Neuropathic pain has been characterized by the sensory abnormalities such as dysesthesia, hyperalgesia and allodynia. The pharmacotherapy for neuropathic pain has limited success with commonly used pain reducing drugs such as opiates and NSAIDS. The neuropathic pain symptoms generated in SNL model mimic the symptoms of human patients suffering from causalgia, developed after an injury to the peripheral nerves. The observational data obtained in the present study showed that the tight ligation produced significant and consistent results in term of development of various symptoms associated with neuropathic pain such as spontaneous pain, mechanical allodynia, cold allodynia and mechanical hyperalgesia. To prevent the learning behaviour of animals to respective tests, schedule of observation was decided to be taken at an alternate day like starting at 3rd day, then 5th, 7th, 9th, 11th and 14th day.

Mechanical allodynia and thermal hyperalgesia test were carried out on Dynamic plantar aesthesiometer and Plantar test, respectively due to variation in reproducibility in different animals. To standardize the

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force and ramp and their correlation, mechanical allodynia was tested at 3 different ramps like 20, 35 and 50 sec with maximum force 50 g but due to same results 20 sec ramp and 50 g maximum force was selected for further validation. To standardize the plantar test instrument, thermal hyperalgesia was assessed at three different intensities 30, 40 and 45 IR (infrared). At 30 IR intensity, the SEM was somewhat higher in comparison to 40 IR and 45 IR. 30 IR intensity was chosen for further validation due to following reasons:

- The development of neuropathic pain was very clearly seen with 30 intensity in initial postoperative days so the reduction of thermal hyperalgesia could be significantly noticed at this intensity
- This intensity is less noxious for animals and so the time taken to recover and chances to get the paw damaged were less at this intensity
- At 30 intensity, already higher SEM was obtained and so it was not possible to further reduce the intensity as the variation were increasing at lower intensity

The L4 nerve contains mainly motor nerve fibers of sciatic nerve. It was very important to avoid damage of L4 nerve during whole surgical procedure. But initially, during standardization, when it was not possible to find and ligate the L5 nerve, the L4 nerve was damaged unintentionally and those animals showed motor impairment like dragging of hind paw, inability to lift the hind paw postoperatively. But at the same time, those animals also showed more robust results in second week especially spontaneous pain, cold allodynia and mechanical hyperalgesia. So, L4 loose ligation with silk 6-0 was performed in addition to L5 and L6 ligation in 2 animals. In 1st postoperative week, they showed mild motor impairment like dragging of hind paw up to some extent but oppositely in 2nd week, they showed robust results in comparison to only L5 and L6 ligation model results. The mechanism for this behavior is still unknown.

After standardization, model was validated with the drug available for neuropathic pain treatment to check that the behavior obtained by spinal nerve ligation was actual neuropathic pain. Validation would also give an idea about pattern and intensity of reduction of neuropathic pain if newly developed molecule would be tested in this model.

Various agents like tricyclic antidepressant, NSAIDs, opioids etc, are used for treatment of neuropathic pain, but due to insufficient efficiency and serious adverse effects, these agents have not gained great importance in treatment of neuropathic pain. Various anti convulsant drugs are also used to treat neuropathic pain symptoms. Consequently, there is a need to explore novel treatment for neuropathic pain. In current study, pregabalin was used to validate the spinal nerve ligation induced neuropathic pain model in rat 30 mg kg<sup>−1</sup> b.i.d. for 7 days from 7th to 13th postoperative day.

Validation data suggested that the 7 days, twice a day dose, pregabalin<sup>19,20</sup> treatment did not cause any significant difference in body weight, food and water consumption in comparison with vehicle group during whole period of study, i.e. post operative day 7-14.

As spontaneous pain is directly related to clinical ongoing pain, it was decided to check spontaneous pain in both groups during validation. Significant decline in spontaneous pain in both the groups with both paw withdrawal duration and frequency was obtained.

Due to having better reproducibility than manual semiquantitative methods during standardization of mechanical allodynia and thermal hyperalgesia were selected for validation. Results suggest that significant difference was seen in treatment groups after 45 and 90 min of dosing with both mechanical allodynia and thermal hyperalgesia in comparison to their vehicle groups on all four days (7th, 9th, 11th and 13th). Apart from the basal readings, those were taken before dosing 9th, 11th, 13th, 14th day reading, also showed the difference in treatment groups in comparison to vehicle group. It was due to effect of pregabalin given on previous day and proves the effectiveness of pregabalin through whole day when taken twice a day.

In this model, the changes in mechanical and thermal sensitivities are robust, substantial, prolonged time and closely mimic many features of clinical neuropathic pain<sup>13</sup>. The surgical procedure for creating this model is relatively easy compared with previous models and also there is relatively lesser variability in degree of damage.

**CONCLUSION**

The future aspect for delivery of drugs with improved analgesic efficacy and patient compliance depend on their successful preclinical and clinical trials. In present study, SNL model for neuropathy pain is standardized and validated successfully in both healthy and diseased animal. The corresponding development of this model is required for better therapeutic effects of drugs for both chronic and acute pain. This model can effectively use for the future treatment of neuropathic pain in humans.
REFERENCES